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MOLECULAR EPIDEMIOLOGY AND NATURAL HYBRIDIZATION IN EMERGING ZOO NOTIC TREMATODIASSES TRANSMITTED BY FRESHWATER SNAILS: FASCIOLIASIS AND SCHISTOSOMIASIS

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A MI TÍA CARMEN Y A MI TÍO CHEMA

*“No es la especie más fuerte la que sobrevive, ni la más inteligente.
Es aquella que mejor se adapta al cambio.”*
Charles Darwin.

ABSTRACT

Fascioliasis and schistosomiasis are two zoonotic trematodiasis of major importance for both human and veterinary health. Both trematodiasis, transmitted by freshwater snails, are strongly influenced by global change, which seems to favor their (re-)emergence and natural hybridization between species of the same genus. This thesis focuses on the natural hybridization of these trematodes in two countries experiencing different global change phenomena. Bangladesh is an endemic country for fascioliasis where livestock is a pillar of the economy. A total of 2,352 fasciolid parasites were collected from the major livestock species in four different agro-ecological zones to assess the prevalence and intensity of infection. The prevalence was remarkably high in all the livestock species studied, reaching 35.37% in goats and 55.56% in buffaloes, the highest ever recorded for these animals in Bangladesh. Due to the detection of intermediate phenotypic forms, the complete intergenic region of nuclear rDNA from 83 samples was sequenced and cloned. Cloning of ITS-1-5.8S-ITS-2 revealed the simultaneous presence of sequences from both *F. hepatica* and *F. gigantica*, suggesting recent hybridization events between imported *F. hepatica* with introduced livestock and local *F. gigantica*. The high proportion of *F. gigantica* x *F. hepatica* hybrids highlights the need for control measures. Schistosomiasis is being addressed in Spain, a non-endemic country that receives a large number of migrants from endemic countries in sub-Saharan Africa. Fifty-one migrant patients diagnosed with urogenital and/or intestinal schistosomiasis from Côte d'Ivoire, The Gambia, Guinea, Guinea-Bissau, Equatorial Guinea, Mali, Mauritania and Senegal were included. A total of 699 eggs were genetically characterized by RD-PCR of *cox1* from mtDNA and sequencing of ITS-2 and 18S from rDNA. The results showed that 16.72% of the eggs detected in urine corresponded to a hybrid profile, including 12.54% with an *S. haematobium* x *S. bovis* profile and 3.01% with an *S. haematobium* x *S. curassoni* profile. Of note is the detection of a three-species hybrid, *S. haematobium* x *S. bovis* x *S. curassoni*, in 0.84% of hybrid eggs. These results allowed us to identify 20 patients carrying hybrid eggs and pure *S. haematobium* eggs simultaneously. The high number of patients carrying *Schistosoma* hybrids underlines the need to study whether there are differences in morbidity and treatment response compared to patients carrying only pure profiles. Finally, five migrant patients from Côte d'Ivoire, Guinea-Bissau, and Senegal diagnosed with urogenital schistosomiasis with ectopic lateral-spined eggs in urine, typical of *S. mansoni*, were analyzed. Forty-two lateral-spined eggs (33 from urine and 9 from feces) and 75 terminal-spined eggs collected from the urine of these five patients were genetically characterized (using RD-PCR of *cox1*, sequencing and cloning of ITS-2, and sequencing of 18S). The results showed that 76.32% of the analyzed lateral-spined eggs were hybrids resulting from the cross between *S. mansoni* x *S. haematobium*. For terminal-spined eggs, 93.15% were identified as *S. haematobium*, while 6.85% were hybrids of *S. haematobium* x *S. bovis* or *S. curassoni*. These results confirmed the simultaneous shedding of *S. mansoni* x *S. haematobium* hybrid eggs and *S. haematobium* pure eggs in the five patients. In addition, the shedding of *S. haematobium* x *S. bovis* or *S. curassoni* hybrids was also confirmed in two of the patients. A subsample of 68 eggs was sequenced to identify combined haplotypes (ITS+*cox1*), describing for the first time a total of 14 different combined haplotypes in patients with ectopic egg shedding in urine and detecting up to six different combined haplotypes within a single patient.

Keywords: *Fasciola hepatica*, *Fasciola gigantica*, Bangladesh, hybrids, sub-Saharan migrants, Spain, *Schistosoma haematobium*, *Schistosoma bovis*, *Schistosoma curassoni*, *cox1*, mtDNA, ITS, 18S, rDNA, cloning, mito-nuclear signature, multigenic haplotyping.

RESUMEN

La fascioliasis y la esquistosomiasis son dos trematodiasis zoonóticas con una gran importancia, tanto en la salud humana, como en el ámbito veterinario. Ambas trematodiasis, transmitidas por caracoles de agua dulce, están fuertemente influenciadas por el cambio global, lo que parece estar favoreciendo su (re-)emergencia, así como la hibridación natural entre especies del mismo género. En esta tesis se estudia la hibridación natural en estos trematodos en dos países sometidos a distintos fenómenos del cambio global. Bangladesh es un país endémico para la fascioliasis donde el ganado es uno de los pilares fundamentales de su economía. Se recolectaron 2.352 fasciólidos de las principales especies ganaderas en cuatro áreas agro-ecológicas diferentes para evaluar las prevalencias e intensidades de la infección. La prevalencia registrada del 35,37% en cabras y 55,56% en búfalos fue la más alta observada en estos animales en Bangladesh. Debido a la detección de formas fenotípicas intermedias, se secuenció y se clonó la región intergénica completa del ADNr nuclear de 83 ejemplares. La clonación de ITS-1-5.8S-ITS-2 reveló la presencia simultánea de secuencias de *F. hepatica* y *F. gigantica*, lo que sugiere eventos de hibridación recientes entre *F. hepatica* importada con el ganado introducido y *F. gigantica* local. La alta proporción de híbridos de *F. gigantica* x *F. hepatica* hallada subraya la necesidad de medidas de control. La esquistosomiasis se aborda en un país no endémico como España, el cual recibe un gran número de migrantes procedentes de países endémicos del África subsahariana. Se incluyeron 51 pacientes migrantes diagnosticados con esquistosomiasis urogenital y/o intestinal procedentes de Costa de Marfil, Gambia, Guinea, Guinea-Bisáu, Guinea Ecuatorial, Malí, Mauritania y Senegal. Se caracterizaron genéticamente 699 huevos mediante RD-PCR de *cox1* del ADNmt y secuenciación de ITS-2 y 18S del ADNr. Los resultados revelaron que el 16,72% de los huevos detectados en la orina correspondían con un perfil híbrido, incluyendo un 12,54% con un perfil *S. haematobium* x *S. bovis* y un 3,01% con un perfil de *S. haematobium* x *S. curassoni*. Se destaca la detección de un híbrido de tres especies, *S. haematobium* x *S. bovis* x *S. curassoni* en un 0,84% de los huevos híbridos. Estos resultados nos permitieron identificar 20 pacientes portadores simultáneamente de huevos híbridos y huevos puros de *S. haematobium*. El elevado número de pacientes portadores de híbridos de *Schistosoma* enfatiza la necesidad de estudiar si existen diferencias en la morbilidad y la respuesta al tratamiento en comparación con los pacientes portadores únicamente de perfiles puros. Por último, se analizaron cinco pacientes migrantes originarios de Costa de Marfil, Guinea-Bisáu y Senegal diagnosticados con esquistosomiasis urogenital con huevos ectópicos en orina de espina lateral, típicos de *S. mansoni*. Se caracterizaron genéticamente (mediante RD-PCR de *cox1*, secuenciación y clonación del ITS-2 y secuenciación del 18S) 42 huevos con espina lateral (33 de orina y 9 de heces) y 75 huevos con espina terminal recolectados de la orina de estos cinco pacientes. Los resultados mostraron que el 76,32% de los huevos con espina lateral analizados eran híbridos resultantes del cruce de *S. mansoni* x *S. haematobium*. En cuanto a los huevos con espina terminal, el 93,15% fueron identificados como *S. haematobium*, mientras que en un 6,85% fueron híbridos de *S. haematobium* x *S. bovis* o *S. curassoni*. Estos hallazgos nos permitieron corroborar la eliminación simultánea de huevos híbridos de *S. mansoni* x *S. haematobium* y huevos puros de *S. haematobium* en los cinco pacientes. Además, en dos de ellos, se confirmó también la eliminación de híbridos de *S. haematobium* x *S. bovis* o *S. curassoni*. Se secuenció una submuestra de 68 huevos para identificar haplotipos combinados (ITSs+*cox1*), describiéndose por primera vez un total de 14 haplotipos combinados diferentes en pacientes con eliminación de huevos ectópicos en orina y detectando hasta seis haplotipos combinados distintos dentro de un mismo paciente.

Palabras claves: *Fasciola hepatica*, *Fasciola gigantica*, Bangladesh, híbridos, migrantes sub-Saharanos, España, *Schistosoma haematobium*, *Schistosoma bovis*, *Schistosoma curassoni*, *cox 1*, ADNmt, ITS, 18S, ADNr, clonación, firma mito-nuclear, haplotipaje multigénico.

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ABBREVIATIONS AND ACRONYMS

bp	Base pairs
<i>cox1</i>	Cytochrome c oxidase subunit 1 (gene)
COXI	Cytochrome c oxidase subunit 1 (protein)
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide triphosphate
EDTA	Ethylenediaminetetraacetic acid
HCl	Hydrogen chloride
h.i.r.	Human infection reported
i.h.	Infected hosts
indels	Insertions/deletions
IPTG	Isopropyl β -d-1-thiogalactopyranoside
ITS	Internal transcribed spacer
IUPAC	International Union Of Pure And Applied Chemistry
LB	Lysogeny Broth
mtDNA	Mitochondrial DNA
NaCl	Sodium chloride
<i>nad1</i>	NADH dehydrogenase subunit 1

NTD	Neglected Tropical Disease
PCR	Polymerase chain reaction
rDNA	Ribosomal DNA
RD-PCR	Rapid diagnostic PCR
rRNA	Ribosomal RNA
TMU	Tropical Medicine Unit
Tris	Tris(hydroxymethyl)aminomethane
TE	Tris EDTA
WHO	World Health Organization
X-Gal	5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside

CHAPTER I. INTRODUCTION



1.1. GLOBAL CHANGE AND PARASITES

There has been considerable concern about global change in recent decades. This term has been defined as the set of environmental changes induced by human activities, with particular emphasis on altering the processes that govern how the Earth System operates. This definition encompasses all activities that, although local, have effects that transcend the local scale and affect the global functioning of the planet (Duarte, 2006).

Although change is inherent to our planet, two features make the modifications associated with global change unique in Earth's history. The first is the speed with which these remarkable changes are occurring, and the second is the fact that a single species, *Homo sapiens*, is driving them all. The rapid growth of the human population and the increase in per capita resource consumption, supported by technological development, have been the main drivers of global change (Duarte, 2006). As a result, many scientists have coined the term "Anthropocene" for the current geological era, emphasizing human impact.

Climate change, biodiversity loss, habitat fragmentation, deforestation, urbanization, pollution, and population movements are some of the interrelated phenomena that are altering the planet and have direct or indirect impacts on societies. The most vulnerable communities are usually disproportionately affected by the effects of these phenomena, exacerbating existing inequalities and posing challenges in

terms of social justice and equitable access to resources. Public health is one of the aspects most impacted by global change, and human parasitic diseases are an interesting group to study the effects.

The majority of human parasitic diseases fall into the category of Neglected Tropical Diseases (NTDs), also known as forgotten diseases. In general, these diseases receive little attention because they primarily affect impoverished communities in low and middle-income countries, mainly in tropical regions. They are concentrated in areas of extreme poverty, urban peripheries, and conflict zones, as transmission cycles are perpetuated by poor living and hygiene conditions. The impoverishment of the population favors the spread of these diseases. The lack of economic resources, as well as information and education, hampers prevention and control efforts. Moreover, the chronic nature of these diseases hinders the potential socio-economic development of these communities, perpetuating the cycle of poverty (WHO, 2023a).

Ecological factors such as extreme climate events (droughts, floods, or severe storms) (Obokata et al., 2014) and economic factors such as income inequality increase forced migration, which can facilitate the spread of these parasitic diseases. Individuals moving from endemic areas to regions where these diseases are not common may carry parasites and their associated diseases. These areas may not be prepared to diagnose and treat them, and under appropriate conditions, outbreaks, epidemics, or even endemicity may occur. All over human history, the introduction of parasites through human displacement has been observed. A good example is the introduction of fascioliasis, into the

American continent as a result of colonization (Bargues et al., 2021; Mas-Coma et al., 2022a). All of this can be amplified when we consider globalization, which facilitates international travel or the import/export of, for example, livestock, pets, food...

In addition, parasitic diseases have a strong social component and are often closely linked to risk factors related to behaviors, traditions, social habits, and/or religious aspects. The flow of information, migration, or international travel promotes cultural exchanges that can lead, for example, to the introduction of new food habits. Japanese cuisine is currently trending in Europe, which includes the consumption of raw fish prepared as sushi and sashimi. A recent study in Portugal linked the popularity of raw fish consumption to the proliferation of Japanese restaurants in the country and the low level of awareness of *Anisakis* among the population. The widespread lack of knowledge about the parasite and, therefore, its preventive measures, such as freezing fish intended for raw consumption when preparing these dishes at home, highlighted the likely increase in the risk of anisakiasis infection in the near (Golden et al., 2022; 2023).

Anthropogenic environmental changes like deforestation, construction of irrigation canals, dams, or urbanization also affect the transmission of these diseases. For example, typical cycles of wild transmission can come into contact with humans as a result of human population growth and its tendency to urbanize, as occurred in the outbreak of leishmaniasis in the Community of Madrid between 2010 and 2012. Although Spain is an endemic area, this outbreak was unusual

since hares were identified as the main reservoir for the transmission of the pathogen. Infrastructure works and environmental changes in the area carried out as part of the conversion of agricultural land into urban parks, appear to have brought humans into contact with hares, which would be very rare under normal conditions. The absence of predators favored the increase of the hare and rabbit populations, which are easily accessible blood sources for sandflies, thus favoring their spread and density. All these factors, along with favorable environmental conditions, explain the high number of infected individuals (Aguado et al., 2013; Carrillo et al., 2013).

Moreover, low and middle-income countries have experienced the greatest urbanization in recent years. These rapid and unregulated urban settlements put a strain on public health programs. Increased human concentration and lack of sanitation intensifies the risk of foodborne protozoan transmission, such as *Cryptosporidium* spp., *Cyclospora cayetanensis*, *Giardia duodenalis*, *Entamoeba* spp. or *Toxoplasma gondii* (Pozio, 2020). The growth of these urban areas creates an increasing demand for products that are not always adequately supplied. In Africa, for example, many of the small-scale pigs raised in rural areas, where *Taenia solium* infection rates range from 30% to 40%, have begun to be slaughtered in national central abattoirs located in peri-urban and urban areas, exposing non-farming populations (Waage et al., 2022).

Finally, climate change also has a direct impact on parasitoses, especially those that have a developmental stage in the external

environment and/or those transmitted by vectors. Studies have focused on the latter, particularly arthropod-borne diseases, and their tendency to spread to more temperate regions where climatic conditions did not previously allow their introduction and colonization, but are currently becoming more favorable (Mordecai et al., 2020; Jambou et al., 2022).

1.2. TREMATODIASES TRANSMITTED BY FRESHWATER SNAILS AND GLOBAL CHANGE

Vector-borne diseases are particularly vulnerable to the effects of global change because the invertebrate vectors that transmit them are particularly sensitive to anthropogenic activities, environmental changes, and climatic variables (Mas-Coma et al., 2009a).

Most studies have focused only on mosquito-borne diseases, underestimating the diseases transmitted by freshwater snails due to a belief in the low dispersal capacity of these animals (Kincaid-Smith et al., 2017).

Two parasitic diseases, fascioliasis and schistosomiasis, stand out among the diseases transmitted by freshwater snails because of the morbidity they cause in humans. Both fascioliasis and schistosomiasis, caused by digenetic trematodes, have one of the most complex life cycles in the animal kingdom, which can be divided into four phases (**Figure 1.1**):

1. **Definitive host phase.** The definitive host is always a vertebrate that harbors the adult stage of the trematode. In the case of *Fasciola* spp., infection of the definitive host occurs through food-borne transmission. Fasciolid adults are hermaphroditic flatworms that parasitize the liver of the definitive vertebrate host. In *Schistosoma* spp., the definitive host is infected by active

1.2. TREMATODIASES TRANSMITTED BY FRESHWATER SNAILS AND GLOBAL CHANGE

transcutaneous transmission. Schistomatid adults have separate sexes with a pronounced sexual dimorphism between the males and females that parasitize the blood vessels of the host. Regardless of the species, *Fasciola* spp. or *Schistosoma* spp., sexual reproduction of the trematode occurs within the definitive host, and, depending on the location of the adult, the resulting eggs are excreted in feces or urine.

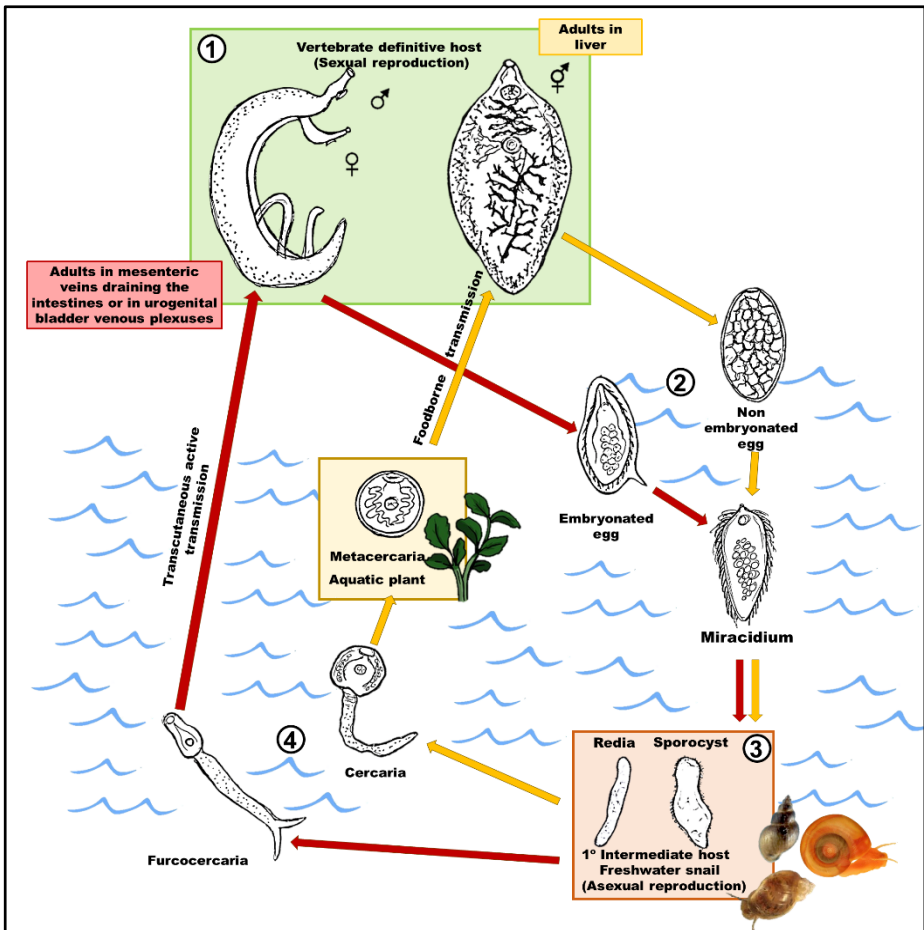


Figure 1.1. Schematic life cycle of the trematodes *Fasciola* spp. (yellow arrows) and *Schistosoma* spp. (red arrows) transmitted by freshwater snails.

- 2. Transition between the definitive host and the intermediate freshwater snail host phase.** In the case of *Fasciola* spp., the transition between the definitive host and the intermediate freshwater snail host involves a long resistance phase of the eggs, since they are not embryonated at the time of laying (they will embryonate under suitable conditions in the external freshwater environment), and a short active phase of the miracidia. In *Schistosoma* spp. the eggs are already embryonated when they leave the definitive host. In both cases, the embryonated eggs hatch and release the miracidia when physicochemical characteristics allow, and they swim rapidly until they find a suitable freshwater snail.
- 3. Intermediate freshwater snail host phase.** Asexual reproduction always occurs in the intermediate freshwater snail host and acts as an amplification of the larval stages. Development in the mollusk includes the penetration of the miracidium, and several developmental stages of the parasite (sporocyst, rediae, cercariae/furcocercariae).
- 4. Transition from intermediate freshwater snail host to definitive host.** The cercariae/furcocercariae leave the intermediate host and, once in the water, in the case of *Fasciola* spp. are deposited on vegetation, lose their tails, and become encysted, transforming into a metacercaria; in the case of *Schistosoma* spp., the furcocercariae penetrate the definitive host and lose their tails during penetration.

1.2. TREMATODIASES TRANSMITTED BY FRESHWATER SNAILS AND GLOBAL CHANGE

Transitions between hosts of these digenetic trematodes (phases 2 and 4) occur in the external freshwater environment so that several larval stages (eggs, miracidia, cercariae/furcocercariae, and metacercariae encysted in vegetation) are directly exposed to environmental features. The developmental stages that occur within a host (phases 1 and 3) are also influenced by external environmental factors, but indirectly. In particular, the developmental phase within the freshwater snail (phase 3) stands out for two reasons: i) freshwater snails are invertebrates that are highly dependent on the environment, and ii) they are poikilothermic animals in which the larval stages of the parasite are at the same temperature as the external environment (Mas-Coma, 2005; Mas-Coma et al., 2008).

1.3. FASCIOLIASIS

1.3.1. CAUASAL AGENT AND TRANSMISSION

Fascioliasis is caused by two species: *Fasciola hepatica* and *F. gigantica*. The adult stages of both *Fasciola* species live in the gallbladder and bile ducts of the liver of their definitive mammalian host (**Figure 1.2.A.1**), where they have an estimated life span of 9 to 13.5 years in humans (Mas-Coma et al., 2022b).

Fasciolids adult worms of both species are large, flat, and have a leaf-shaped or lanceolate body. Dimensions vary between the species: *F. hepatica* can reach a maximum length of 29 mm and a maximum width of 14.10 mm, whereas *F. gigantica* is more elongated (max 52.30 mm) and narrower (max. 11.80 mm). On the ventral side, there are two small, closely spaced suckers situated on the cephalic cone. The digestive tract is incomplete, characterised by two long-branched intestinal caecums. Like most digenetic trematodes, they are hermaphrodites. The male reproductive tract is formed by two branched testes from which two efferent vessels arise, to later unite in a common vas deferens, which widens into a seminal vesicle and ends in a protrusible spined cirrus. The female reproductive tract has a single branched ovary leading to the ootype. The vitellogenic glands are located at the margins and empty their contents into the ootype through the vitelline ducts. The uterus, short and full of eggs, is located before the gonads (Mas-Coma et al., 2019).

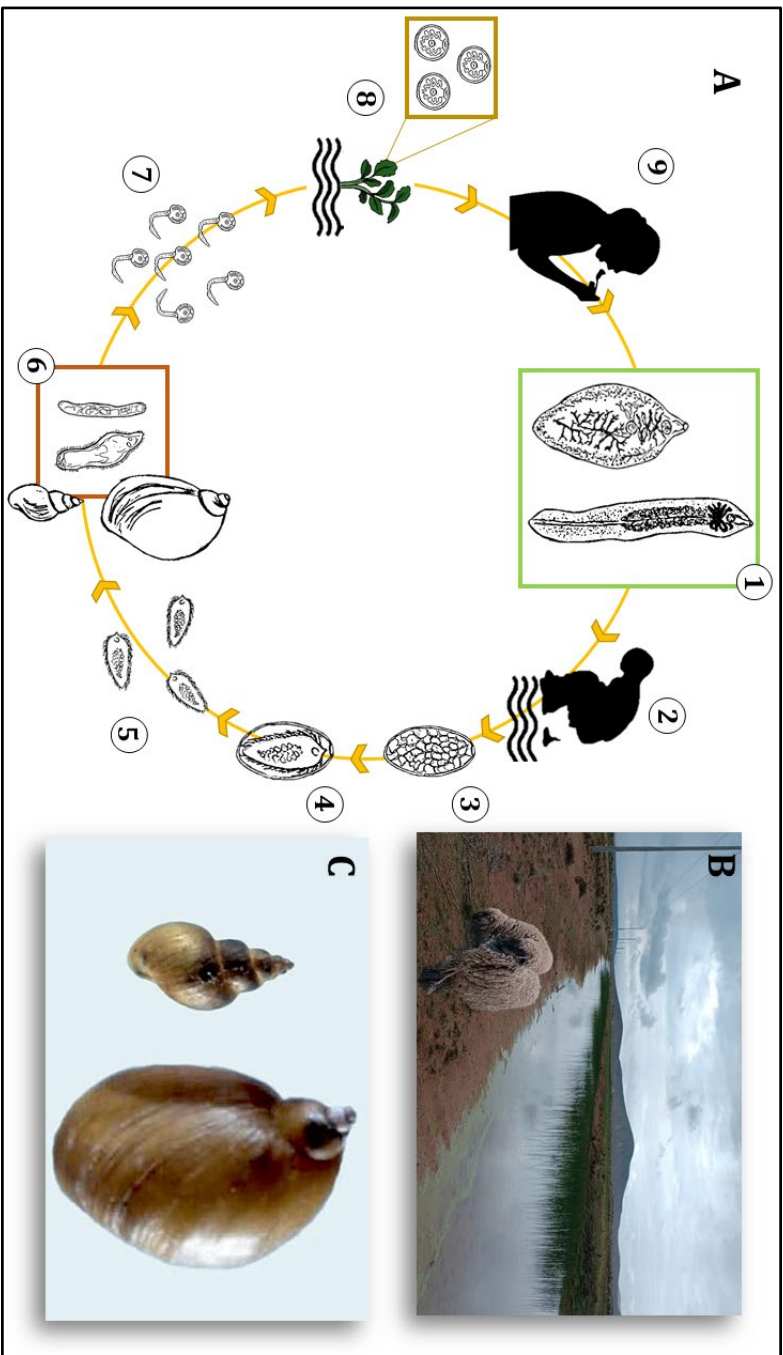


Figure 1.2. A) Life cycle of *Fasciola hepatica* and *F. gigantica*. B) Example of a transmission ecosystem of *Fasciola* spp. C) Main genus of intermediate freshwater snails of fascioliasis: *Galba* and *Radix*, from left to right (image from Mas-Coma et al., 2019).

Inside the definitive host, hermaphroditic *Fasciola* adults reproduce sexually and lay their eggs, which are excreted into the external environment in feces (**Figure 1.2.A.2**). The eggs are large (although the size may vary according to country and intermediate host), operculated, pale yellowish-brown and not embryonated at the time of laying (**Figure 1.2.A.3**) (Valero et al., 2009). Miracidium development (**Figure 1.2.A.4**) and its subsequent hatching (**Figure 1.2.A.5**) will occur between 9 and 21 days after reaching freshwater with the appropriate physico-chemical conditions, such as temperature (15-25°C) or oxygen tension. The miracidium will actively swim until it penetrates the correct intermediate host (**Figure 1.2.A.6**), freshwater lymnaeid snails of the *Galba/fossaria* group in *F. hepatica* and *Radix* group in *F. gigantica*. Inside the lymnaeid freshwater snails, miracidium will continue its development to sporocyst, redial generations, and cercariae production (Mas-Coma et al., 2019). This development at the snail level is temperature dependent, with higher temperatures reducing the prepatent period (15°C: 56-86 days; 20°C: 48-51 days; 25°C: 38 days) (Mas-Coma et al., 2019).

Cercariae are characterised by a single long tail (**Figure 1.2.A.7**), which they swim briefly with and then lose when they encyst in aquatic plants (**Figure 1.2.A.8**). Sometimes they can encyst freely in the water, producing floating metacercariae. The mammal is infected when it ingests plants or drinks water contaminated with metacercariae (**Figure 1.2.A.9**). Within an hour after ingestion, metacercariae excyst in the small intestine and the juvenile flukes penetrate the host's intestinal wall.

Most juveniles are able to reach the liver within the next six days, where they will feed and migrate for the next 5-6 weeks until they finally invade the bile ducts and become sexually mature (Mas-Coma et al., 2019).

1.3.2. GEOGRAPHICAL DISTRIBUTION AND EPIDEMIOLOGY

Fascioliasis is the vector-borne parasitic disease with the widest latitudinal, longitudinal, and altitudinal distribution known to date. The distribution varies between the two species, *F. hepatica* and *F. gigantica*, and is related to their larval specificity at the level of the intermediate freshwater snail host, which in both cases are different species of the family Lymnaeidae (Mas-Coma, 2005).

F. hepatica is transmitted by snails of the *Galba/Fossaria* group, which are very small (less than 1.40 cm in length) and have a pronounced amphibious behavior, usually found in wet mud along the banks of water reservoirs. *F. hepatica* is present in all five continents, reaching an almost global distribution, except for the poles (Mas-Coma, 2005).

F. gigantica is transmitted by longer and wider snails of the *Radix* group, which are usually more aquatic than lymnaeids of the *Galba/Fossaria* group (Mas-Coma et al., 2009b). The distribution of *F. gigantica* is more restricted, being found along the eastern edge of the African continent from Egypt to South Africa, as well as in Asia from the Near East, including Turkey and Syria, to the Far East, including

southern Mongolia, China, Korea and all of Southeast Asia, not forgetting islands such as Japan and Hawaii (Mas-Coma et al., 2022a).

For a long time, the importance of fascioliasis was emphasized primarily in the veterinary field, as infections in animals caused significant economic losses in the livestock industry (such as reduced milk, meat, and wool production, liver condemnation, decreased fertility, or animal deaths) (Charlier et al., 2007; Fiss et al., 2013; Arenal et al., 2018; Nyirenda et al., 2019), and human cases were rarely reported. However, in the last three decades, a new landscape has emerged, highlighting the importance of human fascioliasis and defining it as a unique and highly complex disease. During these years, several endemic areas of human fascioliasis have been described in different countries. These endemic human fascioliasis areas have revealed different transmission patterns and very heterogeneous epidemiological situations related to the *Fasciola* species and lymnaeid snails present in the area, seasonal transmission, human demographics, ethnicities, diets, habits, traditions, and religions, different domestic and wild reservoirs... (see reviews in Mas-Coma et al., 1999; Mas-Coma, 2004, 2005). Currently, more than 2.40 million people in over 70 countries worldwide are estimated to be infected, with several million more at risk (WHO, 2021).

Surprisingly, high human prevalence is not always associated with areas where fascioliasis is a major veterinary concern. Significant human health problems are known to exist in the Andean countries (Bolivia, Peru, Chile, Ecuador), the Caribbean (Cuba), North Africa (Egypt), the Middle East (Iran and neighboring countries), Southeast

Asia (Vietnam), and Western Europe (Portugal, France, and Spain) (Mas-Coma et al., 2022b).

Although cases have been reported in high-income countries, human fascioliasis predominantly affects poor and rural areas. The highest prevalence and intensity have been reported in the hyperendemic area of the northern Bolivian Altiplano, where 72% of people in a community have been found to shed eggs, and up to 100% have been serologically positive. In this hyperendemic zone, the highest prevalence and intensity were found in children, with a peak between 9 and 12 years. In the Bolivian Altiplano, transmission follows a high-altitude transmission pattern of the altiplanic subtype, with *F. hepatica* as the only species involved and transmitted by imported *Galba truncatula* (Esteban et al., 1997; Mas-Coma, 2005).

1.3.3. DEFINITIVE HOST AND RESERVOIRS

The definitive host of *Fasciola* species is usually a ruminant, mainly sheep, goats, and cattle. However, *Fasciola* spp. is characterized by low specificity at the level of definitive hosts. A wide variety of domestic and wild animals, mostly herbivores, have been found to be naturally infected, including buffalo, pigs, horses, donkeys, mules, camelids, deer, wild boar, some marsupial species, rabbits, hares, capybaras, and various rodent species. Humans are also definitive hosts (Gayo et al., 2011; Mas-Coma et al., 2019, 2022).

It has been shown that some of these definitive hosts, especially domestic animals due to their proximity to humans, play a reservoir role in human endemic areas, ensuring long-term transmission of *Fasciola* (Mas-Coma et al., 2009b). These reservoir species may vary slightly between endemic areas and especially between continents (Mera y Sierra et al., 2020). In general, in the human hyperendemic areas of the Americas, cattle and sheep are the most common reservoir species, but donkeys, mules, and pigs should also be considered among the important reservoirs (Mas-Coma et al., 2020a; Mera y Sierra et al., 2020; Mas-Coma et al., 2021). In Africa and Asia, in addition to goats, buffaloes are of great epidemiological importance, especially in areas where *F. gigantica* is present (Afshan et al., 2014a; Sunita et al., 2021). In Europe, the introduced nutria (*Myocastor coypus*) has emerged as a significant reservoir in France and could potentially play a similar role in regions of South America where it is native (Mas-Coma et al., 2022b).

1.3.4. PATHOGENESIS AND SYMPTOMATOLOGY

According to their large dimensions, it is easy to imagine the high pathogenicity of *F. hepatica* and *F. gigantica*, which is mainly related to the mechanical action of the trematode, with *F. gigantica* being the most pathogenic species (Valero et al., 2016).

Four clinical periods are distinguished in human fascioliasis: the incubation period, the invasive or acute period, the latent period, and the biliary, chronic, or obstructive period. Due to the associated clinical manifestations, most diagnoses are usually made in the acute and chronic periods of the disease (Mas-Coma et al., 2009b).

The incubation period ranges from ingestion of the metacercariae to the onset of the first symptoms. This period is uncertain in humans, and the first symptoms may occur anywhere from a few days to three or more months after ingestion (Mas-Coma et al., 2019).

During the invasive or acute period, the juvenile worms migrate through the hepatic parenchyma. In this period, major pathological changes are observed, as the parasites digest the hepatic tissue. The extensive destruction of the hepatic parenchyma with intense hemorrhagic lesions causes localized or generalized toxic and allergic reactions that can last between two and four months (Mas-Coma et al., 2022b). The acute phase is usually accompanied by fever, abdominal pain, gastrointestinal disorders, urticaria, respiratory symptoms, and general discomfort (Ashrafi et al., 2014). Some of the signs that may appear during this phase include hepatomegaly, splenomegaly, jaundice, ascites, chest signs, eosinophilia, and anemia (Mas-Coma et al., 2022b).

The acute period is followed by a latent period, during which the parasites develop into sexually mature adults and begin to lay eggs. The duration of this period can vary from months to years, and it is unknown

how many people may be asymptomatic during this time (Mas-Coma et al., 2019).

Finally, the chronic or obstructive biliary period corresponds to the presence of adult parasites in the bile ducts, which causes inflammation, hyperplasia of the epithelium, and thickening and dilation of the walls of the ducts and gallbladder. The combination of cholangitis and cholecystitis, together with the large size of the trematodes, can cause obstructions. During this period, symptoms such as biliary colic, epigastric pain, intolerance to fatty foods, nausea, jaundice, anemia, pruritus, and sensitivity in the upper right quadrant of the abdomen may occur (Mas-Coma et al., 2022b). In some cases, the bile duct and gallbladder may contain blood mixed with bile (hemobilia), blood clots, and fibrinous plugs. This period can take months or even years to develop after infection. Moreover, acute and chronic phases are accompanied by parasite-induced immunosuppression, which facilitates co-infection with other pathogenic parasites and helminths (Mas-Coma et al., 2022b).

Occasionally, juvenile *Fasciola* parasites may deviate during their migration within the human body and enter in other organs, resulting in ectopic fascioliasis. The most common ectopic sites include the gastrointestinal tract and the subcutaneous tissue, but reports have also indicated occurrences in the heart, blood vessels, lung, pleural cavity, abdominal wall, appendix, pancreas, spleen, inguinal nodes, cervical nodes, skeletal muscle, and epididymis. These ectopic juvenile *Fasciola* parasites rarely mature and cause tissue damage characterized

by inflammation and fibrosis along their migration routes (Mas-Coma et al., 2022b).

Recently, the terms neurofascioliasis and ophthalmofascioliasis have been used to describe the rare, rather than sporadic as previously thought, cases in which juvenile *Fasciola* parasites invade the central nervous system and eye, respectively. Neurofascioliasis and ophthalmofascioliasis are difficult to diagnose because the typical symptoms of fascioliasis may not be present, leading to confusion when neurologic and ocular manifestations are evaluated as other types of neurologic and ocular disorders. Therefore, they are often misdiagnosed, especially in rural, low-income areas where the necessary equipment and diagnostic techniques may not be available (see reviews in Mas-Coma et al., 2013, 2014).

1.3.5. IMPACT OF GLOBAL CHANGE ON FASCIOLIASIS

Several factors included within the broader concept of global change serve as drivers for the (re-)emergence of fascioliasis and contribute to changes in its prevalence, intensity, and geographic spread (Mas-Coma et al., 2008, 2009a; Mas-Coma, 2020).

One of the factors influencing the (re-)emergence of fascioliasis that is included in the concept of global change is the import and export of livestock. Throughout history, human-guided livestock movements

have been critical to the spread and distribution of fascioliasis. Cattle passively transport the vector with mud adhering to their hooves (**Figure 1.3**). These invertebrates are R-strategists, meaning that when a single individual arrives at a new location, it can quickly establish a population by self-fertilization and colonize the new area. This passive transport has mainly favored the expansion of *F. hepatica* due to the ecological preferences of its vector species, leading to its current almost global distribution (Mas-Coma et al., 2022a). The low specificity of *Fasciola* at the intermediate host level allows it to colonize new environments by adapting to available lymnaeids. Moreover, adult fasciolids travel in the livers of infected animals. With this premise, it is easy to understand how the import and export of livestock without adequate safety measures continues to contribute to the current distribution of the disease.



Figure 1.3. Lymnaeid freshwater ecosystems such as **A)** irrigated crop fields, **B)** water troughs, **C)** artificial irrigation channels, and **D)** and **E)** dams. **F)** Lymnaeid found in the footprint of cattle.

The first detection of *F. gigantica* in ovines was recently reported in one of the northwestern Maghreb countries, Algeria. The introduction of *F. gigantica* into this country is suggested by the transhumance of livestock by nomadic pastoralists from Ghana through the Sahel countries of Burkina Faso and Mali into Algeria (Chougar et al., 2020).

Human activity in freshwater environments is another factor within global change that plays a significant role in the (re-)emergence of fascioliasis. *Fasciola* wide range of intermediate host snails can inhabit diverse freshwater ecosystems and adapt to various human-made structures associated with these ecosystems, such as irrigation canals, irrigation ditches, rivers, reservoirs, lakes, and more (**Figure 1.3**). These structures can facilitate the spread of invasive vector species, particularly through the natural and man-made connections between rivers and irrigation canals (Lodge et al., 1998; Loo et al., 2007; Sabourin et al., 2018). In the Peruvian Altiplano, the creation of an irrigation system provided ideal conditions for disease transmission; lymnaeids rapidly colonized this artificial system, resulting in a highly human hyperendemic zone (Esteban et al., 2002). Other aspects such as seasonality, may also be influenced by human activities. An example of this occurred in the Punjab region of Pakistan. Irrigation resulted in an additional period of lymnaeid activity per year, following a bistational pattern of fascioliasis transmission, with one peak associated with natural rainfall and another peak associated with artificial irrigation (Afshan et al., 2014a).

Fascioliasis is strongly linked to climatic factors. Temperature, precipitation, or evapotranspiration affect both the population dynamics of the mollusk vector and the larval stages of the parasite (both those that develop inside the vector and those that are free-living) (**Figure 1.1**). That's why climate change is expected to have a significant impact on the future evolution of this disease. In the high-altitude fascioliasis hyperendemic areas of the Andes, global warming and the El Niño-Southern Oscillation (ENSO) phenomenon are expected to modify the epidemiology of the disease. Global warming in the tropical Andes is evident in the widespread retreat of glaciers observed in recent decades. The rapid disappearance of the Chacaltaya glacier in Bolivia, for example, is consistent with an increase in the 0°C isotherm in the tropical Andes of about 50 meters per decade since 1980. This directly affects the hyperendemic region of fascioliasis in the Bolivian Altiplano, where a long-term reduction in water supply is expected due to glacier retreat (Mas-Coma et al., 2009a). However, the construction of artificial irrigation systems to alleviate water scarcity in this region is risky, given the aforementioned consequences observed in the Peruvian Altiplano (Esteban et al., 2002). Furthermore, an increase in temperature will directly increase parasite transmission, resulting in a shorter pre-patent period of the snail and leading to an increase in cercariae production and infectivity which could mean higher transmission and infection rates, altering current risk areas and expanding endemic areas (Mas-Coma et al., 2009a).

Endemic areas of human and animal fascioliasis in these Andean countries may also be affected by changes in precipitation due to the ENSO phenomenon. Increased rainfall could extend the period of infection risk currently associated with the November to April rainy season. In addition, extreme events such as droughts and floods, which are also associated with the ENSO phenomenon, may also affect the epidemiology of fascioliasis (Mas-Coma et al., 2009a). For example, droughts would reduce the mollusk vector population by decreasing water collection; however, what may initially appear to be a decrease in fascioliasis incidence could be the opposite. The establishment of fewer freshwater sources could concentrate conditions conducive to transmission and potential definitive hosts in search of drinking water, leading to an increase in incidence. In general, climate change causes spatio-temporal variation in risk levels, and the risk of infection will decrease, fluctuate, or increase depending on the particular conditions of each area (Alba et al., 2021). For this reason, models that assess and predict transmission risk based on climate data are of interest (Cuervo et al., 2022).

1.3.6. PREVENTION AND CONTROL

The World Health Organization (WHO) has included fascioliasis in the group of food-borne trematodiasis in the priority list of its NTDs roadmaps for 2020 and 2030. The prevention and control of fascioliasis

depends on the correct and integrated application of various measures with a One Health approach (WHO, 2020).

Due to the heterogeneous epidemiological situations and the different transmission patterns, the control of fascioliasis requires the use of different strategies adapted to the specific characteristics of the endemic area. In some countries, such as Vietnam, control is achieved through passive case detection and treatment in hospitals. In others, active case detection and treatment of positive cases is preferred, especially among school-age children. In hyperendemic areas, such as the Bolivian and Peruvian highlands, mass treatment of school-age children (5-14 years) is used. These preventive chemotherapy interventions use a single dose of 10 mg/kg of triclabendazole. The goal of preventive chemotherapy interventions with triclabendazole is to control the morbidity associated with fascioliasis by reducing the helminth burden in infected individuals (WHO, 2020).

As a zoonotic disease with animal reservoirs, it is essential to implement control and prevention measures in the veterinary field, such as improved animal husbandry, stall feeding, and treatment of domestic animals. At the intermediate host level (vector control), some measures can also be taken, such as the use of molluscicides, biological control, or genetic manipulation (Sokolow et al., 2016).

Water, sanitation, and hygiene practices also play an important role in the prevention and control of fascioliasis. This is because fascioliasis is a food-borne parasitic disease whose transmission may be

closely linked to the quality of water sources and hygiene practices. Access to safe and clean water sources is critical to the prevention of fascioliasis, as drinking contaminated water or washing vegetables, fruits, tubers, kitchen utensils, or other objects with contaminated water is a source of infection (Mas-Coma et al., 2022b). Good hygiene practices, including safe disposal of human and animal waste, are essential since vegetables should be grown in water that is free of fecal contamination. Strict controls on the cultivation and sale of watercress and other edible aquatic plants are needed.

Travelers to endemic areas are advised not to purchase wild plants from uncontrolled street markets and not to consume traditional foods and beverages made from vegetables from endemic areas.

In addition to these measures, there is a need for health education for people living in endemic areas. Therefore, the development of activities that increase knowledge of the disease and its management among at-risk individuals in endemic regions is essential.

1.4. SCHISTOSOMIASIS

1.4.1. CAUSAL AGENT AND TRANSMISSION

The causative agent of schistosomiasis is a blood fluke of the genus *Schistosoma*. Six species have been described to infect humans, five of which parasitize the mesenteric venous system and cause intestinal schistosomiasis, *Schistosoma mansoni*, *S. intercalatum*, *S. guineensis*, *S. mekongi* and *S. japonicum*, while *S. haematobium* lives in the venous plexus around the bladder and causes urogenital schistosomiasis (Boissier et al., 2019; WHO, 2023).

Adult schistosomes are white or grayish worms with a cylindrical body with two terminal suckers, a complex tegument, a blind digestive tract, and reproductive organs. Adult schistosomes can live in the definitive host for an average of 3-5 years, although they can live up to 30 years (Gryseels et al., 2006). They differ from the other trematodes in having separate sexes with marked sexual dimorphism: the longer (~1.50 cm in size) and slender females lodge in the gynecophore canal of the male (~1 cm in size) (**Figure 1.4.A.1**). The peculiarity of having separate sexes is associated with morphological, ecological, behavioral, and molecular differences between the two sexes throughout their life cycle (Moné and Boissier, 2004). The female partially separates from the male when her uterus is mature to reach the finest vessels near the target organ, the bladder or intestine, where she releases all of her eggs (200-3,000 per day, depending on the species). The eggs, embryonated at the time of

laying, are released into the external environment through urine (*S. haematobium*) or feces (*S. japonicum*, *S. mansoni*, *S. intercalatum*, *S. guineensis*, and *S. mekongy*) (**Figure 1.4.A.2**) (LoVerde, 2019). *Schistosoma* eggs are characterized by a spine, which is large and lateral in *S. mansoni*, small and lateral in *S. japonicum*, and large and terminal in *S. haematobium*, *S. intercalatum*, *S. guineensis*, and *S. mekongi* (**Figure 1.4.A.3**). The miracidium (**Figure 1.4.A.4**), a free-swimming ciliated larva, is released when the *Schistosoma* egg comes into contact with fresh water. It swims actively for 1-6 hours in search of a suitable intermediate host (**Figure 1.4.A.5**), which is always a freshwater snail of the genera *Bulinus*, *Biomphalaria*, *Oncomelania* (**Figure 1.4.5.C**), or *Neotricula*, depending on the *Schistosoma* species. Inside the snail, the miracidium becomes a mother sporocyst or sporocyst 1, which produces daughter sporocysts or sporocyst 2. The daughter sporocysts undergo at least two generations of multiplication before producing large numbers of furcocercariae (**Figure 1.4.A.6**). The furcocercariae leave the mollusk 4-6 weeks after infection and circulate in the water for up to 72 hours, seeking the skin of the human host by chemotaxis (**Figure 1.4.A.7**). The release of furcocercariae is light-induced and occurs primarily during daylight hours. A single snail infected with a miracidium can release thousands of furcocercariae for several months. Once inside the definitive host, the furcocercariae transform into a schistosomula, which travels through the blood system from the lungs to the liver, where they mature and mate. They then travel in pairs to the appropriate blood vessels (Gryseels et al., 2006; Boissier et al., 2019).

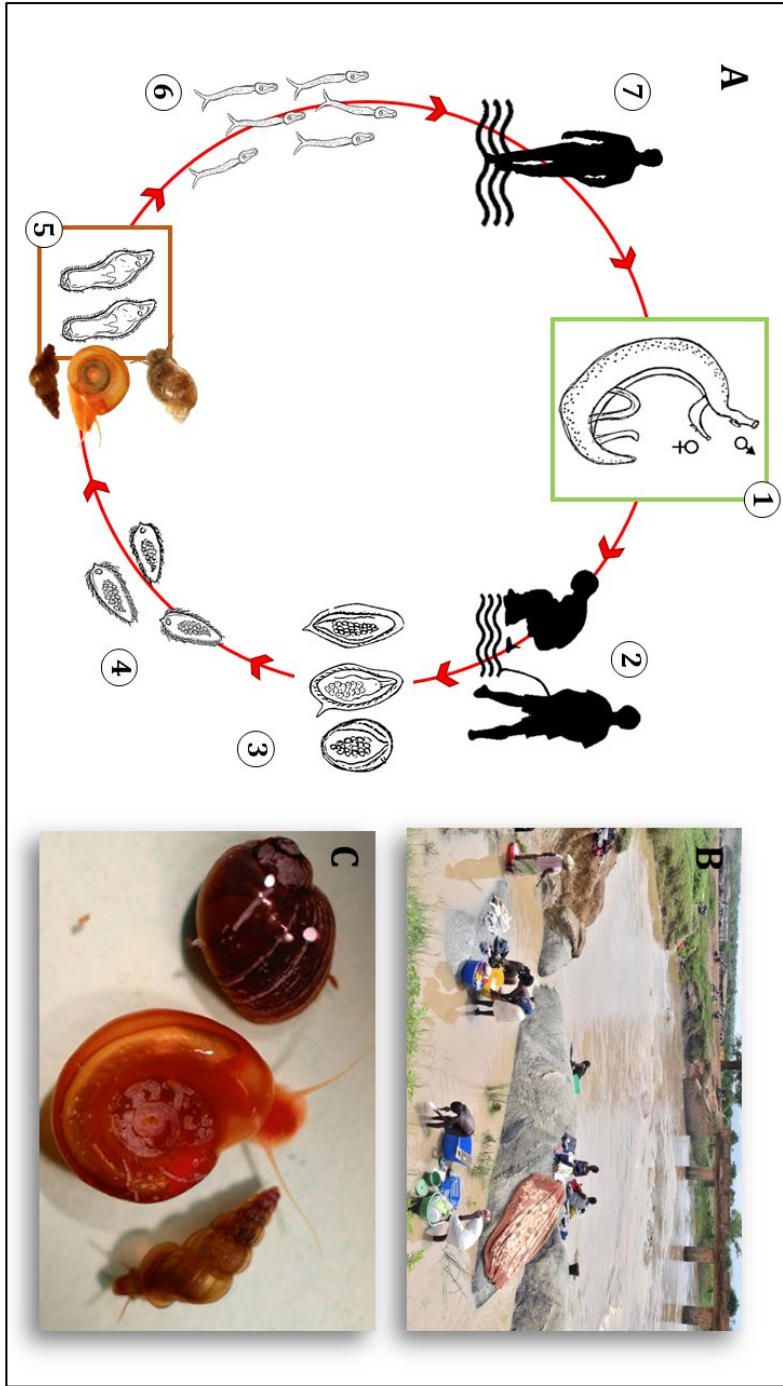


Figure 1.4. A) Life cycle of *Schistosoma* spp. B) Example of a transmission ecosystem of *Schistosoma* spp. C) Main genus of intermediate freshwater snails of schistosomiasis: *Bulinus*, *Biomphalaria* and *Oncomelania*, from left to right (image from LoVerde, 2019).

1.4.2. GEOGRAPHICAL DISTRIBUTION AND EPIDEMIOLOGY

The geographic distribution of *Schistosoma* species is determined by the distribution of their intermediate freshwater snail hosts.

S. mansoni, *S. haematobium*, *S. guineensis*, and *S. intercalatum* are transmitted by different species within the Planorbidae family.

S. mansoni is transmitted by several species within the genus *Biomphalaria*. This species is widespread in Africa and the Middle East, but its presence in the Americas is quite limited, primarily in Brazil, Venezuela, Suriname, and St. Lucia (Boissier et al., 2019).

S. haematobium, *S. guineensis*, and *S. intercalatum* use *Bulinus* snails as intermediate hosts. *S. haematobium* occurs in 37 African countries and 15 countries in the Eastern Mediterranean region (Chitsulo et al., 2000), whereas *S. guineensis* and *S. intercalatum* have a more limited geographic range: *S. guineensis* is found in the Gulf of Guinea region (Cameroon, Equatorial Guinea, Gabon, and São Tomé and Príncipe), while *S. intercalatum* is only found in the Democratic Republic of the Congo (Zaire) (Pagès et al., 2001a; Boissier et al., 2019).

The other two schistosomiasis human species, *S. japonicum*, and *S. mekongi*, are transmitted by snails of the family Pomatiopsidae, and their distribution is restricted to the Asian continent. *S. japonicum* uses snails of the genus *Oncomelania* as vectors and it is found in China (Chen et al., 2016; Yang et al., 2016), the Philippines, and Indonesia

(WHO, 2023b). *S. mekongi* uses only the species *Neotricula aperta* as an intermediate snail host and its distribution is restricted to the Mekong Delta in Laos and Cambodia (Boissier et al., 2019).

Human schistosomiasis is one of the most significant NTDs and the second most important parasitic disease after malaria due to its high socio-economic impact on public health. Although not a high-mortality disease (currently attributed to 11,792 deaths per year worldwide), human schistosomiasis is characterized by the high morbidity and disability that it causes. This chronic and debilitating disease primarily affects poor communities without access to safe drinking water and adequate sanitation in tropical and subtropical regions, with the highest burden on the African continent. The main symptoms of the disease, such as abdominal pain, diarrhea, and blood in the urine, can limit the ability of infected individuals to work, resulting in loss of family income and perpetuating their poverty. In addition, stunted growth, anemia, or learning disabilities may be observed in the infected school-age population, affecting their educational and social development. It is currently estimated that 800 million people are at risk of infection and almost 252 million are infected in 78 countries, although this may be an underestimate due to the difficulty of diagnosis in the early stages (Boissier et al., 2019; WHO, 2023b).

Schistosomiasis is a focal disease determined by the interaction of humans, intermediate freshwater snail hosts, animal reservoirs, and water contact patterns, which can result in significant variation in the prevalence and intensity of infection within a small geographic area

(McManus et al., 2018). In endemic regions, serologic surveys indicate that nearly all long-term residents become infected with schistosomiasis at some point in their lives, with a notable peak in prevalence observed in school-age populations and young adults. In fact, in areas with typical transmission patterns, between 60% and 80% of school-aged children are actively infected. This peak prevalence of schistosomiasis in the school-age and young adult population is attributed to the considerable time they spend swimming or bathing in waters containing infective larval stages, the furcocercariae. The decline in schistosomiasis prevalence in the adult population may be due to one or a combination of: i) death of the worms after peak infection, as they are estimated to live an average of 3-5 years in humans; ii) reduced contact with water as the population ages; and iii) evidence that partial immunity can develop over time. However, exposure to (re-)infection can persist into adulthood through various activities that involve contact with freshwater bodies, whether for domestic tasks such as laundry (**Figure 1.4.B**) or dishwashing, recreational activities, or occupations such as fishing or car washing. (LoVerde, 2019; WHO, 2023b).

1.4.3. DEFINITIVE HOST AND RESERVOIRS

Humans are the primary definitive hosts of the species responsible for human schistosomiasis. However, in addition to humans, other species of vertebrate mammals can serve as definitive hosts. To date, 113 different naturally infected species from 8 different orders have

been described: 16 species of the order Primates, 3 species of Lagomorphs, 58 species of Rodentia, 13 species of Carnivora, 11 species of Cetartiodactyla, 2 species of Perissodactyla, 6 species of Insectivora, 1 species of Xenarthra, and 3 species of Didelphimorphia (Boissier et al., 2019).

S. japonicum and *S. mansoni* are the species with the lowest specificity at the definitive host level. In addition to humans, 56 and 58 different natural host species have been identified for *S. japonicum* and *S. mansoni*, respectively (Boissier et al., 2019).

Surprisingly, 15 species from three different orders (Primates, Rodentia, and Cetartiodactyla) have been found to be naturally infected with *S. haematobium*, which was previously thought to be specific for humans. A possible explanation is that *S. haematobium* can hybridize with other animal species such as *S. bovis* or *S. curassoni*, resulting in introgressed strains of *S. haematobium*. The increase in the definitive host range may be due to hybrid vigor as a result of *S. haematobium* hybridizing with species such as *S. bovis*, which has a wide geographic distribution and variety of definitive and intermediate hosts (Boissier et al., 2019).

For the human species with more limited distribution, *S. mekongi*, *S. intercalatum*, and *S. guineensis*, very few naturally infected hosts have been found. Some authors suggest that this may be due to a lack of research on these species. Only two natural hosts have been reported for *S. mekongi*, *Canis familiaris* and *Sus scrofa*; only *Hybomys univittatus*

has been reported for *S. intercalatum*; and no naturally infected animal hosts have been found for *S. guineensis*, although experimental infection has been demonstrated in *Cetartiodactyla ovis aries* and several species of rodents (Boissier et al., 2019).

Any of the identified definitive hosts could serve as a potential reservoir host for human schistosomiasis transmission. However, to date, zoonotic transmission has only been described for *S. japonicum* and *S. mansoni*, but surprisingly, it has recently also been observed in *S. haematobium*. In the case of *S. japonicum*, its zoonotic potential has been known for years, with ruminants and rodents being the main reservoir hosts, favoring its persistence and reinfection of humans in Asia (Rudge et al., 2013; Gordon et al., 2019). For *S. mansoni*, zoonotic transmission with rodents as reservoirs has been documented in South America and Africa (Gentile et al., 2012; Catalano et al., 2020). Finally, for *S. haematobium* and *S. haematobium* x *S. bovis* hybrids, cattle have been described for the first time as reservoir hosts in Benin (Savassi et al., 2020).

1.4.4. PATHOGENESIS AND SYMPTOMATOLOGY

Human schistosomiasis is an atypical parasitic disease among helminth infections, as the pathology is primarily due to the eggs of the worms and not to the actions of the adults. The progression of the disease

can be divided into three phases: the initial phase or cercarial dermatitis, the acute phase, or Katayama syndrome, and the chronic phase, which can lead to late complications and sequelae due to co-infection with other biological agents (Gryseels et al., 2006).

The initial phase of the disease corresponds to subcutaneous penetration of the furcocercariae, which may result in a transient maculopapular rash within the first 24 hours (Gryseels et al., 2006).

The Katayama syndrome occurs between 14-84 days after exposure. It is a delayed hypersensitivity reaction produced by the immune system in response to the migration of the schistosomula. It is characterized by symptoms such as fever, fatigue, myalgia, skin lesions, pulmonary involvement (non-productive cough, shortness of breath), and eosinophilia. Both rash and Katayama syndrome are often absent in populations continuously exposed to the disease and are mainly observed in first-time infections of tourists, travelers, or accidentally exposed individuals (Bottieau et al., 2006).

The morbidity of the disease occurs in the chronic pathology caused by eggs that become trapped in tissues during perivesical or periintestinal migration or after embolization in the liver, spleen, lungs, or cerebrospinal system. The primary lesion of the disease, the formation of granulomas, occurs around the trapped eggs. If the infection persists for months or years, fibrosis of the organ where the eggs are located occurs. Depending on the species, there are two pathological profiles:

urogenital schistosomiasis and intestinal schistosomiasis (Gryseels et al., 2006).

Urogenital schistosomiasis, caused by *S. haematobium*, is characterized by hematuria (microscopic or macroscopic) associated with increased urinary frequency, dysuria, and renal dysfunction. Cancer has also been associated with *S. haematobium* infection (Boissier et al., 2019). *S. haematobium* infection can also affect the reproductive organs, resulting in genital schistosomiasis. In women, the symptoms include ulcerative lesions of the vulva, vagina, and cervix, which can facilitate the transmission of other sexually transmitted diseases and increase the risk of HIV infection. Other reported sequelae include ectopic pregnancy, infertility, miscarriage, and cervical abnormalities with symptoms that resemble cervical cancer (Swai et al., 2006). Urogenital schistosomiasis also affects male genital function, including cases of sterility or prostate cancer (Figueiredo et al., 2015).

Intestinal schistosomiasis, caused by *S. japonicum*, *S. mansoni*, *S. intercalatum*, *S. guineensis*, and *S. mekongi*, can produce a wide range of symptoms, including fatigue, irregular bowel movements, abdominal pain, diarrhea, and the presence of blood in the stool due to granuloma formation and ulcers in the intestinal wall. In addition, granulomatous inflammation may lead to the development of polyps (Gryseels et al., 2006; Boissier et al., 2019).

Hepatic schistosomiasis can be caused by *S. mansoni*, *S. japonicum*, and *S. mekongi*. A significant number of eggs from these

species (*S. mansoni*, *S. japonicum*, and *S. mekongi*) are transported in the blood to the liver, where they induce granulomatous reactions, and fibrosis, leading to various complications such as hepatomegaly, splenomegaly, and portal hypertension, among others (Gryseels et al., 2006; Boissier et al., 2019).

1.4.5. IMPACT OF GLOBAL CHANGE ON SCHISTOSOMIASIS

Global change has a significant impact on the dynamics and distribution of schistosomiasis. Ecosystem modification due to human activities appears to facilitate the transmission of schistosomiasis, particularly, as in the case of fascioliasis, in relation to changes in freshwater aquatic ecosystems. This has been observed in several African countries where schistosomiasis is endemic due to the implementation of various irrigation projects, changes in agricultural practices, or the construction of dams. One example is the construction of the Diama Dam on the Senegal River. Built to develop and expand rice production, it resulted in reduced salinity and a more stable water flow, creating favorable conditions for outbreaks of human and animal schistosomiasis. Similar effects were observed following the construction of the Kossou and Taabo dams in Côte d'Ivoire (Leger and Webster, 2017). Dam construction can also alter schistosomiasis prevalence in a given area by favoring the development of certain *Schistosoma* species over others, as observed in Egypt after the

construction of the Aswan Dam. The construction of the Aswan Dam is thought to have caused environmental changes that favored the intermediate host of *S. mansoni* (*Biomphalaria alexandrina*) over the snail vectors of *S. haematobium* (*Bulinus truncatus*), resulting in *S. mansoni* replacing *S. haematobium* in critical areas along the river Nile (LoVerde, 2019).

Other ecosystem changes not directly related to freshwater ecosystems, such as deforestation, appear to have affected *Schistosoma* epidemiology, as suggested in Loum, Cameroon. In this area, it was hypothesized that local deforestation led to a series of changes that allowed *B. truncatus*, the intermediate host of *S. haematobium*, to become established (Leger and Webster, 2017).

Population movement is another critical factor influencing the expansion and change in the distribution of *Schistosoma* species. The range of *Schistosoma* species appears to be expanding as a result of the free movement of populations between the Economic Community of West African States (ECOWAS) countries in West Africa (Boissier et al., 2019). This expansion of *Schistosoma* species due to human movement is not limited to Africa but has also reached Europe, where schistosomiasis is not endemic. This was observed in the recent outbreak of urogenital schistosomiasis on the French island of Corsica, caused by a hybrid *S. haematobium* x *S. bovis* of Senegalese origin (Boissier et al., 2015; Noël et al., 2018). In addition, cases of autochthonous transmission have recently been reported in southern Spain (Salas-Coronas et al., 2021).

Similar to *Fasciola*, climatic factors are an important determinant of the *Schistosoma* life cycle and the viability of its freshwater snail hosts. Rising global temperatures are expected to cause a noticeable shift in the transmission patterns of this disease. Some research suggests that high temperatures in several coastal regions of East Africa are responsible for the inability of *Biomphalaria* spp. to survive, and therefore transmission of *S. mansoni* does not occur.(Mas-Coma et al., 2009a). Therefore, in West and Central Africa, some suitable areas for the survival and development of freshwater snail intermediate hosts may be lost due to rising temperatures. This could potentially reduce the range and abundance of these intermediate hosts. However, habitat loss in some parts of Africa may be partially offset by the expansion of schistosome-transmitting freshwater snails into cooler areas of southern Africa. Interestingly, climate change is also leading to an increase in the creation of freshwater habitats through the construction of dams and irrigation systems. This, coupled with an increased reliance on irrigated agriculture and hydropower, may create an environment in which schistosomes and their intermediate hosts can thrive, leading to an increase in disease prevalence (Blum and Hotez, 2018; Stensgaard et al., 2019).

Climate change may also affect the epidemiology of animal schistosomiasis. Desertification, caused by climate change, is shifting livestock movements in West Africa. Animals are forced to travel long distances in search of water and food, increasing the likelihood of contact with multiple potential sources of schistosomiasis transmission. This

could facilitate interspecies encounters and promote hybridization, both among animal *Schistosoma* species and between animal and human *Schistosoma* species. In fact, some authors have already suggested that such changes in livestock movements may have brought *S. bovis* and *S. curassoni* into contact and led to hybridization between them (Leger and Webster, 2017).

1.4.6. PREVENTION AND CONTROL

Schistosomiasis control focuses on prevention and strategies to disrupt the life cycle of the parasite using a One Health approach. Similar to fascioliasis, the effectiveness of schistosomiasis control is greatly enhanced when multiple interventions are combined. The WHO 2021-2030 Roadmap for NTDs has established the ambitious goal of eliminating schistosomiasis as a public health concern (currently characterized by a prevalence of human schistosomiasis infections with a heavy egg-patent intensity below 1%) (Lo et al., 2022). To achieve this goal, some aspects of preventive chemotherapy have been updated, and there is an increased focus on One Health activities in the new guideline for the control and elimination of schistosomiasis in endemic countries published by WHO in 2022 (WHO, 2022).

Praziquantel is the drug of choice because it is safe and effective against adult worms of all human *Schistosoma* species. In endemic communities with a prevalence of *Schistosoma* spp. infection $\geq 10\%$, WHO recommends annual preventive chemotherapy with a single dose

of 40 mg/kg with a treatment coverage of $\geq 75\%$ in all age groups from 2 years, including adults, pregnant women after the first trimester, and lactating women, to reduce morbidity while keeping infection intensity under control. Biannual preventive chemotherapy may be considered instead of annual if there is an inadequate response to preventive chemotherapy in these endemic communities with a prevalence of *Schistosoma* spp. infection $\geq 10\%$ (Lo et al., 2022; WHO, 2022).

Environmental interventions for schistosomiasis control and prevention emphasize vector control. Vector elimination is costly and logistically complex, so efforts are currently focused on vector surveillance. Vector surveillance involves monitoring the presence of the *Schistosoma* intermediate freshwater snail host in its natural habitat. This is very useful because it helps to identify the focus of schistosomiasis transmission or to assess the risk of the water bodies, which helps to define target areas for preventive chemotherapy. New molecular techniques, such as environmental DNA detection, are showing potential in vector surveillance, reducing human error and allowing large areas to be mapped (Sokolow et al., 2016; Boissier et al., 2019).

Health education on schistosomiasis is essential for the reduction of transmission in endemic areas, as human behavior is constitutive for the epidemiology of the disease (Lo et al., 2022; WHO, 2022).

It should be noted that the emergence of hybrids between animal and human *Schistosoma* species, especially those within the *S. haematobium* species group (Lockyer et al., 2003), poses a challenge to

disease control because of the zoonotic component and the potential consequences of hybridization, such as an expanded range of definitive and/or intermediate hosts, increased transmission potential, and altered responses to drug therapy (Boissier et al., 2019).

1.5. HYBRIDIZATION AND INTROGRESSION

Hybridization events in the two major public health trematodiasis, fascioliasis and schistosomiasis, are of concern because of their potential impact on the epidemiology, morbidity, treatment response, and control of these diseases, among others. To understand the issues surrounding the potential consequences of these natural hybridization events, we need to understand hybridization and the conditions that allow hybridization to occur.

Hybridization is defined as the interbreeding of two different species. However, it is a complex phenomenon since, through the speciation process, species develop mechanisms of reproductive isolation that prevent genetic exchange between incipient species, which hybridization must bypass (Vallejo-Marín and Hiscock, 2016). These mechanisms of reproductive isolation have traditionally been classified as pre-zygotic and post-zygotic barriers (Barton and Bengtsson, 1986; Theodosopoulos et al., 2019). Pre-zygotic barriers prevent the mating process itself by avoiding the formation of hybrid zygotes through spatial, temporal, behavioral, gametic, and/or mechanical isolation, while post-zygotic barriers come into play when the pre-zygotic barriers fail, resulting in non-viable hybrids or hybrids with reduced fertility.

If the hybrid offspring is viable and can reproduce with either of the two parent species, gene flow can occur. Gene flow during hybridization events followed by repeated backcrossing can result in the

introduction of genetic material from a donor species into a recipient species, known as introgression (Pfennig, 2021). This introduced genetic variation in the recipient species can enhance its fitness and promote adaptation, causing these genes and/or alleles to be maintained by natural selection in subsequent generations of the population until they eventually become part of the genomic background of the recipient species. This is known as adaptive introgression, which can have significant consequences for ecological and evolutionary dynamics (Barton and Bengtsson, 1986; Theodosopoulos et al., 2019).

In summary, hybridization expands the range of possibilities for natural selection by generating a diversity of phenotypes that may be suitable for adaptation. Thus, the resulting hybrid may improve some phenotypic characteristics compared to the parental species. In the specific case of freshwater snail-transmitted trematodes, the concern is that hybridization may affect phenotypic characteristics such as pathogenicity, host range expansion, infectivity, or drug resistance (King et al., 2015).

1.5.1. HYBRIDIZATION AND GLOBAL CHANGE

Pre-zygotic isolation barriers are the first to come into play during hybridization events and are thought to be the most effective due to their rapid action. In general, these pre-zygotic barriers are expected

to be more pronounced in sympatric species capable of inhabiting the same definitive host. In the case of *Fasciola* and *Schistosoma*, spatial isolation (e.g., living in the same geographic area, the same host individual, or the same tropism within the host) and behavioral isolation (e.g., individuals can choose to mate with individuals of their own species) are pre-zygotic barriers that play an important role (Kincaid-Smith et al., 2017).

Global change appears to alter the incidence of hybridization by affecting spatial isolation barriers (Vallejo-Marín and Hiscock, 2016). Hybridization events within the genus *Fasciola* and the genus *Schistosoma* necessarily occur during their sexual reproduction phase. In both trematodes, sexual reproduction takes place within the definitive host, which harbors the sexually mature adult worms (**Figure 1.1**). This means that for hybridization events to occur, the same definitive host must be simultaneously co-infected by two different species, and, therefore, for co-infection to happen, the species involved must coexist in the same specific area.

Global change appears to affect the distribution of fascioliasis and schistosomiasis (**see sections 1.3.5 and 1.4.5**) by increasing the areas of overlap between closely related species. The loss of ecological and geographic barriers that historically kept these closely related species, which can infect the same definitive host, separate is increasing the chances of encounters between these previously isolated species, creating exceptional opportunities for hybridization (King et al., 2015; Leger and Webster, 2017).

1.5.2. HYBRIDIZATION EVENTS IN *Fasciola*

The existence of hybrids between *F. gigantica* and *F. hepatica* may seem surprising given that the adult stage is hermaphroditic, meaning that a single adult individual (having both male and female reproductive systems leading to the same genital pore) has the ability to self-inseminate and produce eggs in the absence of other individuals. However, the adult stage of *Fasciola* spp. tends to cross-fertilize when coexisting with other individuals of the same species within the bile ducts of the definitive host. The low specificity of *F. gigantica* and *F. hepatica* concerning definitive mammalian host species allows both fasciolids to frequently coexist in the liver of the same definitive host. Preference for cross-fertilization also occurs when these mixed infections occur in the liver of the same animal or human, allowing mating between *F. gigantica* and *F. hepatica* (Mas-Coma et al., 2009b). The absence of pre-zygotic isolation mechanisms between *F. gigantica* and *F. hepatica* when they coexist in the same host, together with the tendency of *F. gigantica* to show a greater preference for heterospecific mating (Itagaki et al., 2011), underlies hybridization events between these two species.

The current distribution of *F. hepatica* and *F. gigantica*, as a consequence of human activities such as livestock domestication, grazing, or transportation, has resulted in overlapping areas in many regions of Africa and Asia where lymnaeid snails are suitable for the development of both species (Mas-Coma et al., 2022a). In these areas, natural hybridization events in *Fasciola* are possible when mixed

infections of *F. gigantica* and *F. hepatica* occur in the same definitive host. In recent decades, the taxonomic determination of *Fasciola* species in overlapping areas has caused controversy and confusion, resulting in various terms such as intermediate forms, spermic, aspermic, diploid, triploid, mixoploid, parthenogenetic, and hybrid flukes.

Specific differentiation of adult specimens can be carried out through morphological studies (Periago et al., 2006) or molecular techniques, typically using a combination of nuclear ribosomal DNA (rDNA) and mitochondrial DNA (mtDNA) markers (Mas-Coma et al., 2009b; Chougar et al., 2020).

Morphological studies in areas where both species are sympatric have revealed the presence of intermediate forms that cannot be attributed to either species (Mas-Coma et al., 2009b).

These intermediate forms show considerable morphological variability and are often the result of hybridization events (Mas-Coma et al., 2022a). However, it should be noted that this is a morphometric term that refers only to a phenotypic characterization, not a molecular one, of adult specimens. These intermediate forms have been detected in Egypt (Periago et al., 2008), Uzbekistan (Gariev, 1970), Pakistan (Afshan et al., 2014b), Bangladesh (Ahasan et al., 2016), and Iran (Ashrafi et al., 2006).

Surprisingly, intermediate morphologies have also been found in Ecuador (Bargues et al., 2021) and Japan (Watanabe, 1965), where only *F. hepatica* occurs.

In Japan, *Fasciola* specimens with an intermediate morphology are called parthenogenetic *Fasciola*. These parthenogenetic *Fasciola* flukes have certain peculiarities, as they may be diploid ($2n = 2 \times = 20$), triploid ($3n = 3 \times = 30$), or mixoploid ($2n/3n = 2 \times / 3 \times = 20/30$), and generally have few or no spermatozoa in their seminal vesicles. Therefore, they have often been referred to as aspermic, suggesting that they can only reproduce by parthenogenesis. It has been hypothesized that these parthenogenetic *Fasciola* originated in central and eastern China, where both species (*F. gigantica* and *F. hepatica*) coexist, through past hybridization (Hayashi et al., 2018; Itagaki et al., 2022).

For the specimens with intermediate morphology detected in Ecuador, a recent phenotyping and genotyping study was conducted (Bargues et al., 2021). The phenotypic results indicated that these large fasciolids resembled *F. gigantica*; however, genotyping using ribosomal (ITS) and mitochondrial (*cox1* and *nad1*) markers showed that these large fasciolids belonged to *F. hepatica*. It is believed that the large fasciolids detected in Ecuador are a consequence of the past introduction of *F. gigantica* through livestock imports. Nevertheless, the vector specificity filter acts as a driving force, accelerating the evolution of hybrid fasciolid lineages. This means that due to the absence of *Radix* (the vector of *F. gigantica*) in the Americas, the hybrids are in the process of reverting to the original *Fasciola* species in this area, *F. hepatica* (Bargues et al., 2021).

Genetic identification of *Fasciola* spp. is performed using a combination of rDNA and mtDNA markers, as described above. The

most recommended approach to correctly catalog a specimen is to use at least one nuclear rDNA sequence and one mtDNA sequence. The most commonly used rDNA markers are internal transcribed spacers (ITSs), ITS-1 and ITS-2, because they are highly conserved in *Fasciola* and have proven useful in the genetic characterization of *Fasciola* species and strains at local and regional levels, including the assessment of transmission routes. In addition, it has been confirmed that nuclear rDNA is consistent with the morphology of adult *Fasciola* (Mas-Coma et al., 2009b). In terms of mtDNA markers, the cytochrome c oxidase subunit 1 (*cox1*) and NADH dehydrogenase subunit 1 (*nad1*) genes are the most commonly used to characterize *Fasciola* flukes. While some studies may use short or incomplete sequences of *nad1* and *cox1* (Ichikawa-Seki et al., 2017; Asadpour et al., 2020; Vudriko et al., 2022), the most advisable approach is the complete sequencing of these genes to allow comparative analyses and to avoid the problems that the short or incomplete sequences may cause in phylogenetic analyses (Mas-Coma et al., 2009b).

The simplest way to detect hybrids is to find an rDNA sequence from one species and an mtDNA sequence from another species in the same individual. Occasionally, however, two different nuclear rDNA sequences can be detected coexisting in the same individual, resulting in double peaks at specific mutation sites that distinguish between species in the chromatogram (Mas-Coma et al., 2009b). Nuclear rDNA is inherited by Mendelian inheritance and undergoes concerted evolution. Concerted evolution means that, given enough time, the nuclear rDNA of a population will homogenize all its copies, eliminating differences

between both homologous and non-homologous chromosomes in the genome. Thus, the detection of two different nuclear rDNA sequences coexisting in the same individual implies recent hybridization, because it indicates that concerted evolution has not had enough time to homogenize the rDNA operon lose the signal from one of the parental species (Mas-Coma and Bargues, 2009).

1.5.3. HYBRIDIZATION EVENTS IN *Schistosoma*

Schistosomes have been described as an evolutionary scandal, the amazing peculiarity of having separate sexes instead of being hermaphrodites makes them unique among the digenetic trematodes (Moné and Boissier, 2004). One of the consequences of having separate sexes is that both male and female individuals are required for sexual reproduction, which occurs within the definitive host. In the majority of *Schistosoma* species, mating is essential for the full development of females and their formation of reproductive structures, as well as for the migration from the hepatic portal vein to the mesenteric veins (Tchuem Tchuenté et al., 1996). In addition, this parasite, which is considered socially monogamous, can change partners and produce rapid offspring, suggesting that it is not genetically monogamous (Beltran and Boissier, 2009).

Male and female schistosomes are highly interdependent since an intimate and permanent association between the two sexes is necessary for reproduction to occur. For this reason, behavior has a significant influence on sexual interactions and hybridization events within the genus *Schistosoma* (Kincaid-Smith et al., 2021). Several studies have described how male and female schistosomes behave in simultaneous or sequential mixed infections, and in general, these studies have revealed three behavioral findings: the existence of a specific mate preference system, the existence of mate competition, and the possibility of mate change (Tchuem Tchuente et al., 1996). In these experimental studies, it has generally been observed that the phylogenetic distance between interacting species determines whether pairings are random or whether there is a predominance of same-species pairings (Onyekwere et al., 2022a). In the pairing and mate choice experiments conducted between species belonging to the monophyletic group of *S. haematobium* (Lockyer et al., 2003), such as *S. haematobium* x *S. bovis* (Kincaid-Smith et al., 2021), *S. bovis* x *S. curassoni* (Rollinson et al., 1990), *S. haematobium* x *S. intercalatum* (Southgate et al., 1998), or *S. intercalatum* x *S. guineensis* (Pagès et al., 2001b), pairings occur randomly. This means that there is no preference for homospecific or heterospecific associations in simultaneous infections. However, when species belong to two different evolutionary lineages, as in crosses between *S. mansoni* x *S. intercalatum* (Tchuem Tchuente et al., 1993), or *S. mansoni* x *S. bovis* (Onyekwere et al., 2022a) mate preference is observed. This means that mating occurs preferentially between pairs of the same species.

In preventing genetic exchange and hybridization, relatedness, also plays a key role. Depending on the phylogenetic distance between sexually interacting species, schistosomes may or may not produce hybrid offspring (Rey et al., 2021a). Laboratory experiments have already demonstrated that closely related *Schistosoma* species can successfully mate, hybridize, and produce viable offspring over multiple generations: *S. mansoni* with *S. rodhaini* (Norton et al., 2008), *S. haematobium* with *S. mattheei* (Southgate et al., 1995), *S. haematobium* with *S. guineensis* (Webster and Southgate, 2003), and *S. haematobium* with *S. bovis* (Kincaid-Smith et al., 2021). However, mating between species with greater phylogenetic distance, such as *S. mansoni* and *S. bovis*, which belong to two different evolutionary lineages, results in viable first generation offspring through parthenogenesis. The miracidia produced by parthenogenesis carry the maternal ITS-2 genotype and consequently, they can only infect the mollusk of the maternal species. Thus, offspring produced by the male *S. mansoni* x female *S. bovis* cross can only infect *Bulinus truncatus* snails, while offspring produced by the female *S. mansoni* x male *S. bovis* cross can only infect *Biomphalaria glabrata* snails (Onyekwere et al., 2022a).

In nature, the geographic and host range overlap of several schistosome species, and the dioecious sexual stage of adult schistosomes favors natural hybridization, which is increasingly being reported (**Table 1.1**). Natural hybridization events have been documented between *Schistosoma* species that typically parasitize humans (**Table 1.1.A**), between *Schistosoma* species that typically

parasitize animals (**Table 1.1.B**), and between *Schistosoma* species that typically parasitize humans and those that typically parasitize animals (**Table 1.1.C**). The latter, hybrids resulting from crosses between typically human and typically animal species, are a major public health concern because of their zoonotic potential. Of particular note are hybrids between the human urogenital schistosomiasis species, *S. haematobium*, and the species of great veterinary importance, *S. bovis*, *S. curassoni*, and *S. mattheei*, which cause intestinal schistosomiasis in livestock (Webster et al., 2013, 2019; Angora et al., 2020). Even the hybrid *S. haematobium* x *S. bovis* has been detected in Europe as it has been implicated in the emergence and persistence of autochthonous transmission of schistosomiasis on the Mediterranean island of Corsica (Rothe et al., 2021; Wellinghausen et al., 2022).

Although the first reports in the scientific literature of possible crosses and hybridizations between *Schistosoma* species were based on phenotypic observations of the eggs (Leger and Webster, 2017), a relationship between the morphology and genotype of hybrid eggs has not yet been established. Therefore, detection of hybrids within the genus *Schistosoma* is now performed using a multi-locus approach combining nuclear and mitochondrial DNA markers, since single-locus approaches are often insufficient to detect hybridization or introgression events (Leger and Webster, 2017).

Table 1.1. Natural hybridizations in the genus *Schistosoma* confirmed by molecular analysis. Table modified from Boissier et al., 2019.

Area	Species	Species	Reference
A) Hybridization between human schistosomes			
Benin, Cameroon	<i>S. haematobium</i>	<i>S. guineensis</i>	(Webster et al., 2005; Moné et al., 2012)
Senegal, Côte d'Ivoire	<i>S. haematobium</i>	<i>S. mansoni</i>	(Huyse et al., 2013; Depaquit et al., 2019; Le Govic et al., 2019)
B) Hybridization between animal schistosomes			
Senegal, Niger, Mali	<i>S. bovis</i>	<i>S. curassoni</i>	(Webster et al., 2013; Léger et al., 2016; Privat et al., 2023)
C) Hybridization between human schistosomes and			
Senegal, France, Mali, Malawi, Côte d'Ivoire, Niger, Benin, Nigeria, Cameroon	<i>S. haematobium</i>	<i>S. bovis</i>	(Webster et al., 2013; Boissier et al., 2016; Soentjens et al., 2016; Webster et al., 2019; Angora et al., 2020; Pennance et al., 2020; Savassi et al., 2020; Onyekwere et al., 2022b; Teukeng et al., 2022; Privat et al., 2023)
Senegal, Mali	<i>S. haematobium</i>	<i>S. curassoni</i>	(Webster et al., 2013; Privat et al., 2023)
Malawi	<i>S. haematobium</i>	<i>S. mattheei</i>	(Webster et al., 2019)
Tanzania, Kenya	<i>S. mansoni</i>	<i>S. rodhaini</i>	(Morgan et al., 2003; Steinauer et al., 2008)

Most studies based on *Schistosoma* species and hybrid identification use a combination of ITSs as a marker for nuclear rDNA and partial *cox1* as a marker for mtDNA. On the one hand, as mentioned above, the ITSs markers are very useful for detecting recent hybridization events. Mixed ITSs profiles, meaning copies from both parental species, suggest first generation or earlier generation hybrids, as this marker tends to genetic homogenization due to concerted evolution (Rey et al., 2021a). On the other hand, the *cox1* gene is part of the genes that encode the protein subunits involved in mitochondrial energy metabolism. Since is a mtDNA marker, it is inherited maternally and it is a good marker to establish evolutionary relationships between species (Mas-Coma and Bargues, 2009). Hybridization is detected when the ITSs and *cox1* markers provide discordant results in species assignment and/or when two different ITSs sequences coexist in the same individual (Djuikwo-Teukeng et al., 2019; Angora et al., 2020; Onyekwere et al., 2022b).

In some species combinations, the use of ITSs and *cox1* is not sufficient for species and hybrid determination, requiring the use of other nuclear regions, such as 18S, for species/hybrid identification (Blin et al., 2023).

1.6. RESEARCH OBJECTIVES AND THESIS OUTLINE

The epidemiology and distribution of parasitic diseases, especially vector-borne diseases, are determined by a complex dynamic of environmental and social factors. Thus, global change, including migration, trade, unplanned urbanization, and climate change, is having a significant impact on the distribution and transmission of parasites and vectors, particularly for diseases of zoonotic origin. These changes in distribution increase the areas of overlap between species that were previously separated by physical barriers, favoring co-infection of parasite species in the same individual and hence the likelihood of natural hybridization events and their potential consequences.

Therefore, taking global change as a framework, this thesis aims to deepen the knowledge of fascioliasis and schistosomiasis, two re-emerging zoonotic trematodiasis transmitted by freshwater snails with enormous importance in both human health and veterinary fields, in which, hybrids have already been reported. To this end, we have divided this thesis into three main chapters: **Chapter II**, **III**, and **IV**.

Chapter II addresses fascioliasis, a disease with almost global distribution, in an endemic country, Bangladesh. Livestock production is a fundamental pillar of the economy of this low-income country, which is experiencing a progressive increase in population and urbanization and is also very vulnerable to climate change due to its

geographical location. In this scenario, the infection rates of fascioliasis in livestock seems to be increasing. The specific objectives of this chapter are as follows:

1. to assess the distribution of fasciolid liver flukes infecting the main livestock species (buffaloes, cattle, goats, and sheep) throughout Bangladesh by including animals from the four different agro-ecological zones (coastal, hilly, Barindh steppe, and floodplains);
2. to perform a genetic characterization of the fasciolids collected by sequencing and cloning the complete intergenic nuclear rDNA region, including the spacers ITS-1 and ITS-2 and the 5.8S gene;
3. analyze the results obtained from the complete intergenic nuclear rDNA region by sequencing and cloning in order to assess the possible reasons for this growing fascioliasis problem in Bangladesh.

Chapter III and **Chapter IV** are dedicated to schistosomiasis, in this case in a non-endemic country such as Spain, but with compatible species of freshwater snails as intermediate hosts (*B. truncatus* and *Planorbarius metidjensis*) of some species of *Schistosoma* spp. and where autochthonous cases of urogenital schistosomiasis have already been described. Due to its geographical location, Spain receives a large number of migrants from sub-Saharan Africa, where schistosomiasis is endemic. In **Chapter III**, 51 migrant

patients diagnosed with urogenital and/or intestinal schistosomiasis are included, and the specific objectives of this chapter are:

4. morphological and molecular identification of *Schistosoma* strains entering our country in sub-Saharan migrant patients;
5. genetic characterization of pure species and haplotype variability;
6. genetic characterization of hybrid between species and haplotype variability;
7. transfer of the molecular identification to the health system for comparison with morbidity and possible problems in treatment.

In **Chapter IV**, we will focus on five patients diagnosed with urogenital schistosomiasis who presented terminal-spined eggs, typical of *S. haematobium*, and ectopic lateral-spined eggs, typical of *S. mansoni*, in their urine. In only one of these five patients, a stool sample was also obtained, which contained only the typical *S. mansoni* eggs, lateral-spined eggs. The specific objectives of this chapter are as follows:

8. determine the pure or hybrid nature of ectopic schistosome lateral-spined eggs, typical of *S. mansoni*, from urine, by genetic characterization;
9. provide a patient-specific description of the different genotypes found in *Schistosoma* eggs, based on their

morphology, lateral-spined or terminal-spined eggs, and the type of sample analyzed;

10. perform a haplotype combined description of terminal-spined and lateral-spined eggs by sequencing ribosomal (ITS-1, 5.8S and ITS-2) and mitochondrial (partial *cox1*) DNA markers.

Finally, the conclusions of **Chapters II, III, and IV** are presented in the last chapter of the thesis, **Chapter V**. **Chapter VI** contains the bibliography used.

**CHAPTER II. RECENT FASCIOLID
HYBRIDS FROM LIVESTOCK IN
BANGLADESH EVALUATED BY THE
COMPLETE INTERGENIC NUCLEAR
RIBOSOMAL DNA REGION
SEQUENCING AND CLONING**



2.1. INTRODUCTION

Bangladesh is a country located in South Asia on the Bay of Bengal. It has an area of 147,570 km² and is bordered on the west, north, and east by a 4,095 km land frontier with India and, in the southeast, by a short land and sea frontier (193 km) with Burma (Myanmar) (CIA, 2021).

It is a low-lying riverine country with a largely marshy jungle coastline of 710 km on the northern littoral of the Bay of Bengal. The country has a tropical monsoon climate characterized by heavy seasonal rainfall, high temperatures, and high humidity with mild winters from October to March, and hot, humid summers from March to June (CIA, 2021).

With approximately 167 million people, Bangladesh is the eighth most populous country in the world and is expected to continue growing at an annual rate of approximately 1% in the coming years. Despite making significant progress in human development and poverty reduction since its independence in 1971, Bangladesh remains on the list of low-income countries. The majority of its population lives in misery and poverty, concentrated in rural areas and directly dependent on livestock and agriculture for their subsistence. As a result, animal husbandry is one of the pillars of the country's economy. Approximately 75% of people depend to some extent on livestock for their livelihood, and it is estimated that it provides full-time employment to 20% of the total population and part-time employment to another 50%, mainly to the

poorest fractions, including landless, marginal, and small farmers (Nath et al., 2022). Most of these animals are raised by small-scale farmers who own 1-2 head of cattle and 2-3 head of goat or sheep. The management system is a subsistence farming type, combining both tethering and scavenging with few or no inputs for breeding, feeding and healthcare, and covering nearly 80% of the livestock farming environment (Bhuiyan, 2014).

Bangladesh's geographical location, socio-environmental conditions, population growth, poverty, and limited economic and technological capacity make it one of the most vulnerable countries to climate change. Frequent extreme weather events such as floods, droughts, salinity intrusion, and waterlogging particularly affect the rural economy, directly and indirectly impacting agriculture and livestock (Ali et al., 2020).

Helminth parasites, strongly influenced by climate change, are one of the most concerning factors affecting livestock health. Among these diseases, fascioliasis stands out as it finds an ideal environment for re-emergence due to the types of crops and ruminants reared in the country (Calvani and Šlapeta, 2021).

Fascioliasis was introduced into South Asia from the Fertile Crescent by taking advantage of the human-guided movements of ruminants, equines and domesticated camelids throughout the Neolithic period up to the present day. Among the intricate network of trade routes that have occurred in Asia throughout history, Bangladesh is strategically

located where several have converged, such the Grand Trunk Road, the Tea-Horse Road, or the Maritime Silk Road (**Figure 2.1**). This allowed *F. gigantica* to expand throughout the southern latitudes of Asia, reaching Southeast Asia and the Pacific Islands, due to its preference for lowlands and warm temperatures. On the contrary, *F. hepatica* was originally unable to spread into these lowlands because of its developmental temperature thresholds (Mas-Coma et al., 2022a).

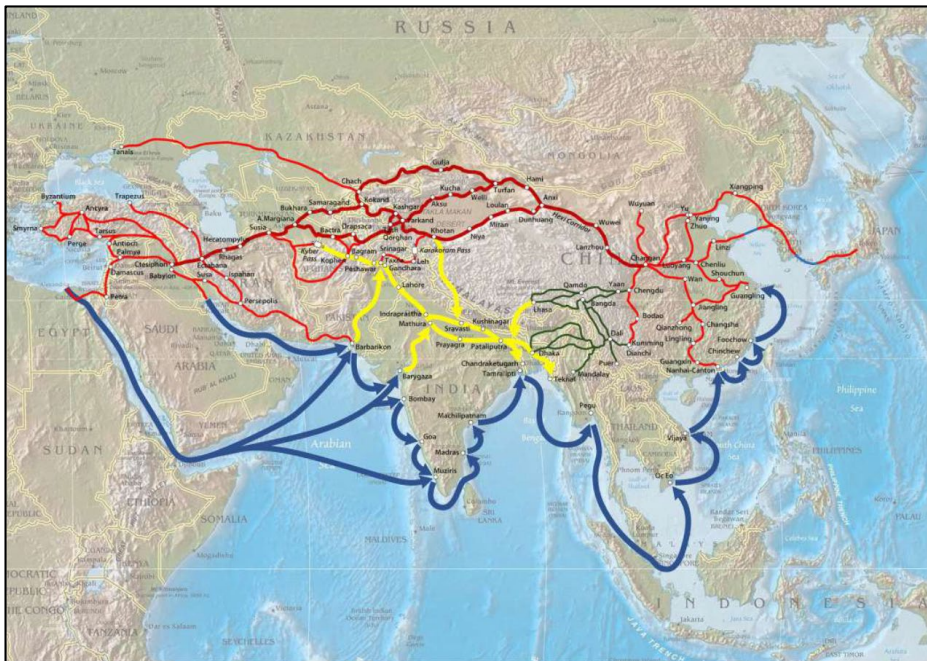


Figure 2.1. Spread of *Fasciola* occurred through the Silk Road (dark red), Grand Trunk Road and its connections (yellow), Tea-Horse Road (green), Maritime Silk Road (blue), and secondary expansion and interconnecting routes (red). Map extracted from Mas-Coma et al., 2022a.

Currently, international livestock trade is playing a key role in the most recent spread of these parasites. Southeast Asia is an example of

this situation. Countries such as Vietnam have experienced rapid growth of the middle class, leading to an increased demand for animal protein. This demand exceeds local production, forcing them to source animals from countries such as Australia or Argentina, which are major livestock exporters and where *F. hepatica* is endemic. The sustained increase in the animal movement from *F. hepatica* endemic regions to non-endemic regions, along with the potential introduction of infected animals, is probably expanding the sympatric ranges of *F. hepatica* and *F. gigantica* (Calvani and Šlapeta, 2021).

In countries surrounding Bangladesh, *F. hepatica* has already been reported from India and Nepal (Sharma et al., 1989; Sah et al., 2017) and hybrids have been reported from India, Nepal and Myanmar (Hayashi et al., 2018). In Bangladesh, a previous phenotypic assessment of fasciolids showed the existence of intermediate forms between *F. gigantica* and *F. hepatica* through morphometric analysis using a computer image analysis system (Ahasan et al., 2016).

In terms of human fascioliasis, the wide south-central region eastward from Afghanistan has been the focus of considerable attention due to the recent increase in cases. Countries such as India, including West Bengal (Ramachandran et al., 2012; Madhumitha et al., 2015), Assam and Arunachal Pradesh (Ramachandran et al., 2012), Nepal (Parelkar et al., 2013; Sah et al., 2017, 2018), and Myanmar (Maeda et al., 2008) have reported human infections caused by both *F. hepatica* and *F. gigantica* (**Figure 2.2**).

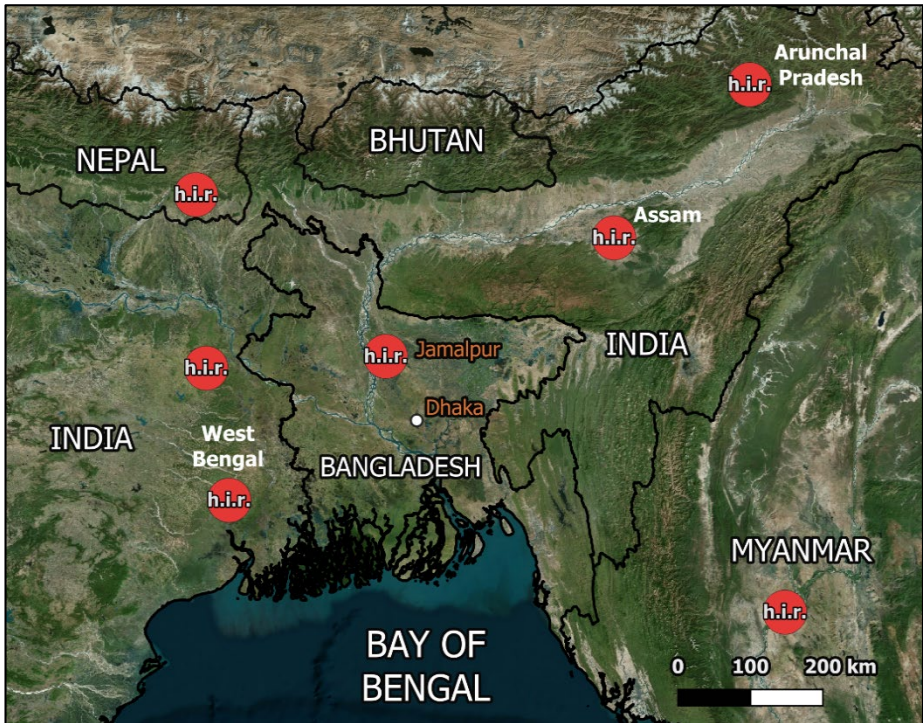


Figure 2.2. Geographic map of Bangladesh and neighboring countries, showing zones where human infection by fasciolid flukes have been reported. h.i.r. = human infection reported (h.i.r. labels noted in zones of reports except in Myanmar). Background from composed satellite map of Asia projection by Bing Satellite.

This scenario suggests that the entire region north of the Bay of Bengal and south of the Himalayan chain is a fascioliasis hotspot, with the risk of human infection in very densely populated areas. However, contrasts with the only two recently reported cases of human fascioliasis in Bangladesh: one involving a 22-year-old Bangladeshi woman diagnosed at London hospital (Behar et al., 2009) and another concerning a 35-year-old housewife from Jamalpur (Das et al., 2019). In both cases, *F. hepatica* is mentioned as the causative agent, although no molecular or morphometric characterization was performed in either case.

This chapter presents the study conducted in Bangladesh, the objectives of which (see **Section 1.6**) are consistent with the One Health approach recently emphasized by WHO for the strategic assessment and control of neglected tropical diseases, particularly those with zoonotic characteristics (WHO, 2020). First, the aim was to assess the distribution of fasciolid liver flukes to determine the regions, animal species, and types of zones where apparent increases in infection rates were occurring. Second, the study focused on the genetic characterization of the collected liver flukes through complete intergenic nuclear rDNA region sequencing and cloning. This is the first time that the complete intergenic nuclear rDNA region, including the spacers ITS-1 and ITS-2 and the 5.8S gene, have been used for fasciolid fluke assessment in Bangladesh. Third, the approach was aimed to investigate potential factors contributing to the aforementioned increasing problem in the country.

2.2. MATERIAL AND METHODS

2.2.1. LIVESTOCK EXAMINED AND LIVER FLUKE COLLECTED

A total of 346 specimens of the four major livestock species in Bangladesh were examined, including: 18 buffaloes, 123 cattle, 164 goats and 41 sheep. Livestock species were sampled throughout the whole of Bangladesh by including animals from the four different agro-ecological zones (coastal, hilly, Barindh steppe and floodplains). The agro-ecological zones, according to Food and Agriculture Organization criteria, are characterized by distinct climatic and environmental conditions and play a crucial role in shaping the agricultural practices and crop production in the country.

The data of the sampled livestock analyzed and their geographical origin in terms of locations, coordinates and agro-ecological zones are presented in **Table 2.1** and **Figure 2.3**.

Adult fasciolids were collected directly from the liver of infected animals of the livestock species studied. Rubber-coated forceps were used to avoid any structural damage to the flukes.

Table 1.1. Agro-ecological zones, coordinates and altitudes of the localities in Bangladesh from which livestock specimens were collected for fasciolid fluke analysis.

No. in map*	Localities (Administrative units)	Agro-ecological zones	Latitude	Longitude	Altitude (m)
1	Chittagong/ Cox's Bazar/Chakaria	Coastal	21°45'41.36"N	92°04'33.45"E	11.21
2	Barisal / Bhola/Bhola Sadar	Coastal	22°41'12.48"N	90°38'30.76"E	7.88
3	Chittagong/ Rangamati/ Rangamati Sadar	Hilly	22°39'28.51"N	92°10'24.54"E	45.45
4	Sylhet/ Sylhet/Sylhet Sadar	Hilly	24°54'23.77"N	91°50'50.29"E	21.1
5	Rajshahi/ Naogaon/Patnitola	Barindh steppe	25°02'40.55"N	88°45'19.68"E	24.24
6	Rajshahi/ Naogaon/Shapahar	Barindh steppe	25°07'30.31"N	88°35'24.50"E	44.85
7	Rajshahi/ Naogaon/ Naogaon Sadar	Barindh steppe	24°48'39.24"N	88°56'37.04"E	20.61
8	Rajshahi/ Pabna/Ishwardi	Floodplains	24°07'12.43"N	89°04'03.56"E	18.48
9	Dhaka/ Mymensingh/ Ishwarganj	Floodplains	24°41'10.20"N	90°35'36.88"E	16.67
10	Rangpur/ Lalmonirhat/ Lalmonirhat Sadar	Floodplains	25°58'26.15"N	89°17'02.26"E	37.58
11	Dhaka/ Mymensingh/ Mymensingh Sadar	Floodplains	24°44'04.60"N	90°25'43.80"E	20.00
12	Rajshahi/ Bogra/Dhunat	Floodplains	24°41'29.12"N	89°32'10.33"E	16.36
13	Khulna/ Jhenaidah/Shailkupa	Floodplains	23°40'45.47"N	89°14'48.28"E	13.03
14	Khulna/ Jhenaidah/ Jhenaidah Sadar	Floodplains	23°32'43.15"N	89°10'28.16"E	14.24

*See map in **Figure 2.3** for the number of locations.

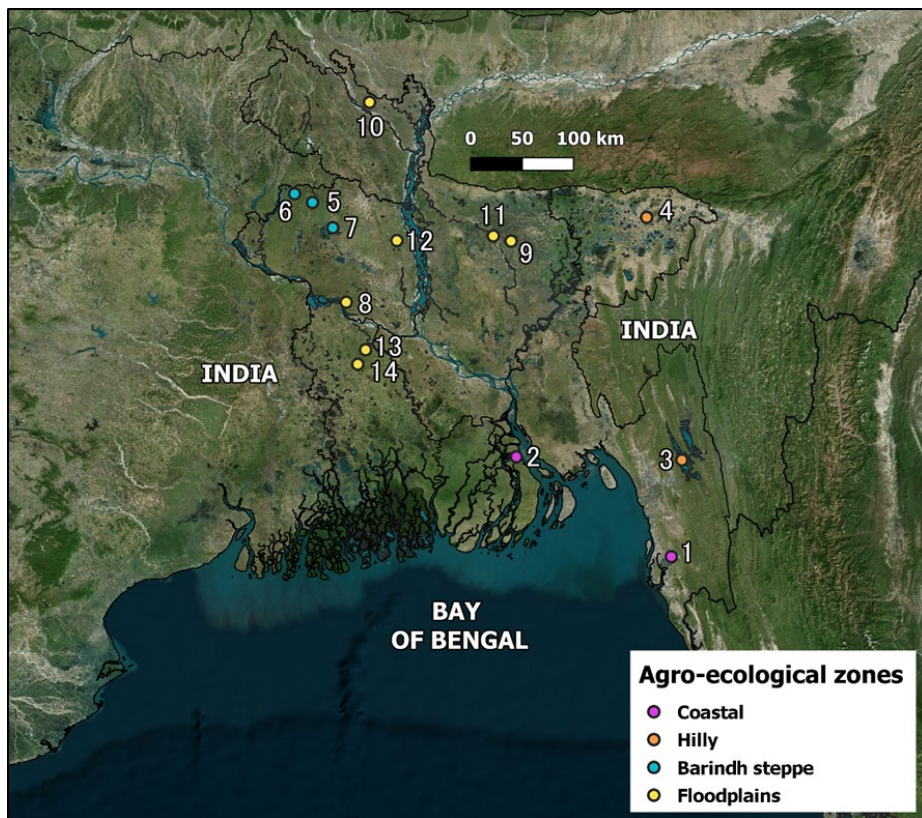


Figure 2.3. Geographic map of Bangladesh, showing the sites where the hosts of the liver flukes analyzed were collected. Coordinates and altitudes of each site are indicated in **Table 2.1**. 1) Chakaria; 2) Bhola Sadar; 3) Rangamati Sadar; 4) Sylhet Sadar; 5) Patnitola; 6) Shapahar; 7) Naogaon Sadar; 8) Ishwardi; 9) Ishwarganj; 10) Lalmonirhat Sadar; 11) Mymensingh Sadar; 12) Dhunat; 13) Shailkupa; 14) Jhenaidah Sadar. Background from composed satellite map of Asia by Bing satellite.

2.2.2. FASCIOLID SPECIMENS FOR MOLECULAR CHARACTERIZATION

A total of 83 adult flukes were used for DNA sequencing purposes, including specimens infecting buffaloes ($n = 6$), cattle ($n = 29$),

goats (n = 42) and sheep (n = 6). Fasciolid specimens were washed extensively in physiological saline (0.85% NaCl) to remove blood and bile and finally preserved in ethanol 70% until DNA extraction.

The sequence of the complete nuclear rDNA, including the ITS-1 and ITS-2 spacers and the 5.8S gene that separates them, has been used as molecular markers for sequencing purposes.

2.2.3. EXTRACTION, AMPLIFICATION AND SEQUENCING OF DNA

For DNA extraction, a small portion of the anterior body region of each fasciolid specimen was individually processed using the phenol-chloroform extraction and ethanol precipitation method according to established protocols as previously described (Mas-Coma et al., 2001; Bargues et al., 2017; Chougar et al., 2020; Bargues et al., 2021). The samples were suspended in 400 μ L of lysis buffer (10 mM Tris-HCl, pH 8.0, 100 mM EDTA, 100 mM NaCl, 1% sodium dodecyl sulfate SDS) containing 500 μ g/mL Proteinase K (Promega, Madison, WI, USA). The digestion was performed for 2 hours at 55 °C, including shaking every 15 min. The phenol-chloroform extraction and ethanol precipitation method was applied for total DNA isolation. Each pellet was dried and resuspended in 30 μ L sterile TE buffer (pH 8.0), and subsequently this suspension was stored at -20°C until needed.

The selected DNA markers were amplified by PCR for each liver fluke individual, by using the following primers: BD1 (5'

GTCGTAACAAGGTTTCCGTA 3') as forward, and BD2 (5' TATGCTTAAATTCAGCGGGT 3') as reverse (Luton et al., 1992). The primers were designed in the regions flanking the ribosomal RNA (rRNA) genes 18S and 28S for the subsequent amplification of the complete ITS-1, 5.8S, ITS-2 region (Mas-Coma et al., 2001; Valero et al., 2018).

For the PCR amplification, the Biotools DNA polymerase (Biotools B&M Labs. S.A., Madrid, Spain) was used in a Verity-96-well Thermal Cycler (Applied Biosystems, Thermo Fisher Scientific, Waltham, MA USA). The program for this rDNA ITS region comprised one cycle of 2 min at 94 °C, 35 cycles of 1 min at 93 °C, 1 min at 55 °C and 1 min at 72 °C each, preceded by 2 min at 72 °C, and followed by a final cooling at 4 °C.

For the purification of the PCR product, the Ultra Clean™ PCR Clean-up DNA Purification System (MoBio, Solana Beach, CA, USA) was used following the manufacturer's protocol and eluted in 50 µL of 10 mM TE buffer (pH 7.6). The final DNA concentration (in µg/ mL) and the absorbance at 260/280 nm were determined in an Eppendorf BioPhotometer (Hamburg, Germany).

Each PCR product was sequenced, for a definitive haplotype characterization, on both strands by the dideoxy chain-termination method performed with the Taq dye-terminator chemistry kit on an Applied Biosystems 3730xl DNA Analyzer (Applied Biosystems, Foster City, CA, USA). The PCR primers were used as sequencing primers.

2.2.4. SEQUENCE ANALYSIS

The software Sequencher v. 5.4.6 (Gene Codes Co. MI, USA) was used to edit and assemble the sequences. The electropherograms (ABI format) of the rDNA intergenic sequence were thoroughly reviewed, especially in the positions that differentiate between *F. hepatica* and *F. gigantica*, using FinchTV v. 1.5 (Geospiza, Inc., Seattle, WA, USA; <http://www.geospiza.com>) and ClustalW to align them by means of default parameters in MEGA X (Kumar et al., 2018).

Corresponding penalties for gaps were included in pairwise and multiple alignments. All changes, comprising transitions (ts), transversions (tv) and insertions/deletions (indels), were considered as character states in MEGA X. By means of the ALTER web server (Glez-Peña et al., 2010), the sequences aligned were collapsed to haplotypes, counting gaps as differences. Closely related sequences were searched by utilizing the BLAST program from Web site of the National Center for Biotechnology Information (<http://www.ncbi.nlm.nih.gov/BLAST>). Comparative analyses were performed with available rDNA ITS-1 and ITS-2 sequences of *F. gigantica*, *F. hepatica* and *Fasciola* spp. downloaded from the GenBank and also with the fasciolid haplotype collection of the Valencia WHO Collaborating Center.

To define *Fasciola* species and “pure” haplotypes, a total of 50 sequences were used for comparison purposes, including 20 sequences of “pure” *F. hepatica* from Europe, the Americas and Algeria representing the haplotypes Fh1A (GenBank Accession No.

MG569980), Fh2A (MG569978, MG569981), Fh3A (MK212150) and Fh1/2A (OQ064782); and 30 sequences of “pure” *F. gigantea* from Africa representing the haplotypes Fg1A (AJ853848), Fg2A (ON661090), and Fg-HtzA (ON661091).

2.2.5. CLONING

Since ITSs markers have several copies, which can lead to intraindividual variation, cloning procedures were applied to PCR products from specimens whose electropherograms obtained by direct sequencing showed unexpected double peaks at relevant positions (**Figure 2.4**).

The purified amplification products from a subsample of 6 specimens with apparent heterozygous sequences and one control of “pure” *F. gigantea*, according to our DNA markers, were cloned.

The ligation procedure was performed using the pGEM®-T vector and T4 DNA ligase enzyme from the pGEM-T Easy Vector System I (Promega, Madison, WI), and the reactions were incubated overnight at 4°C according to the manufacturer's instructions.

For the transformation process, 2 µL of the ligation product was added to a 1.5 mL tube and 50 µL of *Escherichia coli* strain DH5α competent cells were transferred and incubated on ice for 20 minutes.

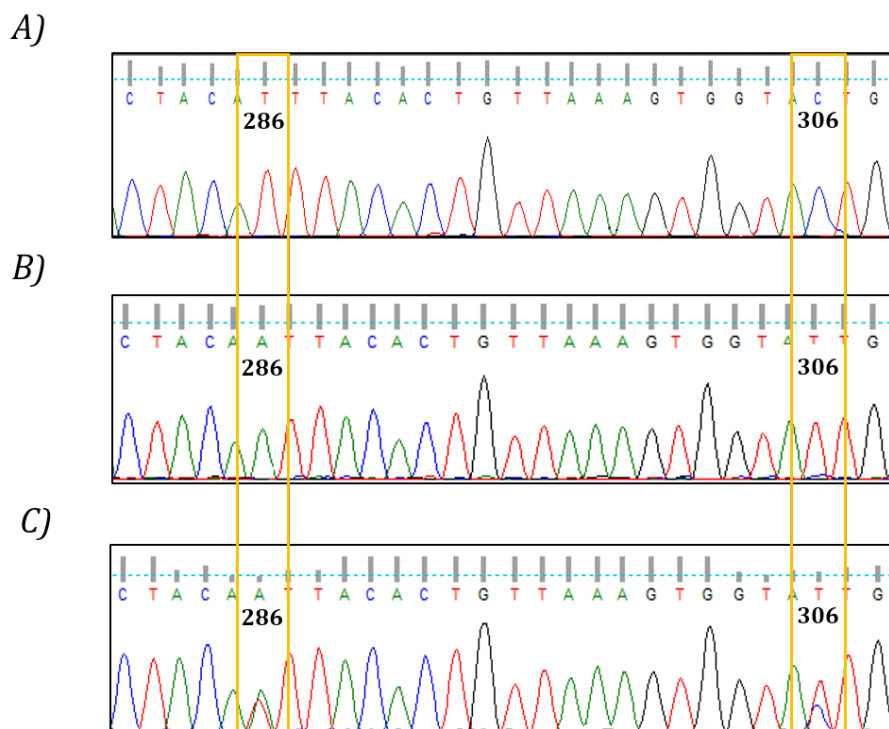


Figure 2.4. ITSs sequencing chromatograms in which: **A)** corresponds to a typical *F. hepatica*; **B)** corresponds to a typical *F. gigantica*; and **C)** shows double peaks at two positions (286 and 306) discriminating between the two *Fasciola* species mentioned above.

Heat shock was applied at 42 °C for 50 seconds and then left on ice for 2 minutes. Next, 950 µL of previously prepared LB/Ampicillin/IPTG/X-Gal medium was added and incubated at 37 °C for 1.5 hours at 300 rpm. Subsequently, the cells were centrifuged for 10 seconds to concentrate them and 100 µL were seeded into plates containing LB/Ampicillin/IPTG/X-Gal medium in duplicate. After overnight incubation at 37 °C, eight white antibiotic-resistant colonies,

thus containing the insert, were selected per cloned sample. A standard PCR was performed on each colony and sequenced individually. DNA sequencing and sequence analysis of the clones were performed as described above.

2.2.6. HAPLOTYPE NOMENCLATURE

The terminology to identify the haplotype (H) of the two aforementioned DNA markers follows the previously proposed combined haplotyping (CH) nomenclature (Bargues and Mas-Coma, 2005; Mas-Coma et al., 2009b). According to this nomenclature, numbers are used to define ITS-2 haplotypes, while capital letters are used for ITS-1 haplotypes.

In combined haplotypes, the ITS-2 code is indicated before the ITS-1 due to the evolutionary significance of ITS-2 in species differentiation. It is important to note that haplotype codes are only definitive when the sequences are complete, meaning they are full-length sequences. In cases where fragments or incomplete sequences are encountered, haplotype codes are considered provisional.

2.2.7. BANKING OF NEW SEQUENCES

The sequences of pure and hybrid fasciolid haplotypes from Bangladesh, obtained in this thesis, have been submitted to the GenBank Data Library and assigned Accession Nos. OQ064778- OQ064781.

2.2.8. ETHICS STATEMENT

All research involving animals was conducted with the approval of the Evaluation of Projects involving Animals at the University of Valencia (Organo Habilitado para la Evaluación de Proyectos de Experimentación Animal de la Universidad de Valencia) (A1263 91,389,140), strictly following the guidelines of the institution based on the Directive 2010/63/EU. Authorization for animal research was additionally obtained from the Servicio de Sanidad y Bienestar Animal, Dirección General de Producción Agraria y Ganadería, Consellería de Presidencia y Agricultura, Pesca, Alimentación y Agua, Generalitat Valenciana, Valencia, Spain (No. 2015/VSC/PEA/00001 tipo 2). Fasciolids were obtained by taking advantage of animals slaughtered for other purposes. Animal ethics guidelines for animal care were strictly adhered to.

2.3. RESULTS

2.3.1. FASCIOLIASIS INFECTING RATES

A total of 2,352 fasciolid adult flukes were collected from 115 infected livestock specimens analyzed including:

- 10 infected from 18 buffaloes dissected (prevalence of 55.56%) showing an infection intensity of 20-61 flukes/animal (mean 38.0 flukes/animal);
- 36 infected from 123 cattle dissected (prevalence of 29.27%) showing an infection intensity of 7-103 flukes/animal (mean 28.30 flukes/animal);
- 58 infected from 164 goats dissected (prevalence of 35.37%) showing an infection intensity of 8-89 flukes/animal (mean 16.60 flukes/animal);
- 11 infected from 41 sheep dissected (prevalence of 26.83%) showing an infection intensity of 3-31 flukes/animal (mean 11.70 flukes/animal).

The distribution of prevalence and intensity by localition, sex, and age of hosts are shown in **Tables 2.2-2.5**.

Infected animals were found in all agro-ecological zones, with higher intensities among the animals sampled in the Barindh steppe and floodplains zones, regardless of the species (**Figure 2.5**).

Table 2.2. Prevalences and intensities of fasciolid flukes collected from buffaloes in Bangladesh. i.h. = infected hosts.

BUFFALOES						
No. in map*	No. Hosts analyzed	Sex i.h.	Age i.h. (years)	No. i.h. (prevalence %)	Total No. flukes collected	Intensity range (mean)
1	5	1 M	6	1 (20%)	121	-
2	-	-	-	-	-	-
3	-	-	-	-	-	-
4	2	2 M	3-4	2 (100%)	42	20-22 (21.0)
5	-	-	-	-	-	-
6	4	1 M	5	1 (25%)	82	--
7	-	-	-	-	-	-
8	6	4 M, 1 F	3-7	5 (83.33%)	224	27-61 (44.88)
9	-	-	-	-	-	-
10	-	-	-	-	-	-
11	-	-	-	-	-	-
12	-	-	-	-	-	-
13	1	1 M	5	1 (100%)	3	-
14	-	-	-	-	-	-
TOTAL	18	9 M, 1 F	3-7	10 (55.56%)	472	20-61 (38.0)

* See Table 2.1 and Figure 2.3 for locations.

Table 2.3. Prevalences and intensities of fasciolid flukes collected from cattle in Bangladesh. i.h. = infected hosts.

CATTLE						
No. in map*	No. hosts analyzed	Sex i.h.	Age i.h. (years)	No. i.h. (prevalence %)	Total No. flukes collected	Intensity range (mean)
1	5	1 F	5	1 (20.0%)	23	-
2	18	3 M, 4 F	3-6	7 (38.89%)	307	26-67 (43.80)
3	11	1 M, 1 F	3-5	2 (18.18%)	28	7-21 (14.0)
4	4	1 M	5	1 (25.0%)	13	-
5	6	1 M	6	1 (16.67%)	8	-
6	7	1 M	6	1 (14.29%)	13	-
7	-	-	-	-	-	-
8	13	2 M, 2 F	3-6	4 (30.77%)	27	4-12 (6.75%)
9	-	-	-	-	-	-
10	5	1 M	1.5	1 (20.0%)	12	-
11	40	6 M, 4 F	1.5-4	10 (25.0%)	44	10-31 (16.40)
12	-	-	-	-	-	-
13	6	1 M, 1 F	4-5	2 (33.33%)	43	9-34 (21.50)
14	18	4 M, 2 F	2-6	6 (33.33%)	267	7-103 (44.50)
TOTAL	123	21 M, 14 F	1.5-6	36 (29.27%)	785	7-103 (28.30)

* See Table 2.1 and Figure 2.3 for locations.

Table 2.4. Prevalences and intensities of fasciolid flukes collected from goats in Bangladesh. i.h. = infected hosts.

GOATS						
No. in map*	No. hosts analyzed	Sex i.h.	Age i.h. (years)	No. i.h. (prevalence %)	Total No. Flukes collected	Intensity range (mean)
1	6	1 M	2	1 (16.67%)	9	-
2	-	-	-	-	-	-
3	-	-	-	-	-	-
4	9	1 M, 2 F	1.5-4	3 (33.33%)	62	11-27 (20.70)
5	5	1 M	2	1 (20.0%)	11	-
6	-	-	-	-	-	-
7	16	2 M, 3 F	1.5-3	4 (25.0%)	85	4-34 (21.30)
8	15	2 M, 3 F	1.5-4	5 (33.33%)	55	6-17 (11.0)
9	18	5 M	1.5-3	5 (27.78%)	118	8-43 (23.60)
10	4	1 M	1.5	1 (25.0%)	26	-
11	63	16 M, 12 F	1.5-3	28 (44.44%)	121	4-34 (21.30)
12	3	1 M	2	1 (33.33%)	124	-
13	5	1 M, 1 F	1.5-2	2 (40.0%)	33	9-34 (15.50)
14	22	6 M, 1 F	1.5-4	7 (31.81%)	322	8-89 (46.0)
TOTAL	164	37 M, 21 F	1.5-4	58 (35.37%)	966	8-89 (16.60)

*See **Table 2.1** and **Figure 2.3** for locations.

Table 2.5. Prevalences and intensities of fasciolid flukes collected from goats in Bangladesh. i.h. = infected hosts.

SHEEP						
No. in map*	No. hosts analyzed	Sex i.h.	Age i.h. (years)	No. i.h. (prevalence %)	Total No. flukes collected	Intensity range (mean)
1	-	-	-	-	-	-
2	-	-	-	-	-	-
3	-	-	-	-	-	-
4	-	-	-	-	-	-
5	5	1 M	2	1 (20.0%)	3	-
6	-	-	-	-	-	-
7	22	4 M, 2 F	1.5-3	6 (27.27%)	65	6-16 (10.30)
8	-	-	-	-	-	-
9	-	-	-	-	-	-
10	4	1 M	1.5	1 (25.0%)	31	-
11	4	1 M	2	1 (25.0%)	8	-
12	-	-	-	-	-	-
13	3	1 M	1.5	1 (33.33%)	11	-
14	3	1 F	2	1 (33.33%)	11	-
TOTAL	41	8 M, 3 F	1.5-3	11 (26.83%)	129	3-31 (11.70)

*See Table 2.1 and Figure 2.3 for locations.

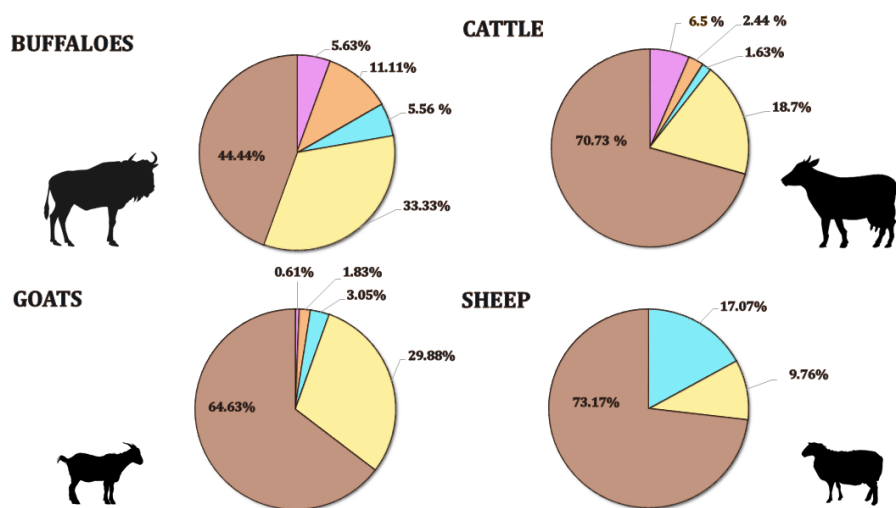


Figure 2.5. Percentage of infected buffaloes, cattle, goats and sheep by agro-ecological area: pink corresponds to coastal, orange to hilly, blue to Barindh steppe and yellow to foodplains. Brown corresponds to the percentage of non-infected animals according to each livestock species.

2.3.2. SEQUENCE ANALYSIS OF THE rDNA ITS-1-5.8S-ITS-2 REGION

The sequencing of the 83 molecularly analyzed fasciolid specimens from the four livestock species provided four distinct haplotypes. Only one of the haplotypes corresponded to “pure” *F. gigantica* and was assigned to the combined haplotype Fg-CH3A (GenBank Accession No. OQ064778), which contains a 950 base pairs (bp) long ITS-1-5.8S-ITS-2 region with a GC content of 50.42%. This haplotype was the most common, occurring in 65 flukes (78.31%) of the total analyzed, and was detected in the four animal species (cattle, buffalo, sheep and goats). The other three combined haplotypes occurred

in the remaining 18 specimens, representing 21.68% of the total fluke samples, and were also detected in the four animal species studied. These three haplotypes were characterized by double peaks observed during the careful inspection of the electropherograms of the rDNA ITS-1-5.8S-ITS-2 sequences of these liver fluke specimens. These double peaks appeared at specific positions known to discriminate between *F. hepatica* and *F. gigantica*, suggesting heterozygotes. Three types of sequences presenting double peaks could be distinguished:

- with double peaks at all such differentiating positions of the ITS-1 and ITS-2;
- with double peaks in some (but not in all) differentiating positions of the ITS-1 and ITS-2;
- with double peaks at differentiating positions only in the ITS-2 and not in the ITS-1.

Based on this distribution, sequences could be classified into three heterozygotic types of combined haplotypes, which we designated as Fg/Fh-Htz1, Fg/Fh-Htz2, and Fg/Fh-Htz3, respectively. The most abundant proved to be the haplotype Fg/Fh-Htz1. The distribution of the combined haplotype Fg-CH3A of “pure” *F. gigantica* and the aforementioned three heterozygotic types of combined haplotypes according to sampling site is noted in **Table 2.6**, and according to host species in **Table 2.7**.

Table 2.6. Number of fasciolids used for DNA sequencing by sampling site and host species, including haplotypes found, where Fg-3A is the *F. gigantica* “pure” haplotype detected and Htz haplot. refers to the heterozygotic haplotypes found.

No. in map*	BUFFALOES		CATTLE		GOATS		SHEEP	
	Fg-3A	Htz haplot.	Fg-3A	Htz haplot.	Fg-3A	Htz haplot.	Pure Fg-3A	Htz haplot.
1	1	-	1	-	1	-	-	-
2	-	-	7	-	-	-	-	-
3	-	-	2	-	-	-	-	-
4	2	-	1	-	3	-	-	-
5	-	-	1	-	1	-	-	1 Htz1
6	-	1 Htz1	1	-	-	-	-	-
7	-	-	-	-	2	-	-	2 Htz1
8	1	1 Htz1	1	-	-	-	-	-
9	-	-	-	-	1	-	-	-
10	-	-	1	-	3	1 Htz1	-	1 Htz1
11	-	-	9	-	17	2 Htz1 6 Htz2	1	-
12	-	-	-	-	1	-	-	-
13	-	-	2	-	1	-	-	-
14	-	-	2	1 Htz3	1	2 Htz1	1	-
TOTAL	4	2 Htz1	29	1 Htz3	31	5 Htz1 6 Htz2	2	4 Htz1

*See Table 2.1 and Figure 2.3 for locations.

Table 2.7. Distribution of rDNA transcribed spacer region combined haplotypes by definitive host species.

Host species	Buffalo	Cattle	Goat	Sheep	TOTAL
Total fasciolid No.= 83	(N= 6)	(N= 29)	(N= 42)	(N= 6)	
Pure <i>F. gigantica</i>	4	28	31	2	65
Fg-CH3A	(66.66%)	(96.55%)	(73.81%)	(33.33%)	(78.31%)
Hybrid Fg/Fh- Htz	2	1	11	4	18
	(33.33%)	(3.44%)	(26.19%)	(66.66%)	(21.69%)
Heterozygotic haplotypes					
Fg/Fh-Htz1	2	-	5	4	11
	(18.18%)		(45.45%)	(36.36%)	(61.11%)
Fg/Fh-Htz2	-	-	6	-	6
			(100%)		(33.33%)
Fg/Fh-Htz3	-	1	-	-	1
		(100%)			(5.55%)

The “pure” Fg-3A combined haplotype was found in 66.66% of buffaloes, 96.55% of cattle, 73.81% of goats and 33.33% of sheep. The heterozygotic combined haplotypes were found in 33.33% of buffaloes, 3.44% cattle, 26.19% goats, and 66.66% of sheep. Among the combined heterozygotic haplotypes, Fg/Fh-Htz1 is the only one shared by three host species (buffaloes, goats and sheep), while the haplotypes Fg/Fh-Htz2 and Fg/Fh-Htz3 were detected in only one host species, namely goats and cattle respectively (**Tables 2.6** and **2.7**). So, the goat is the only animal species in which the “pure” *F. gigantica* combined haplotype plus two types of hybrid haplotypes were detected.

Polymorphic sites in nucleotide positions in the sequence alignment of the whole nuclear rDNA ITSs region including the ITS-1

and ITS-2 between “pure” *F. hepatica*, “pure” *F. gigantica* and Bangladeshi haplotypes are listed in **Table 2.8**.

When the two ITSs markers were analyzed individually, the ITS-1 haplotype HA showed an identical sequence of 432 nucleotides and a GC content of 51.16% in all samples with the combined haplotype Fg-CH3A of the ITSs region. The 5.8S gene was identical in all specimens, consisting of 154 base pairs and a GC content of 53.25%. The 364 bp-long and 48.35% GC ITS-2 was the only one exhibiting a mutation in position 210 of the ITS-2 alignment, that differentiates it from pure haplotypes of Fg-H1, Fg-H2 and Fg-H1/2. This polymorphic position does not differentiate between *F. hepatica* and *F. gigantica* (**Table 2.8**).

Within the identified Fg/Fh hybrid haplotypes, only two ITS-1 sequences had heterozygotic positions. Specifically, Fg/Fh-Htz1 had five positions with heterozygosity, while Fg/Fh-Htz2 had three positions, that discriminated between *F. hepatica* and *F. gigantica* (**Table 2.8**). The hybrid haplotype Fg/Fh-Htz3 presented an ITS-1 base-to-base coinciding with the sequence of “pure” *F. gigantica* (**Table 2.8**).

Regarding the ITS-2, all hybrid Fg/Fh haplotypes detected in Bangladesh presented heterozygotic positions differentiating between *F. hepatica* and *F. gigantica*, namely four positions in the Fg/Fh-Htz1 and three positions in the Fg/Fh-Htz3, whereas in only one position in the Fg/Fh-Htz-2 (**Table 2.8**).

Table 2.8. Polymorphic sites in the sequence comparison of the complete transcribed spacer region of the nuclear rDNA and in the ITS-1 and ITS-2 between haplotypes of 'genetically pure' *Fasciola hepatica* from livestock in Europe, the Americas and Algeria, haplotypes of 'genetically pure' *F. gigantica* from livestock in African countries, and the haplotypes of “pure” and heterozygotic fasciolid samples from Bangladesh (in bold).

<i>Fasciola</i> species	ITS-1, ITS-2 combined haplotypes				Polymorphic sites									
					Intergenic Region (ITS-1, 5.8S, ITS-2)									
					Polymorphic sites ITS-1				Polymorphic sites ITS-2					
	24	114	208	286	306	797	821	834	860	866	874	909	917	924
	Polymorphic sites ITS-1				306	210	234	248	273	279	288	322	330	337
<i>F. hepatica</i>	C	A	C	T	C	T	T	A	C	C	C	T	T	G
<i>F. hepatica</i>	C	A	C	T	C	T	T	A	C	C	T	T	T	G
<i>F. hepatica</i>	C	A	C	T	C	T	T	A	C	C	C/T	T	T	G
<i>F. hepatica</i>	C	A	C	T	C	T	T	A	C	C	C	A	T	G
<i>F. gigantica</i>	T	T	T	A	T	T	C	A	T	T	C	T	-	A
<i>F. gigantica</i>	T	T	T	A	T	T	C	C	T	T	C	T	-	A
<i>F. gigantica</i>	T	T	T	A	T	T	C	C/A	T	T	C	T	-	A
<i>F. gigantica</i>	T	T	T	A	T	C	C	A	T	T	C	T	-	A
<i>Fg/Fh</i>	T/C	T/A	T/C	A/T	T/C	C/T	C/T	A	T/C	T/C	C	T	-	A/G
<i>Fg/Fh</i>	T/C	T	T/C	A/T	T	C	C	A	T	T	C	T	-	A/G
<i>Fg/Fh</i>	T	T	T	A	T	C	C/T	A	T/C	T/C	C	T	-	A

*= heterozygotic in position 874/288 not differentiating between *F. hepatica* and *F. gigantica* (Bargues et al., 2021).

**= heterozygotic in position 834/248 not differentiating between *F. hepatica* and *F. gigantica* (Bargues et al., 2022).

2.3.3. rDNA ITS-1-5.8S-ITS-2 REGION SEQUENCES OBTAINED BY CLONING

A total of seven specimens from five localities were selected for cloning (**Table 2.9**), taking into account their peculiarities of double peak positions in their electropherograms obtained by direct sequencing. In addition, one sample with no double peaks was included as a control. A total of 56 rDNA complete intergenic sequences were finally obtained after individually sequencing eight different colonies per each of the seven aforementioned samples.

Table 2.9. Intraindividual sequence heterogeneity of the ITS-1-5.8S-ITS-2 rDNA region found in the cloned samples showing double peaks in key positions of their electropherograms.

Sample No.	No. in Map*	Localities (Administrative Units)	Host species	Type of clones** (Number of sequences)
1	6	Rajshahi/Naogaon/Shapahar	Buffalo	Fh.a (1); Fg.b (3); Cl-b (1); Cl-c (1); Cl-j (1); Cl-k (1)
2	7	Rajshahi/Naogaon/Naogaon Sadar	Sheep	Fh.a (1); Fg.b (4); Cl-e (1); Cl-f (1); Cl-g (1)
3	7	Rajshahi/Naogaon/Naogaon Sadar	Sheep	Fh.a (1); Fg.a (1); Fg.b (5); Cl-d (1)
4	10	Rangpur/Lalmonirhat/Lalmonirhat Sadar	Goat	Fg.b (4); Cl-a (1), Cl-d (1), Cl-e (1), Cl-m (1)
5	10	Rangpur/Lalmonirhat/Lalmonirhat Sadar	Sheep	Fh.a (2); Fg.b (3); Cl-a (1); Cl-h (1); Cl-i (1)
6	13	Khulna/Jhenaidah/Shailkupa	Cattle	Fg.b (7); Cl-l (1)
7***	14	Khulna/Jhenaidah/Jhenaidah Sadar	Cattle	Fg.b (8)

*See map in **Figure 2.3** for the number of locations.

Clone type nomenclature according to **Table 2.10.

***Positive control

Table 2.10. Haplotypes of the ITS-1, 5.8S and ITS-2 rDNA region found in the clones obtained in the seven cloned specimens, showing nucleotides in the positions differentiating between *Fasciola hepatica* and *F. gigantica*.

Haplotypes (pure and hybrid)	Clone code	Number of clones (n=56*)	Polymorphic sites													
			Intergenic Region (ITS-1, 5.8S, ITS-2)										Polymorphic sites			
			ITS-1					ITS-2								
24	114	208	286	306	797	821	860	866	917	924						
24	114	208	286	306	210	234	273	279	330	337						
<i>F. hepatica</i>																
	Cl-Fh.a	5	C	A	C	T	T	C	T	T	C	C	T	G		
<i>F. gigantica</i>	Cl-Fg.a	1	T	T	T	A	T	T	T	T	C	T	T	A		
<i>F. gigantica</i>	Cl-Fg.b	34*	T	T	T	A	T	C	C	C	T	T	T	A		
<i>Fg-Fh</i>	Cl-a	2	C	T	T	A	T	C	C	C	T	T	T	A		
<i>Fg-Fh</i>	Cl-b	1	C	A	T	A	T	C	C	C	T	T	T	A		
<i>Fg-Fh</i>	Cl-c	1	C	T	C	A	T	C	C	C	T	T	T	A		
<i>Fg-Fh</i>	Cl-d	2	C	A	C	T	C	C	C	C	T	T	T	G		
<i>Fg-Fh</i>	Cl-e	2	C	A	C	T	C	C	C	C	T	T	T	G		
<i>Fg-Fh</i>	Cl-f	1	C	A	C	T	C	C	C	T	T	T	T	A		
<i>Fg-Fh</i>	Cl-g	1	C	A	C	T	T	C	C	C	T	T	T	A		
<i>Fg-Fh</i>	Cl-h	1	T	A	C	T	C	C	C	C	T	T	T	A		
<i>Fg-Fh</i>	Cl-i	1	T	T	C	T	C	C	C	C	T	T	T	A		
<i>Fg-Fh</i>	Cl-j	1	T	T	C	T	C	C	C	C	T	T	T	A		
<i>Fg-Fh</i>	Cl-k	1	T	T	T	T	C	C	C	T	C	C	T	G		
<i>Fg-Fh</i>	Cl-l	1	T	T	T	A	T	C	C	T	C	C	T	G		
<i>Fg-Fh</i>	Cl-m	1	T	T	T	A	T	C	C	T	C	C	T	G		

Color in nucleotide positions: green = nucleotides characterizing *F. hepatica*; purple = nucleotides characterizing *F. gigantica*; black = nucleotides of polymorphic position not differentiating between *F. hepatica* and *F. gigantica*.

* Including the 8 clones of the positive control.

The 8 clones of the positive control proved to be “pure” *F. gigantica*, and among the other 48 clone sequences, 27 proved to be “pure” *F. gigantica* (56.25%), five were “pure” *F. hepatica* (10.42%), and the 16 remaining appeared to be mixed *F. gigantica*-*F. hepatica* (*Fg-Fh*) sequences (33.33%). Polymorphic sites at the positions differentiating *F. hepatica* from *F. gigantica* in the sequences of the 56 clones obtained are shown in **Table 2.10**.

The intra-individual analysis of the clone sequences within each of the six cloned specimens presenting double peaks evidenced a high heterogeneity of mixed sequences in the fluke specimens differing according to localities (**Table 2.9**):

- 6 different sequences (2 “pure” and 4 heterozygotic) in the fluke infecting buffalo from Shapahar;
- 5 different sequences (2 “pure” and 3 heterozygotic) in the fluke infecting a sheep from Naogaon Sadar;
- 4 different sequences (3 “pure” and 1 heterozygotic) in the fluke infecting another sheep from Naogaon Sadar;
- 5 different sequences (1 “pure” and 4 heterozygotic) in the fluke infecting a goat from Lalmonirhat Sadar;
- 5 different sequences (2 “pure” and 3 heterozygotic) in the fluke infecting a sheep from Lalmonirhat Sadar;
- 2 different sequences (1 “pure” and 1 heterozygotic) in the fluke infecting a cattle specimen from Shailkupa.

The eight clones of the control specimen infecting a cattle individual from Jhenaidah Sadar were all identical and proved to correspond to a “pure” *F. gigantea*, thus demonstrating the accuracy of the results of the cloning procedure.

2.4. DISCUSSION

2.4.1. LIVESTOCK HUSBANDRY PRACTICES AND *Fasciola* INFECTION RATES

Livestock is an essential component of Bangladesh's rural economy and the livelihoods of subsistence farmers. However, climate change and the emergence and re-emergence of certain diseases are affecting livestock health and production.

The results of our livestock surveys show that fascioliasis is widespread throughout Bangladesh and in all agro-ecological zones analyzed. It is important to note that we did not find any location where uninfected animals could be found, indicating a high risk of fascioliasis in all areas. Considering that buffalo, cattle, goats, and sheep are known reservoir hosts for liver flukes (Mas-Coma et al., 2020a, 2020b, 2021), it may be concluded that all livestock species are involved in the spread and maintenance of the fascioliasis transmission in the country. In addition, the ability of fasciolid metacercariae to infect different host species independent of the *Fasciola* host species isolate of origin (Valero and Mas-Coma, 2000; Valero et al., 2001, 2002), indicates that each of the four host species studied may act as a reservoir for the other host species.

The prevalence rates observed in the four livestock species prove to be very high and thus confirm the present problem of fascioliasis in Bangladesh. Notably, the prevalence rate of 35.37% detected in goats is the highest recorded for this species in Bangladesh, exceeding the previously reported range of 3.8-32% (Rahman et al., 2017). Similarly, the prevalence rate of 55.56% detected in buffaloes surpasses the previously reported range of 19-51% for this animal in the country (Rahman et al., 2017). Furthermore, our study reveals prevalence rates of 29.27% in cattle and 26.83% in sheep, which, although falling within the respective ranges of 15-66% and 8.40-31% previously reported for these livestock species in Bangladesh (Rahman et al., 2017), are still significantly elevated.

These observed prevalences also appear to be significantly high when compared with lowland plain areas of similar latitude in neighboring India. Epidemiological studies conducted in different geo-climatic zones (hills, tarai and plains) in six states of northern India reported prevalences of 13.90%, 10.79%, 2.78% and 2.35% in buffaloes, cattle, sheep and goats, respectively. Even during seasonal peaks, the highest prevalences recorded in these Indian study (11.84% in cattle, 15.57% in buffaloes, 4.60% in sheep, 2.71% in goats) were significantly lower than those observed in Bangladesh (Garg et al., 2009). In the nearby Indian state of Uttar Pradesh, mean and monthly peak prevalences of 2.02% and 13.30% in goats, and 1.69% and 5.10% in sheep, respectively, were also notably lower compared to Bangladesh (Yadav et al., 2007). Similarly, in the Indian state of Rajasthan,

prevalences were relatively low, with 12.68% in buffaloes, followed by 9.70% in cows, and 7.50% in goats (Swarnakar and Sanger, 2014). In the case of buffaloes, however, the behavioral tendency of this species to congregate in freshwater bodies underlie very high prevalences in some localities, such as 31.14% in northern Indian states (Garg et al., 2009), 37.50% in Uttar Pradesh (Gupta et al., 2008), and even up to a local 94% in the northeastern part of Uttar Pradesh (Singh and Agarwal, 1981), which fits a similarly impressively very high local prevalence of 72.0% found in the lymnaeid vector *Radix acuminata* in one transmission focus in the easternmost area of Gorakhpur of this Indian state, the so-far highest worldwide record of fasciolid infection in a lymnaeid population (Sunita et al., 2021).

An analysis of prevalence and intensity from an ecological and geographic perspective shows that the highest infection rates are observed in floodplains and in the western part of the country. Floodplains provide favorable habitats for fascioliasis transmission foci, given the dependence of aquatic/amphibious lymnaeid snail vectors on surface freshwater. These findings are consistent with the predicted geographic distribution of animal fascioliasis in Bangladesh based on a risk map developed using three years of data (Rahman et al., 2017).

Interestingly, areas of high prevalence and intensity in western Bangladesh coincide with areas of transboundary importation of animals from the state of West Bengal, India. Bangladesh currently has approximately 24.20 million cattle (Bargues and Mas-Coma, 2005), 26.60 million goats (Rahman et al., 2022), 3.40 million sheep (Islam et

al., 2021), and 1.47 million buffaloes (Habib et al., 2017). Despite having one of the highest livestock densities, the country is in a similar situation to Vietnam, unable to meet the growing demand for meat, milk, and leather due to rising incomes, population growth, and urban sprawl.

This is particularly true for cattle, by far one of the most important livestock species in the country. Bangladesh has a density of 145 animals per Km², which is significantly higher compared to 90 animals per Km² in India, 30 in Ethiopia, and 20 in Brazil (Ali et al., 2020). Despite these numbers, many bulls and oxen are regularly smuggled into Bangladesh from India to meet the country's domestic demand. In fact, approximately 40% of the cattle slaughtered annually are imported through cross-border trade. The informal supply chain between India and Bangladesh for cross-border trade in cattle amounts to 1.5 million head per year (approximately 20,000 to 30,000 cattle per day). Traders from both India and Bangladesh bring thousands of cattle to the Putkhali market, which is located close to the border and only five kilometers from the Benapole port station on the Kolkata-Dhaka route (Ali et al., 2020). However, this trade seems to have decreased since 2019.

The situation is similar for goats. Goat production and marketing is one of the oldest and most widespread livestock enterprises in Bangladesh, with smallholder farmers as the main actors in goat farming. Approximately 65% of households are involved in goat rearing as either a primary or secondary occupation. An analysis of the goat supply chain shows that goats from local areas are distributed throughout the country

(Chowdhury et al., 2015). The indigenous Black Bengal goat is a well-adapted and highly productive breed that can be easily reared on low-quality fodder with minimal investment. However, infectious diseases pose a significant threat to productivity, resulting in a mortality rate of nearly 45% (Chowdhury et al., 2015; Rahman et al., 2022). This may lead local farmers to import goats. Imported exotic breeds and/or commercial strains have resulted in crossbreeding with local species/varieties (Bhuiyan, 2014). These crossbred animals can be seen in Bangladesh today (Afroz et al., 2010). Unfortunately, information on goat importation in Bangladesh is not available in the literature or directly from farmers as it is kept under strict confidentiality (Chowdhury et al., 2015).

Imported animals should comply with the Animal Quarantine Act (Act no-VI of 2005) enacted by the Parliament of Bangladesh. Unfortunately, due to lack of manpower and funds, animal quarantine measures are not applied to informal import practices or officially registered imports (Ali et al., 2020). In addition, when an animal is infected most owners are unable to treat it due to lack of resources, and if treated, their choice of medication is based on the cheapest or availability in local pharmacies, without following any veterinary advice (Nath et al., 2022). In the case of fascioliasis, infected imported animals are neither screened nor treated for fascioliasis prior to introduction into local herds, allowing infected specimens of *F. hepatica* to enter the country.

Summing up, high rates of *Fasciola* infection in buffalo, cattle, goats and sheep indicate a high risk of human infection throughout Bangladesh. This is significant given that 84% of the population lives in rural areas and 75% of people depend on livestock for their livelihood and are therefore frequently exposed. This suggests that human fascioliasis may be underestimated in the country and that local physicians should be aware of the cause and diagnosis of this disease. Additionally, the higher pathogenicity of *F. gigantica* compared to *F. hepatica* should be taken into account (Valero et al., 2016).

2.4.2. Fg-CH3A “PURE” *F. gigantica* HAPLOTYPE

The geographical location of Bangladesh has played a crucial role as a crossroads for human-guided movements of domesticated animals in South Asia for thousands of years. In the West, the use of cattle, equines, dromedaries, goats, and sheep as pack and subsistence animals along the Grand Trunk Road enabled the arrival and colonization of Bangladesh by the warm lowland-preferring fasciolid species *F. gigantica* (Mas-Coma et al., 2022a). In the East, Bangladesh traded with southern China and further with South East Asia via the Tea-Horse Road, which had a northern route through Indian Assam and a southern route through Manipur, both passing through the mountainous regions of upper Burma (present-day Myanmar) and ending in Bengal. Equines, cattle and yaks were used as pack animals on the Tea-Horse Road (Mas-

Coma et al., 2022a). The mule, a hardy pack animal well suited for long-distance travel over difficult mountainous terrain, played an important role (Mitchell, 2018). Recent evidence suggests that mules serve as efficient reservoirs for the transmission of fascioliasis (Mera y Sierra et al., 2020). This might explain the presence of the widespread combined haplotype Fg-CH3A in livestock throughout Bangladesh, which is identical to the haplotype recently reported in Vietnam (GenBank Acc. No. MN970009) (Le et al., 2020).

2.4.3. RECENT PAST HYBRIDIZATION EVENT

Surprisingly, "pure" *F. hepatica* haplotypes have been detected in the sequences of the clones obtained. It is possible that "pure" *F. hepatica* could have arrived in the past from the north via the Tea-Horse Road, passing through the Tibetan capital Lhasa, and then via the Indian mountain pass in the state of Sikkim, located between Nepal and Bhutan.

In fact, the Tea-Horse Road played a crucial role in connecting the eastern routes of the Northern Silk Road, facilitating *F. hepatica* dispersal due to the region's more favorable cooler temperatures (Mas-Coma et al., 2022a). It is also worth noting that *Galba truncatula*, the primary lymnaeid vector of *F. hepatica*, is known to be present in both Nepal and Bhutan today. Transhumant agropastoralism has traditionally been practiced in western Bhutan since ancient times, and there was a

secondary south-north road that started at the Jaldhaka River in India, north of present-day Bangladesh (Mas-Coma et al., 2022a). However, the absence of *Galba/Fossaria* snail species in the lowlands of Bangladesh and the surrounding regions of India, together with the high temperatures in these lowlands that do not match the temperature thresholds for *F. hepatica* development, make it difficult to imagine its historical entry into the area.

Nevertheless, the detection of "pure" *F. hepatica* sequences only in clones derived from specimens with heterozygous positions implies the recent evolutionary hybridization events. In other words, the results suggest that native "pure" *F. gigantica* specimens have interbred with introduced *F. hepatica* specimens in the recent past, leaving insufficient time for concerted evolution to homogenize the rDNA operon. The presence of different sequences in clones obtained from several individual fasciolids further supports this phenomenon.

Although the exact time required for the rDNA operon to homogenize different previously coexisting sequences remains unknown, recent studies suggest that it may take several generations over a period of years when only lymnaeid vector species specific to a particular *Fasciola* species are present (Bargues et al., 2021). In Bangladesh, only two lymnaeid species belonging to the *Radix* group, namely *R. acuminata* and *R. luteola*, are present (Mas-Coma et al., 2022a). This means that the evolutionary filter imposed by the vector species will favor the *F. gigantica* genotype (Bargues et al., 2021).

Furthermore, the detection of different sequences coexisting in the rDNA operon of cloned specimens provides clear evidence not only of recent hybridizations but also of crossbreeding between parental specimens that were already hybrids, indicating repeated, overlapping, and rapidly evolving hybridization events.

The various formal and informal procedures for livestock importation in Bangladesh in recent years, driven by the increasing national demand, can explain the wide variation in hybrid fasciolid specimens found. This is consistent with previous reports of intermediate forms of fasciolids in the country (Ahasan et al., 2016). Official records of livestock import during the period 1973-2014 provide information on cattle imported from other countries, as illustrated in **Table 2.11**, where countries, such as Australia and Spain where only *F. hepatica* is endemic. This fact underscores the need to implement appropriate quarantine measures, to include liver fluke diagnosis and treatment for imported animals, and to make efforts to help rural people control the disease in their agricultural management.

Different banding patterns observed by PCR restriction fragment length polymorphism (PCR-RFLP) analysis of the ITS-1 region in fasciolids from livestock in Bangladesh also suggest the presence of both *F. gigantica* and *Fasciola* sp. sequences within individual flukes. However, this technique does not allow for sequence determination or nucleotide position analysis.

Table 2.11. Details of the official importation of cattle into Bangladesh, subject to the necessary government approvals, during the period from 1973 to 2014.

Year	Country of origin	Breed	Cow/Heifer	Bull/Bullcalf	Total no. of head	Remarks	Information source*
1973	Australia	Friesian	62	2	64	Gift	1
1973	Australia	Jersey	58	3	61	Gift	2
1987	Pakistan	Sahiwal	95	5	100	Import	3
1989	Spain	Friesian	69	3	72	Gift	4
1990	Pakistan	Sahiwal	80	20	100	Import	5
1992	Pakistan	Sahiwal	96	-	96	Import	6
1993	Australia	AFS**	150	-	150	Import	7
1995	Australia	Friesian	250	4	254	Import	8
2006	Australia	Friesian	25 (Pregnant)	-	25	Import	9
2012	India***	-	-	-	1287800	Influx^	-
2013	India***	-	-	-	1796904	Influx^	-
2013	Australia		-	7	7	Import	10
2013	Australia	Friesian	-	17	17	Import	11
2014	Australia	Friesian	-	2	2	Import	12
2014	India***	-	-	-	1746514	Influx^	-

* 1-7 and 9: Central Cattle Breeding Station, Department of Livestock Services, Bangladesh;

8: Personal communication with Maj. (Rt.) Aktaruzzaman, owner of the Gochhata Dairy & Fish Farm;

10-12: Department of Livestock Services, Bangladesh;

** AFS: Australian Friesian;

*** Information from National Board of Revenue, Segunbaagicha, Dhaka, earned influx revenue but details data not available.

^ influx: local term used in Bangladesh to refer to an indiscriminately, randomly importation (lacking records and carried out without following standard importation procedures)

Furthermore, a 535 bp fragment of the mtDNA *nad1* gene was found to represent *F. gigantica* sequences similar to those known from other Asian countries, suggesting no introgression by hybridization (Mohanta et al., 2014). Interestingly, cloning and sequencing of a fasciolid specimen infecting cattle from Bangladesh revealed a combination of an *F. gigantica* haplotype and an *F. hepatica* in the nuclear intron 4 of domain 2 of the taurocyamine kinase gene (TkD2Int4), indicating hybridization (Saijuntha et al., 2018).

The presence of heterozygotic sequences concentrated in the western parts of Bangladesh (with the exception of the two central localities around Mymensingh, which can be attributed to the demands of a large city) (compare **Table 2.6** and **Figure 2.3**) further suggests the role of livestock importation from neighboring India. In addition, the analysis of the distribution of heterozygotic hybrid combined haplotypes by host species suggests that goats may play a critical role in the spread of hybrid specimens, mainly through unregulated small-scale household practices and the goat supply chain that distributes animals throughout the country.

The high proportion of hybrid fasciolids found in the livestock analyzed also indicates a potential increasing trend of infection in animals, taking into account the usually higher dispersal capacity of hybrid forms in cases where they prove to be viable, i.e. transmissible by the locally available lymnaeid snail vector species. Consequently, human infection risk may also increase, similar to what has recently occurred in Vietnam (Nguyen et al., 2018).

**CHAPTER III. MOLECULAR GENOTYPING
OF *Schistosoma* STRAINS INFECTING SUB-
SAHARAN MIGRANTS**



3.1. INTRODUCTION

Human schistosomiasis is considered an imported disease in Europe. The reported cases in the European continent are associated with travellers arriving from tropical countries (Witzenrath et al., 2017; Marchese et al., 2018) or with migrants from endemic countries who are settled in Europe (Beltrame et al., 2017; Brindicci et al., 2017; Salas-Coronas et al., 2020). This second group represents the largest burden of diagnosed schistosomiasis in Europe, as the percentage of infected individuals is remarkably higher among migrants originating from endemic countries, particularly sub-Saharan Africa. This significant proportion reflects the high levels of transmission and the limited access to diagnosis and treatment they have been through in their respective countries of origin (Gabrielli and Garba Djirmay, 2023).

In general, patients from endemic areas are exposed to constant reinfections throughout their lives, making chronic schistosomiasis the most prevalent form of the disease in those countries. Therefore its diagnosis predominates among their medical visits in Europe (Norman et al., 2020).

Chronic schistosomiasis can lead to severe long-term complications if it remains untreated, highlighting the critical importance of accurate diagnosis and treatment. However, several factors continue to hinder the control of imported schistosomiasis and perpetuate its morbidity in Europe. These include barriers to health care faced by migrants, differences in knowledge of the disease among health workers

in non-endemic countries, and heterogeneity in diagnostic pathways and therapeutic doses (Comelli et al., 2020).

The lack of treatment of patients with schistosomiasis could potentially lead to the emergence of autochthonous transmission foci in areas of the European continent where competent intermediate hosts are present. In fact, during the last two centuries, three foci have been reported in three different countries: Portugal, France, and Spain (Gabrielli and Garba Djirmay, 2023).

In the early 20th century, autochthonous transmission was reported in Estoi, a locality in southern Portugal. The transmission continued until 1966, and it is believed that the introduction may have occurred by travelers from Angola, which was a Portuguese colony at the time. Autochthonous transmission was possible because Portugal harbors snail vectors for *S. haematobium*, such as *B. truncatus*, *B. contortus*, and *P. metidjensis* (Gabrielli and Garba Djirmay, 2023).

In the 21st century, specifically in 2013, new cases of urogenital schistosomiasis were detected in European patients who had never traveled to an endemic area. This infection, which reached up to 106 confirmed cases, was acquired in the Cavu River, a popular bathing spot for tourists in southern Corsica, France (Boissier et al., 2015; Noël et al., 2018). The infection was caused by a hybrid strain of *S. haematobium*-*S. bovis* imported from Senegal (Boissier et al., 2016). In 2014, French authorities banned bathing in the Cavu River, and no cases related to infections acquired on the French island were notified that summer.

However, new infections associated with the river were reported in 2015 and 2016 (Berry et al., 2016), and consequently, schistosomiasis became a notifiable disease in the country in 2017. Surprisingly, new infections were reported in 2019, this time associated with the Solenzara River in the southeastern part of the island. Genotyping revealed the involvement of the same hybrid strain described in previous outbreaks, suggesting ongoing transmission rather than reintroduction and indicating possible endemicity (Rothe et al., 2021).

Several hypotheses have been developed to understand the maintenance of transmission on the Mediterranean island. One of them was the persistence of infected snails from one year to another. Experimental studies demonstrated the ability of the intermediate snail, *B. truncatus*, to hibernate with the infection and produce viable cercariae when optimal temperatures are reached (Mulero et al., 2019). Nevertheless, this would not explain the appearance of the parasite in another river. Another hypothesis was the existence of reservoir animals that could introduce the disease into a nearby river. Unfortunately, no infections were detected in local livestock, and the only infected animals found were two rats (Oleaga et al., 2019), so there was insufficient evidence to assign them a significant role in transmission. While it is true that there may be other animal reservoirs that have not yet been analyzed, such as *Ovis aries musimon*, a species of wild sheep native of Corsica, the most likely option seems to be that infected humans continue to maintain infection in the island (Rothe et al., 2021; Wellinghausen et al., 2022).

The most recent report of autochthonous transmission occurred in Spain in 2019. In this case, it was a retrospective report, as the infection specifically occurred in 2003. Four farmers were infected when bathing in an artificial irrigation pond used for watering crops in surrounding greenhouses in Almeria (a province in southern Spain) (Salas-Coronas et al., 2021). Parasitological samples for molecular studies of the parasite involved in this outbreak were not obtained. However, the presence of *B. truncatus* has been documented in these irrigation ponds (Martínez-Ortí et al., 2015, 2019, 2022), as has its ability to transmit both *S. haematobium* and the hybrid *S. haematobium* x *S. bovis* strain reported in Corsica (Mulero et al., 2019).

The presence of *B. truncatus* has been reported in other Spanish provinces, such as Girona, Barcelona, Tarragona, Alicante, Malaga, Huelva, Pontevedra, A Coruña, and the Balearic Islands (**Figure 3.1**) (Silva et al., 1977; Muñoz-Antoli et al., 2006; Soler et al., 2006; Martínez-Ortí et al., 2015; Kincaid-Smith et al., 2017; Martínez-Ortí et al., 2022). Furthermore, like its neighboring country Portugal, Spain is the other European country where the presence of *P. metidjensis* has been reported (Kincaid-Smith et al., 2017) (**Figure 3.1**).

As demonstrated by the 2003 schistosomiasis outbreak, Spain, like other Mediterranean regions, is a country susceptible to autochthonous transmission due to the presence of competent intermediate hosts. It is also one of the European countries with the highest number of reported schistosomiasis cases in recent years. This is due to the significant flow of migrants from sub-Saharan countries, many

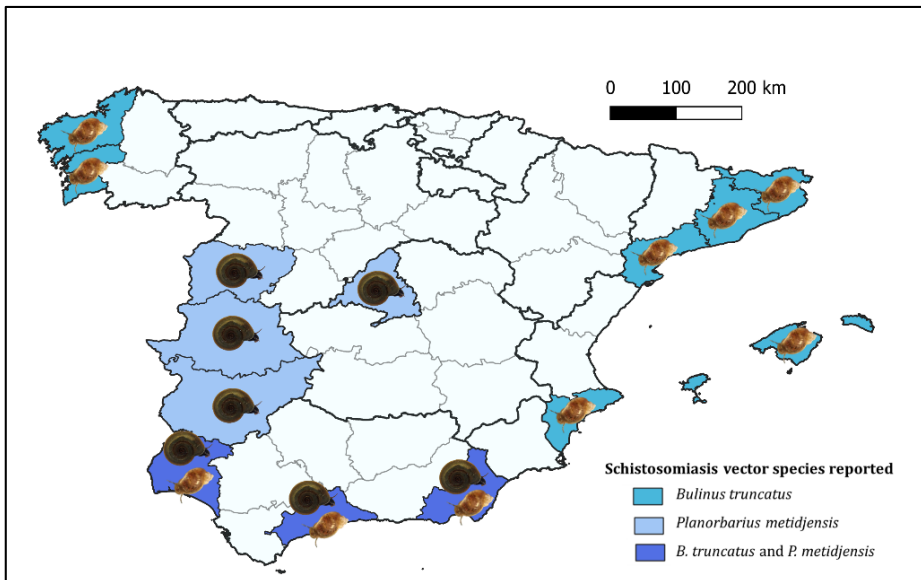


Figure 3.1. Spanish provinces where the presence of *B. truncatus* and *P. metidjensis* have been reported.

of them irregular, compared to other European countries (Gabrielli and Garba Djirmay, 2023). The most commonly reported infections in Spain are those caused by *S. mansoni* and *S. haematobium* (Leblanc et al., 2017; Roure et al., 2017, 2022; Salas-Coronas et al., 2020; Castillo-Fernández et al., 2021), the two species that represent the highest burden of infection in sub-Saharan Africa, with some sporadic reports of *S. intercalatum* (Salas-Coronas et al., 2018; Mendoza-Palomar et al., 2020), also present on the African continent. Species determination in these Spanish studies, as in most reports from other European countries, is based only on the position of the spine within the egg, lateral or terminal, and the site of egg excretion, urine or feces. These two characteristics do not allow to detect infections produced by hybrids, which are becoming increasingly common in Africa. As discussed in

Section 1.5, hybrids produced by *S. haematobium* with livestock species of high veterinary importance stand out due to their potential zoonotic transmission (Gower et al., 2017; Léger and Webster, 2017).

Here we aimed to know the different species of *Schistosoma* arriving in Spain. To this end, we analyzed 699 *Schistosoma* eggs obtained from urine and stool of 51 migrant patients from 8 different sub-Saharan countries residing in Spain. Molecular characterization of these 699 eggs was performed, including mitochondrial (*cox1*) and nuclear (ITSs and 18S) markers, allowing the identification of pure and hybrid species. In addition, a description of the different genetic haplotypes detected for these markers was made.

3.2. MATERIAL AND METHODS

3.2.1. MATERIAL

A total of 699 eggs collected from 51 migrants, 50 males and 1 female (aged 17-52 years, mean 26.43), with a mean residence in Spain of 23.92 (1-192) months, were analyzed. The migrants came from 8 different sub-Saharan African countries (**Figure 3.2**), with Mali (n = 23), Senegal (n = 16), and Mauritania (n = 6) being the most common countries of origin. All patients were diagnosed and treated at the Tropical Medicine Unit (TMU) of Hospital de Poniente (El Ejido, Almería, Spain) or at the TMU of Vall d'Hebron University Hospital, Barcelona (Spain) between October 2016 and June 2022.

Sub-Saharan migrant patients will be referred to the TMU when an imported disease is suspected or diagnosed in primary care. Both TMUs have a very similar screening protocol for newly arrived migrants from sub-Saharan Africa. The protocol includes medical history, epidemiological data, complete physical examination, and several tests: complete blood count, liver and kidney function tests, serology for syphilis, HIV, HBV, and HCV, tuberculin skin test, parasitology in stool (three concentrated stool samples using the Ritchie method) and urine (one concentrated urine sample [10 cc]), serology for *Strongyloides* and *Schistosoma*, and Knott and/or saponin tests for microfilariae (Salas-Coronas et al., 2013, 2018, 2020).



Figure 3.2. Countries of origin of migrants diagnosed with schistosomiasis in Spain (Almeria or Barcelona).

Only urine or fecal samples from patients serologically positive for schistosomiasis and diagnosed with genitourinary or intestinal schistosomiasis in one of the TMUs were sent to the Department of Parasitology, University of Valencia, for molecular identification.

3.2.2. URINE AND STOOL EXAMINATION

Urine samples were filtered using a sterilised nylon cell filter with a pore size of 40 μm (Falcon®, Durham, NC, USA) using a standard 20 ml plastic Pasteur pipette. The eggs were pulled out from the filter with pressurised 0.9% NaCl saline solution into a glass Petri dish and examined under a stereomicroscope. The maximum number of eggs per patient was collected with a 10 μl micropipette for individual photography of each using a fluorescence light microscope (Leica DMR 72-89663) and subsequent individualization in 70% ethanol for molecular analysis. A low percentage of these eggs hatched after having been photographed, so their miracidia were individually fixed. The remaining pool of eggs, was collected and stored in 70% ethanol (**Figure 3.3**).

Stool samples were appropriately filtered, using the same saline solution, through a column of decreasing pore size metal sieves (0.5 mm, 0.25 mm, 0.125 mm, 0.1 mm and 0.04 mm) to isolate parasite eggs from the debris. All eggs trapped on the last sieve were recovered in a Petri dish. From here, the eggs were collected and individualised to be photographed and later fixed in 70% ethanol for molecular analyses.

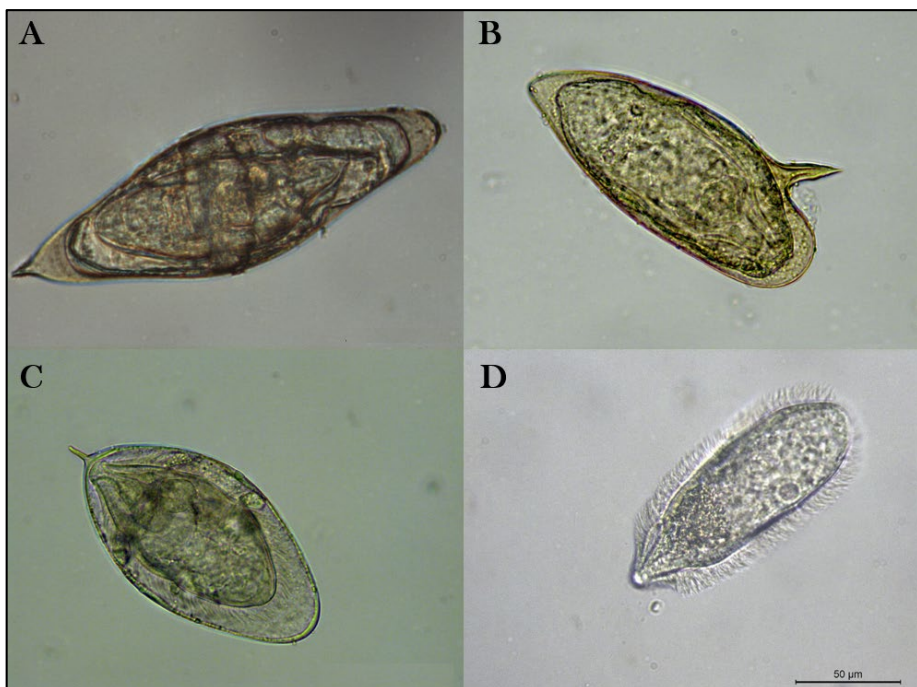


Figure 3.3. *Schistosoma* spp. eggs and miracidia: **A)** *Schistosoma guineensis* and **B)** *S. mansoni* both recovered in stool samples; **C)** *S. haematobium* recovered in urine; and **D)** a newly hatched miracidium after taking the photograph.

3.2.3. MOLECULAR METHODS

3.2.3.1. DNA EXTRACTION

Genomic DNA was extracted individually from each of 699 eggs, of which 654 terminal-spined eggs recovered from 49 urine samples, and 35 lateral-spined and 10 terminal-spined eggs from 3 stool samples (**Table 3.1**), using the InstaGene™ Matrix kit (Bio-Rad Laboratories® CA, USA) according to the manufacturer's instructions, and stored at -20 °C until further analysis.

Table 3.1. Number of eggs processed according to the country of origin of the patients and the type of sample from which they were recovered.

Patients		Type of sample analyzed	Eggs processed	
Country of origin	Number of patients		Terminal-spined eggs	Lateral-spined eggs
Guinea-Bissau	2	Urine	24	-
Côte d'Ivoire	1	Urine	13	-
Mali	23	Urine	273	-
Mauritania	6	Urine	120	-
Senegal	15	Urine	192	-
Senegal	1*	Urine	16	-
The Gambia	1	Urine	16	-
Guinea	1	Stool	-	12
Equatorial Guinea	1	Stool	10	-
Senegal	1*	Stool	-	23
Total	51		664	35

*Both urine and stool samples were received from a single patient originally from Senegal.

3.2.3.2. RAPID DIAGNOSTIC PCR

To identify the species-specific mitotype of each sample, a rapid diagnostic PCR (RD-PCR) based on a multiplex one-step PCR of the *cox1* marker was performed from each of the 699 eggs (**Table 3.2**).

Table 3.2. Total number of eggs processed and finally successfully genotyped for the creation of a mito-nuclear signature or haplotype identification, along with the markers and techniques used for each purpose. bp = base pairs.

MITO-NUCLEAR SIGNATURE						HAPLOTYPE IDENTIFICATION							
cox1 RD-PCR			ITS-2 sequencing (313 bp)			18S sequencing (1369 bp)		ITS-1, 5.8S, ITS-2 sequencing (927 bp)			cox1 sequencing (1024/1052 bp)		
Urine	Stool		Urine	Stool		Urine		Urine	Stool		Urine	Stool	
654	10	35	654	10	35	27	222	10	20	222	10	20	
Processed eggs						27				20			
Total			699			27	252			252			
Genotyped eggs	598	Under*	35	598	9	35	25	203	9	20	203	9	20
Total	633		642		25	232			232				

*Undetermined eggs by RD-PCR

For this purpose, a single universal forward primer and three species-specific reverse primers amplifying different length for *S. bovis* (306 bp), *S. mansoni* (375 bp) and *S. haematobium* (543 bp) were used (Webster et al., 2010; Van den Broeck et al., 2011) (**Appendix B.1**). The PCR conditions were 3 min at 95 °C, followed by 35 cycles of 30 secs at 94 °C, 1.30 min at 58 °C and 1.30 min at 72 °C followed by a final cycle at 72 °C for 7 min. The *cox1* RD-PCR products were visualized by electrophoresis on a 2.5% agarose gel stained with GelRed (Biotium) and photographed using the UVP gel documentation system (Reguera-Gomez et al., 2021, 2023).

3.2.3.3. rDNA AND mtDNA PCR AMPLIFICATION AND SEQUENCING

The information provided by the maternally inherited mtDNA profiling by RD-PCR was complemented with the complete ITS-2 of the rDNA to create a mito-nuclear signature as has been performed in other studies (Angora et al., 2022; Onyekwere et al., 2022b). PCR amplification and sequencing were performed independently on each of the 699 eggs (**Table 3.2**). Primers 3Sbis and A28S (**Appendix B.1**), which partially amplified the 5.8S gene (139 bp) and the entire ITS-2 (313 bp) were used. The PCR was programmed for an initial activation phase of the DNA polymerase at 94 °C for 4 min, followed by 32 amplification cycles consisting of a denaturation step at 94 °C for 55 secs, followed by primer annealing at 55-62 °C for 1 min and elongation

step at 72 °C for 1.30 min each, and a final extension step at 72 °C for 5 min followed by a final cooling at 4 °C (Reguera-Gomez et al., 2021, 2023).

A complementary partial amplification and sequencing of the 5' end of the 18S nuclear ribosomal gene (1369 bp) was performed only in eggs where double peaks were found at polymorphic positions of the ITS-2 sequence discriminating *S. haematobium* from *S. bovis* or *S. curassoni* (**Table 3.3**). For this partial amplification of the 18S, two sets of primers BUG1 and BUG608 and Lim463 and Lim1283 (**Appendix B.1**) were used, amplifying two "concatenated" 18S fragments (626 bp + 844 bp). PCR conditions were carried out as previously described (Bargues et al., 2022), with an initial step of 3 minutes at 94 °C, followed by 35 cycles of 30 secs at 94 °C, 40 secs at 50 °C and 1.50 min at 72 °C each, ending with a 7-minute cycle at 72 °C.

A random subsample of 252 eggs was used for haplotype identification by full ITS-1-5.8S-ITS-2 (927 bp) and partial *cox1* mtDNA (1024 bp/1052 bp) amplification and sequencing. Of this subsample of 252 eggs: 222 were terminal-spined eggs collected from the urine representing the 6 countries of origin of 49 sub-Saharan migrants diagnosed with urinary schistosomiasis; and 30 (10 eggs per patient) were eggs recovered from the stool of the three patients diagnosed with intestinal schistosomiasis, of which 20 were lateral and 10 terminal-spined eggs. For the complete ITS-1-5.8S-ITS-2, primers BD1 (Luton et al., 1992) and 4S (**Appendix B.1**) were used to amplify the complete ITS-1 (458 bp) and the partial 5.8S gene (97 bp) in order to complete the

whole region by joining it with the already sequenced partial of 5.8S gene and ITS-2, and using the same PCR conditions described above for partial 5.8S and the entire ITS-2 (Reguera-Gomez et al., 2021, 2023).

For the partial amplification and sequencing of *cox1*, the primers Cox1_schist F and Cox1_schist R (Lockyer et al., 2003) (**Appendix B.1**) were used, and PCR conditions were performed with an activation step of 3 min at 95 °C, continued by 35-40 cycles of 1 min at 95 °C, 30 secs at 45-50 °C, and 2 min at 72 °C, and a final extension of 7 min at 72 °C (Moné et al., 2015).

Table 3.3. rDNA polymorphic positions. **A)** The complete intergenic region (including ITS-1, 5.8S and ITS-2) and **B)** 18S polymorphic positions discriminating the three *Schistosoma* species: *Schistosoma haematobium*, *S. bovis* and *S. curassoni* (Table modified from Blin et al., 2023).

A		ITS-1, 5.8S and ITS-2 polymorphic positions				
<i>specie</i>	GenBank Acc N°	ITS-1		ITS-2		
		51	703	758	808	878
<i>S. haematobium</i>	OX103963	A	G	C	G	C
<i>S. bovis</i>	OX104095	G	A	T	A	T
<i>S. curassoni</i>	MT580946	A	A	T	A	T

B		18S polymorphic positions				
<i>specie</i>	GenBank Acc N°	225	250	297	685	1184
<i>S. haematobium</i>	Z11976	T	C	T	T	A
<i>S. haematobium</i>	OX103963	T	C	T	T	G
<i>S. bovis</i>	OX104095	C	T	C	C	G
<i>S. curassoni</i>	AY157236	T	T	T	T	G

Each of the DNA markers (ITS-2, 18S, ITS-1-5.8S-ITS-2 and *cox1*) was PCR amplified independently for each egg. PCR amplifications were performed in a Veriti 96-well thermal cycler (Applied Biosystems, Thermo Fisher Scientific, Waltham, MA USA) and PCR products were purified using the Ultra Clean™ PCR Clean-up DNA Purification System (MoBio, Solana Beach, CA, USA) according to the manufacturer's protocol and resuspended in 50 µl of 10 mM TE buffer (pH 7.6). Final DNA concentration (in µg /ml) and the absorbance at 260/280 nm were determined using an Eppendorf BioPhotometer (Eppendorf, Hamburg, Germany).

The purified PCR product from each PCR amplification was used for sequencing on both strands by the dideoxy chain termination method. This was performed using the Taq dye terminator chemistry kit on an Applied Biosystems 3730xl DNA Analyzer (Applied Biosystems, Foster City, CA, USA) using the same amplification PCR primers.

3.2.4. SEQUENCE ANALYSIS

The software Sequencher v. 5.4.6 (Gene Codes Co. MI, USA) was used to edit and assemble the sequences of the 18S, ITS-1-5.8S-ITS-2 and *cox1* markers, and ClustalW to align them by means of default parameters in MEGA X software (Kumar et al., 2018). Corresponding penalties for gaps were included in pairwise and multiple alignments. Total character differences were used to measure the divergence of the sequences within and among each one of the markers. All changes,

comprising transitions (ts), transversions (tv) and insertions/deletions (indels), were considered as character states in MEGA X.

In the case of ITSs rDNA sequences, a careful inspection of all nucleotide positions in the raw sequence chromatograms allowing the detection of sequence polymorphisms between *S. haematobium* and *S. bovis* or *S. curassoni* was checked to identify the presence of possible heterozygosity, especially at the polymorphic positions that differentiate between those species, as previously described (**Table 3.3.A**) (Webster et al., 2013; Angora et al., 2020; Savassi et al., 2020). In the case of the 18S rDNA sequence, the target conserved positions of the alignment allowing the distinction between *S. haematobium*, *S. bovis* and *S. curassoni* were carefully checked for final species adscription (**Table 3.3.B**) (Blin et al., 2023). The corresponding symbol of the International Union of Pure and Applied Chemistry (IUPAC) code for incomplete nucleic acid specification was used when chromatograms showed clearly overlapping secondary peaks.

The aligned sequences were collapsed into haplotypes using the ALTER web server (Glez-Peña et al., 2010), with gaps counted as differences. Homologies were determined using the BLAST program from the National Center for Biotechnology Information website (<http://www.ncbi.nlm.nih.gov/BLAST>).

Comparative sequence analyses of the ITS-1, 5.8S and ITS-2 rDNA region, were performed using complete sequences or sufficiently

long fragments obtained from GenBank and belonging to *Schistosoma* species in the same clade as our sequences (**Appendix B.2**).

For the 18S, seven sequences were used for comparison purposes: three from *S. haematobium* (Z11976, OX103963 and OX104046), three from *S. bovis* (OX104095, OX103954 and AY318828), and one from *S. curassoni* (AY157236). These seven sequences were long enough to compare the five conserved target positions (225, 250, 297, 685 and 1184) included in our partial 18S amplification that allow differentiation between the three species (**Table 3.3.B**).

For the comparative analysis of the *cox1* sequences, due to the variability of this marker, the sequence comparisons were restricted to those sequences that showed 100% coverage and greater than 99% similarity in BLAST (**Appendix B.3**) with our obtained sequences.

3.2.5. MITO-NUCLEAR SIGNATURE CODE AND HYBRID DEFINITION

A mito-nuclear signature based on RD-PCR of *cox1* and ITS-2 sequencing analysis was used to determine species and identify hybrids. In the mito-nuclear signature, the species identity is indicated by a two-letter abbreviation (e.g., *S. haematobium* = *Sh*). It was assumed that the *cox1* gene is a mitochondrial marker of haploid inheritance, so only one allele is indicated by the abbreviation of the species involved. Whereas

ITS-2 is a marker of biparental inheritance, so each allele is indicated by the 2-letter abbreviation of the identified species. For instance, the mitochondrial signature for defining the genetic profile of *S. haematobium* will be *S. haematobium* *cox1* x *S. haematobium* ITS-2 = *ShxShSh*.

Hybrid eggs are defined when the *cox1* and ITS-2 markers are read together and the results are discordant in species assignment (e.g., *S. bovis* *cox1* x *S. haematobium* ITS-2 = *SbxShSh*) and/or when heterozygotic positions are observed in the ITS-2 sequences at points that discriminate between species (e.g., *S. haematobium* *cox1* x *S. haematobium* x *S. bovis* ITS-2 = *ShxShSb*).

In the specific case of *S. haematobium*, *S. bovis*, and *S. curassoni*, the ITS-2 allows differentiation between *S. haematobium* and *S. bovis* or *S. curassoni*, but not between *S. bovis* and *S. curassoni*. Therefore, when obtaining *S. haematobium* x *S. bovis/curassoni* ITS-2, the 18S marker is used for hybrid identification.

If no discordance is observed in mito-nuclear signature, eggs are considered pure.

3.2.6. HAPLOTYPE CODE NOMENCLATURE

The haplotype nomenclature used is organized by first identifying the species with a two-letter abbreviation (or, in the case of hybrids, the species involved), second the genetic marker used (18S,

ITSs, or *cox1*), and third the haplotype number (in the case of hybrid haplotypes, these are indicated by Htz).

As explained above, an example of a pure *S. curassoni* haplotype from partial 18S sequencing would be Sc-18S-H1, while a hybrid *S. haematobium* x *S. curassoni* haplotype for this marker would be ShxSc-18S-Htz1. An example of a pure *S. haematobium* haplotype from the complete intergenic region sequencing would be Sh-ITSs-H1, and a hybrid *S. haematobium* x *S. curassoni* haplotype would be ShxSc-ITSs-Htz1. Finally, an example of a pure *S. haematobium* haplotype from partial sequencing of *cox1* would be Sh-*cox1*-H1.

3.2.7. PHYLOGENETIC ANALYSIS

Phylogenetic analysis was carried out including all the *S. haematobium*, *S. bovis*, *S. guineensis* and *S. mansoni* *cox1* haplotypes identified in this study plus reference haplotypes obtained from GenBank Nucleotide Database (**Appendix B.4**). The data matrix included 73 sequences (59 haplotypes from this study + 14 reference sequences) and 1024 positions in the final data set. *S. japonicum* (KY196417) was used as outgroup. The best substitution model selection analysis was run in MEGA X, considering the BIC (Bayesian Information Criterion) scores, the AICc (Akaike Information Criterion, corrected) value, the Maximum Likelihood (lnL) value, and the number of parameters (including branch lengths) for each model. The evolutionary history was inferred by using the Maximum Likelihood (ML) method. The initial tree(s) for the

heuristic search were automatically obtained using the Nearest-Neighbor-Interchange (NNI) method by applying the Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with the superior log likelihood value. In order to assess the reliability of the nodes in the trees, a bootstrap analysis with 1,000 replicates was carried out using the Bootstrap method in MEGA X.

3.2.8. ETHICS STATEMENT

This study was approved by the Ethics Committee of each hospital (protocol Schis-01-UMT-2018 and CPMP/ICH/135/95). The informed consent form was signed by all participants enrolled in the study. All individuals were invited to learn about their parasitologic results, and participants found positive for schistosomiasis were offered free treatment (single 40 mg/kg dose of praziquantel).

3.3. RESULTS

3.3.1. GENETIC PROFILE BY MITO-NUCLEAR SIGNATURE

Mito-nuclear profiling was performed using the combination of the mitochondrial marker (*cox1*) obtained by RD-PCR and ITS-2 sequences, as a nuclear marker. Out of a total of 699 genetically processed eggs, mito-nuclear profiling could be performed on 642 eggs, 598 terminal-spined eggs from urine and 44 eggs (35 lateral-spined eggs and 9 terminal-spined eggs) from stool.

Of the 598 successfully genotyped terminal-spined eggs collected from urine, 518 (518/598, 86.62%) exhibited an *S. haematobium cox1* profile by RD-PCR, while 80 (80/598, 13.38%) showed an *S. bovis* profile (**Figure 3.4**). Among the same 598 terminal-spined eggs, 571 (571/598, 95.48%) yielded ITS-2 sequences corresponding to *S. haematobium*, while in 27 eggs (27/598, 4.52%) double chromatogram peaks were found at the polymorphic positions discriminating between *S. haematobium* and *S. bovis* or *S. curassoni* in their resulting ITS-2 sequences (**Appendix B.5**).

Partial sequencing of the 18S gene was performed on these 27 eggs that showed double chromatogram peaks. Unfortunately, only 25 eggs were successfully genotyped because we did not have enough DNA from two eggs to perform PCR (**Table 3.2**).

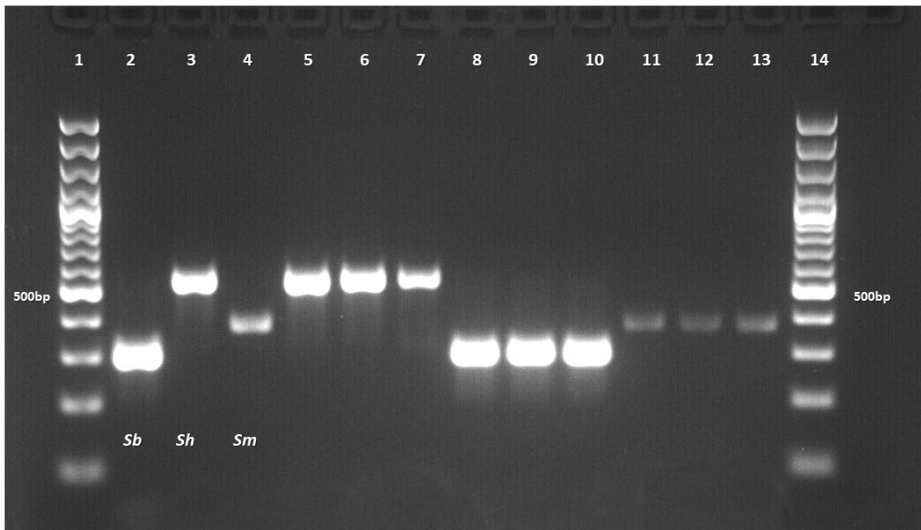


Figure 3.4. Mitochondrial *cox1* profile by rapid diagnostic multiplex one-step polymerase chain reaction (RD-PCR). Lane 1 and 14: DNA ladder (100-3000 bp); lane 2: *S. bovis* (306 bp); lane 3: *S. haematobium* (543 bp); and lane 4: *S. mansoni* (375 bp). Each of the following lanes (5-13) corresponds to a separate multiplex PCR containing DNA extracted from a single egg: lanes 5-10 are terminal-spined eggs from urine; lanes 11-13 are lateral-spined eggs from stool.

Partial 18S sequence analysis revealed three 1369 bp-long haplotypes, Sh-18S-H1 (48.87% GC), Sc-18S-H1 (49.59% GC) and ShxSc-18S-Htz1 (48.83% GC). The three haplotypes detected, differ only at one of the target positions, position 250, used to discriminate the three species, *S. haematobium*, *S. bovis* and *S. curassoni* (**Appendix B.6**):

- the Sh-18S-H1 haplotype showed a "C" at position 250, confirming a pure *S. haematobium* genetic profile for this marker, and it was found to be identical to a sequence

detected in Tanzania (OX103963) and Egypt (OX104046). It was detected in eggs collected from a patient from Mauritania.

- the Sc-18S-H1 haplotype presented "T" at position 250, confirming a pure *S. curassoni* genetic profile for this marker and proving to be identical to a sequence found in *Mesocricetus auratus* in Senegal (AY157236). It was detected in eggs collected from a patient from Côte d'Ivoire and from a patient from Senegal.
- the ShxSc-18S-Htz1 showed a double peak "C/T" (Y) at position 250, indicating a hybrid profile of *S. haematobium* x *S. curassoni*. It was detected in eggs collected from patients from Mali, Mauritania and Senegal.

The ShxSc-18S-Htz1 haplotype was the most frequent haplotype detected in 18 out of 25 successfully genotyped eggs (18/25, 72.00%) (**Appendix B.7**).

When all three markers (*cox1*, ITS-2, and 18S when necessary), were read together 5 mito-nuclear signatures were obtained (**Appendices B.5 and B.7**):

- *ShxShSh* (498/598, 83.28%), corresponding to *S. haematobium*. This mito-nuclear signature was detected in eggs collected from patients from The Gambia, Guinea-Bissau, Côte d'Ivoire, Mali, Mauritania and Senegal.

- *ShxShSc* (18/598, 3.01%), resulting from a hybrid between *S. haematobium* and *S. curassoni*. This mito-nuclear signature was identified in eggs collected from patients from Côte d'Ivoire, Mali, Mauritania and Senegal.
- *SbxShSh* (73/598, 12.21%), resulting from a hybrid between *S. haematobium* and *S. bovis*. This mito-nuclear signature was identified in eggs collected from patients from Mali, Mauritania and Senegal.
- *SbxShSb* (2/598, 0.33%), resulting from a hybrid between *S. haematobium* and *S. bovis*. This mito-nuclear signature was detected in eggs collected from a single patient from Mauritania.
- *SbxShSc* (5/598, 0.84%) resulting from a three-species hybrid, as *S. bovis cox1* was detected by RD-PCR and confirmed by sequencing, double peaks were detected in ITS-2 at positions discriminating between *S. haematobium* and *S. bovis* or *S. curassoni*, and the ShxSc-18S-Htz1 haplotype was detected in 18S. This mito-nuclear signature was detected in eggs collected from a single patient from Mali.

Unfortunately, the signature of two hybrid eggs (2/598, 0.33%) could not be identified, so we designated them as ShxShSb/Sc.

Two mito-nuclear signatures were found in eggs collected from stools, associated with their morphology. While the 35 lateral-spined eggs provided a profile of *S. mansoni* in both *cox1* and ITS-2 markers,

giving an *SmxSmSm* signature (detected in patients from Senegal and Guinea), the 9 terminal-spined eggs presented a *S. guineensis* profile with an *SgxSgSg* signature (identified in a patient from Equatorial Guinea) (**Appendix B.5**). In this case, the 9 *cox1* profiles were obtained by sequencing due to the reverse primers used in RD-PCR were specific for *S. haematobium*, *S. bovis* and *S. mansoni* and not for *S. guineensis*. It is noteworthy that no hybrid mito-nuclear signature was detected in stool samples (**Appendix B.8**).

3.3.1.1. ESTIMATION OF PATIENTS WITH UROGENITAL SCHISTOSOMIASIS CONTAINING HYBRID EGGS

In 29 of 49 patients (29/49, 59.18%) diagnosed with urogenital schistosomiasis, only pure *S. haematobium* eggs, corresponding to the mito-nuclear signature *ShxShSh*, were detected in their urine. These patients came from five different countries: 1 from The Gambia, 1 from Guinea-Bissau, 11 from Mali, 3 from Mauritania and 13 from Senegal. (**Appendix B.8**). It is noteworthy that one of the aforementioned 13 patients from Senegal corresponds to the patient who was also diagnosed with intestinal schistosomiasis, shedding *S. mansoni* lateral-spined eggs with a *SmxSmSm* mito-nuclear signature in the stool (**Table 3.1** and **Appendix B.8**), in which no ectopic or hybrid eggs were detected.

In the remaining 20 patients (20/49, 40.82%) diagnosed with urogenital schistosomiasis, both pure *S. haematobium* (*ShxShSh*) and

hybrid eggs were simultaneously detected in their urine. These patients came from 5 different countries: 1 from Guinea-Bissau, 1 from Côte d'Ivoire, 12 from Mali, 3 from Mauritania and 3 from Senegal (**Appendix B.8**).

Among these 20 patients carrying both pure and hybrid eggs: in eleven patients (11/20, 55.0%), including 9 patients from Mali, 1 from Mauritania and 1 from Senegal, exclusively *S. haematobium* x *S. bovis* hybrid eggs (*SbxShSh* and/or *SbxShSb*) were detected; in six patients (6/20, 30.0%), including 1 patient from Côte d'Ivoire, 2 from Mali, 2 from Mauritania and 1 from Senegal, only *S. haematobium* x *S. curassoni* (*ShxShSc*) hybrid eggs were observed; and in a single Senegalese patient (1/20, 5.0%), both *S. haematobium* x *S. bovis* (*SbxShSh*) and *S. haematobium* x *S. curassoni* (*ShxShSc*) hybrid eggs were observed. Notably, three-species hybrid eggs (*SbxShSc*) involving the interaction of *S. haematobium*, *S. bovis* and *S. curassoni* were identified in only one patient (1/20, 5.0%) from Mali.

In **Appendix B.8**, the patients are grouped according to their country of origin and the different mito-nuclear signatures or combinations of mito-nuclear signatures detected in the eggs found in their urines.

3.3.2. MOLECULAR HAPLOTYPES FROM rDNA ITS-1-5.8S-ITS-2 SEQUENCING

3.3.2.1. *Schistosoma* spp. EGGS FROM URINE

The complete sequence of the nuclear rDNA intergenic region of 222 subsampled terminal-spined eggs processed from urine was successful for 203. It resulted in the identification of five haplotypes corresponding to a pure *S. haematobium* sequence (Sh-ITSs-H1, Sh-ITSs-H2, Sh-ITSs-H3, Sh-ITSs-H4 and Sh-ITSs-H5) and 3 different sequences with double peaks at positions discriminating between *S. haematobium* and *S. bovis* or *S. curassoni*, conforming 3 hybrid haplotypes (ShxSc-ITSs-Htz1, ShxSc-ITSs-Htz2 and ShxSc-ITSs-Htz3).

The five *S. haematobium* haplotypes provided a sequence of 927 bp-long with an average GC content of 49.75%, differing only in two polymorphic sites located at positions 19 (T, C or C/T) and 535 (C or C/T) of their alignment (**Appendix B.9**), belonging to ITS-1 and 5.8S gene, respectively. No variation was found in ITS-2. We assigned the haplotypes Sh-ITSs-H1 (49.73% GC) and Sh-ITSs-H2 (49.84% GC) to those sequences that did not contain a heterozygotic signal. Meanwhile, for those sequences that were heterozygotic, we designated:

- Sh-ITSs-H3 (49.78% GC), when the heterozygotic (C/T) signal was at position 19.
- Sh-ITSs-H4 (49.68% GC) when the heterozygotic (C/T) signal was at position 535.

- Sh-ITSs-H5 (49.73% GC) when both positions mentioned (19 and 535) were heterozygotic (C/T).

None of these polymorphisms corresponded to a discriminating position between other *Schistosoma* species (**Appendix B.9**). The five haplotypes described for this intergenic region, showed identity with previously described *S. haematobium* isolates or haplotypes available in the Genbank database (**Appendix B.10**).

The Sh-ITSs-H4 haplotype, previously described in Côte d'Ivoire (MG554667), Benin (MT158876, MW027655) and France (MW130296), proved to be the most frequent haplotype in all our patients regardless of their country of origin, being detected in 94 out of 203 successfully genotyped eggs (94/203, 46.31%) (**Appendices B.9 and B.10**).

The three hybrid haplotypes (927 bp-long and 49.5% GC mean content) present four heterozygotic signals at positions 704 (G/A), 759 (C/T), 809 (G/A) and 879 (C/T) and located in the ITS-2 (**Appendix B.9**). These four positions discriminate between *S. haematobium* and *S. bovis* or *S. curassoni*, but not between *S. bovis* and *S. curassoni* (**Appendices B.9 and B.10**). Finally, these three ribosomal haplotypes were confirmed as *S. haematobium* x *S. curassoni* and ascribed to the codes ShxSc-ITSs-Htz1, ShxSc-ITSs-Htz2 and ShxSc-ITSs-Htz3 after 18S determination.

The rDNA ITS-1, 5.8S and ITS-2 sequence alignment including our 8 haplotypes and 59 sequences representing 21 haplotypes/isolates of the same or proximal species included in the *S. haematobium* clade collected from GenBank (**Appendix B.2.A**), was 928 bp in length, of which 18 were variable positions (including heterozygotic positions), 6 parsimony informative (p-info) and no singleton sites (**Appendix B.9**). A detailed and summarized results of haplotype identities found is shown in **Appendix B.10**.

3.3.2.2. *Schistosoma* spp. EGGS FROM STOOL

3.3.2.2.1. TERMINAL-SPINED EGGS

The ITS-1, 5.8S and ITS-2 region provided one 927 bp-long haplotype among the 9 successfully genotyped of 10 terminal-spined eggs processed collected from the stool of an Equatorial Guinean patient. This haplotype was adscribed as Sg-ITSs-H1 (49.41% GC) and showed 100% identity with a *S. guineensis* sequence available in GenBank (OX103898) reported from Sao Tome and Principe (**Appendices B.9 and B.10**).

3.3.2.2.2. LATERAL-SPINED EGGS

The ITS-1, 5.8S and ITS-2 region yielded a unique 927 bp-long haplotype among the 20 genotyped lateral-spined eggs collected from

the stool of two patients from Senegal and Guinea. The haplotype was adscribed as Sm-ITSs-H1 (48.4% GC). Our Sm-ITSs-H1 haplotype proved to be identical to *S. mansoni* haplotypes/isolates from GenBank (AF531314, FJ750523, JQ289742-50, MF776590) and reported from various African countries such as Egypt, Kenya, Mali, Nigeria, Tanzania and Uganda, as well as in Brazil, Saudi Arabia and Oman (**Appendices B.11 and B.2.B**).

For comparative purposes, an alignment was performed with our *S. mansoni* haplotype and 27 other *S. mansoni* sequences, corresponding to 4 haplotypes, available in GenBank (**Appendix B.2.B**). The alignment was 927 bp-long and contained 3 variable positions, of which 3 were singleton sites (**Appendix B.11**).

3.3.3. MOLECULAR HAPLOTYPES FROM mtDNA SEQUENCING

3.3.3.1. *Schistosoma* spp. EGGS FROM URINE

Partial sequencing of the mitochondrial *cox1* gene from the 203 succesfully genotyped of 232 terminal-spined eggs processed collected from urine yielded a total of 40 haplotypes of 1024 bp length, of which 35 had an *S. haematobium* profile and 5 had an *S. bovis* profile.

The 35 haplotypes obtained with an *S. haematobium* profile showed 39 mutation sites in their alignment (11 p-info and 28 singleton

sites) (**Appendix B.12**) and a mean AT content of 70.25%. Haplotypes were designated as Sh-*cox1*-H1 to Sh-*cox1*-H35. The resulting COXI protein was 340 aa long, and provided eight different haplotypes due to eight amino acid changes (**Appendix B.12**).

Four of the 35 haplotypes here described showed 100% sequence identity with *S. haematobium* haplotypes/isolates available in GenBank (**Appendix B.12**). The Sh-*cox1*-H14 and Sh-*cox1*-H15 haplotypes were previously reported in *M. auratus* from Benin (KT354661) and Mali (NC_008074), respectively; the Sh-*cox1*-H22 haplotype was reported in *M. auratus* from Egypt (OX104052 and MW067222-27); and the Sh-*cox1*-H35 haplotype was found to be identical to other haplotypes or isolates previously reported in human samples from Tanzania, (MK253577) and the Democratic Republic of the Congo (KY967520), *M. auratus* from Gabon (KT354659-60), and *B. globosus* from Malawi (EU567128) (**Appendix B.12**). The aforementioned Sh-*cox1*-H35 haplotype proved to be the most common among the 167 terminal-spined eggs sequenced with a *S. haematobium cox1* profile, being detected in 96 (96/167, 57.5%).

Four haplotypes were found to be shared among patients from different countries of origin: Sh-*cox1*-H1 haplotype, which was described in the only patient from The Gambia and in one patient from Mauritania; the Sh-*cox1*-H9 haplotype, which was detected in one patient from Guinea-Bissau and in three from Mali; the Sh-*cox1*-H15 haplotype, which was found in two patients, one from Mali and one from Senegal; and finally, the Sh-*cox1*-35 haplotype, which was present in

patients from all the countries described in this study except in The Gambia.

Our 35 *S. haematobium* haplotypes were compared with 19 published nucleotide sequences of *S. haematobium* in GenBank (BLAST = 100% coverage; >99% similarity) representing 9 different haplotypes (**Appendix B.3.A**). The alignment was 1024 bp-long, of which 44 were variable position (8 p-info and 36 singleton sites) (Table not shown).

The five haplotypes obtained with an *S. bovis* profile showed 10 mutation sites in their alignment (1 p-info and 9 singleton sites (**Appendix B.13**) and a mean AT content of 67.56%. Haplotypes were designated as Sb-*cox1*-H1 to Sb-*cox1*-H5. The resulting COXI protein was 340 aa long, and provided three different haplotypes due to 2 amino acid changes (**Appendix B.13**). Only one (Sb-*cox1*-H5) of the 5 haplotypes here described showed 100% sequence identity with one *S. bovis cox1* haplotype (KT354658) previously reported in humans from the locality of Ste. Lucie de Porto-Vecchio in Corsica (France) (**Appendix B.13**). This Sb-*cox1*-H5 haplotype was the most common among the 36 terminal-spined eggs sequenced with a *S. bovis cox1* profile being detected in 27 eggs (27/36, 75.0%).

Using the same criteria as for *S. haematobium* profile sequences, the five *S. bovis* haplotypes obtained were aligned with 48 sequences deposited as *S. bovis* or *S. haematobium* x *S. bovis*, according to the authors species description, in GenBank (BLAST = 100% coverage; >99% similarity) representing 48 different haplotypes (**Appendix**

B.3.B). The alignment of those 53 sequences was clustered into 52 haplotypes, according to ALTER, and showed 951 conserved positions and 73 variables, of which 21 were p-informative and 52 singleton sites (Table not shown).

3.3.3.2. *Schistosoma* spp. EGGS FROM STOOL

3.3.3.2.1. TERMINAL-SPINED EGGS

Partial sequencing of the mitochondrial *cox1* gene from 9 successfully genotyped of 10 terminal-spined eggs processed from the stool of Equatorial Guinean patient, provided 2 haplotypes of 1052 bp-long. These two haplotypes showed two differences in their alignment, corresponding to two singleton sites, and had an average AT content of 66.44%. They were designated as Sg-*cox1*-H1 and Sg-*cox1*-H2. These two haplotypes produced a protein of 350 amino acids in length, and provided two protein haplotypes due to two amino acid changes. The Sg-*cox1*-H1 haplotype was found to be identical to two sequences of *S. guineensis* reported in São Tomé and Príncipe (AJ519517 and OX103896) (**Appendix B.14**).

The comparative alignment was performed with our two *S. guineensis* haplotypes and four other sequences deposited in GenBank belonging to *S. guineensis* (BLAST = 100% coverage; >99% similarity) and representing 3 haplotypes (**Appendix B.3.C**). The comparative

alignment clustered into 4 haplotypes and yielded 11 variable positions, of which 2 were p-informative and 9 singleton sites (Table not shown).

3.3.3.2.2. LATERAL-SPINED EGGS

Sequencing of the partial *cox1* gene of the 20 lateral-spined eggs collected from two stool samples from two patients of Senegal and Guinea resulted in 17 haplotypes of 1024 bp-long.

The alignment of the 17 haplotypes showed 31 variable positions, of which 18 were p-info and 13 singleton sites, and an average AT content of 66.62%. The haplotypes were described as Sm-*cox1*-H1 to Sm-*cox1*-H17. The resulting COXI protein showed a 340 aa long and three haplotypes differing by two amino acids (**Appendix B.15**). Two haplotypes, Sm-*cox1*-H11 and Sm-*cox1*-H16, both found in the Guinean patient, proved to be identical to two *S. mansoni* sequences, MG562512 and MG562513, respectively, previously reported in humans from Côte d'Ivoire (**Appendix B.15**).

The comparative alignment was performed with our 17 *S. mansoni* haplotypes and other 6 *S. mansoni* sequences, corresponding to 5 haplotypes, which showed 100% coverage and more than 99% similarity to our haplotypes in BLAST (**Appendix B.3.D**). The sequences were grouped into 20 haplotypes in which 35 variable positions were observed, of which 21 were p-informative and 14 singleton sites (Table not shown).

3.3.4. *cox1* PHYLOGENIES

The ML model that best fitting our *cox1* data set was the Hasegawa-Kishino-Yano model with discrete Gamma distribution (HKY+G). The resulting ML tree (log likelihood = -4279.75) was inferred with +G, parameter = 0.1916, and the rate variation model allowed for some sites to be evolutionarily invariant (**Figure 3.4**). This analysis involved 74 nucleotide sequences, including *S. haematobium*, *S. bovis*, *S. guineensis*, and *S. mansoni* *cox1* haplotypes and other *Schistosoma* spp. isolates or haplotypes from GenBank (**Appendix B.4**). There were a total of 1024 positions in the final data set.

In the ML tree, the 35 *cox1* haplotypes of *S. haematobium* cluster together in a monophyletic clade, in which another representative haplotypes of group 1 of *S. haematobium* are included. This clade is closely related and well supported (98%) with the clade including *S. haematobium* from Mauritius, Zanzibar and Coastal Kenya, representing *cox1* haplotypes of *S. haematobium* of group 2. Thus, all haplotypes detected in migrants in Spain arriving from Mali, Mauritania, The Gambia, Senegal, Côte d'Ivoire, and Guinea-Bissau grouped together in the monophyletic clade corresponding to *S. haematobium* group 1.

The haplotypes corresponding to *S. guineensis*, *S. curassoni* and *S. bovis*, cluster together in a well supported (98%) monophyletic clade that includes a monophyletic branch for each species.

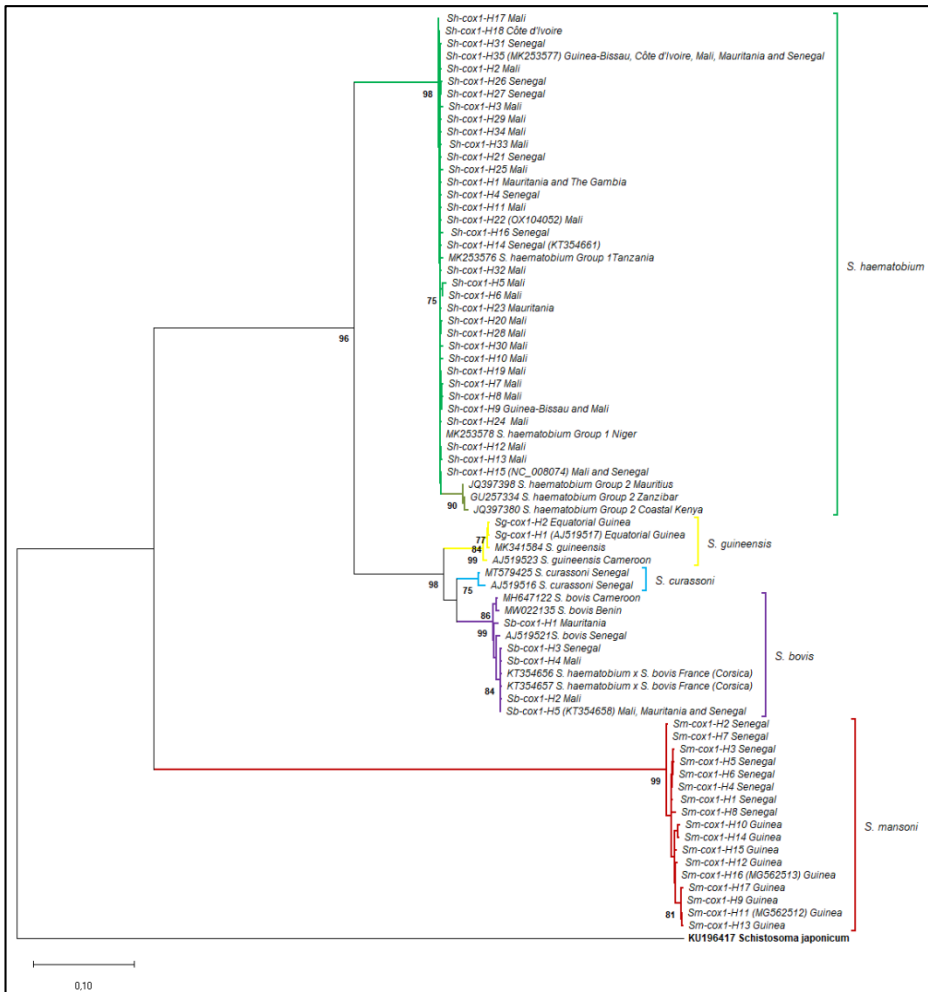


Figure 3.4. Phylogenetic tree of mtDNA *cox1* sequences of *Schistosoma haematobium* (Sh-cox1-H1 to Sh-cox1-H35), *S. bovis* (Sb-cox1-H1 to Sb-cox1-H5), *S. guineensis* (Sg-cox1-H1 and Sg-cox1-H2) and *S. mansoni* (Sm-cox1-H1 to Sm-cox1-H17) haplotypes and data from GenBank, based on maximum-likelihood (ML) method and Hasegawa-Kishino-Yano model (HKY) +G, parameter = 0.1916. The tree with the highest log likelihood (-4279.75) is shown. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The tree was rooted using the sequence of *S. japonicum* (KU196417) as outgroup. Supports for nodes was made with Bootstrap method and 1000 replications in MEGA X.

Within the *S. bovis* branch, four *S. bovis* *cox1* haplotypes detected in migrants in Spain arriving from Mali, Mauritania and Senegal cluster together with *S. bovis* haplotypes from Europe (France: Corsica) with an 84% of support. The two *S. guineensis* *cox1* haplotypes from Equatorial Guinea, cluster inside a monophyletic branch of *S. guineensis* that includes other *S. guineensis* *cox1* haplotypes from Cameroon.

A basal clade of the tree includes *S. mansoni* haplotypes showing high support (99%). Although clustered in the same clade, the Guinea *S. mansoni* *cox1* haplotypes detected in migrants in Spain are grouped in the same branch, appearing as a sister branch of all haplotypes from Senegal.

3.4. DISCUSSION

Molecular techniques combining the mitochondrial *cox1* gene and the nuclear ITSs are commonly used to distinguish *Schistosoma* species and identify hybrids in patients diagnosed in endemic countries (Webster et al., 2013; Boon et al., 2018; Angora et al., 2020; Savassi et al., 2020). However, this type of genetic studies using the combination of mitochondrial and nuclear markers, are very scarce in patients diagnosed in Europe. They have only been performed in specific cases, such as migrants diagnosed with schistosomiasis with ectopic eggs elimination (Depaquit et al., 2019; Le Govic et al., 2019) or travelers returning from endemic countries with shedding of *Schistosoma* eggs with an unusual morphology (Soentjens et al., 2016).

To our knowledge, this is the first study based on the combination of nuclear and mitochondrial DNA markers in sub-Saharan migrants diagnosed with schistosomiasis and residing in Spain.

Our study revealed that 20 out of 49 (40.82%) patients diagnosed with urogenital schistosomiasis were carriers of pure and hybrid eggs in their urine (**Appendix B.8**). This simultaneous detection of both pure and hybrid genetic profiles in the same patient is in line with observations made in patients from endemic countries such as Senegal and Côte d'Ivoire (Huyse et al., 2009; Webster et al., 2013; Angora et al., 2020).

The presence of *S. haematobium* x *S. bovis* hybrid eggs were confirmed in 12 (including the Senegalese patient in which both

S. haematobium x *S. bovis* and *S. haematobium* x *S. curassoni* hybrid eggs were observed) out of the 20 patients (12/20, 60.0%) shedding hybrid eggs (**Appendix B.8**). Specifically, this profile was observed in patients from Senegal, Mauritania and Mali. The *S. haematobium* x *S. bovis* hybrid is the most prevalent and extensively studied in West African countries with reports of human infections in Benin (Savassi et al., 2020), Cameroon (Teukeng et al., 2022), Côte d'Ivoire (Angora et al., 2020), Mali (Privat et al., 2023), Nigeria (Onyekwere et al., 2022b) and Senegal (Webster et al., 2013; Boon et al., 2018, 2019).

In the literature all possible mito-nuclear combinations of *S. haematobium* x *S. bovis* have been detected in eggs collected from patients with urogenital schistosomiasis in sub-Saharan endemic countries: *ShxShSb*, *ShxSbSb*, *SbxShSh*, *SbxShSb* (Webster et al., 2013; Angora et al., 2020; Onyekwere et al., 2022b; Teukeng et al., 2022). However, hybrids in which *cox1* shows an *S. bovis* profile and ITSs exhibits *S. haematobium* alleles (*SbxShSh*), have proven to be the most common mito-nuclear signature reported, being detected in Benin, (Savassi et al., 2020), Cameroon (Teukeng et al., 2022), Côte d'Ivoire (Angora et al., 2020), Nigeria (Onyekwere et al., 2022b), Senegal (Webster et al., 2013), and even in France (Moné et al., 2015). This mito-nuclear signature proves the mitochondrial introgressive hybridization of *S. haematobium cox1* by *S. bovis* (Moné et al., 2015). In our migrant patients, we identified two distinct mito-nuclear signatures corresponding to a *S. haematobium* x *S. bovis*: *SbxShSh* and *SbxShSb*. Consistent with previous reports, the mito-nuclear signature *SbxShSh*

was the most frequent in our *S. haematobium* x *S. bovis* hybrid eggs, detected in 73 out of 75 eggs (73/75, 97.33%) (**Appendix B.5**), and corresponding to 12 patients (including 9 patients from Mali, 1 from Mauritania and 2 from Senegal) (**Appendix B.8**).

Although some studies have reported the presence of miracidia with a "pure" *S. bovis* signature (*SbxSbSb*) in patients' urine (Boissier et al., 2016; Angora et al., 2020; Onyekwere et al., 2022b), we did not observe such cases in our patients.

S. haematobium x *S. curassoni* hybrids were identified in 7 (including the Senegalese patient in which both *S. haematobium* x *S. bovis* and *S. haematobium* x *S. curassoni* hybrid eggs were observed) out of the 20 migrant patients (7/20, 35.0%) with urogenital schistosomiasis shedding hybrid eggs (**Appendix B.8**). In this case, only one mitochondrial signature, *ShxShSc*, was observed. These six patients in whom we confirmed the presence of *S. haematobium* x *S. curassoni* hybrids were from Côte d'Ivoire, Mali, Mauritania, and Senegal. Except for one of these patients, who also reported being exposure in a river in another endemic country, the rest of the patients were presumably infected in their respective countries of origin, which could mean that the interaction between these two species, so far reported in the literature only in patients diagnosed in Senegal (Webster et al., 2013) and Mali (Privat et al., 2023), could occur in more West African countries.

Surprisingly, three-species hybrid eggs were identified in a single patient from Mali (1/20, 5.0%) (**Appendix B.8**). The detection of five

eggs in the urine of the Malian patient with a mito-nuclear signature of *SbxShSc* (**Appendix B.5**), in which an *S. bovis* was detected in *cox1* and an *S. haematobium* x *S. curassoni* profile was observed in ITS-2 and 18S, supports the potential interaction between the three species: *S. haematobium*, *S. bovis* and *S. curassoni*. These three-species hybrids have been previously reported in Niger (Léger et al., 2016) and Mali (Privat et al., 2023). The genetic profile of the three-species hybrid eggs observed in our study supports the hypothesis proposed in the other two studies (Léger et al., 2016; Privat et al., 2023) that they are not first-generation hybrids, but the result of parental backcrosses and/or relatively recent hybrids.

The low frequency of *S. haematobium* x *S. curassoni* hybrids reported in the literature compared to hybrids resulting from *S. haematobium* and *S. bovis*, as well as the recently identified three-species hybrids, has been attributed to the rarity of these interactions due to the limited distribution of *S. curassoni*, which is found in specific foci in some West African countries, with the majority of transmission occurring in Senegal (Rey et al., 2021a). However, it is possible that these hybrids are being underestimated because ITS-2, the marker used in most field studies, does not discriminate between *S. bovis* and *S. curassoni*, requiring the use of another nuclear marker, such as the recently proposed 18S rRNA gene (Léger et al., 2016; Privat et al., 2023), for correct identification.

The 18S rRNA gene, which is highly conserved, appears to be a useful marker to differentiate *S. haematobium*, *S. curassoni* and *S. bovis*.

However, the small number of 18S sequences available in Genbank, coupled with the small number of positions that distinguish *S. haematobium* from *S. curassoni* in this marker (**Table 3.3.B**), highlights the need for further study of 18S variability in these two species.

Partial mitochondrial *cox1* sequencing analysis of terminal-spined eggs collected from urine of patients with urogenital schistosomiasis revealed 35 haplotypes of *S. haematobium* and 5 haplotypes of *S. bovis* (**Appendices B.12 and B.13**). Across the sub-Saharan African region, *S. haematobium cox1* was confirmed to cluster into two groups, group 1, which included parasites from mainland Africa, and group 2, which included parasites from Indian Ocean islands and neighboring African coastal regions (Webster et al., 2012). Our phylogenetic tree, based on 1024 bp of *cox1*, grouped the 35 *S. haematobium* haplotypes from our study into group 1, as expected based on the country of origin of our patients (**Figure 3.4**). Interestingly, regarding the *S. bovis cox1* haplotypes, our phylogenetic tree based on 1024 bp, grouped the Sb-*cox1*-H2, Sb-*cox1*-H3, Sb-*cox1*-H4, and Sb-*cox1*-H5 haplotypes with those from Corsica (KT354656 and KT354657), with the Sb-*cox1*-H5 haplotype being exactly identical to the Sh_Corsica3 haplotype (KT354658). Our Sb-*cox1*-H2 and Sb-*cox1*-H4 haplotypes have been both detected in patients from Mali, Sb-*cox1*-H3 in a patient from Senegal, and Sb-*cox1*-H5 in patients from Mali, Mauritania, and Senegal. Initially, the origin of Corsican hybrids was attributed to Senegal (Boissier et al., 2016), which is also supported by our results considering the country of origin of our patients. However,

our tree also places haplotypes detected in patients originating from Mali or Mauritania in the same cluster which could mean that it is difficult to establish a single country as origin of these hybrids found in Corsica. In fact, a recent study has suggested that the origin of these hybrids could also be attributed to Benin, based on an 881 bp partial *cox1* phylogenetic tree (Savassi et al., 2020).

Among our patient cases, we also analyzed eggs collected from the stool of three patients diagnosed with intestinal schistosomiasis. On the one hand, infection with *S. guineensis* was confirmed in the patient from Equatorial Guinea who excreted terminal-spined eggs in the stool. This species is restricted to Lower Guinea (Moné et al., 2012), which includes the country of origin of our patient. Interestingly, the possible involvement of *B. truncatus* in its transmission has recently been suggested in Gabon (Dejon Agobé et al., 2021), which, if confirmed, could represent a risk of autochthonous transmission of this species in Spain, where *B. truncatus* is present. On the other hand, *S. mansoni* was identified in the lateral-spined eggs collected from the stools of the patients from Guinea and Senegal. It should be noted that the patient from Senegal was also diagnosed with urogenital schistosomiasis and excreted pure *S. haematobium* eggs in his urine, indicating co-infection. *S. mansoni* is widely distributed across the African continent, with both Guinea and Senegal being endemic countries (Boissier et al., 2019). Furthermore, in Senegal, due to the overlap between the two main species, *S. haematobium* and *S. mansoni*, has led to increased reports of mixed infections in the northern region of

the country in recent years (Meurs et al., 2012a). In principle, *S. mansoni* would not pose a risk of autochthonous transmission in Spain due to the absence of a competent vector, freshwater snails of the genus *Biomphalaria*.

In Spain, as in the rest of Europe, the main profile of sub-Saharan migrants diagnosed with schistosomiasis corresponds to young men mainly from West Africa, with Mali and Senegal standing out as countries of origin (Witzenrath et al., 2017; Comelli et al., 2020). Given the high prevalence of hybrids between typical human species, such as *S. haematobium*, and typical animal species, such as *S. bovis* and *S. curassoni*, in their origin countries, it could be expected that these type of hybrids would be observed in our migrant patients, as demonstrated in this study. Even though crosses between *S. haematobium* and *S. bovis* or *S. haematobium* and *S. curassoni*, are thought to generate lineages with increased virulence compared to the parental species (Blin et al., 2023), very little is known about the impact of these hybrids on pathology, morbidity, and treatment response. Recently, a study from Senegal observed a different morbidity profile in hybrid *S. haematobium* x *S. bovis* infections compared to *S. haematobium* infections. Individuals infected with *S. haematobium* x *S. bovis* hybrids had an increase in hepatic morbidity and a decrease in urogenital morbidity, as well as a reduction in improvement after treatment with praziquantel, compared with patients without hybrids (Fall et al., 2021). These data may be similar for hybrid infections of *S. haematobium* x *S. curassoni*, as the latter is also an animal species that causes intestinal schistosomiasis.

However, further specific studies are needed, including simultaneous infection with different types of hybrids, considering that simultaneous shedding of pure *S. haematobium* eggs together with *S. haematobium* x *S. bovis* and *S. haematobium* x *S. curassoni* hybrid eggs has been reported in Senegal and also, as observed in one of our patients. The significant percentage of our migrant patients showing hybrids (40.82%), especially those from Mali, highlight the importance of monitoring and detecting these hybrid infections. Collaboration between researchers and Spanish physicians is essential to understand the impact of these hybrids on morbidity and treatment response.

Diagnosis and treatment of patients with schistosomiasis are essential not only to prevent serious complications, but also to prevent possible future autochthonous transmission. The public health impact in European countries, as observed in Corsica, is not as devastating as in other endemic countries, since infections are usually associated with recreational activities. Banning bathing in these areas should stop transmission. However, the potential zoonotic nature of the parasite could complicate its control, especially considering that the presence of *S. haematobium* x *S. bovis* hybrids was recently confirmed for the first time in cattle, rodents (*M. natalensis*) and schoolchildren in the same area in Kessounou, Benin (Savassi et al., 2020, 2021).

In summary, this is the first genetic study based on nuclear (18S and ITSs) and mitochondrial (*cox1*) markers carried out in migrants from 8 different countries (Côte d'Ivoire, Equatorial Guinea, Guinea, Guinea-Bissau, Mali, Mauritania, Senegal and The Gambia) diagnosed with

schistosomiasis in Spain. In this study, we genotyped 642 eggs, of which 598 were collected from 49 urine samples of patients diagnosed with urogenital schistosomiasis, and 44 from 3 stool samples of patients diagnosed with intestinal schistosomiasis.

The results revealed that 83.28% (498/598 eggs) of the terminal-spined eggs collected from urine corresponded to a pure *S. haematobium* profile, while the remaining eggs presented hybrid profiles, with 12.54% (75/598) showing a *S. haematobium* x *S. bovis* profile and 3.01% (18/598) showing a *S. haematobium* x *S. curassoni* profile (**Appendix B.5**). Notably, a three-species hybrid *S. haematobium* x *S. bovis* x *S. curassoni* was detected in 0.84% (5/598) of the eggs collected from urine. In the eggs collected from stool samples, 79.54% (33/44 eggs) showed a *S. mansoni* profile, and 20.45% (9/44) exhibited a *S. guineensis* profile (**Appendix B.5**).

These findings allowed us to confirm 28 patients with urogenital schistosomiasis, in which *S. haematobium* was identified as the causative agent; 20 patients with urogenital schistosomiasis in which both pure *S. haematobium* and hybrids, *S. haematobium* x *S. bovis* and/or *S. haematobium* x *S. curassoni*, were detected (11 patients with *S. haematobium* x *S. bovis* hybrids, 6 with *S. haematobium* x *S. curassoni*, 1 with *S. haematobium* x *S. bovis* or *S. curassoni*, 1 with *S. haematobium* x *S. bovis* and *S. haematobium* x *S. curassoni*, and 1 with *S. haematobium* x *S. bovis* x *S. curassoni* three-species hybrid); 1 patient with intestinal schistosomiasis due to *S. guineensis*; 1 patient with intestinal schistosomiasis caused by *S. mansoni*; and 1 patient with urogenital and

intestinal schistosomiasis caused by a co-infection of *S. haematobium* and *S. mansoni* (**Figure 3.5**).

Taking into account our results, the patients from Mali (12 out of 23 patients, 52.17%), are the ones who presented the most hybrids, followed by patients from Mauritania (3/6, 50%) and Guinea-Bissau (1/2, 50%) and, finally, those from Senegal (3/16, 18.75%). In the remaining patients, only one patient could be analyzed, with pure species being detected in the patients from The Gambia, Guinea and Equatorial Guinea, and in the patient from Côte d'Ivoire, pure *S. haematobium* and the *S. haematobium* x *S. curassoni* hybrid (**Figure 3.5**).

Regarding the sequencing results, the ITS-1, 5.8S and ITS-2 region revealed 7 pure haplotypes: 5 haplotypes of *S. haematobium*, 1 of *S. guineensis*, and 1 of *S. mansoni*; and 3 hybrid haplotypes of *S. haematobium* x *S. curassoni*. The partial *cox1* sequencing detected a total of 59 haplotypes of which 35 haplotypes correspond to *S. haematobium*; 5 to *S. bovis*; 2 to *S. guineensis*; and 17 to *S. mansoni*.

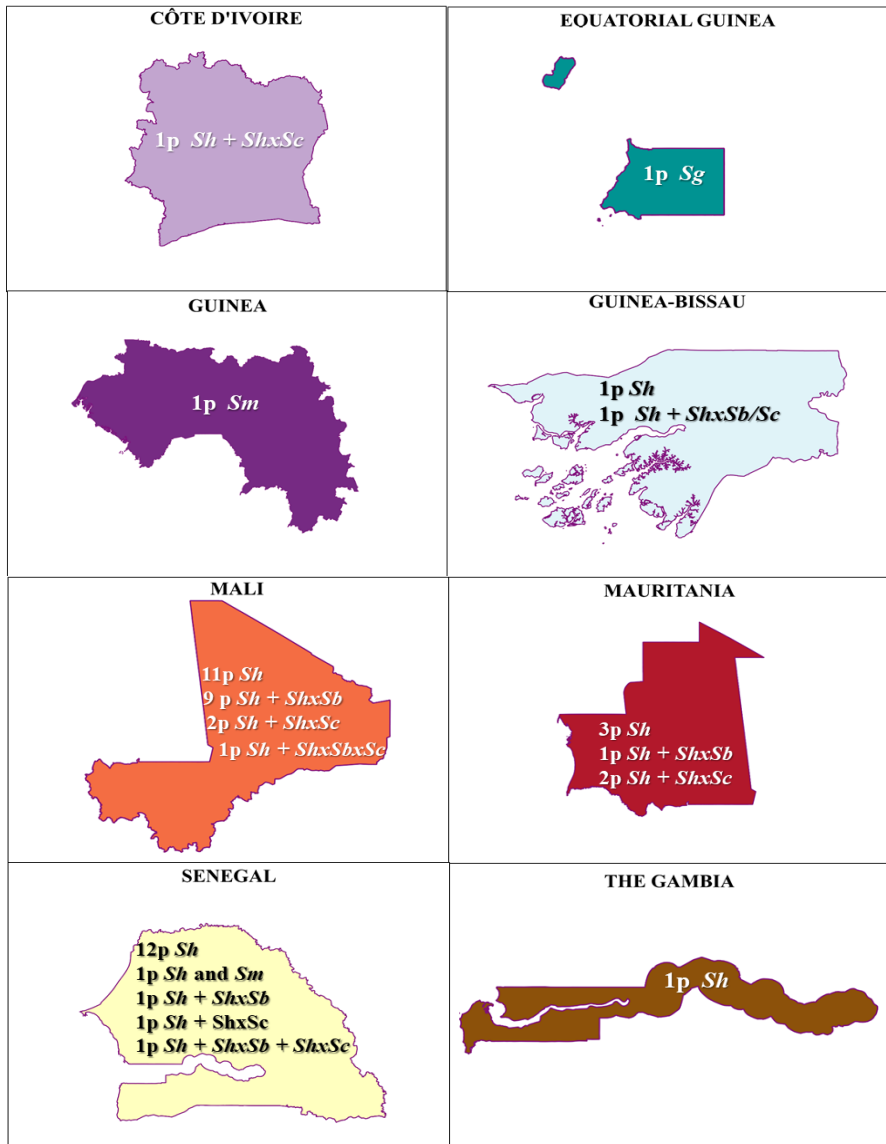
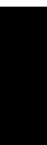


Figure 3.5. Graphical summary of pure and hybrid *Schistosoma* species organized by country of origin of migrant patients (p) in whom it has been detected. When the same patient is a carrier of pure and hybrid eggs, a “+” symbol is included. *Sh*=*Schistosoma haematobium*; *Sb*=*S. bovis*; *Sc*=*S. curassoni*; *Sm*=*S. mansoni*; *Sg*=*S. guineensis*; *ShxSb*=*S. haematobium* x *S. bovis*; *ShxSc*=*S. haematobium* x *S. curassoni*; *ShxSb/Sc*=*S. haematobium* x *S. bovis* or *S. curassoni*; *ShxSbxSc*=*S. haematobium* x *S. bovis* x *S. curassoni*.

**CHAPTER IV. EVIDENCE OF *S.*
haematobium AND *S. mansoni* HYBRIDS IN
SUBSAHARAN MIGRANTS DIAGNOSED
IN SOUTHERN SPAIN**



4.1. INTRODUCTION

S. haematobium and *S. mansoni* are responsible for the majority of human schistosomiasis infections. These species share host specificity for humans, but have different tropisms within their definitive hosts, causing two pathological profiles, urogenital schistosomiasis and intestinal schistosomiasis, respectively (Gryseels et al., 2006). The morphology of the eggs also differs between these two species: the eggs of *S. haematobium*, which are detected in the urine, are characterized by a terminal spine, whereas the eggs of *S. mansoni*, found in the stool, have a lateral spine (Gryseels et al., 2006).

It is known that these two *Schistosoma* species are co-endemic in 35 African countries (Gryseels, 2012; Boissier et al., 2019) and can potentially infect the same human host (Garba et al., 2010; Meurs et al., 2012a; Gouvras et al., 2013; Sang et al., 2014). Thus, mixed infections have been frequently documented in different countries of the African continent (Garba et al., 2010; Koukounari et al., 2010; Meurs et al., 2012a; Gouvras et al., 2013; Sang et al., 2014; Onzo-Aboki et al., 2019; Ojo et al., 2021). Furthermore, in some endemic countries such as Nigeria (Ojo et al., 2021), Senegal (Meurs et al., 2012a), and Cameroon (Cunin et al., 2003), the presence of lateral-spined eggs, typical of *S. mansoni*, in urine or, conversely, terminal-spined eggs, typical of *S. haematobium*, in stool, have been reported. The high frequency of ectopic *S. mansoni*-like eggs in urine and *S. haematobium*-like eggs in stool may be due to interspecific competition between the two

schistosomes or, the most widely accepted explanation until now, a spillover from high infection loads (Cunin et al., 2003).

The high phylogenetic distance between them, since the species belong to two different evolutionary lineages; the *S. mansoni* and *S. haematobium* group respectively, and physiological barriers in the adult's location in the definitive host, would prevent heterospecific crosses with a viable offspring (Jourdane and Southgate, 1992; Lockyer et al., 2003). Hence, it was surprising that *S. mansoni* x *S. haematobium* hybrids have been recently genetically documented, in patients diagnosed in Senegal (Huyse et al., 2013) and in two migrants diagnosed in France eliminating ectopic *S. mansoni*-like eggs from Côte d'Ivoire (Depaquit et al., 2019; Le Govic et al., 2019)

The study carried out in patients diagnosed in Senegal was the first to report these *S. mansoni* x *S. haematobium* hybrids. The discovery occurred while researchers were conducting a broader study of the epidemiology and control of schistosomiasis in schoolchildren in Senegal. During genotyping, they noticed an atypical allele in the genetic profile of the microsatellite marker SMD28. These findings led them to clone the ITSs nuclear DNA region and partial fragments of the mitochondrial gene *cox1* in 10 eggs/miracidia with an atypical profile for the SMD28 microsatellite. Of the 10 eggs/miracidia, 7 yielded clones with two genotypes of ITSs within individual eggs and miracidia, one identical to *S. mansoni* and the other identical to *S. haematobium*. While the *cox1* clones always belonged exclusively to one of the parental

species, either *S. haematobium* or *S. mansoni*. This study does not refer to the morphology of the eggs analyzed.

In the case of the two migrants from Côte d'Ivoire residing in France, both were 14-year-old boys, one diagnosed in 2008 (Depaquit et al., 2019) and the other in 2017 (Le Govic et al., 2019). In the boy diagnosed in 2008, only lateral-spined eggs were found in his urine, and no evidence of *Schistosoma* eggs in his stool. Meanwhile, in the migrant diagnosed in 2017, the presence of both lateral-spined and terminal-spined eggs was detected in his urine, as well as the presence of lateral-spined eggs in his stool. In both cases, double peaks in the nuclear markers used (28S and ITS) were detected in lateral-spined eggs, confirming a hybrid profile of *S. mansoni* x *S. haematobium* in these eggs. The *cox1* of these lateral-spined eggs gave a single profile corresponding to one of the parental species, either *S. haematobium* or *S. mansoni*.

In this chapter, we present the genetic characterization, by RD-PCR of the *cox1* mtDNA gene and by sequencing and cloning of the ITS-2 nuclear rDNA, of schistosome lateral-spined eggs, typical of *S. mansoni*, detected in five urine samples from five young migrants from three different countries (Senegal, Guinea-Bissau and Côte d'Ivoire) and diagnosed with urogenital schistosomiasis in Spain. The aim is to determine whether the ectopic lateral-spined eggs (*S. mansoni*-like eggs), are pure or hybrid, based on the mito-nuclear signature generated by combining the results of *cox1* RD-PCR and ITS-2 sequencing and cloning. The study also includes the genetic characterization of the

lateral-spined eggs detected in the only stool sample obtained from one of the five patients studied.

These five patients also carried terminal-spined eggs (*S. haematobium*-like eggs) in their urine. Their genetic characterization is included in this chapter for comparison purposes and for a global analysis of the genotypes of both pure and hybrid eggs. In addition, a combined haplotype description was performed on a subsample of eggs using the sequencing results of ribosomal (ITS-1, 5.8S and ITS-2) and mitochondrial (*cox1*) DNA markers.

4.2. MATERIAL AND METHODS

4.2.1. MATERIAL

This chapter includes five patients diagnosed with urogenital schistosomiasis, in whom ectopic lateral-spined schistosome eggs (*S. mansoni*-like eggs), were found in their urine (**Figure 4.1**).



Figure 4.1. Ectopic lateral-spined eggs (*S. mansoni*-like eggs) found in urine.

All these patients were diagnosed at the TMU of the Hospital de Poniente. They were young men, aged between 19 and 25 years, who had lived in Spain for between 3 and 48 months. None of them had returned to an endemic area. Three migrant patients were from Senegal, one from Guinea-Bissau (this patient reported bathing activities in the Senegal River), and one from Côte d'Ivoire (**Table 4.1**).

A 24-hour urine sample was obtained from each of the five patients, but an additional stool sample could only be obtained from one of the three Senegalese patients.

Table 4.1. Schistosome infected patients living in Spain with ectopic shedding of lateral-spined eggs (*S. mansoni*-like eggs): demographic and clinical data. M = male; Eos = eosinophils.

Patient code	Years, gender	Country of origin	Time living in Spain (months)		Migratory route	Eos/mm ³	Clinical manifestations	Other associated infections	Imaging findings
1Se	25, M	Senegal	4		Mauritania, Morocco, Spain	1456	Hematuria	<i>Mansonella persians</i>	Not performed
2Gb	21, M	Guinea-Bissau	8		Senegal*, Mali, Burkina Faso, Niger, Algeria, Morocco, Spain	655	Terminal hematuria and urinary discomfort for 2 years	-	1 focal thickening of the bladder and 2 parietal bladder nodules **Score pre-treatment: 5
3Se	19, M	Senegal	3		Mali, Burkina Faso, Niger, Libya, Italy (3 years), Spain	1574	Terminal hematuria for 2 years	<i>Entamoeba coli</i> , <i>Blastocystis hominis</i>	Bladder nodule of 11x6 mm **Score pre-treatment: 2
4Ci	23, M	Côte d'Ivoire	48		Burkina Faso, Niger, Algeria, Morocco, Spain	1140	Terminal hematuria for 5 years	-	Bladder nodule of 11x17 mm **Score pre-treatment: 2
5Se	23, M	Senegal	13		Morocco, Spain	796	Terminal hematuria for 5 months	-	Focal thickening of the bladder and 2 parietal bladder nodules **Score pre-treatment: 5

*Baths in rivers of Senegal **WHO-Niaamey Score for the assessment of schistosomiasis-related morbidity (Richter et al., 2000).

The five urine and stool samples were filtered and the eggs found in them individualized, photographed, and then fixed for subsequent genetic analysis as described in **Section 3.2.2**.

4.2.2. MOLECULAR METHODS

A total of 117 *Schistosoma* eggs were individually used for genetic characterization, of which 33 lateral-spined eggs (*S. mansoni*-like eggs) and 75 terminal-spined (*S. haematobium*-like eggs) were from urine samples and 9 lateral-spined eggs (*S. mansoni*-like eggs) were from the stool sample of one of the Senegalese patients (**Table 4.2**).

The mito-nuclear signature of each of the 117 *Schistosoma* eggs, collected from urine and stool, was determined by RD-PCR of *cox1* and ITS-2 sequencing analysis as previously described **Sections 3.2.3.2 and 3.2.3.3 (Table 4.2)**.

When sequencing the ITS-2 of the 42 lateral-spined eggs (*S. mansoni*-like eggs), the decision to go for ITS-2 cloning was taken when double peaks were observed in many positions in the electropherograms of the sequences obtained for this fragment by direct sequencing of some lateral-spined eggs. To confirm the identity of the heterozygotic sequences, the amplification products from 15 eggs, including 13 lateral-spined eggs (3 from feces and 10 from urine), and 2 controls, pure *S. haematobium* and pure *S. mansoni*, according to our DNA markers, were cloned with pGEM-T Easy Vector System I (Promega, Madison, WI) and introduced in *Escherichia coli* DH5 α competent cells. After the

growth of colonies, standard PCR of 7-8 different colonies per sample was performed and individually sequenced. This cloning allowed for the obtaining of a DNA sequence including the major part of the 5.8S gene (139 bp) followed by the complete ITS-2 sequence (314 bp), in its turn followed by 69 nucleotides of the initial 5' region of the 28S gene.

Table 4.2. Number of eggs processed for the creation of a mito-nuclear signature according to the migrant's country of origin, morphology of shedding eggs, and type of sample analyzed (urine or stool). Sh = *Schistosoma haematobium*; Sm = *S. mansoni*.

MITO-NUCLEAR SIGNATURE					
Human sample code	Country	Egg morphology			Total
		Sm-like (urine)	Sm-like (Stool)	Sh-like (urine)	
1Se	Senegal	3	9	7	19
2Gb	Guinea-Bissau	2	-	22	24
3Se	Senegal	16	-	24	40
4Ci	Côte d'Ivoire	2	-	13	15
5Se	Senegal	10	-	9	19
Total		33	9	75	117

A random subsample of 30 lateral-spined eggs (*S. mansoni*-like eggs), 24 collected from urine and 6 collected from stool, and 38 terminal-spined eggs (*S. haematobium*-like eggs) from urine (**Table 4.3**) was used for haplotype identification by full ITS-1-5.8S-ITS-2 (927 bp)

and partial *cox1* (1024 bp) sequencing as previously described in **Chapter III (Section 3.2.3.3)**.

Table 4.3. Number of eggs selected for haplotype identification by full ITS-1-5.8S-ITS-2 rDNA (927 bp) and partial *cox1* mtDNA (1024 bp) sequencing according to the migrant's country of origin, morphology of shedding eggs and type of sample processed (urine or stool). Sh = *Schistosoma haematobium*; Sm = *S. mansoni*.

HAPLOTYPE IDENTIFICATION					
Human sample code	Country	Egg morphology			Total
		Sm-like (urine)	Sm-like (Stool)	Sh-like (urine)	
1Se	Senegal	3	6	7	16
2Gb	Guinea-Bissau	1	-	8	9
3Se	Senegal	11	-	5	16
4Ci	Côte d'Ivoire	1	-	9	10
5Se	Senegal	8	-	9	17
Total		24	6	38	68

4.2.3. SEQUENCE ANALYSIS

For the editing and assembly of ITS-1-5.8S-ITS-2 and *cox1* sequences, we used Sequencher v. 5.4.6 software (Gene Codes Co. MI, USA) and ClustalW for their alignment using default parameters in MEGA X software (Kumar et al., 2018).

In the case of ITS-1-5.8S-ITS-2 rDNA sequences, a careful inspection of all nucleotide positions in the raw sequence chromatograms that allowed the detection of sequence polymorphisms between *S. haematobium* and *S. mansoni* was checked to identify the presence of possible heterozygosity, especially at the polymorphic positions that differentiate between these species (**Table 4.4**). When chromatograms revealed clear secondary overlapping peaks, the corresponding symbol of the IUPAC code for incomplete nucleic acid specification was used.

The determination of haplotypes was carried out using the ALTER web server (Glez-Peña et al., 2010), and the establishment of homologies were performed using the BLAST program from the National Centre for Biotechnology Information website (<http://www.ncbi.nlm.nih.gov/BLAST>). Only complete sequences or sufficiently long fragments of the same markers were selected for comparison purposes (**Appendices B.2 and B.3**).

4.2.4. MITO-NUCLEAR SIGNATURE CODE AND HYBRID DEFINITION

The code used for the mito-nuclear signature obtained from the lateral-spined eggs (*S. mansoni*-like eggs) follows the same criteria established in **Section 3.2.6 of Chapter III**. Species identity is indicated by a two-letter abbreviation.

Table 4.4. The complete intergenic region (including ITS-1, 5.8S, and ITS-2) polymorphic positions discriminating the two *Schistosoma* species (sp). Numbers (to be read vertically) refer to variable positions obtained in the alignment of these two species made with MEGA X. Sh = *Schistosoma haematobium*; Sm = *S. mansoni*.

species	rDNA polymorphic positions				
	ITS-1, 5.8S, ITS-2				
	ITS-1	ITS-2			
	11122222 233344	6666677777	8888888888	889999	
	1244577 7800500356 809933	3477800246	1222344667	890011	
	3792745505 6001534742 397849	2136256320	0013878783	044779	
Sm	AGACCGCCAA ACATGAATGT CAGATG	AATATAGATT ATATTAA--T	TTTTAT		
Sh	TACATTTTGT T-GAAGCAAC -TAGAA	GGGTCGAGCC GAGACGGATC	CGACGA		

AF531314 and MG554667 were used as reference sequences, for *S. mansoni* and *S. haematobium*, respectively.

For the mitochondrial marker *cox1*, only one allele is indicated because it is maternally inherited, whereas for the nuclear marker ITS-2, both alleles are indicated. Lateral-spined eggs (*S. mansoni*-like eggs) were classified as hybrids when the results obtained from the *cox1* and ITS-2 markers were discordant in species assignment and/or when heterozygotic positions were observed in the ITS-2 sequences at locations that differentiate between *S. mansoni* and *S. haematobium* (Table 4.4).

4.2.5. HAPLOTYPE CODE

The nomenclature code used for the haplotypes follows the same criteria as defined in Section 3.2.6 of Chapter III. The abbreviations Sm for *S. mansoni* and SmxSh for *S. mansoni* x *S. haematobium* have been used in the haplotypes obtained from lateral-spined eggs in this Chapter IV.

4.2.6. PHYLOGENETIC ANALYSIS

Phylogenetic analysis was performed using all *S. mansoni* and *S. haematobium cox1* haplotypes identified in this study, as well as reference haplotypes obtained from the GenBank Nucleotide Database (Appendix B.16). The data matrix included 27 sequences (9 haplotypes from this study+18 reference sequences) and 604 positions in the final

data set. *S. japonicum* (KY196417) was used as outgroup. The best substitution model selection analysis was performed in MEGA X, taking into account the BIC (Bayesian Information Criterion) scores, the AICc (Akaike Information Criterion, corrected) value, the Maximum Likelihood (lnL) value, and the number of parameters (including branch lengths) for each model. The Maximum Likelihood (ML) method was used to infer the evolutionary history. The initial tree(s) for the heuristic search were automatically obtained using the Nearest-Neighbor-Interchange (NNI) method by applying the Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach and then selecting the topology with the superior log likelihood value. To assess the reliability of the nodes in the trees, a bootstrap analysis using 1,000 replicates was made using the Bootstrap method in MEGA X.

4.3. RESULTS

4.3.1. GENETIC PROFILE BY MITO-NUCLEAR SIGNATURE

4.3.1.1. LATERAL-SPINED EGGS

Out of a total of 42 processed lateral-spined eggs (*S. mansoni*-like eggs), successful mito-nuclear profiling was obtained from 38 eggs, 30 of which were collected from urine and 8 from stool. All 38 successfully genotyped eggs showed an *S. mansoni cox1* profile by RD-PCR (Figure 4.2).

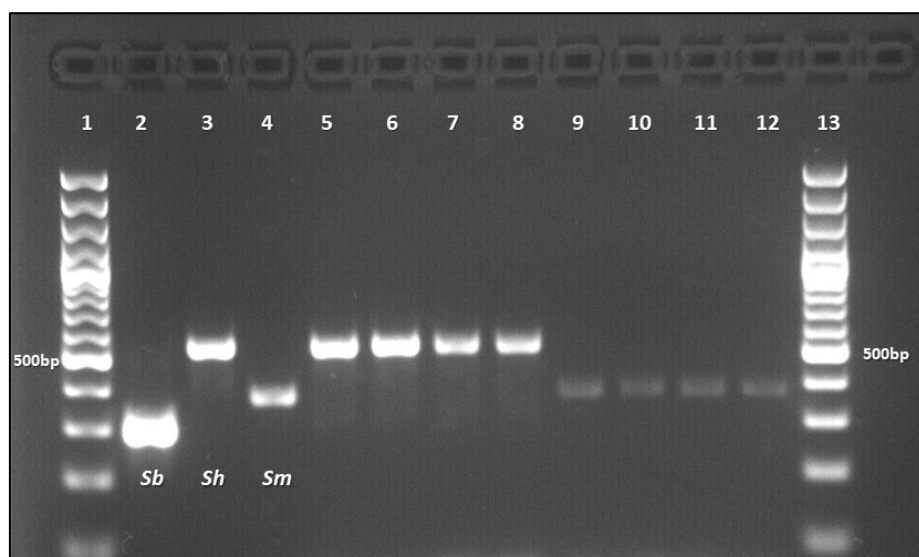


Figure 4.2. Mitochondrial *cox1* profile by RD-PCR. Lane 1 and 13: DNA ladder (100-3000 bp); lane 2: *S. bovis* (306 bp); lane 3: *S. haematobium* (543 bp); and lane 4: *S. mansoni* (375 bp). Each of the following lanes (5-12) corresponds to a separate multiplex PCR containing DNA extracted from a single egg: lanes 5-8 are terminal-spined eggs (*S. haematobium*-like eggs) from urine; lanes 9-10 are lateral-spined eggs (*S. mansoni*-like eggs) from urine; and lanes 11-12 are lateral-spined eggs (*S. mansoni*-like eggs) from stool.

Among the same 38 lateral-spined eggs, 9 (9/38, 23.68%) provided ITS-2 sequences corresponding to *S. mansoni*, while in 29 eggs (29/38, 76.32%), double chromatogram peaks were found at the polymorphic positions that discriminate between *S. mansoni* and *S. haematobium* in their resulting ITS-2 sequences (**Appendix B.17 and Figure 4.3**).

When the two markers were read together, two mito-nuclear signatures were obtained (**Appendix B.17**): *SmxSmSm* and *SmxSmSh*. The *SmxSmSm* mito-nuclear signature, corresponding to a pure *S. mansoni* genetic profile, was detected in 9 eggs (9/38, 23.68%), 3 from urine and 6 from stool, all collected from a single Senegalese patient, while the *SmxSmSh* mito-nuclear signature, corresponding to a *S. mansoni* x *S. haematobium* hybrid, was identified in 29 eggs, of which 27 (27/29, 93.10%) were collected from urine and 2 (2/29, 6.90%) from stool.

4.3.1.1.1. ITS-2 rDNA REGION SEQUENCES OBTAINED BY CLONING

The presence of mixed ITS-2 profiles between *S. mansoni* and *S. haematobium* (**Figure 4.3**), was confirmed by cloning. Both, *S. mansoni* and *S. haematobium* profiles were obtained for each of the 13 lateral-spined eggs (*S. mansoni*-like eggs) cloned. These clone sequences were found to be identical to the GenBank sequences AY446082 and MG554667 of *S. mansoni* and *S. haematobium*, respectively. It is worth.

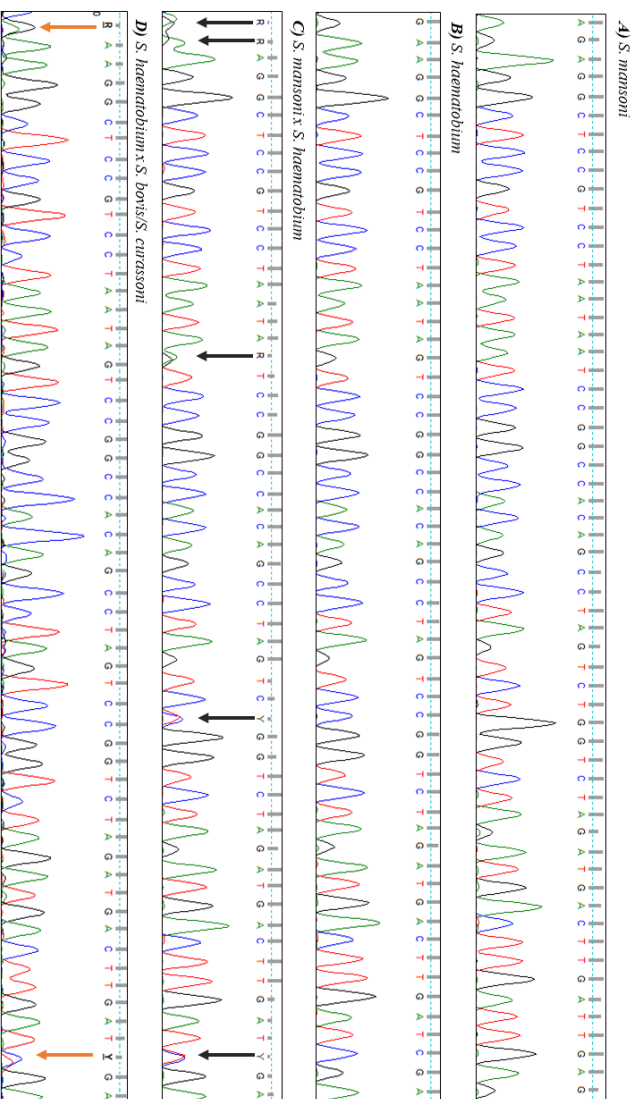


Figure 4.3. Selected fragment of ITS-2 sequences chromatograms obtained in the present study showing: a pure signal of **A)** *S. mansoni* detected in lateral-spined eggs from urine and stool; a pure signal of **B)** *S. haematobium* detected in terminal-spined eggs from urine; a hybrid signal of **C)** *S. mansoni* x *S. haematobium* showing double peaks (marked with black arrows) at positions that discriminate between *S. mansoni* and *S. haematobium* detected in lateral-spined eggs from urine and stool; and a hybrid signal of **D)** *S. haematobium* x *S. bovis/S. curassoni* presenting double peaks (marked with orange arrows) at positions that discriminate between *S. haematobium* and *S. bovis* or *S. curassoni* detected in terminal-spined eggs from urine.

mentioning that among the 99 clonal sequences analyzed, 54.55% (54/99) correspond to a pure ITS-2 of *S. mansoni* and 43.43% (43/99) to a pure *S. haematobium*. No intraindividual variation was detected among the clone sequences obtained from each single egg. Only a sporadically different result was obtained in two clone sequences analyzed (2/99, 2.02%) from 2 lateral-spined eggs of Senegalese origin, which presented a mixed sequence, starting at the 5' end as one of the two *Schistosoma* species (*S. mansoni* or *S. haematobium*), and when sequence reading arrives to positions 127 or 145 of the ITS-2 alignment, the reading continues as the other *Schistosoma* species (*S. haematobium* or *S. mansoni*) (**Appendix B.18**). Both positive controls, *S. mansoni* and *S. haematobium*, showed a pure *S. mansoni* or a pure *S. haematobium* profile, respectively, in 100% of their clone sequences obtained.

4.3.1.2. TERMINAL-SPINED EGGS

The mito-nuclear signature of 75 terminal-spined eggs processed (*S. haematobium*-like eggs), collected in the urine of the five patients with ectopic lateral-spined eggs (*S. mansoni*-like eggs) elimination, could be performed on 73. The 73 successfully genotyped terminal-spined eggs showed an *S. haematobium* *cox1* profile by RD-PCR (**Figure 4.2**). Out of the same 73 terminal-spined eggs, 68 (68/73, 93.15%) yielded ITS-2 sequences corresponding to *S. haematobium*, while in the other 5 (5/73, 6.85%), double chromatogram peaks were found at the polymorphic positions that discriminate between *S.*

haematobium and *S. bovis* or *S. curassoni* in their resulting ITS-2 sequences (**Appendix B.17** and **Figure 4.3**).

Two mito-nuclear signatures were obtained by combining the results of the two markers (**Appendix B.17**): *ShxShSh* (68/73, 93.15%), corresponding to a pure *S. haematobium*, and *ShxShSc* (4/73, 5.48%), resulting from a hybrid between *S. haematobium* and *S. curassoni*, confirmed by 18S sequencing. Due to the lack of DNA, the mito-nuclear signature of one of the hybrid terminal-spined eggs found in the Guinea-Bissau patient (1/73, 1.37%) could not be identified, so it was designated as *ShxShSb/Sc*.

It is worth mentioning that the patients from Côte d'Ivoire (4Ci) and Guinea-Bissau (2Gb), simultaneously presented two different hybrid mito-nuclear signatures: *SmxSmSh* in lateral-spined eggs from urine, and *ShxShSc* (in the case of patient 4Ci) or the undetermined *ShxShSb/Sc* (in the case of 2Gb) in terminal-spined eggs from urine (**Appendix B.17**).

4.3.2. MOLECULAR HAPLOTYPES FROM rDNA ITS-1-5.8S-ITS-2 SEQUENCING

4.3.2.1. LATERAL-SPINED EGGS

The complete sequence of the nuclear rDNA intergenic region of the 30 subsampled lateral-spined eggs (*S. mansoni*-like eggs) collected from the urine and stool of the five migrants diagnosed with urogenital schistosomiasis in Spain with ectopic lateral-spined eggs (*S. mansoni*-like eggs) provided two pure *S. mansoni* haplotypes and a hybrid *S. mansoni* x *S. haematobium* haplotype.

The two pure *S. mansoni* haplotypes yielded a 927 bp-long sequence of 48.49% GC mean content and differed between them at two heterozygotic positions located in the ITS-1, at positions 166 and 262 of their alignment. We have designated them as Sm-ITSs-H2 (48.49% GC) and as Sm-ITSs-H3 (48.49% GC). Sm-ITSs-H2 has a double peak "C/T"(Y) at position 166 and was detected in lateral-spined eggs collected from the urine of the Senegalese patient 1Se, while Sm-ITSs-H3 has a double peak "C/T" (Y) at position 262, and was detected in lateral-spined eggs collected from the stool of the same patient, 1Se. They were considered to be pure *S. mansoni* haplotypes since the heterozygotic positions they showed did not correspond to any position that discriminates between species (**Appendix B.19**).

The *S. mansoni* x *S. haematobium* haplotype showed variants of the typical *S. mansoni* profile with double peaks in the chromatograms (**Figure 4.3**), both in the ITS-1 and in the ITS-2 at positions that

distinguish it from *S. haematobium* (**Appendix B.19**). The sequence of this heterozygotic haplotype, including heterozygotic positions with their corresponding IUPAC codes, is indicated in **Appendices B.19** and **B.20**. Nucleotide assignment from positions 80 to 283 of the 5' region of the ITS-1 was not possible with either primer due to the considerable overlap of the two sequences (*S. mansoni* and *S. haematobium*) in this fragment, caused by two deletions in *S. haematobium* sequence, or two insertions in *S. mansoni*, at positions 80 and 284, in their alignment (**Appendix B.19**). A deletion or insertion means that the sequences no longer match, and therefore they overlap in the chromatogram reading (**Figure 4.4**). This haplotype pattern was designated as SmxSh-ITSs-Htz1.

For comparative purposes, an alignment was performed with our two *S. mansoni* haplotypes and 27 other *S. mansoni* sequences, corresponding to 4 haplotypes, available in GenBank and one reference *S. haematobium* sequence from GenBank (OX103963). The alignment was 929 bp-long and contained 55 variable positions (including heterozygotic positions), of which 1 was p-info, 4 were indels and 50 were singleton sites (**Appendix B.19**). Although the *S. mansoni* x *S. haematobium* hybrid pattern, SmxSh-ITSs-Htz1, is shown in **Appendix B.19**, it was not included in the comparison because nearly 200 bp of ITS-1 could not be determined due to overlapping sequences in the chromatogram reading.

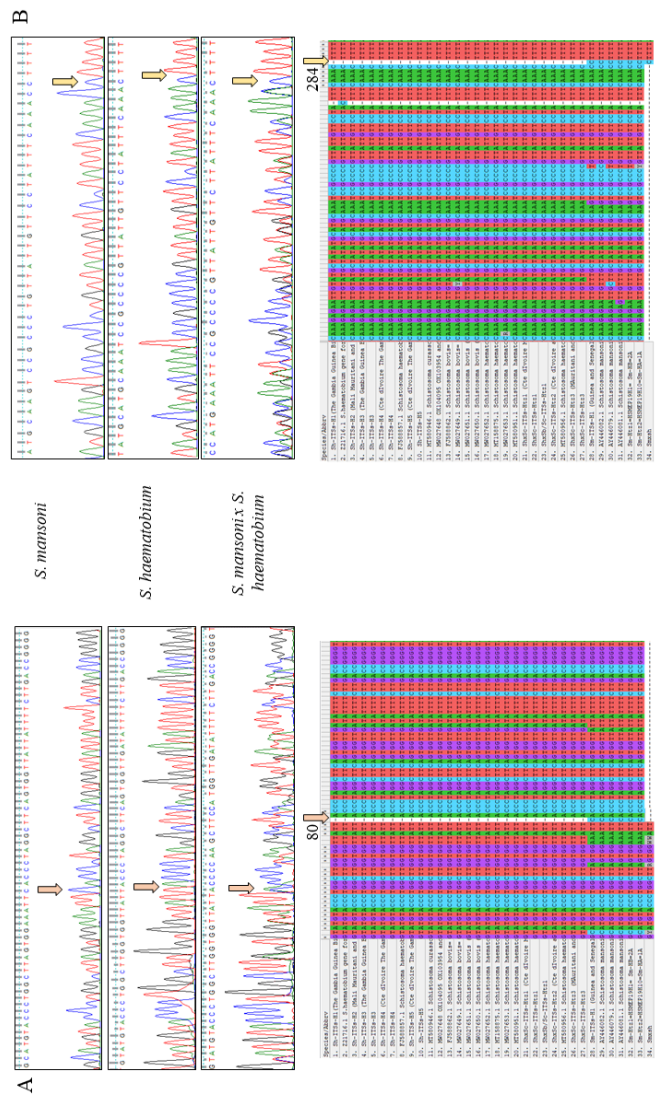


Figure 4.4. Two different fragments of ITSs chromatograms showing the onset of the overlapping reading with the A) forward and the B) reverse primers, respectively, in *S. mansoni* x *S. haematobium* sequences. The overlapping chromatograms reading with both primers is caused by two deletions in the *S. haematobium* sequence or two insertions in the *S. mansoni* sequence at positions 80 to 283 of the 5' ITS-1 region (orange arrow) and 284 (yellow arrow) of their alignment. This does not allow the nucleotide assignment from positions 80 to 283 of the 5' ITS-1 region in eggs containing an *S. mansoni* x *S. haematobium* ITSs sequence.

4.3.2.2. TERMINAL-SPINED EGGS

The complete intergenic nuclear rDNA region sequence of the 38 subsampled terminal-spined eggs (*S. haematobium*-like eggs) collected from the urine of migrants diagnosed with urogenital schistosomiasis in Spain with ectopic lateral-spined eggs (*S. mansoni*-like eggs) in their urine, yielded five haplotypes: Sh-ITSs-H3, Sh-ITSs-H4, and Sh-ITSs-H5, corresponding to pure *S. haematobium*, and ShxSc-ITSs-Htz1 and ShxSc-ITSs-Htz2, corresponding to hybrid *S. haematobium* x *S. curassoni* (**Appendix B.21**).

The three pure *S. haematobium* haplotypes provided a 927 bp-long sequence of 49.73% GC mean content and only differ at two polymorphic sites located in positions (19, of the ITS-1 and 534, within the 5.8S gene) of their alignment (**Appendix B.21**). No variation was detected in ITS-2. The haplotype Sh-ITSs-H4, previously reported in Côte d'Ivoire (MG554667), Benin (MT158876, MW027655) and Corsica (MW130296), was found to be the most frequent haplotype in the five patients with ectopic lateral-spined eggs (*S. mansoni*-like eggs) in urine from Senegal, Guinea-Bissau, and Côte d'Ivoire. The haplotype Sh-ITSs-H3 was found only in one of our patients from Senegal and in the patient from Guinea-Bissau, and the haplotype Sh-ITSs-H5, found in the patient from Côte d'Ivoire, correspond to the haplotypes MT158878 and MW027657, respectively, both previously described in schoolchildren from Benin.

The haplotypes with hybrid profile *S. haematobium* x *S. bovis* or *S. curassoni* were confirmed by the sequenced fragment of the 18S ribosomal gene, allowing the distinction between *S. bovis* and *S. curassoni*. Two haplotypes were confirmed as *S. haematobium* x *S. curassoni* (ShxSc-ITSs-Htz1 and ShxSc-ITSs-Htz2) found in the patient from Côte d'Ivoire (4Ci). Unfortunately, in the case of the patient from Guinea-Bissau (2Gb) no more DNA was available for 18S rDNA sequencing. For this reason, the haplotype in this case was ascribed as ShxSb/Sc-ITSs-Htz1. These hybrid haplotypes provided a 927 bp-long sequence of 49.54% GC mean content and only differ at nucleotide position 19 (ITS-1) where haplotype ShxSc-ITSs-Htz1 presents a “T” and haplotype ShxSc-ITSs-Htz2 has a heterozygotic mutation “C/T” (Y). The ITS-2 sequence of the aforementioned two haplotypes showed double peaks at positions where *S. haematobium* and *S. curassoni* differ. Both haplotypes have been previously reported in Beninese schoolchildren corresponding to accession numbers MT158877, MW027654, and MT158882, MW027656, respectively.

For comparison purposes an alignment of the complete intergenic nuclear rDNA region was made with our five haplotypes obtained in terminal-spined eggs, the three haplotypes obtained in lateral-spined eggs and 86 other ITSs rDNA sequences representing 25 haplotypes or isolates of the same or proximal species collected from GenBank. Sequences were collapsed into 28 different haplotypes and their alignment was 930 bp-long of which 63 were polymorphic sites in nucleotide positions (including heterozygotic positions), of which 49

were parsimony informative (p-info), 3 singleton sites, 5 indels (**Appendix B.20**). Although the *S. mansoni* x *S. haematobium* hybrid pattern, SmxSh-ITSs-Htz1, is shown in **Appendix B.20**, it was not included in the comparison because of the nearly 200 bp undetermined of ITS-1 due to overlapping sequences.

4.3.3. MOLECULAR HAPLOTYPES FROM PARTIAL mtDNA *cox1* GENE SEQUENCING

4.3.3.1. LATERAL-SPINED EGGS

Partial sequencing of the mitochondrial *cox1* gene from the 30 subsampled lateral-spined eggs (*S. mansoni*-like eggs) collected from the urine and stool of migrants diagnosed with urogenital schistosomiasis in Spain with ectopic lateral-spined eggs (*S. mansoni*-like eggs) in their urine was successful for 28. It provided five different haplotypes of 1024 bp-long.

These 5 haplotypes had an average AT content of 66.60% and showed 12 nucleotide differences in their alignment, corresponding to 6 p-info and 6 singleton sites. One of the 5 haplotypes described here had 100% sequence identity with the Sm-*cox1*-H2 haplotype detected in lateral-spined eggs collected from the stool of the Senegalese patient co-infected with *S. haematobium* and *S. mansoni* described in **Chapter III (Appendix B.13)**. The other four haplotypes detected were designated

as Sm-*cox1*-H18 to Sm-*cox1*-H21. The resulting *COXI* protein was 340 aa long and provided three different haplotypes due to 2 amino acid changes.

In the comparative alignment performed, including our five haplotypes described here, and 6 proximal sequences of *S. mansoni* according to BLAST (100% coverage and >99% similarity) representing 5 haplotypes (**Appendix B.3.D**), 1001 conserved positions and 23 variables were detected, of which 15 were parsimony informative and 8 singleton sites. The resulting *COXI* protein was 340 aa long and provided six different haplotypes due to 5 amino acid changes (**Appendix B.22**).

4.3.3.2. TERMINAL-SPINED EGGS

Partial sequencing of the mitochondrial *cox1* gene from the 38 terminal-spined eggs (*S. haematobium*-like eggs) collected from the urine of the five migrants diagnosed in Spain with ectopic lateral-spined eggs (*S. mansoni*-like eggs) in their urine was successful for 37. The 37 successful terminal-spined eggs (*S. haematobium*-like eggs) showed a *cox1* mtDNA sequence of 1024 bp-long and an average AT content of 70.31%. The analysis of the sequences obtained allowed us to collapse them into 3 different *S. haematobium* haplotypes: Sh-*cox1*-H9, Sh-*cox1*-H18, and Sh-*cox1*-H35 (**Appendix B.23**). These three haplotypes showed three nucleotide differences in their alignment, corresponding to three singleton sites. The *COXI* protein was 340 aa long and provided only one haplotype that was identical in all of our 37 successfully subsampled terminal-spined eggs (**Appendix B.23**).

Sh-*cox1*-H35 proved to be the most frequent haplotype in our samples found in patients from the three countries. This haplotype was identical to the other haplotypes or isolates previously reported in: human samples from Tanzania, (MK253577) and the Democratic Republic of the Congo (KY967520); *M. auratus* from Gabon (KT354659-60); and *B. globosus* from Malawi (EU567128) (**Appendices B.23**). The Sh-*cox1*-H9 haplotype was detected only in patient 2Gb from Guinea-Bissau, while the Sh-*cox1*-H18 haplotype was detected only in patient 4Ci from Côte d'Ivoire.

A comparative sequence analysis was performed including all *cox1* mtDNA haplotypes of *S. mansoni* (5 haplotypes) and *S. haematobium* (3 haplotypes) obtained from lateral-spined and terminal-spined eggs collected from the urine and stool of the five migrant patients with ectopic lateral-spined eggs (*S. mansoni*-like eggs) in their urine, and other 25 *cox1* sequences of the same or proximal species available in the GenBank database showing highly similar sequences according to BLAST (**Appendices B.3.A and B.3.D**). This alignment contains 203 variable positions, of which, 196 p-info and 7 singleton sites, and allows us to distinguish 21 different *cox1* haplotypes (Table not shown).

4.3.4. *cox1* PHYLOGENIES

The Hasegawa-Kishino-Yano model with discrete gamma distribution was the ML model that best fit our *cox1* data set. The resulting ML tree (log likelihood = -1908,73) was inferred with 5

categories and +G, parameter = 2,2569, and the rate variation model allowed for some sites to be evolutionarily invariant (**Figure 4.5**). This analysis involved 27 nucleotide sequences, including our *S. mansoni* and *S. haematobium* *cox1* haplotypes and other *Schistosoma* spp. isolates or haplotypes from GenBank (**Appendix B.16**). There were a total of 604 positions in the final data set.

The five *S. mansoni* *cox1* haplotypes (Sm-*cox1*-H2, Sm-*cox1*-H18, Sm-*cox1*-H19, Sm-*cox1*-H20, and Sm-*cox1*-H21) obtained from lateral-spined eggs (*S. mansoni*-like eggs) from urine and stool of migrant patients from Senegal and Côte d'Ivoire clustered in a well supported monophyletic clade (99%) with another representative haplotypes of *S. mansoni*. Within the monophyletic clade, our five haplotypes grouped in a well supported branch (94%) with other *S. mansoni* haplotypes from Senegal and Côte d'Ivoire.

The three *S. haematobium* *cox1* haplotypes (Sh-*cox1*-H9, Sh-*cox1*-H18, Sh-*cox1*-H35) obtained from terminal-spined eggs (*S. haematobium*-like eggs) from the urine of our five migrant patients grouped with another representative haplotypes of *S. haematobium* with high support (99%). All three *S. haematobium* *cox1* haplotypes obtained in this study clustered with group 1.

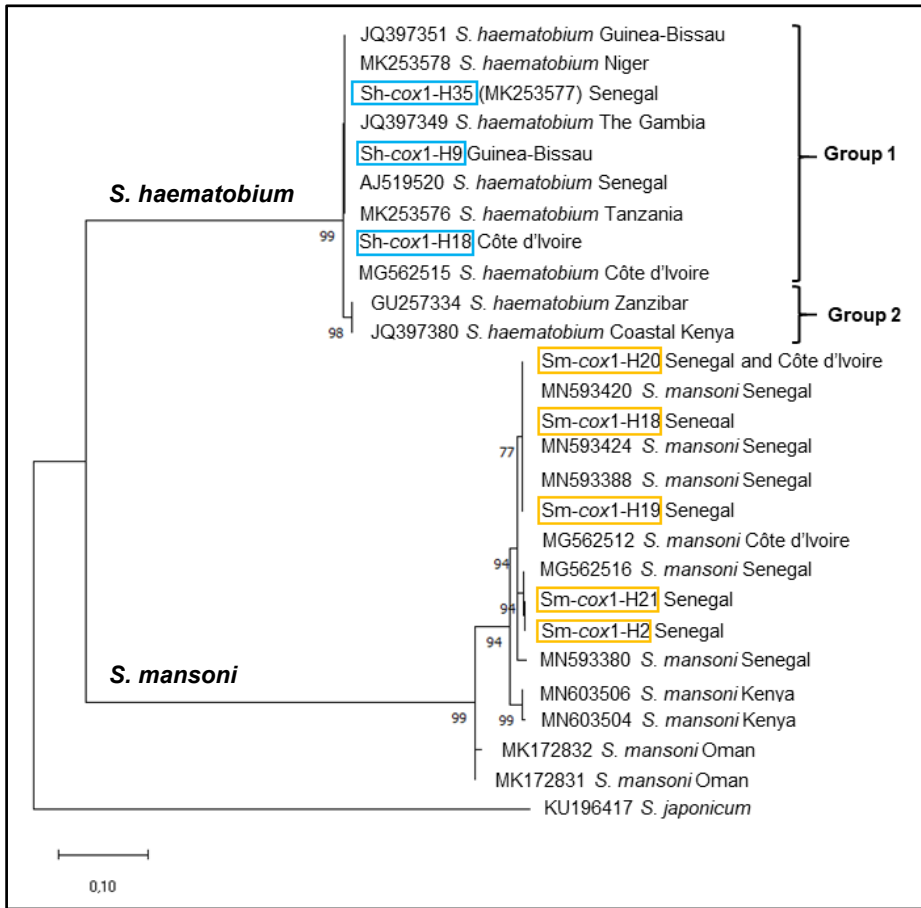


Figure 4.5. Phylogenetic tree of *cox1* mtDNA *cox1* sequences of *Schistosoma mansoni* (Sm-*cox1*-H2, Sm-*cox1*-H18, Sm-*cox1*-H19, Sm-*cox1*-H20, and Sm-*cox1*-H21) and *S. haematobium* (Sh-*cox1*-H9, Sh-*cox1*-H18, Sh-*cox1*-H35) haplotypes and data from GenBank, based on maximum-likelihood (ML) method and Hasegawa-Kishino-Yano model 5 categories +G, parameter = 2,2569. The tree with the highest log likelihood (-1908,73) is shown. The tree is drawn to scale. Branch lengths are measured in number of substitutions per site. The *S. japonicum* sequence (KU196417) was used as an outgroup to root the tree. The support for the nodes was obtained using the Bootstrap method with 1000 replications in MEGA X.

4.3.5. RIBOSOMAL AND MITOCHONDRIAL COMBINED HAPLOTYPES

The combination of the complete intergenic region of nuclear rDNA (ITS-1, 5.8S and ITS-2) and partial mtDNA *cox1* gene sequencing of 68 subsampled eggs, of which 30 were lateral-spined eggs (*S. mansoni*-like eggs) collected from urine and stool samples and 38 were terminal-spined eggs (*S. haematobium*-like eggs) collected from urine (**Table 4.3**), resulted in 14 different combined haplotypes (**Appendix B.24**).

A total of 7 combined haplotypes were described for lateral-spined eggs (*S. mansoni*-like eggs): 4 in urine, of which 3 (3/4, 75.0%) were hybrids (*S. mansoni* x *S. haematobium*) and a single combined haplotype (1/4, 25.0%) was pure *S. mansoni*; and, 3 in stool, of which the three were pure *S. mansoni* haplotypes (**Appendix B.24**). The SmxSh-ITSs-Htz1 and Sm-*cox1*-H21 combined haplotype was the most frequent among our 30 subsampled lateral-spined eggs, detected in 13 eggs (13/30, 43.33%) from the urine of two Senegalese patients (**Appendix B.25**).

For terminal-spined eggs (*S. haematobium*-like eggs), we described a total of 7 combined haplotypes, of which, 5 (5/7, 71.43%) were pure *S. haematobium*, and 2 (2/7, 28.57%) were hybrids (*S. haematobium* x *S. curassoni*) (**Appendix B.24**). The Sh-ITSs-H4 and Sh-*cox1*-H35 combined haplotype was the most common among our 38 subsampled terminal spined-eggs, detected in 24 eggs (24/38, 46.16%)

from the urine of three Senegalese and one Ivorian patient (**Appendix B.25**).

Unfortunately, it was not possible to provide the combined haplotype of 3 subsampled eggs, one terminal and one lateral-spined egg from the urine of patient 2Gb, from Guinea-Bissau, and one terminal-spined egg from the stool of patient 1Se, from Senegal, because we were unable to determine the *cox1* haplotype after sequencing them due to the bad quality of the sequences obtained (**Appendices B.24 and B.25**).

It is worth noting that within the same patient, we have detected up to 6 different combined haplotypes, as seen in the Ivorian patient (4Ci) (**Appendix B.25**).

4.4. DISCUSSION

Many studies have reported the presence of ectopic lateral-spined eggs (*S. mansoni*-like eggs) in urine (Cunin et al., 2003; Meurs et al., 2012a; Depaquit et al., 2019; Le Govic et al., 2019; Ojo et al., 2021), but very few have characterized them genetically (Depaquit et al., 2019; Le Govic et al., 2019). In the few studies that focused on genetic characterization of these ectopic *S. mansoni*-like eggs (Depaquit et al., 2019; Le Govic et al., 2019), a low number of eggs were genotyped and all were collected from a single patient.

In our study, we successfully genotyped 30 ectopic lateral-spined eggs (*S. mansoni*-like eggs) collected from the urine of five migrant patients residing in Spain from three different countries (Côte d'Ivoire, Guinea-Bissau, and Senegal) using a combination of nuclear and mitochondrial DNA markers.

The presence of ectopic lateral-spined eggs in urine can be explained through sexual interaction between *S. mansoni* and *S. haematobium* species, or due to a “spilling-over” caused by high *S. mansoni* infection loads (Cunin et al., 2003). Surprisingly, all five patients reported in this study showed *S. mansoni* x *S. haematobium* hybrid eggs, demonstrating a heterospecific cross between these two species and not a simple co-infection as the one described in **Chapter III** in one of the Senegalese patients, in whom no ectopic eggs or any hybrid eggs were detected.

These five patients are included in a larger investigation of imported urogenital schistosomiasis in collaboration with only two Spanish hospitals and represent 9.80% of the samples received in our laboratory in a short period. Although sporadic interactions between *S. mansoni* and *S. haematobium* have been observed in Senegalese and Ivorian children (Panzner and Boissier, 2021), our results lead us to believe that the heterospecific crosses between these two species and the resulting hybrids may be common in areas where they are co-endemic and, as already suggested (Le Govic et al., 2019), probably more widespread than previously observed. Most importantly, considering the significant number of mixed *S. mansoni* and *S. haematobium* infections studies conducted in different African countries and reporting the elimination of ectopic eggs (Cunin et al., 2003; Meurs et al., 2012a; Ojo et al., 2021) without providing genetic data on the nature of these eggs.

The fact that we have only detected *S. mansoni* x *S. haematobium* hybrid eggs in lateral-spined eggs, a typical *S. mansoni* morphology, is in line with the previous observations made in the two Ivorian children diagnosed in French hospitals (Depaquit et al., 2019; Le Govic et al., 2019). All hybrids identified in lateral-spined eggs from urine showed a *SmxSmSh* mito-nuclear signature, which means a *S. mansoni* genetic profile in the maternally inherited *cox1* marker and a *S. mansoni* x *S. haematobium* profile in ITS-2 (**Appendix B.17**). These results suggest that *S. haematobium* males have mated and migrated with *S. mansoni* females to the veins surrounding the bladder, resulting in the production of lateral-spined eggs in urine which seems consistent with the

competitiveness and the higher proportion of *S. haematobium* adult males compared to *S. mansoni* (Webster et al., 1999; Cunin et al., 2003).

Our data also indicate, a low percentage of *S. mansoni* x *S. haematobium* hybrid eggs in stool (2/29, 6.90%) (**Appendix B.17**). Unfortunately, stool samples from the other four patients could not be collected for further investigation. Both in northern Senegal (Huyse et al., 2013) and in one of the aforementioned Ivorian children (Le Govic et al., 2019), hybrids were detected in stool. While the study conducted in Senegal does not refer to egg morphology, the French study alludes to *S. mansoni* morphology which is in concordance with our results. Traditionally, it has always been assumed that it is the male that determines the location of oviposition due to its critical role in carrying the female (Nation et al., 2020), while egg morphology has been associated with the female, as it is in the ootype that the shape is determined (Tchuem Tchuente et al., 1993; Webster et al., 1999; LoVerde, 2019). Nevertheless, a hybrid lateral-spined egg in stool does not support either of these aforementioned assumptions. Although we cannot exclude the possibility that stool samples were contaminated with urine during collection, it would be interesting to conduct further experiments with these two species with clearly visually different eggs to better understand the inheritance of morphology. Especially since it has been suggested that hybrid schistosome eggs may exhibit different morphotypes (Moné et al., 2015).

Bidirectional inheritance has been described in *S. mansoni* x *S. haematobium* hybrids, since some eggs/miracidia with a mixed ITSs

profile (*S. mansoni* x *S. haematobium*) had a *S. mansoni* *cox1* haplotype, while others displayed a *S. haematobium* haplotype (Huyse et al., 2013; Le Govic et al., 2019). However, we did not identify an *S. haematobium* *cox1* profile among our analyzed hybrid lateral-spined eggs.

All haplotyped *S. mansoni* x *S. haematobium* lateral-spined eggs showed the SmxSh-ITSs-Htz1 haplotype pattern for ITSs, with double peaks at positions discriminating between *S. mansoni* and *S. haematobium*, while by partial sequencing of the *cox1*, we detected three haplotypes, Sm-*cox1*-H2, Sm-*cox1*-H20, and Sm-*cox1*-H21. The phylogenetic tree of the *cox1* sequences clustered these haplotypes (Sm-*cox1*-H2, Sm-*cox1*-H20, and Sm-*cox1*-H21) with other sequences from Senegal and Côte d'Ivoire (**Figure 4.5**), supporting the fact that hybrid parasite responsible of the infections have originated in West Africa, where the countries of origin of the migrant patients are encompassed.

The production of *S. mansoni* x *S. haematobium* hybrids is still quite surprising since they belong to two different evolutionary lineages of schistosomes. According to their phylogeny, it was traditionally expected that their pairings would result in predominantly parthenogenetic egg production and, if viable, would only be able to infect the mollusk host spectrum of the maternal schistosome species, as recently demonstrated experimentally between *S. mansoni* and *S. bovis* (Onyekwere et al., 2022a). Although *S. bovis* and *S. haematobium* are closely related species, both belonging to the monophyletic *S. haematobium* clade, and a large unidirectional introgression of *S. bovis* into *S. haematobium* has been demonstrated (Rey et al., 2021b), they do

not interact equally with *S. mansoni*. On the one hand, *S. mansoni* and *S. bovis* can share the same rodent host, have the same tropism for the oviposition site, and their combination shows mate choice recognition, which is behavioral isolation associated with species belonging to two different evolutionary lineages (Leger and Webster, 2017). On the other hand, *S. mansoni* and *S. haematobium*, which can also share the same host but different tropism in the vertebrate host, can easily mate without preference (Webster et al., 1999) which is associated with closely related species interaction. It appears that pre-zygotic reproductive isolating traits have not been strongly selected for in *S. mansoni* and *S. haematobium* as they have been in *S. mansoni* and *S. bovis*, and, despite both being crosses between phylogenetically distant species, the former combination results in hybrid progeny and the latter in parthenogenetic offspring (Onyekwere et al., 2022a).

The viability of the resulting *S. mansoni* x *S. haematobium* progeny has not yet been evaluated. However, the study conducted in Senegal suggests that these hybrids may be viable (Huyse et al., 2013), at least in the early generations, based on their ITSs cloning results. They found no intra-individual variation in cloned ITSs copies and a high number of *S. haematobium* copies compared to *S. mansoni* clone copies within the two free-living miracidia they analyzed. These results may indicate that the two miracidia are further generations or backcross hybrids.

Our data do not allow us to infer the viability of the lateral-spined hybrid eggs detected in this study, in which we detected a motile

miracidia inside them. Given the minimal intra-individual variation that we detected in cloned copies of ITS-2, it is possible that our *S. mansoni* x *S. haematobium* lateral-spined eggs are first or early generation hybrids. Experimental studies are definitely needed to assess the viability and transmission of the offspring resulting from crosses between *S. mansoni* and *S. haematobium*, which would help to understand their potential impact on the epidemiology of the disease.

No *S. mansoni* x *S. haematobium* hybrids were found regarding the terminal-spined eggs (*S. haematobium*-like eggs) analyzed in this study, all of which were collected from urine samples. All these terminal-spined eggs showed a pure *S. haematobium* profile on *cox1* by RD-PCR and most of them also showed a pure *S. haematobium* profile on ITS-2 sequencing, being the *ShxShSh*, the mito-nuclear more frequent among these eggs. However, in two patients (2Gb and 4Ci) (**Appendix B.17**), eggs with double peaks at the polymorphic sites between *S. haematobium* and *S. bovis* or *S. curassoni* were detected on ITS-2. The combined haplotype Sh-ITSs-H4 and Sh-*cox1*-H35, corresponding to a pure genetic profile of *S. haematobium*, was the most frequent in our genotyped terminal-spined eggs and was identified in four of the five patients analyzed (1Se, 3Se, 4Ci and 5Se) (**Appendix B.25**). The Sh-ITSs-H4 haplotype had previously been detected in West Africa, in Côte d'Ivoire and Benin, as well as in the Corsican outbreak. However, the Sh-*cox1*-H35 haplotype has only been reported in countries corresponding to Central and East Africa. The fact that we have detected this haplotype

in migrant patients from West African countries may suggest that this *cox1* haplotype may be widespread throughout the African continent.

To our knowledge, this is the first time to report patients with urinary ectopic lateral-spined eggs elimination simultaneously carrying two types of hybrid mito-nuclear signature: *ShxShSc* or *ShxShSb/Sc* (in *S. haematobium*-like eggs) and *SmxSmSh* (in *S. mansoni*-like eggs).

Based on the current data, it is not possible to know what impact *S. mansoni* x *S. haematobium* hybrids (*SmxSmSh*), as well as the presence of two types of hybrids (*SmxSmSh* and *ShxShSc* or *SmxSmSh* and *ShxShSb/Sc*) involving three *Schistosoma* species (*S. mansoni*, *S. haematobium* and *S. curassoni*/*S. bovis*) in the same patient, may have on morbidity and treatment response. Notably, all five patients had macroscopic haematuria and high eosinophilia, and except for one patient, who was unable to undergo imaging analysis, the rest presented nodules in the bladder (**Table 4.1**).

The five patients reported here are from countries where schistosomiasis is endemic: Senegal, Guinea-Bissau, and Côte d'Ivoire. Both *S. haematobium* and *S. mansoni* known to be established in Senegal (Stelma et al., 1993; Frigerio et al., 2016; Catalano et al., 2020) and Côte d'Ivoire (Assaré et al., 2015, 2020; Angora et al., 2020; Diakité et al., 2021) where, even mixed infection prevalence (De Clercq et al., 1999; Meurs et al., 2012a), morbidity (Meurs et al., 2012b) and model-based estimation co-distribution studies (Chammartin et al., 2014) have already been conducted. Furthermore, as mentioned above, these two countries

are the only ones where the presence of *S. mansoni* x *S. haematobium* hybrids has already been genetically confirmed (Huyse et al., 2013; Depaquit et al., 2019; Le Govic et al., 2019). However, these hybrids have never been reported from Guinea-Bissau. Although it is impossible to verify that the patient 2Gb was infected in his country of origin, since he confirmed that he also bathed in the Senegal River (**Table 4.1**), this could suggest a new localization for the hybrids resulting from *S. mansoni* and *S. haematobium* crosses. Knowledge of the epidemiology of schistosomiasis in Guinea-Bissau is scarce, with only one study on prevalence and morbidity due to *S. haematobium* infection (Botelho et al., 2016) found in the literature, and nothing regarding other *Schistosoma* species.

In summary, hybridization studies using a mito-nuclear signature applied to each of the 111 successfully genotyped *Schistosoma* eggs (73 terminal-spined eggs and 30 lateral-spined eggs from urine, and 8 lateral-spined eggs from stools) from the five selected patients with ectopic elimination of lateral-spined eggs included in this chapter, allow us to corroborate the frequent hybridization of *S. mansoni* x *S. haematobium*. Furthermore, the simultaneous presence of *S. haematobium* x *S. bovis* or *S. curassoni* hybrids was confirmed in two of the five patients included in the study.

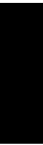
The majority of lateral-spined eggs analyzed (76.32%) presented a hybrid *SmxSmSh* mito-nuclear signature and only 23.68% had a pure *SmxSmSm* mito-nuclear signature (**Appendix B.17**). Regarding the terminal-spined eggs in urine, the majority (93.15%) showed a pure

ShxShSh mito-nuclear signature, while only 6.85%, presented a hybrid mito-nuclear signature (*ShxShSc* or *ShxShSb/Sc*) (**Appendix B.17**).

This is the first time that a great number of haplotypes are described in patients with lateral and terminal-spined eggs in urine. The complete intergenic region of the nuclear rDNA (ITS-1, 5.8S and ITS-2) and the partial mtDNA *cox1* gene sequencing results revealed a total of 14 different combined haplotypes, with up to six different haplotypes were detected within the same patient.

Four combined haplotypes corresponding to *S. mansoni* and three corresponding to *S. mansoni* x *S. haematobium* were detected in lateral-spined eggs. The genetic haplotyping performed here shows no variability within the *S. mansoni* x *S. haematobium* hybrids in the nuclear marker, all of them showing the same haplotype pattern (SmxSh-ITSs-Htz1). In terminal-spined eggs, five pure combined haplotypes corresponding to *S. haematobium* and two hybrid combined haplotypes corresponding to *S. haematobium* x *S. curassoni* were described (**Appendix B.24**).

CHAPTER V. CONCLUSIONS





This chapter outlines the main conclusions drawn from the three original works presented in the main chapters of this thesis (**Chapters II, III, and IV**). These conclusions respond to the specific objectives set for each of them.

At the time of submission of this thesis, the original work presented in **Chapter II**, based on the distribution of fascioliasis infecting the main livestock species in Bangladesh and the first study using the complete intergenic nuclear region of rDNA to assess fascioliasis in the country, has been published in the international open-access journal *One Health*. The mentioned article is attached as an appendix (**Appendix A**). The main conclusions of this chapter are presented below.

Regarding the results obtained on the distribution of fascioliasis infecting the main livestock species in Bangladesh, it can be concluded that:

- Fascioliasis is widespread throughout the country, being present in all the agro-ecological zones studied: coastal, hilly, Barindh steppe and floodplains, and infected animals were found in all the livestock species analyzed: buffaloes, cattle, goats and sheep.
- The prevalence found in goats (35.37%) and buffaloes (55.56%) is the highest ever observed in these animals in Bangladesh.
- The highest infection rates are observed in the floodplains, which are favorable habitats for fascioliasis transmission foci, and in the

western part of the country, coinciding with areas of transboundary livestock importation from the state of West Bengal (India).

- The high rates of *Fasciola* infection in the species studied indicate a high risk of human infection in Bangladesh, where the majority of the population is rural and livestock dependent.

In relation to the results obtained from the genetic characterization by sequencing and cloning of ITS-1-5.8S-ITS-2, the main conclusions are:

- The usefulness of ITS-1 and ITS-2 markers in the identification of pure and hybrid *Fasciola* species is confirmed, highlighting the suitability of using these markers in regions where the distributions of *F. hepatica* and *F. gigantica* overlap.
- The pure combined Fg-CH3A haplotype is widely distributed in cattle in Bangladesh. This distribution may be explained by the country's historical connection to the western Grand Trunk Road and the eastern Tea-Horse Road.
- Recent hybridization between *F. hepatica* and the local *F. gigantica* is confirmed in adult fasciolids showing heterozygotic positions in ITS-1 and ITS-2 at positions that differentiate the two species.
- The detection of up to six different ITS-1-5.8S-ITS-2 sequences coexisting in the cloned fasciolids provides clear evidence not only that this is a recent hybridization, but also that the parental specimens were already hybrids, indicating repeated, overlapping, and rapidly evolving hybridization events.

- The detection of three combined hybrid haplotypes could be explained by the high prevalence rates and infection intensity detected, which would facilitate repeated encounters and crosses between *F. hepatica* and *F. gigantica* within the bile canal or gallbladder of the definitive host.
- Recent hybridization between *F. hepatica* and local *F. gigantica* could be explained by the introduction of *F. hepatica* through livestock importation into Bangladesh, emphasizing the need for appropriate quarantine measures.
- Based on the distribution of combined hybrid haplotypes by host species, goats may play a critical role in the spread of hybrid specimens, underlining the need for control measures in small-scale domestic goat husbandry.
- The proportion of hybrids detected suggests a trend of increasing animal infection and risk to humans, given the potentially greater ability of hybrids to spread.

Chapter III presents the first genetic characterization study based on mitochondrial and nuclear markers of *Schistosoma* eggs obtained from urine and/or stool of 51 migrant patients from 8 different sub-Saharan countries residing in Spain, in order to evaluate the different *Schistosoma* species entering the country.

With regard to the genetic markers used, the following conclusions were drawn:

- The usefulness of combining the mtDNA marker *cox1* with rDNA, ITS-2 and, if necessary, 18S markers for the identification of *Schistosoma* species and hybrids is corroborated.
- ITS-2 was found to be a useful marker for distinguishing *S. haematobium* from *S. bovis* or *S. curassoni*, but not for distinguishing *S. bovis* from *S. curassoni*.
- 18S was found to be a useful marker for discriminating between *S. bovis*, *S. haematobium* and *S. curassoni*.
- Further studies are needed to know the variability of the 18S marker in *S. haematobium* and *S. curassoni* species.

Regarding terminal-spined eggs collected from the urine of patients diagnosed with urogenital schistosomiasis:

- The high percentage of patients (40.82%) harboring *Schistosoma* hybrids highlights the need to study whether there are differences in morbidity and treatment response compared to patients with pure genetic profiles.
- The presence of *S. haematobium* x *S. bovis* hybrids was confirmed in migrants from Mali, Mauritania and Senegal, while the presence of *S. haematobium* x *S. curassoni* hybrids was confirmed in migrants from Côte d'Ivoire, Mali, Mauritania and Senegal.
- The simultaneous presence of more than one hybrid genetic profile in the same patient was confirmed by the detection of *S. haematobium* x *S. bovis* and *S. haematobium* x *S. curassoni* hybrid eggs in the urine of a patient from Senegal.

- Repeated interactions and crosses between *S. haematobium*, *S. bovis* and *S. curassoni* were confirmed by the identification of eggs resulting from the interaction of these three species in a patient from Mali.

In relation to the terminal and lateral-spined eggs collected from the stool of patients diagnosed with intestinal schistosomiasis, it is concluded that:

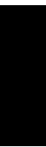
- The presence of genetically pure *S. guineensis* by the markers used was confirmed in terminal-spined eggs from stool of a migrant patient from Equatorial Guinea.
- The presence of genetically pure *S. mansoni* by the markers used was confirmed in eggs with lateral-spined eggs collected from the stool of migrant patients from Senegal and Guinea.
- Co-infection with *S. haematobium* and *S. mansoni* was confirmed in a patient from Senegal by the identification of pure terminal-spined eggs with a genetic profile of *S. haematobium* in urine and lateral-spined eggs with a genetic profile of *S. mansoni* in stool.

Chapter IV presents one of the few studies focused on the genetic characterization, using mitochondrial and nuclear markers, of ectopic lateral-spined eggs, typical of *S. mansoni*, in urine. In this study, for the first time, five migrant patients living in Spain and presenting with this type of ectopic eggs shedding were analyzed. In addition, the work includes the genetic characterization of terminal-spined eggs collected from the urine of the same patients, as well as lateral-spined

eggs collected from the stool of one of them. The conclusions that can be drawn from the results of this chapter are the following:

- All five patients included in the study are confirmed carriers of *S. mansoni* x *S. haematobium* hybrid lateral-spined eggs.
- All *S. mansoni* x *S. haematobium* hybrid eggs showed a *S. mansoni* *cox1* profile and double peaks in ITS-2 at positions that discriminate between *S. mansoni* and *S. haematobium*.
- It is confirmed that the five patients included in the study are also carriers of terminal-spined eggs in the urine with a pure *S. haematobium* genetic profile by the markers used.
- The simultaneous shedding of eggs with different pure and hybrid genetic profiles in the same patient is confirmed by the detection of:
 - *S. mansoni* x *S. haematobium*, *S. mansoni* and *S. haematobium* eggs in one of the Senegalese patients;
 - *S. mansoni* x *S. haematobium*, *S. haematobium* x *S. curassoni*, and *S. haematobium* in the patient from Côte d'Ivoire;
 - and the presence of *S. mansoni* x *S. haematobium*, *S. haematobium* x *S. bovis* or *S. curassoni* and *S. haematobium* eggs in the patient from Guinea-Bissau.
- Experimental studies are needed to assess the viability of *S. mansoni* x *S. haematobium* hybrids.

CHAPTER VI. BIBLIOGRAPHY





A

- Afroz, M.F., Faruque, M.O., Husain, S.S., Han, J.L., Paul, B., 2010. Genetic variation in relations in different goat populations of Bangladesh. *Bangladesh J. Anim. Sci.* 39, 1–8. <https://doi.org/10.3329/bjas.v39i1-2.9672>.
- Afshan, K., Fortes-Lima, C.A., Artigas, P., Valero, M.A., Qayyum, M., Mas-Coma, S., 2014a. Impact of climate change and man-made irrigation systems on the transmission risk, long-term trend and seasonality of human and animal fascioliasis in Pakistan. *Geospatial Health* 8, 317–334. <https://doi.org/10.4081/gh.2014.22>.
- Afshan, K., Valero, M.A., Qayyum, M., Peixoto, R.V., Magraner, A., Mas-Coma, S., 2014b. Phenotypes of intermediate forms of *Fasciola hepatica* and *F. gigantica* in buffaloes from Central Punjab, Pakistan. *J. Helminthol.* 88, 417–426. <https://doi.org/10.1017/S0022149X13000369>.
- Aguado, M., Espinosa, P., Romero-Maté, A., Tardío, J.C., Córdoba, S., Borbujo, J., 2012. Outbreak of cutaneous leishmaniasis in Fuenlabrada, Madrid. *Actas Dermosifiliogr.* 4, 334–342. <https://doi.org/10.1016/j.adengl.2013.03.005>
- Ahasan, S.A., Valero, M.A., Chowdhury, E.H., Islam, M.T., Islam, M.R., Hussain Mondal, M.M., Peixoto, R.V., Berinde, L., Panova, M., Mas-Coma, S., 2016. CIAS detection of *Fasciola hepatica*/*F. gigantica* intermediate forms in bovines from Bangladesh. *Acta Parasitol.* 61, 267–277. <https://doi.org/10.1515/ap-2016-0037>.
- Alba, A., Vazquez, A.A., Hurtrez-Boussès, S., 2021. Towards the comprehension of fasciolosis (re-)emergence: an integrative overview. *Parasitology* 148, 385–407. <https://doi.org/10.1017/S0031182020002255>.
- Ali, M.Z., Carlile, G., Giasuddin, M., 2020. Impact of global climate change on livestock health: Bangladesh perspective. *Open Vet. J.* 10, 178–188. <https://doi.org/10.4314/ovj.v10i2.7>.

BIBLIOGRAPHY

- Angora, E.K., Allienne, J.-F., Rey, O., Menan, H., Touré, A.O., Coulibaly, J.T., Raso, G., Yavo, W., N’Goran, E.K., Utzinger, J., Balmer, O., Boissier, J., 2020. High prevalence of *Schistosoma haematobium* × *Schistosoma bovis* hybrids in schoolchildren in Côte d’Ivoire. *Parasitology* 147, 287–294.
<https://doi.org/10.1017/S0031182019001549>.
- Angora, E.K., Vangraefschep, A., Allienne, J.-F., Menan, H., Coulibaly, J.T., Meïté, A., Raso, G., Winkler, M.S., Yavo, W., Touré, A.O., N’Goran, E.K., Zinsstag, J., Utzinger, J., Balmer, O., Boissier, J., 2022. Population genetic structure of *Schistosoma haematobium* and *Schistosoma haematobium* × *Schistosoma bovis* hybrids among school-aged children in Côte d’Ivoire. *Parasite* 29, 1–13.
<https://doi.org/10.1051/parasite/20222023>.
- Arenal, A., García, Y., Quesada, L., Velázquez, D., Sánchez, D., Peña, M., Suárez, A., Díaz, A., Sánchez, Y., Casaert, S., van Dijk, J., Vercruysse, J., Charlier, J., 2018. Risk factors for the presence of *Fasciola hepatica* antibodies in bulk-milk samples and their association with milk production decreases, in Cuban dairy cattle. *BMC Vet. Res.* 14, 1–8.
<https://doi.org/10.1186/s12917-018-1654-2>.
- Asadpour, M., Sharifiyazdi, H., Moazeni, M., Malekpour, S.H., 2020. Molecular characterization of *Fasciola* spp. from a donkey (*Equus asinus*) using partial sequencing of *cox1* and *nad1*. *Iran. J. Parasitol.* 15, 549–558. <https://doi.org/10.18502/ijpa.v15i4.4860>.
- Ashrafi, K., Bargues, M.D., O’Neill, S., Mas-Coma, S., 2014. Fascioliasis: a worldwide parasitic disease of importance in travel medicine. *Travel Med. Infect. Dis.*, special issue: zoonoses and travel medicine 12, 636–649. <https://doi.org/10.1016/j.tmaid.2014.09.006>.
- Ashrafi, K., Valero, M.A., Panova, M., Periago, M.V., Massoud, J., Mas-Coma, S., 2006. Phenotypic analysis of adults of *Fasciola hepatica*, *Fasciola gigantica* and intermediate forms from the endemic region of Gilan, Iran. *Parasitol. Int.* 55, 249–260.
<https://doi.org/10.1016/j.parint.2006.06.003>.

- Assaré, R.K., Lai, Y.-S., Yapi, A., Tian-Bi, Y.-N.T., Ouattara, M., Yao, P.K., Knopp, S., Vounatsou, P., Utzinger, J., N’Goran, E.K., 2015. The spatial distribution of *Schistosoma mansoni* infection in four regions of western Côte d’Ivoire. *Geospatial Health* 10, 69–79. <https://doi.org/10.4081/gh.2015.345>.
- Assaré, R.K., N’Tamon, R.N., Bellai, L.G., Koffi, J.A., Mathieu, T.-B.I., Ouattara, M., Hürlimann, E., Coulibaly, J.T., Diabaté, S., N’Goran, E.K., Utzinger, J., 2020. Characteristics of persistent hotspots of *Schistosoma mansoni* in western Côte d’Ivoire. *Parasit. Vectors* 13, 1–16. <https://doi.org/10.1186/s13071-020-04188-x>.

B

- Bargues, M.D., Gayo, V., Sanchis, J., Artigas, P., Khoubbane, M., Birriel, S., Mas-Coma, S., 2017. DNA multigene characterization of *Fasciola hepatica* and *Lymnaea neotropica* and its fascioliasis transmission capacity in Uruguay, with historical correlation, human report review and infection risk analysis. *PLoS Negl. Trop. Dis.* 11, 1–33. <https://doi.org/10.1371/journal.pntd.0005352>.
- Bargues, M.D., Halajian, A., Artigas, P., Luus-Powell, W.J., Valero, M.A., Mas-Coma, S., 2022. Paleobiogeographical origins of *Fasciola hepatica* and *F. gigantica* in light of new DNA sequence characteristics of *F. nyanzae* from hippopotamus. *Front. Vet. Sci.* 9, 1–28. <https://doi.org/10.3389/fvets.2022.990872>.
- Bargues, M.D., Mas-Coma, S., 2005. Reviewing lymnaeid vectors of fascioliasis by ribosomal DNA sequence analyses. *J. Helminthol.* 79, 257–267. <https://doi.org/10.1079/JOH2005297>.
- Bargues, M.D., Valero, M.A., Trueba, G.A., Fornasini, M., Villavicencio, A.F., Guamán, R., De Elías-Escribano, A., Pérez-Crespo, I., Artigas, P., Mas-Coma, S., 2021. DNA Multi-Marker genotyping and CIAS morphometric phenotyping of *Fasciola gigantica*-sized flukes from Ecuador, with an analysis of the *Radix* absence in the New World and the evolutionary Lymnaeid snail vector filter. *Animals* 11, 1–28. <https://doi.org/10.3390/ani11092495>.

BIBLIOGRAPHY

- Barton, N., Bengtsson, B.O., 1986. The barrier to genetic exchange between hybridising populations. *Heredity* 57, 357–376.
<https://doi.org/10.1038/hdy.1986.135>.
- Behar, J.M., Winston, J.S., Borgstein, R., 2009. Hepatic fascioliasis at a London hospital - the importance of recognising typical radiological features to avoid a delay in diagnosis. *Br. J. Radiol.* 82, e189–e193.
<https://doi.org/10.1259/bjr/78759407>.
- Beltrame, A., Buonfrate, D., Gobbi, F., Angheben, A., Marchese, V., Monteiro, G.B., Bisoffi, Z., 2017. The hidden epidemic of schistosomiasis in recent African immigrants and asylum seekers to Italy. *Eur. J. Epidemiol.* 32, 733–735. <https://doi.org/10.1007/s10654-017-0259-6>.
- Beltran, S., Boissier, J., 2009. Are schistosomes socially and genetically monogamous? *Parasitol. Res.* 104, 481–483.
<https://doi.org/10.1007/s00436-008-1225-8>.
- Berry, A., Fillaux, J., Martin-Blondel, G., Boissier, J., Iriart, X., Marchou, B., Magnaval, J.F., Delobel, P., 2016. Evidence for a permanent presence of schistosomiasis in Corsica, France, 2015. *Eurosurveillance* 21, 1–4.
<https://doi.org/10.2807/1560-7917.ES.2016.21.1.30100>.
- Bhuiyan, A.K.F.H., 2014. Farm animal genetic resources in Bangladesh: diversity, conservation and management. In: farm animal genetic resources in SAARC countries: diversity, conservation and management. 2014. SAARC Agriculture Centre (SAC), Dhaka 1215, Bangladesh, pp 1-74.
- Blin, M., Dametto, S., Agniwo, P., Webster, B.L., Angora, E., Dabo, A., Boissier, J., 2023. A duplex tetra-primer ARMS-PCR assay to discriminate three species of the *Schistosoma haematobium* group: *Schistosoma curassoni*, *S. bovis*, *S. haematobium* and their hybrids. *Parasit. Vectors* 16, 1–11. <https://doi.org/10.1186/s13071-023-05754-9>.
- Blum, A.J., Hotez, P.J., 2018. Global “worming”: climate change and its projected general impact on human helminth infections. *PLoS Negl. Trop. Dis.* 12, 1–6.
<https://doi.org/10.1371/journal.pntd.0006370>.

- Boissier, J., Grech-Angelini, S., Webster, B.L., Allienne, J.-F., Huyse, T., Mas-Coma, S., Toulza, E., Barré-Cardi, H., Rollinson, D., Kincaid-Smith, J., Oleaga, A., Galinier, R., Foata, J., Rognon, A., Berry, A., Mouahid, G., Henneron, R., Moné, H., Noel, H., Mitta, G., 2016. Outbreak of urogenital schistosomiasis in Corsica (France): an epidemiological case study. *Lancet Infect. Dis.* 16, 971–979. [https://doi.org/10.1016/S1473-3099\(16\)00175-4](https://doi.org/10.1016/S1473-3099(16)00175-4).
- Boissier, J., Moné, H., Mitta, G., Bargues, M.D., Molyneux, D., Mas-Coma, S., 2015. Schistosomiasis reaches Europe. *Lancet Infect. Dis.* 15, 757–758. [https://doi.org/10.1016/S1473-3099\(15\)00084-5](https://doi.org/10.1016/S1473-3099(15)00084-5).
- Boissier, J., Mouahid, G., Moné, H., 2019. *Schistosoma* spp., in: Water and sanitation for the 21st century: health and microbiological aspects of excreta and wastewater management (global water pathogen project). Michigan State University. <https://doi.org/10.14321/waterpathogens.45>.
- Boon, N.A.M., Mbow, M., Paredis, L., Moris, P., Sy, I., Maes, T., Webster, B.L., Sacko, M., Volckaert, F.A.M., Polman, K., Huyse, T., 2019. No barrier breakdown between human and cattle schistosome species in the Senegal River Basin in the face of hybridisation. *Int. J. Parasitol.* 49, 1039–1048. <https://doi.org/10.1016/j.ijpara.2019.08.004>.
- Boon, N.A.M., Van Den Broeck, F., Faye, D., Volckaert, F.A.M., Mboup, S., Polman, K., Huyse, T., 2018. Barcoding hybrids: heterogeneous distribution of *Schistosoma haematobium* × *Schistosoma bovis* hybrids across the Senegal River Basin. *Parasitology* 145, 634–645. <https://doi.org/10.1017/S0031182018000525>.
- Botelho, M.C., Machado, A., Carvalho, A., Vilaça, M., Conceição, O., Rosa, F., Alves, H., Richter, J., Bordalo, A.A., 2016. *Schistosoma haematobium* in Guinea-Bissau: unacknowledged morbidity due to a particularly neglected parasite in a particularly neglected country. *Parasitol. Res.* 115, 1567–1572. <https://doi.org/10.1007/s00436-015-4891-3>.
- Bottieau, E., Clerinx, J., de Vega, M.R., Van den Enden, E., Colebunders, R., Van Esbroeck, M., Vervoort, T., Van Gompel, A., Van den Ende, J., 2006. Imported Katayama fever: clinical and biological features at presentation and during treatment. *J. Infect.* 52, 339–345. <https://doi.org/10.1016/j.jinf.2005.07.022>.

Brindicci, G., Santoro, C.R., De Laurentiis, V., Capolongo, C., Solarino, M.E., Papagni, R., Ciraci, E., Gatti, P., Loconsole, D., Monno, R., Monno, L., Miragliotta, G., Angarano, G., 2017. Prevalence of urinary schistosomiasis in migrants in Apulia, a region of southern Italy, in the Years 2006–2016. *Biomed Res Int* 2017, 1–6.
<https://doi.org/10.1155/2017/8257310>.

C

Calvani, N.E.D., Šlapeta, J., 2021. *Fasciola* species introgression: just a fluke or something more? *Trends Parasitol.* 37, 25–34.
<https://doi.org/10.1016/j.pt.2020.09.008>.

Carrillo, E., Moreno, J., Cruz, I., 2013. What is responsible for a large and unusual outbreak of leishmaniasis in Madrid. *Trends Parasitol.* 29, 579–580. <https://doi.org/10.1016/j.pt.2013.10.007>.

Castillo-Fernández, N., Soriano-Pérez, M.J., Lozano-Serrano, A.B., Sánchez-Sánchez, J.C., Villarejo-Ordóñez, A., Cuenca-Gómez, J.A., Vázquez-Villegas, J., Cabeza-Barrera, M.I., Salas-Coronas, J., 2021. Usefulness of ultrasound in sub-Saharan patients with a serological diagnosis of schistosomiasis. *Infection* 49, 919–926.
<https://doi.org/10.1007/s15010-021-01612-x>.

Catalano, S., Léger, E., Fall, C.B., Borlase, A., Diop, S.D., Berger, D., Webster, B.L., Faye, B., Diouf, N.D., Rollinson, D., Sène, M., Bâ, K., Webster, J.P., 2020. Multihost transmission of *Schistosoma mansoni* in Senegal, 2015–2018. *Emerg. Infect. Dis.* 26, 1234–1242.
<https://doi.org/10.3201/eid2606.200107>.

Chammartin, F., HOUNGBEDJI, C.A., HÜRLIMANN, E., YAPI, R.B., SILUÉ, K.D., SORO, G., KOUAMÉ, F.N., N GORAN, E.K., UTZINGER, J., RASO, G., VOUNATSOU, P., 2014. Bayesian risk mapping and model-based estimation of *Schistosoma haematobium*-*Schistosoma mansoni* co-distribution in Côte d'Ivoire. *PLoS Negl. Trop. Dis.* 8, 1–10.
<https://doi.org/10.1371/journal.pntd.0003407>.

- Charlier, J., Duchateau, L., Claerebout, E., Williams, D., Vercruysse, J., 2007. Associations between anti-*Fasciola hepatica* antibody levels in bulk-tank milk samples and production parameters in dairy herds. *Prev. Vet. Med.* 78, 57–66. <https://doi.org/10.1016/j.prevetmed.2006.09.010>.
- Chen, L., Zhong, B., Xu, J., Li, R.-Z., Cao, C.-L., 2016. Health education as an important component in the national schistosomiasis control programme in the People's Republic of China. *Adv. Parasitol.* 92, 307–339. <https://doi.org/10.1016/bs.apar.2016.03.004>.
- Chitsulo, L., Engels, D., Montresor, A., Savioli, L., 2000. The global status of schistosomiasis and its control. *Acta Trop.* 77, 41–51. [https://doi.org/10.1016/s0001-706x\(00\)00122-4](https://doi.org/10.1016/s0001-706x(00)00122-4).
- Chougar, L., Mas-Coma, S., Artigas, P., Harhoura, K., Aissi, M., Agramunt, V.H., Bargues, M.D., 2020. Genetically ‘pure’ *Fasciola gigantica* discovered in Algeria: DNA multimarker characterization, trans-Saharan introduction from a Sahel origin and spreading risk into north-western Maghreb countries. *Transbound. Emerg. Dis.* 2190–2205. <https://doi.org/10.1111/tbed.13572>.
- Chowdhury, S.D., Hossain, M.M., Rahman, K.M.M., Saha, M.K., Hossain, S., 2015. Study on goat value chain in Bangladesh. Final report. Heifer International Bangladesh, Dhaka, Bangladesh 1–64.
- CIA, 2021. Bangladesh. The world factbook 2021. Washington, DC: Central Intelligence Agency. <https://www.cia.gov/the-world-factbook/countries/bangladesh/#geography> (accessed 5.12.23).
- Comelli, A., Riccardi, N., Canetti, D., Spinicci, M., Cenderello, G., Magro, P., Nicolini, L.A., Marchese, V., Zammarchi, L., Castelli, F., Bartoloni, A., Di Biagio, A., Caligaris, S., Gaiera, G., 2020. Delay in schistosomiasis diagnosis and treatment: a multicenter cohort study in Italy. *J. Travel Med.* 27, 1–7. <https://doi.org/10.1093/jtm/taz075>.
- Cuervo, P.F., Artigas, P., Mas-Coma, S., Bargues, M.D., 2022. West Nile virus in Spain: forecasting the geographical distribution of risky areas with an ecological niche modelling approach. *Transbound. Emerg. Dis.* 69, e1113–e1129. <https://doi.org/10.1111/tbed.14398>.

BIBLIOGRAPHY

Cunin, P., Tchuem Tchuente, L.-A., Poste, B., Djibrilla, K., Martin, P.M.V., 2003. Interactions between *Schistosoma haematobium* and *Schistosoma mansoni* in humans in north Cameroon. *Trop. Med. Int. Health* 8, 1110–1117.
<https://doi.org/10.1046/j.1360-2276.2003.01139.x>.

D

Das, B.C., Khan, A.S., Uddin, M.S., Chowdhury, M.M., Khan, M.Z.R., 2019. Fascioliasis- an uncommon cause of recurrent cholangitis. *J. Surg. Sci.* 19, 35–38. <https://doi.org/10.3329/jss.v19i1.43776>.

Dejon Agobé, J.C., Kariuki, H.C., Zinsou, J.F., Honkpehedji, Y.J., Grobusch, M.P., Adegnika, A.A., 2021. Pilot malacology surveys for the intermediate hosts of schistosomiasis in rural and semi-urban areas of the Moyen-Ogooué province, Gabon. *Trop. Med. Infect. Dis.* 7, 1–7.
<https://doi.org/10.3390/tropicalmed7010001>.

Depaquit, J., Akhoundi, M., Haouchine, D., Mantelet, S., Izri, A., 2019. No limit in interspecific hybridization in schistosomes: observation from a case report. *Parasite* 26, 1–5. <https://doi.org/10.1051/parasite/2019010>.

De Clercq, D., Vercruysse, J., Picquet, M., Shaw, D.J., Diop, M., Ly, A., Gryseels, B., 1999. The epidemiology of a recent focus of mixed *Schistosoma haematobium* and *Schistosoma mansoni* infections around the “Lac de Guiers” in the Senegal River Basin, Senegal. *Trop. Med. Int. Health* 4, 544–550.
<https://doi.org/10.1046/j.1365-3156.1999.00444.x>.

Diakité, N.R., Ouattara, M., Bassa, F.K., Coulibaly, J.T., Tian-Bi, Y.-N.T., Meïté, A., Hattendorf, J., Utzinger, J., N’Goran, E.K., 2021. Baseline and impact of first-year intervention on *Schistosoma haematobium* infection in Seasonal transmission foci in the Northern and Central Parts of Côte d’Ivoire. *Trop. Med. Infect. Dis.* 6, 1–16.
<https://doi.org/10.3390/tropicalmed6010007>.

Djuikwo-Teukeng, F.F., Kouam Simo, A., Allienne, J.-F., Rey, O., Njayou Ngapagna, A., Tchuem-Tchuente, L.A., Boissier, J., 2019. Population genetic structure of *Schistosoma bovis* in Cameroon. *Parasit. Vectors* 12, 1–11. <https://doi.org/10.1186/s13071-019-3307-0>.

Duarte, C.M. (Ed.), 2006. Cambio global: impacto de la actividad humana sobre el sistema tierra, Colección Divulgación. Consejo Superior de Investigaciones Científicas, Madrid.

E

Esteban, J.G., Flores, A., Aguirre, C., Strauss, W., Angles, R., Mas-Coma, S., 1997. Presence of very high prevalence and intensity of infection with *Fasciola hepatica* among Aymara children from the Northern Bolivian Altiplano. *Acta Trop.* 66, 1–14. [https://doi.org/10.1016/S0001-706X\(97\)00669-4](https://doi.org/10.1016/S0001-706X(97)00669-4).

Esteban, J.G., González, C., Barges, M.D., Angles, R., Sánchez, C., Náquira, C., Mas-Coma, S., 2002. High fascioliasis infection in children linked to a man-made irrigation zone in Peru. *Trop. Med. Int. Health* 7, 339–348. <https://doi.org/10.1046/j.1365-3156.2002.00870.x>.

F

Fall, C.B., Lambert, S., Léger, E., Yasenev, L., Garba, A.D., Diop, S.D., Borlase, A., Catalano, S., Faye, B., Walker, M., Sene, M., Webster, J.P., 2021. Hybridized zoonotic *Schistosoma* infections result in hybridized morbidity profiles: a clinical morbidity study amongst co-infected human populations of Senegal. *Microorganisms* 9, 1–18. <https://doi.org/10.3390/microorganisms9081776>.

Fiss, L., de Lourdes Adrien, M., Marcolongo-Pereira, C., Assis-Brasil, N.D., Sallis, E.S.V., Riet-Correa, F., Ruas, J.L., Schild, A.L., 2013. Subacute and acute fasciolosis in sheep in southern Brazil. *Parasitol. Res.* 112, 883–887. <https://doi.org/10.1007/s00436-012-3096-2>.

BIBLIOGRAPHY

- Figueiredo, J.C., Richter, J., Borja, N., Balaca, A., Costa, S., Belo, S., Grácio, M.A., 2015. Prostate adenocarcinoma associated with prostatic infection due to *Schistosoma haematobium*. Case report and systematic review. *Parasitol. Res.* 114, 351–358. <https://doi.org/10.1007/s00436-014-4250-9>.
- Frigerio, S., Macario, M., Iacovone, G., Dussey-Comlavi, K.J., Narcisi, P., Ndiaye, A.T., Moramarco, S., Alvaro, R., Palombi, L., Buonomo, E., 2016. *Schistosoma haematobium* infection, health and nutritional status in school-age children in a rural setting in Northern Senegal. *Minerva Pediatr.* 68, 282–287.

G

- Gabrielli, A.F., Garba Djirmay, A., 2023. Schistosomiasis in Europe. *Curr. Trop. Med. Rep.* <https://doi.org/10.1007/s40475-023-00286-9>.
- Garba, A., Barkiré, N., Djibo, A., Lamine, M.S., Sofu, B., Gouvras, A.N., Bosqué-Oliva, E., Webster, J.P., Stothard, J.R., Utzinger, J., Fenwick, A., 2010. Schistosomiasis in infants and preschool-aged children: Infection in a single *Schistosoma haematobium* and a mixed *S. haematobium*-*S. mansoni* foci of Niger. *Acta Trop.* 115, 212–219. <https://doi.org/10.1016/j.actatropica.2010.03.005>.
- Garg, R., Yadav, C.L., Kumar, R.R., Banerjee, P.S., Vatsya, S., Godara, R., 2009. The epidemiology of fasciolosis in ruminants in different geo-climatic regions of north India. *Trop. Anim. Health Prod.* 41, 1695–1700. <https://doi.org/10.1007/s11250-009-9367-y>.
- Gariev, B.G., 1970. The first occurrence of *Fasciola indica* (Trematoda) in the USSR. *Zool. Zhurnal* 49.
- Gayo, V., Cuervo, P., Rosadilla, D., Birriel, S., Dell'Oca, L., Trelles, A., Cuore, U., Mera y Sierra, R. Natural *Fasciola hepatica* infection in nutria (*Myocastor coypus*) in Uruguay. *J. Zoo Wildl. Med.* 2011 Jun;42(2):354-6. doi: 10.1638/2010-0226.1.

- Gentile, R., Soares, M., Barreto, M., Gonçalves, M., D'Andrea, P., 2012. The role of wild rodents in the transmission of *Schistosoma mansoni* in Brazil. pp. 231–254. <https://doi.org/10.5772/25909>.
- Glez-Peña, D., Gómez-Blanco, D., Reboiro-Jato, M., Fdez-Riverola, F., Posada, D., 2010. ALTER: program-oriented conversion of DNA and protein alignments. *Nucleic Acids Res.* 38, W14–W18. <https://doi.org/10.1093/nar/gkq321>.
- Golden, O., 2023. Raw fish consumption in Portugal: commonly consumed fish species and associated risk factors for anisakiosis. *Food control* 145, 1–10. <https://doi.org/10.1016/j.foodcont.2022.109457>.
- Golden, O., Caldeira, A.J.R., Santos, M.J., 2022. Raw fish consumption in Portugal: a survey on trends in consumption and consumer characteristics. *Food control* 135, 1–9. <https://doi.org/10.1016/j.foodcont.2022.108810>.
- Gordon, C.A., Kurscheid, J., Williams, G.M., Clements, A.C.A., Li, Y., Zhou, X.-N., Utzinger, J., McManus, D.P., Gray, D.J., 2019. Asian schistosomiasis: current status and prospects for control leading to elimination. *Trop. Med. Infect. Dis.* 4, 1–29. <https://doi.org/10.3390/tropicalmed4010040>.
- Gouvras, A.N., Kariuki, C., Koukounari, A., Norton, A.J., Lange, C.N., Ileri, E., Fenwick, A., Mkoji, G.M., Webster, J.P., 2013. The impact of single versus mixed *Schistosoma haematobium* and *S. mansoni* infections on morbidity profiles amongst school-children in Taveta, Kenya. *Acta Trop.* 128, 309–317. <https://doi.org/10.1016/j.actatropica.2013.01.001>.
- Gower, C.M., Vince, L., Webster, J.P., 2017. Should we be treating animal schistosomiasis in Africa? The need for a One Health economic evaluation of schistosomiasis control in people and their livestock. *Trans. R. Soc. Trop. Med. Hyg.* 111, 244–247. <https://doi.org/10.1093/trstmh/trx047>.
- Gryseels, B., 2012. Schistosomiasis. *Infect. Dis. Clin. North Am., Tropical Diseases* 26, 383–397. <https://doi.org/10.1016/j.idc.2012.03.004>.

BIBLIOGRAPHY

- Gryseels, B., Polman, K., Clerinx, J., Kestens, L., 2006. Human schistosomiasis. *Lancet Lond. Engl.* 368, 1106–1118. [https://doi.org/10.1016/S0140-6736\(06\)69440-3](https://doi.org/10.1016/S0140-6736(06)69440-3).
- Gupta, S., Ghosh, S., Raina, O.K., Joseph, D., Rawat, P., Singh, B.P., Mishra, A.K., Chandra, D., Samanta, S., 2008. Status and prevalence of fasciolosis in cattle and buffaloes in different agro-climatic zones of Uttar Pradesh, India. *J. Vet. Parasitol.* 22, 59–63.

H

- Habib, M.R., Haque, M.N., Rahman, A., Aftabuzzaman, M., Ali, M.M., Shahjahan, M., 2017. Dairy buffalo production scenario in Bangladesh: a review. *Asian J. Med. Biol. Res.* 3, 305–316. <https://doi.org/10.3329/ajmbr.v3i3.34518>.
- Hayashi, K., Ichikawa-Seki, M., Mohanta, U.K., Shoriki, T., Chaichanasak, P., Itagaki, T., 2018. Hybrid origin of Asian aspermic *Fasciola* flukes is confirmed by analyzing two single-copy genes, *pepck* and *pold*. *J. Vet. Med. Sci.* 80, 98–102. <https://doi.org/10.1292/jvms.17-0406>.
- Huyse, T., Van den Broeck, F., Hellems, B., Volckaert, F.A.M., Polman, K., 2013. Hybridisation between the two major African schistosome species of humans. *Int. J. Parasitol.* 43, 687–689. <https://doi.org/10.1016/j.ijpara.2013.04.001>.
- Huyse, T., Webster, B.L., Geldof, S., Stothard, J., Russell, S., Diaw, O.T., Polman, K., Rollinson, D., 2009. Bidirectional introgressive hybridization between a cattle and human schistosome species. *PLoS Pathog.* 5, 1–9. <https://doi.org/10.1371/journal.ppat.1000571>.

I

- Ichikawa-Seki, M., Tokashiki, M., Opara, M.N., Iroh, G., Hayashi, K., Kumar, U.M., Itagaki, T., 2017. Molecular characterization and phylogenetic analysis of *Fasciola gigantica* from Nigeria. *Parasitol. Int.* 66, 893–897. <https://doi.org/10.1016/j.parint.2016.10.010>.
- Islam, S.S., Hasan, M.S., Ghosh, N., Islam, M.M., 2021. Prospects and problems of indigenous sheep production in South-Western Coastal regions of Bangladesh. *J. Agric. Sci. – Sri Lanka* 16, 54. <https://doi.org/10.4038/jas.v16i1.9183>.
- Itagaki, T., Hayashi, K., Ohari, Y., 2022. The causative agents of fascioliasis in animals and humans: parthenogenetic *Fasciola* in Asia and other regions. *Infect. Genet. Evol.* 99, 1–11. <https://doi.org/10.1016/j.meegid.2022.105248>.
- Itagaki, T., Ichinomiya, M., Fukuda, K., Fusyuku, S., Carmona, C., 2011. Hybridization experiments indicate incomplete reproductive isolating mechanism between *Fasciola hepatica* and *Fasciola gigantica*. *Parasitology* 138, 1278–1284. <https://doi.org/10.1017/S0031182011000965>.

J

- Jambou, R., Njedanoun, M., Panthou, G., Descroix, L., 2022. Malaria transmission in Sahelian African regions, a witness of climate changes. *Int. J. Environ. Res. Public. Health* 19, 1–5. <https://doi.org/10.3390/ijerph191610105>.
- Jourdane, J., Southgate, V.R., 1992. Genetic exchanges and sexual interactions between species of the genus *Schistosoma*. *Res. Rev. Parasitol.* 52, 21–26.

K

- Kincaid-Smith, J., Mathieu-Bégné, E., Chaparro, C., Reguera-Gomez, M., Mulero, S., Allienne, J.-F., Toulza, E., Boissier, J., 2021. No pre-zygotic isolation mechanisms between *Schistosoma haematobium* and *Schistosoma bovis* parasites: from mating interactions to differential gene expression. PLoS Negl. Trop. Dis. 15, 1–24. <https://doi.org/10.1371/journal.pntd.0009363>
- Kincaid-Smith, J., Rey, O., Toulza, E., Berry, A., Boissier, J., 2017. Emerging schistosomiasis in Europe: a need to quantify the risks. Trends Parasitol. 33, 600–609. <https://doi.org/10.1016/j.pt.2017.04.009>.
- King, K.C., Stelkens, R.B., Webster, J.P., Smith, D.F., Brockhurst, M.A., 2015. Hybridization in parasites: consequences for adaptive evolution, pathogenesis, and public health in a changing world. PLOS Pathog. 11, 1–12. <https://doi.org/10.1371/journal.ppat.1005098>.
- Koukounari, A., Donnelly, C.A., Sacko, M., Keita, A.D., Landouré, A., Dembelé, R., Bosqué-Oliva, E., Gabrielli, A.F., Gouvras, A., Traoré, M., Fenwick, A., Webster, J.P., 2010. The impact of single versus mixed schistosome species infections on liver, spleen and bladder morbidity within Malian children pre- and post-praziquantel treatment. BMC Infect. Dis. 10, 1–14. <https://doi.org/10.1186/1471-2334-10-227>.
- Kumar, S., Stecher, G., Li, M., Knyaz, C., Tamura, K., 2018. MEGA X: Molecular evolutionary genetics analysis across computing platforms. Mol. Biol. Evol. 35, 1547–1549. <https://doi.org/10.1093/molbev/msy096>.

L

- Le, T.H., Pham, K.L.T., Doan, H.T.T., Le, T.K.X., Nguyen, K.T., Lawton, S.P., 2020. Description and phylogenetic analyses of ribosomal transcription units from species of Fasciolidae (Platyhelminthes: Digenea). *J. Helminthol.* 94, 2–6. <https://doi.org/10.1017/S0022149X20000164>.
- Le Govic, Y., Kincaid-Smith, J., Allienne, J.-F., Rey, O., Gentile, L. de, Boissier, J., 2019. *Schistosoma haematobium*–*Schistosoma mansoni* hybrid parasite in migrant boy, France, 2017. *Emerg Infect Dis* 25, 365–367. <https://doi.org/10.3201/eid2502.172028>.
- Leblanc, C., Pham, L., Mariani, P., Titomanlio, L., El Ghoneimi, A., Paris, L., Escoda, S., Lottmann, H., Toubiana, J., Paugam, A., Ulinski, T., Bouchaud, O., Brun, S., Izri, A., Faye, A., De Pontual, L., 2017. Imported schistosomiasis in children: clinical, diagnostic aspects and outcome in 5 tertiary hospitals in France. *Pediatr. Infect. Dis. J.* 36, e349–e351. <https://doi.org/10.1097/INF.0000000000001679>.
- Léger, E., Garba, A., Hamidou, A.A., Webster, B.L., Pennance, T., Rollinson, D., Webster, J.P., 2016. Introgressed animal schistosomes *Schistosoma curassoni* and *S. bovis* naturally infecting humans. *Emerg. Infect. Dis.* 22, 2212–2214. <https://doi.org/10.3201/eid2212.160644>.
- Léger, E., Webster, J.P., 2017. Hybridizations within the Genus *Schistosoma*: implications for evolution, epidemiology and control. *Parasitology* 144, 65–80. <https://doi.org/10.1017/S0031182016001190>.
- Lo, N.C., Bezerra, F.S.M., Colley, D.G., Fleming, F.M., Homeida, M., Kabatereine, N., Kabole, F.M., King, C.H., Mafe, M.A., Midzi, N., Mutapi, F., Mwanga, J.R., Ramzy, R.M.R., Satrija, F., Stothard, J.R., Traoré, M.S., Webster, J.P., Utzinger, J., Zhou, X.-N., Danso-Appiah, A., Eusebi, P., Loker, E.S., Obonyo, C.O., Quansah, R., Liang, S., Vaillant, M., Murad, M.H., Hagan, P., Garba, A., 2022. Review of 2022 WHO guidelines on the control and elimination of schistosomiasis. *Lancet Infect. Dis.* 22, e327–e335. [https://doi.org/10.1016/S1473-3099\(22\)00221-3](https://doi.org/10.1016/S1473-3099(22)00221-3).

BIBLIOGRAPHY

- Lockyer, A.E., Olson, P.D., Østergaard, P., Rollinson, D., Johnston, D.A., Attwood, S.W., Southgate, V.R., Horak, P., Snyder, S.D., Le, T.H., Agatsuma, T., Mcmanus, D.P., Carmichael, A.C., Naem, S., Littlewood, D.T.J., 2003. The phylogeny of the Schistosomatidae based on three genes with emphasis on the interrelationships of *Schistosoma* Weinland, 1858. *Parasitology* 126, 203–224. <https://doi.org/10.1017/S0031182002002792>.
- Lodge, D.M., Stein, R.A., Brown, K.M., Covich, A.P., Brönmark, C., Garvey, J.E., Klosiewski, S.P., 1998. Predicting impact of freshwater exotic species on native biodiversity: challenges in spatial scaling. *Aust. J. Ecol.* 23, 53–67. <https://doi.org/10.1111/j.1442-9993.1998.tb00705.x>.
- Loo, S.E., Keller, R.P., Leung, B., 2007. Freshwater invasions: using historical data to analyse spread. *Divers. Distrib.* 13, 23–32. <https://doi.org/10.1111/j.1366-9516.2006.00313.x>.
- LoVerde, P.T., 2019. Schistosomiasis. *Adv. Exp. Med. Biol.* 1154, 45–70. https://doi.org/10.1007/978-3-030-18616-6_3.
- Luton, K., Walker, D., Blair, D., 1992. Comparisons of ribosomal internal transcribed spacers from two congeneric species of flukes (Platyhelminthes: Trematoda: Digenea). *Mol. Biochem. Parasitol.* 56, 323–327. [https://doi.org/10.1016/0166-6851\(92\)90181-I](https://doi.org/10.1016/0166-6851(92)90181-I).

M

- Madhumitha, R., Gohel, S., Vishwanathan, L., Gopalakrishnan, R., 2015. Liver lesions, fever and eosinophilia caused by *Fasciola hepatica* in a 15-year-old girl. *Indian J. Pediatr.* 82, 967–968. <https://doi.org/10.1007/s12098-015-1745-z>.
- Maeda, T., Yamada, H., Akao, N., Iga, M., Endo, T., Koibuchi, T., Nakamura, T., Odawara, T., Iwamoto, A., Fujii, T., 2008. Unusual radiological findings of *Fasciola hepatica* infection with huge cystic and multilocular lesions. *Intern. Med.* 47, 449–452. <https://doi.org/10.2169/internalmedicine.47.0626>.

- Marchese, V., Beltrame, A., Angheben, A., Monteiro, G.B., Giorli, G., Perandin, F., Buonfrate, D., Bisoffi, Z., 2018. Schistosomiasis in immigrants, refugees and travellers in an Italian referral centre for tropical diseases. *Infect. Dis. Poverty* 7, 55. <https://doi.org/10.1186/s40249-018-0440-5>.
- Martínez-Ortí, A., Adam, S., Garippa, G., Boissier, J., Bargues, M.D., Mas-Coma, S., 2022. Morpho-anatomical characterization of the urogenital schistosomiasis vector *Bulinus truncatus* (Audouin, 1827) (Heterobranchia: Bulinidae) from Southwestern Europe. <https://doi.org/10.48550/arXiv.2210.02451>.
- Martínez-Ortí, A., Bargues, M.D., Mas-Coma, S., 2015. Dos nuevas localizaciones para España de *Bulinus truncatus* (Audouin, 1827) (Gastropoda, Planorbidae), hospedador intermediario de schistosomiasis urinaria. *Arx. Miscel·lània Zoològica* 13, 25–31. <https://doi.org/10.32800/amz.2015.13.0025>.
- Martínez-Ortí, A., Vilavella, D., Bargues, M.D., Mas-Coma, S., 2019. Risk map of transmission of urogenital schistosomiasis by *Bulinus truncatus* (Audouin, 1827) (Mollusca Gastropoda, Bulinidae) in Spain and Portugal. *Anim. Biodivers. Conserv.* 257–266. <https://doi.org/10.32800/abc.2019.42.0257>.
- Mas-Coma, S., 2005. Epidemiology of fascioliasis in human endemic areas. *J. Helminthol.* 79, 207–216. <https://doi.org/10.1079/JOH2005296>.
- Mas-Coma, S., 2020. Human fascioliasis emergence risks in developed countries: from individual patients and small epidemics to climate and global change impacts. *Enfermedades Infecc. Microbiol. Clin. Engl.* Ed 38, 253–256. <https://doi.org/10.1016/j.eimc.2020.01.014>.
- Mas-Coma, S., 2004. Human fascioliasis: epidemiological patterns in human endemic areas of South America, Africa and Asia. *Southeast Asian J Trop Med Public Health.* 35, 1-11.
- Mas-Coma, S., Agramunt, V.H., Valero, M.A., 2013. Direct and indirect affection of the central nervous system by *Fasciola* infection. *Handb. Clin. Neurol.* 114, 297–310. <https://doi.org/10.1016/B978-0-444-53490-3.00024-8>.

BIBLIOGRAPHY

- Mas-Coma, S., Agramunt, V.H., Valero, M.A., 2014. Neurological and ocular fascioliasis in humans. *Adv. Parasitol.* 84, 27–149.
<https://doi.org/10.1016/B978-0-12-800099-1.00002-8>.
- Mas-Coma, S., Bargues, M.D., 2009. Populations, hybrids and the systematic concepts of species and subspecies in Chagas disease triatomine vectors inferred from nuclear ribosomal and mitochondrial DNA. *Acta Trop.* 110, 112–136. doi: 10.1016/j.actatropica.2008.10.013.
- Mas-Coma, S., Bargues, M.D., Valero, M.A., 2022b. Fascioliasis, in: Bruschi, F. (Ed.), *Helminth Infections and their impact on global public health*. Springer International Publishing, Cham, pp. 185–219.
https://doi.org/10.1007/978-3-031-00303-5_6.
- Mas-Coma, S., Buchon, P., Funatsu, I.R., Angles, R., Artigas, P., Valero, M.A., Bargues, M.D., 2020b. Sheep and cattle reservoirs in the highest human fascioliasis hyperendemic area: experimental transmission capacity, field epidemiology, and control within a One Health initiative in Bolivia. *Front. Vet. Sci.* 7, 1–14.
- Mas-Coma, S., Buchon, P., Funatsu, I.R., Angles, R., Mas-Bargues, C., Artigas, P., Valero, M.A., Bargues, M.D., 2020a. Donkey fascioliasis within a One Health control action: transmission capacity, field epidemiology, and reservoir role in a human hyperendemic area. *Front. Vet. Sci.* 7, 1–17. <https://doi.org/10.3389/fvets.2020.591384>.
- Mas-Coma, S., Esteban, J.G., Bargues, M.D., 1999. Epidemiology of human fascioliasis: a review and proposed new classification. *Bull. World Health Organ.* 77, 340–346.
- Mas-Coma, S., Funatsu, I.R., Angles, R., Buchon, P., Mas-Bargues, C., Artigas, P., Valero, M.A., Bargues, M.D., 2021. Domestic pig prioritized in one health action against fascioliasis in human endemic areas: experimental assessment of transmission capacity and epidemiological evaluation of reservoir role. *One Health* 13, 1–14.
<https://doi.org/10.1016/j.onehlt.2021.100249>.
- Mas-Coma, S., Funatsu, I.R., Bargues, M.D., 2001. *Fasciola hepatica* and lymnaeid snails occurring at very high altitude in South America. *Parasitology* 123, 115–127.
<https://doi.org/10.1017/S0031182001008034>

- Mas-Coma, S., Valero, M.A., Bargues, M.D., 2008. Effects of climate change on animal and zoonotic helminthiasis. *Rev. sci. tech. Off. int. Epiz.* 27, 443–452.
- Mas-Coma, S., Valero, M.A., Bargues, M.D., 2009a. Climate change effects on trematodiasis, with emphasis on zoonotic fascioliasis and schistosomiasis. *Vet. Parasitol.* 4, 264–280.
<https://doi.org/10.1016/j.vetpar.2009.03.024>.
- Mas-Coma, S., Valero, M.A., Bargues, M.D., 2009b. Chapter 2. *Fasciola*, lymnaeids and human fascioliasis, with a global overview on disease transmission, epidemiology, evolutionary genetics, molecular epidemiology and control. *Adv. Parasitol.* 69, 41–146.
[https://doi.org/10.1016/S0065-308X\(09\)69002-3](https://doi.org/10.1016/S0065-308X(09)69002-3).
- Mas-Coma, S., Valero, M.A., Bargues, M.D., 2019. Fascioliasis. *Adv. Exp. Med. Biol.* 1154, 71–103. https://doi.org/10.1007/978-3-030-18616-6_4.
- Mas-Coma, S., Valero, M.A., Bargues, M.D., 2022a. Human and animal fascioliasis: origins and worldwide evolving scenario. *Clin. Microbiol. Rev.* 35, 1–96. <https://doi.org/10.1128/cmr.00088-19>.
- McManus, D.P., Dunne, D.W., Sacko, M., Utzinger, J., Vennervald, B.J., Zhou, X.-N., 2018. Schistosomiasis. *Nat. Rev. Dis. Primer* 4, 1–19.
<https://doi.org/10.1038/s41572-018-0013-8>.
- Mendoza-Palomar, N., Sulleiro, E., Perez-Garcia, I., Espiau, M., Soriano-Arandes, A., Martín-Nalda, A., Espasa, M., Zarzuela, F., Soler-Palacin, P., 2020. Schistosomiasis in children: review of 51 imported cases in Spain. *J. Travel Med.* 27, 1–3. <https://doi.org/10.1093/jtm/taz099>.
- Mera y Sierra, R., Neira, G., Bargues, M.D., Cuervo, P.F., Artigas, P., Logarzo, L., Cortiñas, G., Ibaceta, D.E.J., Lopez Garrido, A., Bisutti, E., Mas-Coma, S., 2020. Equines as reservoirs of human fascioliasis: transmission capacity, epidemiology and pathogenicity in *Fasciola hepatica*-infected mules. *J. Helminthol.* 94, 1–12.
<https://doi.org/10.1017/S0022149X20000693>.

BIBLIOGRAPHY

- Meurs, L., Mbow, M., Vereecken, K., Menten, J., Mboup, S., Polman, K., 2012a. Epidemiology of mixed *Schistosoma mansoni* and *Schistosoma haematobium* infections in northern Senegal. *Int. J. Parasitol.* 42, 305–311. <https://doi.org/10.1016/j.ijpara.2012.02.002>.
- Meurs, L., Mbow, M., Vereecken, K., Menten, J., Mboup, S., Polman, K., 2012b. Bladder morbidity and hepatic fibrosis in mixed *Schistosoma haematobium* and *S. mansoni* infections: a population-wide study in Northern Senegal. *PLoS Negl. Trop. Dis.* 6, 1–8. <https://doi.org/10.1371/journal.pntd.0001829>.
- Mitchell, P., 2018. The donkey in human history: an archaeological perspective. Oxford University Press.
- Mohanta, U.K., Ichikawa-Seki, M., Shoriki, T., Katakura, K., Itagaki, T., 2014. Characteristics and molecular phylogeny of *Fasciola* flukes from Bangladesh, determined based on spermatogenesis and nuclear and mitochondrial DNA analyses. *Parasitol. Res.* 113, 2493–2501. <https://doi.org/10.1007/s00436-014-3898-5>.
- Moné, H., Boissier, J., 2004. Sexual biology of schistosomes. *Adv Parasitol* 57, 89–189. [https://doi.org/10.1016/S0065-308X\(04\)57002-1](https://doi.org/10.1016/S0065-308X(04)57002-1).
- Moné, H., Holtfreter, M.C., Allienne, J.-F., Mintsu-Nguéma, R., Ibikounlé, M., Boissier, J., Berry, A., Mitta, G., Richter, J., Mouahid, G., 2015. Introgressive hybridizations of *Schistosoma haematobium* by *Schistosoma bovis* at the origin of the first case report of schistosomiasis in Corsica (France, Europe). *Parasitol. Res.* 114, 4127–4133. <https://doi.org/10.1007/s00436-015-4643-4>.
- Moné, H., Minguez, S., Ibikounlé, M., Allienne, J.-F., Massougbodji, A., Mouahid, G., 2012. Natural Interactions between *S. haematobium* and *S. guineensis* in the Republic of Benin. *Sci. World J.* 2012, 1–8. <https://doi.org/10.1100/2012/793420>.
- Mordecai, E.A., Ryan, S.J., Caldwell, J.M., Shah, M.M., LaBeaud, A.D., 2020. Climate change could shift disease burden from malaria to arboviruses in Africa. *Lancet Planet. Health* 4, e416–e423. [https://doi.org/10.1016/S2542-5196\(20\)30178-9](https://doi.org/10.1016/S2542-5196(20)30178-9).

- Morgan, J.A.T., DeJong, R.J., Lwambo, N.J.S., Mungai, B.N., Mkoji, G.M., Loker, E.S., 2003. First report of a natural hybrid between *Schistosoma mansoni* and *S. rodhaini*. J. Parasitol. 89, 416–418. [https://doi.org/10.1645/0022-3395\(2003\)089\[0416:FROANH\]2.0.CO;2](https://doi.org/10.1645/0022-3395(2003)089[0416:FROANH]2.0.CO;2).
- Mulero, S., Rey, O., Arancibia, N., Mas-Coma, S., Boissier, J., 2019. Persistent establishment of a tropical disease in Europe: the preadaptation of schistosomes to overwinter. Parasit. Vectors 12, 379. <https://doi.org/10.1186/s13071-019-3635-0>.
- Muñoz-Antoli, C., Trelis, M., Toledo, R., Esteban, J.G., 2006. Infectivity of *Echinostoma friedi* miracidia to different snail species under experimental conditions. J. Helminthol. 80, 323–325.

N

- Nath, T.C., S. Eom, K., Choe, S., Islam, S., Sabuj, S.S., Saha, E., Tuhin, R.H., Ndosi, B.A., Kang, Y., Kim, S., Bia, M.M., Park, H., Lee, D., 2022. Insights to helminth infections in food and companion animals in Bangladesh: occurrence and risk profiling. Parasite Epidemiol Control 17, 1–8. <https://doi.org/10.1016/j.parepi.2022.e00245>.
- Nation, C.S., Da'dara, A.A., Marchant, J.K., Skelly, P.J., 2020. Schistosome migration in the definitive host. PLoS Negl. Trop. Dis. 14, 1–12. <https://doi.org/10.1371/journal.pntd.0007951>.
- Nguyen, T.B.N., De, N.V., Nguyen, T.K.L., Quang, H.H., Doan, H.T.T., Agatsuma, T., Le, T.H., 2018. Distribution status of hybrid types in large liver flukes, *Fasciola* species (Digenea: Fasciolidae), from ruminants and humans in Vietnam. Korean J. Parasitol. 56, 453–461. <https://doi.org/10.3347/kjp.2018.56.5.453>.
- Noël, H., Ruello, M., Maccary, A., Pelat, C., Sommen, C., Boissier, J., Barré-Cardi, H., Fillaux, J., Termignon, J.-L., Debruyne, M., Chyderiotis, G., Durand, J., Ramalli, L., Chiappini, J.-D., Malfait, P., Berry, A., 2018. Large outbreak of urogenital schistosomiasis acquired in Southern Corsica, France: monitoring early signs of endemicization. Clin. Microbiol. Infect. 24, 295–300. <https://doi.org/10.1016/j.cmi.2017.06.026>.

BIBLIOGRAPHY

- Norman, F.F., Comeche, B., Chamorro, S., López-Vélez, R., 2020. Overcoming challenges in the diagnosis and treatment of parasitic infectious diseases in migrants. *Expert Rev. Anti Infect. Ther.* 18, 127–143. <https://doi.org/10.1080/14787210.2020.1713099>.
- Norton, A.J., Webster, J.P., Kane, R.A., Rollinson, D., 2008. Inter-specific parasite competition: mixed infections of *Schistosoma mansoni* and *S. rodhaini* in the definitive host. *Parasitology* 135, 473–484. <https://doi.org/10.1017/S0031182007004118>.
- Nyirenda, S.S., Sakala, M., Moonde, L., Kayesa, E., Fandamu, P., Banda, F., Sinkala, Y., 2019. Prevalence of bovine fascioliasis and economic impact associated with liver condemnation in abattoirs in Mongu district of Zambia. *BMC Vet. Res.* 15, 1–8. <https://doi.org/10.1186/s12917-019-1777-0>.

O

- Obokata, R., Veronis, L., McLeman, R., 2014. Empirical research on international environmental migration: a systematic review. *Popul. Environ.* 36, 111–135. <https://doi.org/10.1007/s11111-014-0210-7>.
- Oleaga, A., Rey, O., Polack, B., Grech-Angelini, S., Quilichini, Y., Pérez-Sánchez, R., Boireau, P., Mulero, S., Brunet, A., Rognon, A., Vallée, I., Kincaid-Smith, J., Allienne, J.-F., Boissier, J., 2019. Epidemiological surveillance of schistosomiasis outbreak in Corsica (France): are animal reservoir hosts implicated in local transmission? *PLoS Negl. Trop. Dis.* 13, 1–15. <https://doi.org/10.1371/journal.pntd.0007543>.
- Ojo, J.A., Adedokun, S.A., Akindele, A.A., Olorunfemi, A.B., Otutu, O.A., Ojurongbe, T.A., Thomas, B.N., Velavan, T.P., Ojurongbe, O., 2021. Prevalence of urogenital and intestinal schistosomiasis among school children in South-west Nigeria. *PLoS Negl. Trop. Dis.* 15, 1–15. <https://doi.org/10.1371/journal.pntd.0009628>.

- Onyekwere, A.M., De Elias-Escribano, A., Kincaid-Smith, J., Dametto, S., Allienne, J.-F., Rognon, A., Bargues, M.D., Boissier, J., 2022a. Mating Interactions between *Schistosoma bovis* and *S. mansoni* and compatibility of their F1 progeny with *Biomphalaria glabrata* and *Bulinus truncatus*. *Microorganisms* 10, 1251. <https://doi.org/10.3390/microorganisms10061251>.
- Onyekwere, A.M., Rey, O., Allienne, J.-F., Nwanchor, M.C., Alo, M., Uwa, C., Boissier, J., 2022b. Population genetic structure and hybridization of *Schistosoma haematobium* in Nigeria. *Pathogens* 11, 1–15. <https://doi.org/10.3390/pathogens11040425>.
- Onzo-Aboki, A., Ibikounlé, M., Boko, P.M., Savassi, B.S., Doritchamou, J., Siko, E.J., Daré, A., Batcho, W., Massougboji, A., Tougoue, J.J., Kaboré, A., 2019. Human schistosomiasis in Benin: countrywide evidence of *Schistosoma haematobium* predominance. *Acta Trop.* 191, 185–197. <https://doi.org/10.1016/j.actatropica.2019.01.004>.

P

- Pagès, J.R., Durand, P., Southgate, V.R., Tchuem Tchuente, L.A., Jourdan, J., 2001a. Molecular arguments for splitting of *Schistosoma intercalatum*, into two distinct species. *Parasitol. Res.* 87, 57–62. <https://doi.org/10.1007/s004360000301>.
- Pagès, J.R., Southgate, V.R., Tchuem Tchuente, L.A., Jourdan, J., 2001b. Lack of prezygotic isolation by assortative mating between the two cryptic species of the polytypic *Schistosoma intercalatum* taxon. *Parasitol. Res.* 87, 888–890. <https://doi.org/10.1007/s004360100477>.
- Parelkar, S., Oak, S., Maydeo, A., Sanghvi, B., Joshi, P., Chaubal, N., Patil, R., Sahoo, S., Lal, P., Sampath, N., Koticha, A., 2013. Biliary fascioliasis: management in a child using endoscopic retrograde cholangio pancreatography. *J. Indian Assoc. Pediatr. Surg.* 18, 23–23. <https://doi.org/10.4103/0971-9261.107012>.

BIBLIOGRAPHY

- Panzner, U., Boissier, J., 2021. Natural intra- and interclade human hybrid schistosomes in Africa with considerations on prevention through vaccination. *Microorganisms* 9, 1465. <https://doi.org/10.3390/microorganisms9071465>.
- Pennance, T., Allan, F., Emery, A., Rabone, M., Cable, J., Garba, A.D., Hamidou, A.A., Webster, J.P., Rollinson, D., Webster, B.L., 2020. Interactions between *Schistosoma haematobium* group species and their *Bulinus* spp. intermediate hosts along the Niger River Valley. *Parasit. Vectors* 13, 268. <https://doi.org/10.1186/s13071-020-04136-9>.
- Periago, M.V., Valero, M.A., El Sayed, M., Ashrafi, K., El Wakeel, A., Mohamed, M.Y., Desquesnes, M., Curtale, F., Mas-Coma, S., 2008. First phenotypic description of *Fasciola hepatica*/*Fasciola gigantica* intermediate forms from the human endemic area of the Nile Delta, Egypt. *Infect. Genet. Evol. J. Mol. Epidemiol. Evol. Genet. Infect. Dis.* 8, 51–58. <https://doi.org/10.1016/j.meegid.2007.10.001>.
- Periago, M.V., Valero, M.A., Panova, M., Mas-Coma, S., 2006. Phenotypic comparison of allopatric populations of *Fasciola hepatica* and *Fasciola gigantica* from European and African bovines using a computer image analysis system (CIAS). *Parasitol. Res.* 99, 368–378. <https://doi.org/10.1007/s00436-006-0174-3>.
- Pfennig, K.S., 2021. Biased Hybridization and its impact on adaptive introgression. *Trends Ecol Evol.* 6, 488–497. <https://doi.org/10.1016/j.tree.2021.02.010>.
- Pozio, E., 2020. How globalization and climate change could affect foodborne parasites. *Exp. Parasitol.* 208, 1–7. <https://doi.org/10.1016/j.exppara.2019.107807>.
- Privat, A., Jérôme, B., Bakary, S., Laurent, D., Assitan, D., Niaré, D.S., Ahristode, A., Hassim, G., Manon, B., Sarah, D., Moudachirou, I., Thomas, S., Abdoulaye, D., 2023. Genetic profiles of *Schistosoma haematobium* parasites from Malian transmission hotspot areas. *Parasit. Vectors* 16, 263. <https://doi.org/10.1186/s13071-023-05860-8>.

R

- Rahman, A.K.M.A., Islam, S.S., Talukder, Md.H., Hassan, Md.K., Dhand, N.K., Ward, M.P., 2017. Fascioliasis risk factors and space-time clusters in domestic ruminants in Bangladesh. *Parasit. Vectors* 10, 228. <https://doi.org/10.1186/s13071-017-2168-7>.
- Rahman, M., Akther, S., Ali, M., Hassan, M., 2022. Incidence of diseases in goats in Bangladesh. *Bangladesh Vet.* 37, 14–20. <https://doi.org/10.3329/bvet.v37i1-2.59180>.
- Ramachandran, J., Ajampur, S., Chandramohan, A., Varghese, G., 2012. Cases of human fascioliasis in India: tip of the iceberg. *J. Postgrad. Med.* 58, 150–150. <https://doi.org/10.4103/0022-3859.97180>.
- Reguera-Gomez, M., Valero, M.A., Artigas, P., De Elías-Escribano, A., Fantozzi, M.C., Luzón-García, M.P., Salas-Coronas, J., Boissier, J., Mas-Coma, S., Bargues, M.D., 2023. Geographical influence on morphometric variability of genetically “pure” *Schistosoma haematobium* eggs from Sub-Saharan migrants in Spain. *Trop. Med. Infect. Dis.* 8, 1-20. <https://doi.org/10.3390/tropicalmed8030144>.
- Reguera-Gomez, M., Valero, M.A., Oliver-Chiva, M.C., de Elias-Escribano, A., Artigas, P., Cabeza-Barrera, M.I., Salas-Coronas, J., Boissier, J., Mas-Coma, S., Bargues, M.D., 2021. First morphogenetic analysis of parasite eggs from *Schistosomiasis haematobium* infected sub-Saharan migrants in Spain and proposal for a new standardised study methodology. *Acta Trop.* 223, 1-16. <https://doi.org/10.1016/j.actatropica.2021.106075>.
- Rey, O., Toulza, E., Chaparro, C., Allienne, J.-F., Kincaid-Smith, J., Mathieu-Begn , E., Allan, F., Rollinson, D., Webster, B.L., Boissier, J., 2021b. Diverging patterns of introgression from *Schistosoma bovis* across *S. haematobium* African lineages. *PLOS Pathog.* 17, 1–20. <https://doi.org/10.1371/journal.ppat.1009313>.

BIBLIOGRAPHY

- Rey, O., Webster, B.L., Huyse, T., Rollinson, D., Van Den Broeck, F., Kincaid-Smith, J., Onyekwere, A., Boissier, J., 2021a. Population genetics of African *Schistosoma* species. *Infect. Genet. Evol.* 89, 1–14. <https://doi.org/10.1016/j.meegid.2021.104727>.
- Rollinson, D., Southgate, V.R., Vercruysse, J., Moore, P.J., 1990. Observations on natural and experimental interactions between *Schistosoma bovis* and *S. curassoni* from West Africa. *Acta Trop.* 47, 101–114. [https://doi.org/10.1016/0001-706x\(90\)90072-8](https://doi.org/10.1016/0001-706x(90)90072-8).
- Rothe, C., Zimmer, T., Schunk, M., Wallrauch, C., Helfrich, K., Gültekin, F., Bretzel, G., Allienne, J.-F., Boissier, J., 2021. Developing endemicity of schistosomiasis, Corsica, France. *Emerg. Infect. Dis.* 27, 319–321. <https://doi.org/10.3201/eid2701.204391>.
- Roure, S., Pérez-Quílez, O., Vallès, X., Valerio, L., López-Muñoz, I., Soldevila, L., Torrella, A., Fernández-Rivas, G., Chamorro, A., Clotet, B., 2022. Schistosomiasis among female migrants in non-endemic countries: neglected among the neglected? A pilot study. *Front. Public Health* 10, 1–6. <https://doi.org/10.3389/fpubh.2022.778110>.
- Roure, S., Valerio, L., Pérez-Quílez, O., Fernández-Rivas, G., Martínez-Cuevas, O., Alcántara-Román, A., Viasus, D., Pedro-Botet, M.L., Sabrià, M., Clotet, B., 2017. Epidemiological, clinical, diagnostic and economic features of an immigrant population of chronic schistosomiasis sufferers with long-term residence in a non-endemic country (North Metropolitan area of Barcelona, 2002-2016). *PLOS ONE* 12, 1–12. <https://doi.org/10.1371/journal.pone.0185245>.
- Rudge, J.W., Webster, J.P., Lu, D.-B., Wang, T.-P., Fang, G.-R., Basáñez, M.-G., 2013. Identifying host species driving transmission of schistosomiasis japonica, a multihost parasite system, in China. *Proc. Natl. Acad. Sci. U. S. A.* 110, 11457–11462. <https://doi.org/10.1073/pnas.1221509110>.

S

- Sabourin, E., Alda, P., Vázquez, A., Hurtrez-Boussès, S., Vittecoq, M., 2018. Impact of human activities on fasciolosis transmission. *Trends Parasitol.* 34, 891–903. <https://doi.org/10.1016/j.pt.2018.08.004>.
- Sah, R., Khadka, S., Khadka, M., Gurubacharya, D., Sherchand, J.B., Parajuli, K., Shah, N.P., Kattel, H.P., Pokharel, B.M., Rijal, B., 2017. Human fascioliasis by *Fasciola hepatica*: the first case report in Nepal. *BMC Res. Notes* 10, 439. <https://doi.org/10.1186/s13104-017-2761-z>.
- Sah, R., Khadka, S., Lakhey, P.J., Pradhan, S., Shah, N.P., Singh, Y.P., Mas-Coma, S., 2018. Human case of *Fasciola gigantica*-like infection, review of human fascioliasis reports in Nepal, and epidemiological analysis within the South Central Asia. *Acta Parasitol.* 63, 435–443. <https://doi.org/10.1515/ap-2018-0053>.
- Saijuntha, W., Tantrawatpan, C., Agatsuma, T., Wang, C., Intapan, P.M., Maleewong, W., Petney, T.N., 2018. Revealing genetic hybridization and DNA recombination of *Fasciola hepatica* and *Fasciola gigantica* in nuclear introns of the hybrid *Fasciola* flukes. *Mol. Biochem. Parasitol.* 223, 31–36. <https://doi.org/10.1016/j.molbiopara.2018.06.004>.
- Salas-Coronas, J., Bargues, M.D., Lozano-Serrano, A.B., Artigas, P., Martínez-Ortí, A., Mas-Coma, S., Merino-Salas, S., Abad Vivas-Pérez, J.I., 2021. Evidence of autochthonous transmission of urinary schistosomiasis in Almeria (southeast Spain): an outbreak analysis. *Travel Med. Infect. Dis.* 44, 1–6. <https://doi.org/10.1016/j.tmaid.2021.102165>.
- Salas-Coronas, J., Cabezas-Fernández, M.T., Lozano-Serrano, A.B., Soriano-Pérez, M.J., Vázquez-Villegas, J., Cuenca-Gómez, J.Á., 2018. Newly arrived African migrants to Spain: epidemiology and burden of disease. *Am. J. Trop. Med. Hyg.* 98, 319–325. <https://doi.org/10.4269/ajtmh.17-0604>.

BIBLIOGRAPHY

- Salas-Coronas, J., Vázquez-Villegas, J., Lozano-Serrano, A.B., Soriano-Pérez, M.J., Cabeza-Barrera, I., Cabezas-Fernández, M.T., Villarejo-Ordóñez, A., Sánchez-Sánchez, J.C., Abad Vivas-Pérez, J.I., Vázquez-Blanc, S., Palanca-Giménez, M., Cuenca-Gómez, J.A., 2020. Severe complications of imported schistosomiasis, Spain: A retrospective observational study. *Travel Med. Infect. Dis.* 35, 1–7.
<https://doi.org/10.1016/j.tmaid.2019.101508>.
- Salas-Coronas, J., Vázquez-Villegas, J., Villarejo-Ordóñez, A., Sánchez-Sánchez, J.C., Espada-Chavarría, J., Soriano-Pérez, M.J., Cabeza-Barrera, M.I., Cabezas-Fernández, M.T., 2013. Hallazgos radiológicos en pacientes con esquistosomiasis importada. *Enfermedades Infecc. Microbiol. Clínica* 31, 205–209.
<https://doi.org/10.1016/j.eimc.2012.04.003>.
- Sang, H.C., Muchiri, G., Ombok, M., Odieri, M.R., Mwinzi, P.N., 2014. *Schistosoma haematobium* hotspots in south Nyanza, western Kenya: prevalence, distribution and co-endemicity with *Schistosoma mansoni* and soil-transmitted helminths. *Parasit. Vectors* 7, 125.
<https://doi.org/10.1186/1756-3305-7-125>.
- Savassi, B.A.E.S., Dobigny, G., Etougbéché, J.R., Avocegan, T.T., Quinsou, F.T., Gauthier, P., Ibikounlé, M., Moné, H., Mouahid, G., 2021. *Mastomys natalensis* (Smith, 1834) as a natural host for *Schistosoma haematobium* (Bilharz, 1852) Weinland, 1858 x *Schistosoma bovis* Sonsino, 1876 introgressive hybrids. *Parasitol. Res.* 120, 1755–1770.
<https://doi.org/10.1007/s00436-021-07099-7>.
- Savassi, B.A.E.S., Mouahid, G., Lasica, C., Mahaman, S.-D.K., Garcia, A., Courtin, D., Allienne, J.-F., Ibikounlé, M., Moné, H., 2020. Cattle as natural host for *Schistosoma haematobium* (Bilharz, 1852) Weinland, 1858 x *Schistosoma bovis* Sonsino, 1876 interactions, with new cercarial emergence and genetic patterns. *Parasitol. Res.* 119, 2189–2205. <https://doi.org/10.1007/s00436-020-06709-0>.
- Sharma, R.L., Dhar, T. late D.N., Raina, O.K., 1989. Studies on the prevalence and laboratory transmission of fascioliasis in animals in the Kashmir valley. *Br. Vet. J.* 145, 57–61.
[https://doi.org/10.1016/0007-1935\(89\)90010-9](https://doi.org/10.1016/0007-1935(89)90010-9).

- Silva, M.L., Vicente, F.S., Avelino, I.C., Martin, V.R., 1977. Susceptibility of *Planorbarius metidjensis* from Portugal and Spain to *Schistosoma bovis* from Salamanca, Spain. *Malacologia* 16, 251–254.
- Singh, O., Agarwal, R.A., 1981. Toxicity of certain pesticides to two economic species of snails in Northern India. *J. Econ. Entomol.* 74, 568–571. <https://doi.org/10.1093/jee/74.5.568>.
- Soentjens, P., Cnops, L., Huyse, T., Yansouni, C., De Vos, D., Bottieau, E., Clerinx, J., Van Esbroeck, M., 2016. Diagnosis and clinical management of *Schistosoma haematobium* – *Schistosoma bovis* hybrid infection in a cluster of travelers returning from Mali. *Clin. Infect. Dis.* 63, 1626–1629. <https://doi.org/10.1093/cid/ciw493>.
- Sokolow, S.H., Wood, C.L., Jones, I.J., Swartz, S.J., Lopez, M., Hsieh, M.H., Lafferty, K.D., Kuris, A.M., Rickards, C., De Leo, G.A., 2016. Global assessment of schistosomiasis control over the past century shows targeting the snail intermediate host works best. *PLoS Negl. Trop. Dis.* 10, 1–19. <https://doi.org/10.1371/journal.pntd.0004794>.
- Soler, J., Moreno, D., Araujo, R., Ramos, M.A., 2006. Diversidad y distribución de los moluscos de agua dulce en la Comunidad de Madrid (España). *Graellsia* 62, 201–252. <https://doi.org/10.3989/graellsia.2006.v62.iExtra.119>.
- Southgate, V.R., Jourdane, J., Tchuente, L.A., 1998. Recent studies on the reproductive biology of the schistosomes and their relevance to speciation in the Digenea. *Int. J. Parasitol.* 28, 1159–1172. [https://doi.org/10.1016/s0020-7519\(98\)00021-6](https://doi.org/10.1016/s0020-7519(98)00021-6).
- Southgate, V.R., Tchuente, L.A., Vercruyse, J., Jourdane, J., 1995. Mating behaviour in mixed infections of *Schistosoma haematobium* and *S. mattheei*. *Parasitol. Res.* 81, 651–656. <https://doi.org/10.1007/BF00931841>.
- Steinauer, M.L., Hanelt, B., Mwangi, I.N., Maina, G.M., Agola, L.E., Kinuthia, J.M., Mutuku, M.W., Mungai, B.N., Wilson, W.D., Mkoji, G.M., Loker, E.S., 2008. Introgressive hybridization of human and rodent schistosome parasites in western Kenya. *Mol. Ecol.* 17, 5062–5074. <https://doi.org/10.1111/j.1365-294X.2008.03957.x>.

BIBLIOGRAPHY

- Stelma, F.F., Talla, I., Polman, K., Niang, M., Sturrock, R.F., Deelder, A.M., Gryseels, B., 1993. Epidemiology of *Schistosoma mansoni* infection in a recently exposed community in northern Senegal. *Am. J. Trop. Med. Hyg.* 49, 701–706. <https://doi.org/10.4269/ajtmh.1993.49.701>.
- Stensgaard, A.-S., Vounatsou, P., Sengupta, M.E., Utzinger, J., 2019. Schistosomes, snails and climate change: current trends and future expectations. *Acta Trop.* 190, 257–268. <https://doi.org/10.1016/j.actatropica.2018.09.013>.
- Sunita, K., Mas-Coma, S., Bargues, M.D., Sadaf, Khan, M.A., Habib, M., Mustafa, S., Husain, S.A., 2021. Buffalo infection by *Fasciola gigantica* transmitted by *Radix acuminata* in Uttar Pradesh, India: a molecular tool to improve snail vector epidemiology assessments and control surveillance. *Acta Parasitol.* 66, 1396–1405. <https://doi.org/10.1007/s11686-021-00414-3>.
- Swai, B., Poggensee, G., Mtweve, S., Krantz, I., 2006. Female genital schistosomiasis as an evidence of a neglected cause for reproductive ill-health: a retrospective histopathological study from Tanzania. *BMC Infect. Dis.* 6, 134. <https://doi.org/10.1186/1471-2334-6-134>.
- Swarnakar, G., Sanger, B., 2014. Epidemiological study of liver fluke (Trematoda: Digenea) in domestic ruminants of Udaipur district. *Int. J. Curr. Microbiol. Appl. Sci.* 3, 632–640.

T

- Tchuem Tchuente, L.A., Imbert-Establet, D., Delay, B., Jourdane, J., 1993. Choice of mate, a reproductive isolating mechanism between *Schistosoma intercalatum* and *S. mansoni* in mixed infections. *Int. J. Parasitol.* 23, 179–185. [https://doi.org/10.1016/0020-7519\(93\)90139-p](https://doi.org/10.1016/0020-7519(93)90139-p).
- Tchuem Tchuente, L.-A., Southgate, V.R., Combes, C., Jourdane, J., 1996. Mating behaviour in schistosomes: are paired worms always faithful? *Parasitol. Today.* 12, 231–236. [https://doi.org/10.1016/0169-4758\(96\)10020-X](https://doi.org/10.1016/0169-4758(96)10020-X).

- Teukeng, F.F.D., Blin, M., Bech, N., Gomez, M.R., Zein-Eddine, R., Simo, A.M.K., Allienne, J.-F., Tchuem-Tchuente, L.A., Boissier, J., 2022. Hybridization increases genetic diversity in *Schistosoma haematobium* populations infecting humans in Cameroon. *Infect. Dis. Poverty* 11, 1–11. <https://doi.org/10.1186/s40249-022-00958-0>.
- Theodosopoulos, A.N., Hund, A.K., Taylor, S.A., 2019. Parasites and host species barriers in animal hybrid zones. *Trends Ecol. Evol.* 34, 19–30. <https://doi.org/10.1016/j.tree.2018.09.011>.

V

- Valero, M.A., Bargues, M.D., Calderón, L., Artigas, P., Mas-Coma, S., 2018. First phenotypic and genotypic description of *Fasciola hepatica* infecting highland cattle in the state of Mexico, Mexico. *Infect. Genet. Evol.* 64, 231–240. <https://doi.org/10.1016/j.meegid.2018.06.032>.
- Valero, M.A., Bargues, M.D., Khoubbane, M., Artigas, P., Quesada, C., Berinde, L., Ubeira, F.M., Mezo, M., Hernandez, J.L., Agramunt, V.H., Mas-Coma, S., 2016. Higher physiopathogenicity by *Fasciola gigantica* than by the genetically close *F. hepatica*: experimental long-term follow-up of biochemical markers. *Trans. R. Soc. Trop. Med. Hyg.* 110, 55–66. <https://doi.org/10.1093/trstmh/trv110>.
- Valero, M.A., Darce, N.A., Panova, M., Mas-Coma, S., 2001. Relationships between host species and morphometric patterns in *Fasciola hepatica* adults and eggs from the northern Bolivian Altiplano hyperendemic region. *Vet. Parasitol.* 102, 85–100. [https://doi.org/10.1016/S0304-4017\(01\)00499-X](https://doi.org/10.1016/S0304-4017(01)00499-X).
- Valero, M.A., Mas-Coma, S., 2000. Comparative infectivity of *Fasciola hepatica* metacercariae from isolates of the main and secondary reservoir animal host species in the Bolivian Altiplano high human endemic region. *Folia Parasitol. (Praha)* 47, 17–22. <https://doi.org/10.14411/fp.2000.004>.

BIBLIOGRAPHY

- Valero, M.A., Panova, M., Comes, A.M., Fons, R., Mas-Coma, S., 2002. Patterns in size and shedding of *Fasciola hepatica* eggs by naturally and experimentally infected murid rodents. *J. Parasitol.* 88, 308–313. <https://doi.org/10.1645>.
- Valero, M.A., Perez-Crespo, I., Periago, M.V., Khoubbane, M., Mas-Coma, S., 2009. Fluke egg characteristics for the diagnosis of human and animal fascioliasis by *Fasciola hepatica* and *F. gigantica*. *Acta Trop.* 111, 150–159. <https://doi.org/10.1016/j.actatropica.2009.04.005>.
- Vallejo-Marín, M., Hiscock, S.J., 2016. Hybridization and hybrid speciation under global change. *New Phytol.* 211, 1170–1187. <https://doi.org/10.1111/nph.14004>.
- Van den Broeck, F., Geldof, S., Polman, K., Volckaert, F.A.M., Huyse, T., 2011. Optimal sample storage and extraction protocols for reliable multilocus genotyping of the human parasite *Schistosoma mansoni*. *Infect. Genet. Evol.* 11, 1413–1418. <https://doi.org/10.1016/j.meegid.2011.05.006>.
- Vudriko, P., Echodu, R., Tashiro, M., Oka, N., Hayashi, K., Ichikawa-Seki, M., 2022. Population structure, molecular characterization, and phylogenetic analysis of *Fasciola gigantica* from two locations in Uganda. *Infect. Genet. Evol.* 104, 105359. <https://doi.org/10.1016/j.meegid.2022.105359>.

W

- Waage, J., Grace, D., Fèvre, E.M., McDermott, J., Lines, J., Wieland, B., Naylor, N.R., Hassell, J.M., Chan, K., 2022. Changing food systems and infectious disease risks in low-income and middle-income countries. *Lancet Planet. Health* 6, e760–e768. [https://doi.org/10.1016/S2542-5196\(22\)00116-4](https://doi.org/10.1016/S2542-5196(22)00116-4).
- Watanabe, S., 1965. A revision of genus *Fasciola* in Japan, with particular reference to *F. hepatica* and *F. gigantica* (ed. Morishita, K., Komiya, Y. and Matsubayashi, H.) *Progress of Medical Parasitology in Japan*, 2, 359–381. Meguro Parasitol. Mus. Tokyo.

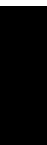
- Webster, B.L., Alharbi, M.H., Kayuni, S., Makaula, P., Halstead, F., Christiansen, R., Juziwele, L., Stanton, M.C., LaCourse, E.J., Rollinson, D., Kalua, K., Stothard, J.R., 2019. Schistosome interactions within the *Schistosoma haematobium* group, Malawi. *Emerg. Infect. Dis.* 25, 1245–1247.
<https://doi.org/10.3201/eid2506.190020>.
- Webster, B.L., Diaw, O.T., Seye, M.M., Webster, J.P., Rollinson, D., 2013. Introgressive hybridization of *Schistosoma haematobium* group species in Senegal: species barrier breaks down between ruminant and human schistosomes. *PLoS Negl. Trop. Dis.* 7, 1–9.
<https://doi.org/10.1371/journal.pntd.0002110>.
- Webster, B.L., Emery, A.M., Webster, J.P., Gouvras, A., Garba, A., Diaw, O., Seye, M.M., Tchuente, L.A.T., Simoonga, C., Mwanga, J., Lange, C., Kariuki, C., Mohammed, K.A., Stothard, J.R., Rollinson, D., 2012. Genetic diversity within *Schistosoma haematobium*: DNA barcoding reveals two distinct groups. *PLoS Negl. Trop. Dis.* 6, 1–10.
<https://doi.org/10.1371/journal.pntd.0001882>.
- Webster, B.L., Rollinson, D., Stothard, J.R., Huyse, T., 2010. Rapid diagnostic multiplex PCR (RD-PCR) to discriminate *Schistosoma haematobium* and *S. bovis*. *J. Helminthol.* 84, 107–114.
<https://doi.org/10.1017/S0022149X09990447>.
- Webster, B.L., Southgate, V.R., 2003. Compatibility of *Schistosoma haematobium*, *S. intercalatum* and their hybrids with *Bulinus truncatus* and *B. forskalii*. *Parasitology* 127, 231–242.
<https://doi.org/10.1017/S0031182003003597>.
- Webster, B.L., Southgate, V.R., Tchuente, L.-A., 1999. Mating interactions between *Schistosoma haematobium* and *S. mansoni*. *J. Helminthol.* 73, 351–356.
<https://doi.org/10.1017/S0022149X99000591>.
- Webster, B.L., Tchuente, L.A., Jourdan, J., Southgate, V.R., 2005. The interaction of *Schistosoma haematobium* and *S. guineensis* in Cameroon. *J. Helminthol.* 79, 193–197.
<https://doi.org/10.1079/joh2005306>.

BIBLIOGRAPHY

- Wellinghausen, N., Moné, H., Mouahid, G., Nebel, A., Tappe, D., Gabriel, M., 2022. A family cluster of schistosomiasis acquired in Solenzara River, Corsica (France). Solenzara River is clearly a transmission site for schistosomiasis in Corsica. *Parasitol. Res.* 121, 2449–2452. <https://doi.org/10.1007/s00436-022-07574-9>.
- WHO, 2020. Ending the neglect to attain the sustainable development goals: a road map for neglected tropical diseases 2021–2030. Geneva, Switzerland: World Health Organ. <https://www.who.int/publications-detail-redirect/9789240010352> (accessed 19.5.23).
- WHO, 2021. Foodborne parasitic infections: fascioliasis (Liver fluke). Geneva, Switzerland: World Health Organ. <https://www.who.int/publications-detail-redirect/WHO-UCN-NTD-VVE-2021.4> (accessed 7.6.23).
- WHO, 2022. WHO guideline on control and elimination of human schistosomiasis. Geneva, Switzerland: World Health Organ. <https://www.who.int/publications-detail-redirect/9789240041608> (accessed 9.9.23).
- WHO, 2023a. Neglected tropical diseases. Geneva, Switzerland: World Health Organ. <https://www.who.int/news-room/questions-and-answers/item/neglected-tropical-diseases> (accessed 17.9.23).
- WHO, 2023b. Schistosomiasis. Geneva, Switzerland: World Health Organ. <https://www.who.int/news-room/fact-sheets/detail/schistosomiasis> (accessed 2.6.23).
- Witzenrath, M., Bouchaud, O., Kern, P., Saraiva da Cunha, J., TropNet Schistosomiasis Investigator Group, Beltrame, A., Zammarchi, L., Paul, M., Kosina, P., Marocco, S., Schunk, M., Just-Nübling, G., Clerinx, J., Mørch, K., Trevino, B., Ramos, J.M., Fry, G., Schmid, M.L., Develoux, M., Jänisch, T., Grobusch, M.P., Pongratz, P., Puente, S., Suttorp, N., Hellgren, U., Calleri, G., Nielsen, H., Zoller, T., Hatz, C., Kurth, F., Myrvang, B., Lingscheid, T., Jelinek, T., Stich, A., Rochat, L., Gjørup, I.E., Siikamäki, H., Muñoz, J., Neumayr, A., Björkman, A., 2017. Schistosomiasis in European travelers and migrants: analysis of 14 years TropNet surveillance data. *Am. J. Trop. Med. Hyg.* 97, 567–574. <https://doi.org/10.4269/ajtmh.17-0034>.

Y

- Yadav, Vatsya, Garg, Kumar, R.R., Banerjee, P.S., Godara, R., 2007. Seasonal prevalence of *Fasciola gigantica* infection in sheep and goats in western Uttar Pradesh. J. Parasit. Dis, 31, 141–144.
- Yang, Y., Zhou, Y.-B., Song, X.-X., Li, S.-Z., Zhong, B., Wang, T.-P., Bergquist, R., Zhou, X.-N., Jiang, Q.-W., 2016. Integrated control strategy of schistosomiasis in the People's Republic of China: projects involving agriculture, water conservancy, forestry, sanitation and environmental modification. Adv Parasitol. 92, 237–68.
<https://doi.org/10.1016/bs.par.2016.02.004>. Epub 2016 Apr 7.4.



APPENDIX A. PUBLISHED DATA





Two papers are included in this **Appendix A**.

The first, entitled "**Wide variation of heterozygotic genotypes of recent fasciolid hybrids from livestock in Bangladesh assessed by rDNA internal transcribed spacer region sequencing and cloning**", includes the results obtained and presented in **Chapter II** of this thesis.

The second article, "**Mating interactions between *Schistosoma bovis* and *S. mansoni* and compatibility of their F1 progeny with *Biomphalaria glabrata* and *Bulinus truncatus***", is the result of the collaboration with the "Interactions Hôtes-Pathogènes-Environnements" (IHPE) laboratory of the University of Perpignan (France), WHO Collaborating Center for Schistosomiasis, in the team of Dr. Jérôme Boissier during my predoctoral stay.

APPENDIX A





Wide variation of heterozygotic genotypes of recent fasciolid hybrids from livestock in Bangladesh assessed by rDNA internal transcribed spacer region sequencing and cloning

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ABSTRACT

Fascioliasis causes high economic losses in livestock and underlies public health problems in rural areas, mainly of low-income countries. The increasing animal infection rates in Bangladesh were assessed, by focusing on host species, different parts of the country, and rDNA sequences. Fasciolid flukes were collected from buffaloes, cattle, goats and sheep from many localities to assess prevalences and intensities of infection. The nuclear rDNA internal transcribed spacer (ITS) region including ITS-1 and ITS-2 spacers was analyzed by direct sequencing and cloning, given the detection of intermediate phenotypic forms in Bangladesh. The 35.4% prevalence in goats and 55.5% in buffaloes are the highest recorded in these animals in Bangladesh. In cattle (29.3%) and sheep (26.8%) prevalences are also high for these species. These prevalences are very high when compared to lowlands at similar latitudes in neighboring India. The high prevalences and intensities appear in western Bangladesh where cross-border importation of animals from India occur. The combined haplotype CH3A of *Fasciola gigantica* widely found in all livestock species throughout Bangladesh fits its historical connections with the western Grand Trunk Road and the eastern Tea-Horse Road. The “pure” *F. hepatica* sequences only in clones from specimens showing heterozygotic positions indicate recent hybridization events with local “pure” *F. gigantica*, since concerted evolution did not yet have sufficient time to homogenize the rDNA operon. The detection of up to six different sequences coexisting in the cloned specimens evidences crossbreeding between hybrid parents, indicating repeated, superimposed and rapidly evolving hybridization events. The high proportion of hybrids highlights an increasing animal infection trend and human infection risk, and the need for control measures, mainly concerning goats in household farming management. ITS-1 and ITS-2 markers prove to be useful for detecting recent hybrid fasciolids. The introduction of a *Fasciola* species with imported livestock into a highly prevalent area of the other *Fasciola* species may lead to a high nucleotide variation in the species-differing positions in the extremely conserved fasciolid spacers. Results suggest that, in ancient times, frequent crossbreeding inside the same *Fasciola* species gave rise to the very peculiar characteristics of the present-day nuclear genome of both fasciolids.

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1. Background

Two liver fluke species of the genus *Fasciola* cause the zoonotic trematodiasis called fascioliasis. This disease is of great veterinary importance due to the high economic losses in livestock husbandry [1] and has been demonstrated to underlie public health problems in many rural areas, mainly in low-income countries of different continents. The World Health Organization has therefore included this disease among the group of foodborne trematodiasis within the Neglected Tropical Diseases (NTDs) to which priority should be given [2,3]. This is based on its pathogenicity and the increasing emergence of this disease apparent in the 1990 and 2000 decades. Recent studies have shown that infection may affect the sufferer very early in life [4] and that severe clinical pictures also develop during the long chronic phase and not only in the short initial acute phase [5,6].

Both climate change, among which the global warming phenomenon but also rainfall-rate changes, and other anthropogenic effects, including man-made environmental modifications such as irrigation projects, human and animal movements, and livestock importation/exportation, are driving forces for the potential emergence of fascioliasis in many areas, both in developed and developing countries [7,8]. Numerous studies have analyzed climate change impacts on animal

fascioliasis, and others have also demonstrated the influence of both climate and anthropogenic modifications of the milieu on human fascioliasis [9,10].

Fasciola hepatica originated in cooler areas and mountainous foothills of the Asian Near East in the latest Miocene to Early Pliocene, around 6.0 to 4.0 mya and perhaps shortly afterwards. *Fasciola gigantica* originated in warm lowlands of southeastern Africa in the mid-Miocene, around 13.5 mya and later spread northward to the same Asian Near East through the eastern Mediterranean Levantine Corridor [11]. Thus, both fasciolids were present in the Fertile Crescent and took advantage of human-guided movements of domesticated ruminants, equines and camelids for subsequent further spread throughout the Neolithic and up to the present day, *F. hepatica* expanding worldwide and *F. gigantica* eastward into Asia [8]. The preference for warmer lowlands allowed *F. gigantica* to spread along all southern latitudes of Asia up to South East Asia and Pacific islands. On the contrary, multidisciplinary analyses indicate that *F. hepatica* did not expand throughout these southern Asian warm lowlands in the past, because of the development temperature thresholds of this fasciolid [8].

From the public health point of view, *F. gigantica* was traditionally only given secondary importance, but its higher pathogenicity [12], and the increasing number of reports of human infection are leading to a

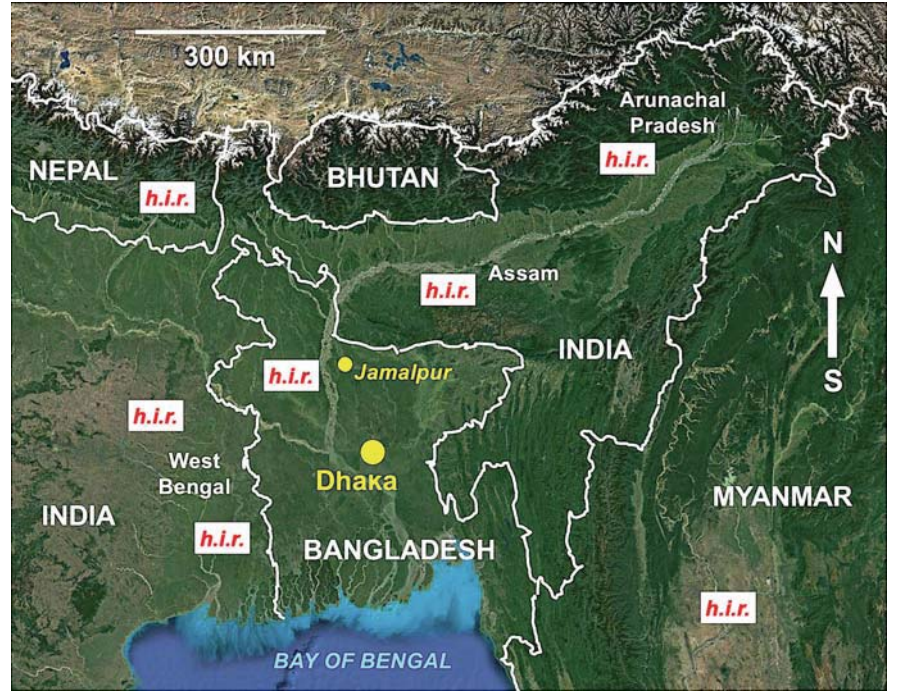


Fig. 1. Geographic map of Bangladesh and neighboring countries, showing zones where human infection by fasciolid flukes have been reported. h.i.r. = human infection reported (h.i.r. labels noted in zones of reports except in Myanmar). Background from composed satellite map of Asia orthographic projection by NASA (full resolution of 1887 × 1962 pixels; public domain) via Wikimedia Commons.

reconsideration of the scenario in Africa [13–15] and Asia [8,16].

In Asia, the very wide south-central region eastward from Afghanistan has become a focus for studies on fascioliasis after recent reports of human infection. In Bangladesh, there have only been two human fascioliasis reports, one of a 22-year-old Bangladeshi women diagnosed at a London hospital [17] and another of a 35-year-old housewife from Jamalpur [18]. This contrasts, however, with the number of human infection cases reported in zones of other countries neighboring Bangladesh, such as India including West Bengal [19,20], Assam [21] and Arunachal Pradesh [19], Nepal [22–24] and Myanmar [25] (Fig. 1). This scenario suggests the entire region of northward from the Bay of Bengal and southward from the Himalayan chain to be a hot spot of fascioliasis with human infection risk throughout very high-density populated areas.

The present study had two objectives in agreement with the One-Health focus recently prioritized by WHO for the strategic assessment and control of neglected tropical diseases, mainly those with zoonotic characteristics [26]. First, the purpose was to assess the distribution of fasciolid liver flukes infecting the main livestock species (buffaloes, cattle, goats and sheep) throughout the whole of Bangladesh by including animals from the four different agro-ecological zones, i.e. coastal, hilly, Barindh steppe, and floodplains, to verify the apparently increasing rates, in which parts of the country, which animal species, and in which types of zones this might be occurring. Second, the study has focused on the genetic characterization of the liver flukes collected, by means of DNA sequencing and cloning in the way to assess the potential reasons for the aforementioned increasing problem in Bangladesh.

A previous phenotypic assessment of fasciolid flukes from Bangladesh demonstrated the existence of intermediate forms between *F. gigantica* and *F. hepatica* by means of CIAS morphometry analyses [27]. Therefore, the nuclear ribosomal DNA (rDNA) internal transcribed spacers (ITS-1 and ITS-2) were used as molecular markers for sequencing purposes, because the nuclear rDNA has been verified to agree with fluke adult morphology whereas the mitochondrial DNA does not always do so, potentially indicating hybrid origins of introgression [28]. This is the first time that the complete sequences of both rDNA ITSs have been used for fasciolid fluke assessment in Bangladesh.

2. Material and methods

2.1. Liver fluke materials

A total of 2295 adult flukes of fasciolids were collected directly from the livers of the livestock species studied. Rubber-coated forceps were used to avoid any structural damage to the flukes. Details of the specimens of livestock species analyzed and their geographical origins of

localities, coordinates and agro-ecological zones are given in Table 1 and Fig. 2.

Bangladesh has an area of 147,570 km² and is bordered on the west, north, and east by a 4095-km land frontier with India and, in the southeast, by a short land and water frontier (193 km) with Burma (Myanmar). It is a low-lying riverine country vulnerable to flood and drought, located in South Asia with a largely marshy jungle coastline of 710 km on the northern littoral of the Bay of Bengal. The country has a tropical monsoon climate characterized by heavy seasonal rainfall, high temperatures, and high humidity with mild winters from October to March, and hot, humid summers from March to June.

2.2. Fasciolid specimens and DNA markers for molecular characterization

A total of 83 adult flukes were used for DNA sequencing purposes, including specimens infecting buffaloes ($n = 6$), cattle ($n = 29$), goats ($n = 42$) and sheep ($n = 6$). Fasciolid specimens were washed extensively in physiological saline (0.85% NaCl) to remove blood and bile and finally preserved in ethanol 70% until DNA extraction.

The sequence of the complete nuclear ribosomal DNA (rDNA) internal transcribed spacer region, including the spacers ITS-1 and ITS-2 and the 5.8S gene, which separate these was selected to characterize the flukes. These two ITS markers have already proved to be useful for the genetic characterization of *Fasciola* species and strains at local and regional levels as well as in worldwide analyses, including the assessment of the routes of spread [28].

2.3. Extraction, amplification and sequencing of DNA

For DNA extraction, a small part of the anterior body region of each fasciolid was individually processed using the phenol-chloroform extraction and ethanol precipitation method as previously described [29–31]. Materials were suspended in 400 μ l of lysis buffer (10 mM Tris-HCl, pH 8.0, 100 mM EDTA, 100 mM NaCl, 1% sodium dodecyl sulfate SDS) containing 500 μ g/ml Proteinase K (Promega, Madison, WI, USA). The digestion was performed for 2 h at 55 °C, including shaking every 15 min. Methods previously outlined were followed concerning the procedure steps [32]. The phenol-chloroform extraction and ethanol precipitation method was applied for total DNA isolation. Each pellet was dried and resuspended in 30 μ l sterile TE buffer (pH 8.0), and subsequently this suspension was stored at –20 °C until needed.

The selected DNA marker was amplified by PCR for each liver fluke individual. Forward and reverse primers were designed in the regions flanking the rRNA genes 18S and 28S for the subsequent amplification of the complete ITS-1, 5.8S, ITS-2 region [29,33].

For the PCR amplification, the Biotools DNA polymerase® (Biotools

Table 1
Agro-ecological zones, coordinates and altitudes of the localities in Bangladesh from where livestock specimens were analyzed for fasciolid fluke collection.

No. in map ^a	Localities (Administrative Units)	Agro-eco-logical zones	Latitude	Longitude	Altitude (m)
1	Chittagong/Cox's Bazar/Chakaria	Coastal	21°45'41.36"N	92°04'33.45"E	11.21
2	Barisal /Bhola/Bhola Sadar	Coastal	22°41'12.48"N	90°38'30.76"E	7.88
3	Chittagong/Rangamati/Rangamati Sadar	Hilly	22°39'28.51"N	92°10'24.54"E	45.45
4	Sylhet/Sylhet/Sylhet Sadar	Hilly	24°54'23.77"N	91°50'50.29"E	21.21
5	Rajshahi/Naogaon/Patniola	Barindh steppe	25°02'40.55"N	88°45'19.68"E	24.24
6	Rajshahi/Naogaon/Shaiphar	Barindh steppe	25°07'30.31"N	88°35'24.50"E	44.85
7	Rajshahi/Naogaon/Naogaon Sadar	Barindh steppe	24°48'39.24"N	88°56'37.04"E	20.61
8	Rajshahi/Pabna/Ishwardi	Floodplains	24°07'12.43"N	89°04'03.56"E	18.48
9	Dhaka/Mymensingh/Ishwarganj	Floodplains	24°41'10.20"N	90°35'36.88"E	16.67
10	Rangpur/Lalmonirhat/Lalmonirhat Sadar	Floodplains	25°58'26.15"N	89°17'02.26"E	37.58
11	Dhaka/Mymensingh/Mymensingh Sadar	Floodplains	24°44'04.60"N	90°25'43.80"E	20.00
12	Rajshahi/Bogra/Dhunat	Floodplains	24°41'29.12"N	89°32'10.33"E	16.36
13	Khulna/Jhenaidah/Shailkupa	Floodplains	23°40'45.47"N	89°14'48.28"E	13.03
14	Khulna/Jhenaidah/Jhenaidah Sadar	Floodplains	23°32'43.15"N	89°10'28.16"E	14.24

^a For numbers of localities see map in Fig. 2.

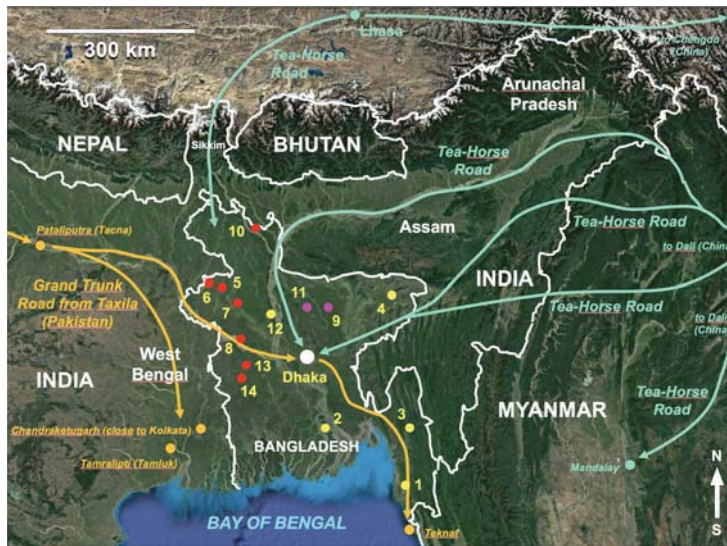


Fig. 2. Geographic map of Bangladesh, showing the sites where the hosts of the liver flukes analyzed were collected. Coordinates and altitudes of each site are indicated in Table 1. 1) Chakaria; 2) Bholahar; 3) Rangamati; 4) Sylhet; 5) Patnita; 6) Shapahar; 7) Naogaon; 8) Ishwardi; 9) Ishwarganj; 10) Lalmonirhat; 11) Mymensingh; 12) Dhunat; 13) Shailkupa; 14) Jhenaidah. Yellow dots = rural localities where only rDNA ITS region sequences of the combined haplotype Fg-CH3A of “pure” *F. gigantica* were found; red dots = rural localities where heterozygotic sequences of the rDNA ITS region were found; pink dots = localities surrounding Mymensingh city where heterozygotic sequences of the rDNA ITS region were also found; pumpkin lines = routes from the West followed by *F. gigantica* along the Grand Trunk Road; green lines = routes from the North and the East followed by *F. gigantica* along the Tea-Horse Road (for more details about the Grand Trunk Road and the Tea-Horse Road, see [8]). Background from composed satellite map of Asia orthographic projection by NASA (full resolution of 1887 × 1962 pixels; public domain) via Wikimedia Commons. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

B&M Labs. S.A., Madrid, Spain) was used in a Verity-96-well Thermal Cycler (Applied Biosystems, Thermo Fisher Scientific, Waltham, MA USA). The program for this rDNA ITS region comprised one cycle of 2 min at 94 °C, 35 cycles of 1 min at 93 °C, 1 min at 55 °C and 1 min at 72 °C each, preceded by 2 min at 72 °C, and followed by a final cooling at 4 °C.

For the purification of the PCR product, the Ultra Clean™ PCR Clean-up DNA Purification System (MoBio, Solana Beach, CA, USA) was used following the manufacturer's protocol and eluted in 50 µl of 10 mM TE buffer (pH 7.6). The final DNA concentration (in µg/ml) and the absorbance at 260/280 nm were determined in an Eppendorf Bio-Photometer (Hamburg, Germany).

Each PCR product was sequenced, for a definitive haplotype characterization, on both strands by the dideoxy chain-termination method performed with the Taq dye-terminator chemistry kit on an Applied Biosystems 3730xl DNA Analyzer (Applied Biosystems, Foster City, CA, USA). The PCR primers were used as sequencing primers.

2.4. Sequence analyses

The software Sequencher v. 5.4.6 (Gene Codes Co. MI, USA) was used to edit and assemble the sequences. The electropherograms (ABI format) of the rDNA intergenic sequence were thoroughly reviewed, especially in the positions that differentiate between *F. hepatica* and *F. gigantica*, using FinchTV v. 1.5 (Geospiza, Inc., Seattle, WA, USA; <http://www.geo>

[spiza.com](http://www.geospiza.com)) and ClustalW to align them by means of default parameters in MEGA X [34].

Corresponding penalties for gaps were included in pairwise and multiple alignments. All changes, comprising transitions (ts), transversions (tv) and insertions/deletions (indels), were considered as character states in MEGA X. By means of the ALTER web server [35], the sequences aligned were collapsed to haplotypes, counting gaps as differences. Closely related sequences were searched by utilizing the BLASTN programme from the National Center for Biotechnology Information website (<http://www.ncbi.nlm.nih.gov/BLAST>). Comparative analyses were performed with available rDNA ITS-1 and ITS-2 sequences of *F. gigantica*, *F. hepatica* and *Fasciola* spp. downloaded from the GenBank and also with the fasciolid haplotype collection of the Valencia WHO Collaborating Center.

To define *Fasciola* species and “pure” haplotypes, a total of 50 sequences were used for comparison purposes, including 20 sequences of “pure” *F. hepatica* from Europe, the Americas and Algeria representing the haplotypes Fh1A (GenBank Accession No. MG569980), Fh2A (MG569978, MG569981), Fh3A (MK212150) and Fh1/2A (OQ064782); and 30 sequences of “pure” *F. gigantica* from Africa representing the haplotypes Fg1A (AJ853848), Fg2A (ON661090), and Fg-HtzA (ON661091). The term “pure” is here used to refer to haplotypes of fasciolid specimens appearing within the intraspecific variability shown by populations of a *Fasciola* species where the other *Fasciola* species is absent, as described and proposed previously [28]. These haplotypes are

almost always homozygotes, but in a very few specimens a heterozygote may be found (i.e., presenting a double peak in a given position indicating the existence of two different nucleotides in that position depending on the rDNA operon copy).

2.5. Cloning

Cloning procedures were applied to PCR products from specimens whose electropherograms obtained by direct sequencing showed unexpected double peaks in relevant positions. The amplification products from a subsample of 6 specimens with apparent heterozygotic sequences and 1 control of "pure" *F. gigantica*, according to our DNA markers, were cloned with pGEM-T Easy Vector System I (Promega, Madison, WI) and introduced in *Escherichia coli* DH5 α competent cells, to confirm the identity of the heterozygous sequences. After the growth of colonies, standard PCR of 8 different colonies per sample was performed and individually sequenced. DNA sequencing and sequence analyses of the clones were performed as described above.

2.6. DNA haplotype nomenclature

The terminology to identify the haplotype (H) of the two

aforementioned DNA markers follows the previously proposed combined haplotyping (CH) nomenclature [28,36]. According to this nomenclature, ITS-2 haplotypes are defined by numbers, and ITS-1 haplotypes by capital letters. In combined haplotypes, the ITS-2 code is noted before that of the ITS-1 because of the evolutionary usefulness of ITS-2 for species differentiation. Worth mentioning is that haplotype codes are only definitive when the sequences are complete, i.e. full-length sequences. When dealing with fragments or incomplete sequences, haplotype codes are considered only provisional.

2.7. New sequences deposited in DNA repositories

Sequences of pure and hybrid haplotypes of fasciolids from Bangladesh obtained in this study have been deposited in the GenBank Data Library under Accession Nos. OQ064778- OQ064781.

3. Results

3.1. Fascioliasis infection rates

A total of 2352 fasciolid adult flukes were collected from 115 infected livestock specimens analyzed, including: (i) 10 infected from 18

Table 2
Prevalences and intensities of fasciolid flukes collected from buffaloes and cattle in Bangladesh.

No. in map ^a	Localities (Administrative Units)	Buffaloes							Cattle						
		No. hosts analyzed	Sex i.h.	Age (years)	No. i.h. (prevalence %)	Total No. flukes collected	Intensity range (mean)		No. hosts analyzed	Sex i.h.	Age (years)	No. i.h. (prevalence %)	Total No. flukes collected	Intensity range (mean)	
1	Chittagong/Cox's Bazar/Chakaria	5	1 M	6 y	1 (20%)	121	–		5	1 F	5 y	1 (20.0%)	23	–	
2	Barisal/Bhola/Bhola Sadar	–	–	–	–	–	–		18	3 M, 4 F	3–6 y	7 (38.8%)	307	26–67, (43.8)	
3	Chittagong/Rangamati/Rangamati Sadar	–	–	–	–	–	–		11	1 M, 1 F	3–5 y	2 (18.1%)	28	7–21 (14.0)	
4	Sylhet/Sylhet/Sylhet Sadar	2	2 M	3–4 y	2 (100%)	42	20–22 (21.0)		4	1 M	5 y	1 (25.0%)	13	–	
5	Rajshahi/Naogaon/Patnitola	–	–	–	–	–	–		6	1 M	6 y	1 (16.6%)	8	–	
6	Rajshahi/Naogaon/Shapahar	4	1 M	5 y	1 (25%)	82	–		7	1 M	6 y	1 (14.3%)	13	–	
7	Rajshahi/Naogaon/Naogaon Sadar	–	–	–	–	–	–		–	–	–	–	–	–	
8	Rajshahi/Pabna/Ishwardi	6	4 M, 1 F	3–7 y	5 (83.3%)	224	27–61 (44.88)		13	2 M, 2 F	3–6 y	4 (30.7%)	27	4–12 (6.75)	
9	Dhaka/Mymensingh/Ishwarganj	–	–	–	–	–	–		–	–	–	–	–	–	
10	Rangpur/Lalmonirhat/Lalmonirhat Sadar	–	–	–	–	–	–		5	1 M	1.5 y	1 (20.0%)	12	–	
11	Dhaka/Mymensingh/Mymensingh Sadar	–	–	–	–	–	–		40	6 M, 4 F	1.5–4 y	10 (25.0%)	44	10–31 (16.4)	
12	Rajshahi/Bogra/Dhunat	–	–	–	–	–	–		–	–	–	–	–	–	
13	Khulna/Jhenaidah/Shailkupa	1	1 M	5 y	1 (100%)	3	–		6	1 M, 1 F	4–5 y	2 (33.3%)	43	9–34, (21.5)	
14	Khulna/Jhenaidah/Jhenaidah Sadar	–	–	–	–	–	–		18	4 M, 2 F	2–6 y	6 (33.3%)	267	7–103 (44.5)	
	TOTAL	18	9 M, 1 F	3–7 y	10 (55.5%)	472	20–61 (38.0)		123	21 M, 14 F	1.5–6 y	36 (29.3%)	785	7–103 (28.3)	

i.h. = infected hosts.

^a For numbers of localities see map in Fig. 2.

buffaloes dissected (prevalence of 55.5%) showing an infection intensity of 20–61 flukes/animal (mean 38.0 flukes/animal); (ii) 36 infected from 123 cattle dissected (prevalence of 29.3%) showing an infection intensity of 7–103 flukes/animal (mean 28.3 flukes/animal); (iii) 58 infected from 164 goats dissected (prevalence of 35.4%) showing an infection intensity of 8–89 flukes/animal (mean 16.6 flukes/animal); and (iv) 11 infected from 41 sheep dissected (prevalence of 26.8%) showing an infection intensity of 3–31 flukes/animal (mean 11.7 flukes/animal).

The distribution of prevalences and intensities according to the localities, sex and age of the hosts for buffaloes and cattle are noted in Table 2 and those of goats and sheep in Table 3.

3.2. Sequence analysis of the rDNA ITS1–5.8S–ITS2 region

The geographical origin of the 83 flukes molecularly analyzed infecting the four animal species according to localities in Bangladesh is noted in Table 4. The sequencing of these fasciolid specimens provided four different haplotypes.

Only one of the haplotypes corresponded to “pure” *F. gigantica* and is

ascribed to the combined haplotype Fg-CH3A (GenBank Accession No. OQ064778), including a 950-bp-long ITS1–5.8S–ITS2 region of 50.42% GC content. This haplotype was the most abundant, occurring in 65 flukes (78.31%) of the total flukes analyzed, and was detected in the four animal species (cattle, buffalo, sheep and goats).

The other three combined haplotypes occurred in the remaining 18 specimens, i.e. 21.68% of the total fluke samples, and were also detected in the four animal species studied. These three haplotypes are characterized by showing double peaks which were detected in the careful inspection of the electropherograms of the rDNA ITS1, 5.8S, ITS2 sequences of these liver fluke specimens. These double peaks appeared in the specific positions known to differentiate between *F. hepatica* and *F. gigantica*, suggesting heterozygotes. Three types of sequences presenting double peaks could be distinguished:

- a) with double peaks in all such differentiating positions of the ITS-1 and ITS-2;
- b) with double peaks in some (but not in all) such positions of the ITS-1 and ITS-2;

Table 3
Prevalences and intensities of fasciolid flukes collected from goats and sheep in Bangladesh.

No. in map ^a	Localities (Administrative Units)	Goats						Sheep					
		No. hosts analyzed	Sex i.h.	Age i.h. (years)	No. i.h. (prevalence %)	Total No. flukes collected	Intensity range (mean)	No. hosts analyzed	Sex i.h.	Age i.h. (years)	No. i.h. (prevalence %)	Total No. flukes collected	Intensity range (mean)
1	Chittagong/Cox's Bazar/Chakaria	6	1 M	2 y	1 (16.6%)	9	–	–	–	–	–	–	–
2	Barisal/Bhola/Bhola Sadar	–	–	–	–	–	–	–	–	–	–	–	–
3	Chittagong/Rangamati/Rangamati Sadar	–	–	–	–	–	–	–	–	–	–	–	–
4	Sylhet/Sylhet/Sylhet Sadar	9	1 M, 2 F	1.5–4 y	3 (33.3%)	62	11–27 (20.7)	–	–	–	–	–	–
5	Rajshahi/Naogaon/Patnitola	5	1 M	2 y	1 (20.0%)	11	–	5	1 M	2 y	1 (20%)	3	–
6	Rajshahi/Naogaon/Shapahar	–	–	–	–	–	–	–	–	–	–	–	–
7	Rajshahi/Naogaon/Sadar	16	2 M, 2 F	1.5–3 y	4 (25.0%)	85	4–34 (21.25)	22	4 M, 2 F	1.5–3 y	6 (27.3%)	65	6–16 (10.3)
8	Rajshahi/Pabna/Ishwardi	15	2 M, 3 F	1.5–4 y	5 (33.3%)	55	6–17 (11.0)	–	–	–	–	–	–
9	Dhaka/Mymensingh/Ishwarganj	18	5 M	1.5–3 y	5 (27.8%)	118	8–43 (23.6)	–	–	–	–	–	–
10	Rangpur/Lalmonirhat/Lalmonirhat Sadar	4	1 M	1.5 y	1 (25.0%)	26	–	4	1 M	1.5 y	1 (25%)	31	–
11	Dhaka/Mymensingh/Mymensingh Sadar	63	16 M, 12 F	1.5–3 y	28 (44.4%)	121	4–34 (21.25)	4	1 M	2 y	1 (25%)	8	–
12	Rajshahi/Bogra/Dhulna/Khulna	3	1 M	2 y	1 (33.3%)	124	–	–	–	–	–	–	–
13	Jhenaidah/Jhenaidah/Shailkupa	5	1 M, 1 F	1.5–2 y	2 (40.0%)	33	9–34 (15.5)	3	1 M	1.5 y	1 (33.3%)	11	–
14	Khulna/Jhenaidah/Jhenaidah Sadar	22	6 M, 1 F	1.5–4 y	7 (31.8%)	322	8–89 (46.0)	3	1 F	2 y	1 (33.3%)	11	–
	TOTAL	164	21 M, 3 F	1.5–4 y	58 (35.4%)	966	8–89 (16.6)	41	8 M, 3 F	1.5–3 y	11 (26.8%)	129	3–31 (11.7)

i.h. = infected hosts.

^a For numbers of localities see map in Fig. 2.

Table 4

Number of fasciolid flukes used for DNA sequencing according to localities and host species, including haplotypes found.

No. in map ^a	Localities (Administrative Units)	Buffaloes			Cattle			Goats			Sheep		
		No. flukes studied	No. pure Fg-3A	No.heterozygotic haplot.	No. flukes studied	No. pure Fg-3A	No.heterozygotic haplot.	No. flukes studied	No. pure Fg-3A	No.heterozygotic haplot.	No. flukes studied	No. pure Fg-3A	No.heterozygotic haplot.
1	Chittagong/Cox's Bazar/ Chakaria	1	1	–	1	1	–	1	1	–	–	–	–
2	Barisal /Bhola/Bhola Sadar	–	–	–	7	7	–	–	–	–	–	–	–
3	Chittagong/Rangamati/Rangamati Sadar	–	–	–	2	2	–	–	–	–	–	–	–
4	Sylhet/Sylhet/Sylhet Sadar	2	2	–	1	1	–	3	3	–	–	–	–
5	Rajshahi/Naogaon/Patnola	–	–	–	1	1	–	1	1	–	1	–	1 Htz1
6	Rajshahi/Naogaon/Shapahar	1	–	1 Htz1	1	1	–	–	–	–	–	–	–
7	Rajshahi/Naogaon/Naogaon Sadar	–	–	–	–	–	–	2	2	–	2	–	2 Htz1
8	Rajshahi/Pabna/Ishwardi	2	1	1 Htz1	1	1	–	–	–	–	–	–	–
9	Dhaka/Mymensingh/Ishwarganj	–	–	–	–	–	–	1	1	–	–	–	–
10	Rangpur/Lalmonirhat/Lalmonirhat Sadar	–	–	–	1	1	–	4	3	1 Htz1	1	–	1 Htz1
11	Dhaka/Mymensingh/Mymensingh Sadar	–	–	–	9	9	–	25	17	2 Htz1 6 Htz2	1	1	–
12	Rajshahi/Bogra/Dhunat	–	–	–	–	–	–	1	1	–	–	–	–
13	Khulna/Jhenaidah/Shailkupa	–	–	–	2	2	–	1	1	–	–	–	–
14	Khulna/Jhenaidah/Jhenaidah Sadar	–	–	–	3	2	1 Htz3	3	1	2 Htz1	1	1	–
TOTAL		6	4	2 Htz1	29	28	1 Htz3	42	31	5 Htz1 6 Htz2	6	2	4 Htz1

^a For numbers of localities see map in Fig. 2.

c) with double peaks in such positions only in the ITS-2 and not in the ITS-1.

This distribution allows us to group the aforementioned sequences in three heterozygotic types of combined haplotypes, for which ones we assigned the haplotype nomenclature Fg/Fh-Htz1, Fg/Fh-Htz2, Fg/Fh-Htz3, respectively. The most abundant proved to be the haplotype Fg/Fh-Htz1. The distribution of the combined haplotype Fg-CH3A of "pure" *F. gigantica* and the aforementioned three heterozygotic types of combined haplotypes according to localities is noted in Table 4, and according to host species in Table 5.

The "pure" Fg-3A combined haplotype was found in 66.66% of buffaloes, 96.55% of cattle, 73.81% of goats and 33.33% of sheep. The

heterozygotic combined haplotypes were found in 33.33% of buffaloes, 3.44% cattle, 26.19% goats, and 66.66% of sheep. Among the combined heterozygotic haplotypes, Fg/Fh-Htz1 is the only one shared by three host species (buffaloes, goats and sheep), while the haplotypes Fg/Fh-Htz2 and Fg/Fh-Htz3 were detected in only one host species, namely goats and cattle respectively (Tables 4 and 5). So, the goat is the only animal species in which the "pure" *F. gigantica* combined haplotype plus two types of hybrid haplotypes were detected.

Polymorphic sites in nucleotide positions in the sequence alignment of the whole nuclear rDNA ITS region including the ITS-1 and ITS-2 between "pure" *F. hepatica*, "pure" *F. gigantica* and Bangladeshi haplotypes are listed in Table 6.

When analyzing the two ITS markers individually, the ITS-1

Table 5

Distribution of the combined haplotypes of the rDNA transcribed spacer region according to definitive host species.

Host species	Buffalo	Cattle	Goat	Sheep
Total fasciolid No. = 83	No. = 6	No. = 29	No. = 42	No. = 6
"Pure" <i>F. gigantica</i> Fg-CH3A	4 (66.66%)	28 (96.55%)	31 (73.81%)	2 (33.33%)
No. = 65				
Hybrid Fg/Fh-Htz	2 (33.33%)	1 (3.44%)	11 (26.19%)	4 (66.66%)
No. = 18				
Heterozygotic haplotypes				
Fg/Fh-Htz1	2 (18.18%)	–	5 (45.45%)	4 (36.36%)
No. = 11 (61.11%)				
Fg/Fh-Htz2	–	–	6 (100%)	–
No. = 6 (33.33%)				
Fg/Fh-Htz3	–	1 (100%)	–	–
No. = 1 (5.55%)				

Table 6

Polymorphic sites in the sequence comparison of the complete transcribed spacer region of the nuclear rDNA and in the ITS-1 and ITS-2 between haplotypes of 'genetically pure' *Fasciola hepatica* from livestock in Europe, the Americas and Algeria, haplotypes of 'genetically pure' *F. gigantica* from livestock in African countries, and the haplotypes of "pure" and heterozygotic fasciolid samples from Bangladesh (in bold).

Fasciola species	ITS-2, ITS-1 Combined haplotypes	Polymorphic sites														
							Intergenic Region (ITS-1, 5.8S, ITS-2)									
		24	114	208	286	306	797	821	834	860	866	874	909	917	924	
		Polymorphic sites ITS-1					Polymorphic sites ITS-2									
		24	114	208	286	306	210	234	248	273	279	288	322	330	337	
<i>F. hepatica</i>	1A	C	A	C	T	C	T	T	A	C	C	C	T	T	G	
<i>F. hepatica</i>	2A	C	A	C	T	C	T	T	A	C	C	T	T	T	G	
<i>F. hepatica</i>	1/2A*	C	A	C	T	C	T	T	A	C	C	C/T	T	T	G	
<i>F. hepatica</i>	3A	C	A	C	T	C	T	T	A	C	C	C	A	T	G	
<i>F. gigantica</i>	1A	T	T	T	A	T	T	C	A	T	T	C	T	–	A	
<i>F. gigantica</i>	2A	T	T	T	A	T	T	C	C	T	T	C	T	–	A	
<i>F. gigantica</i>	1/2A**	T	T	T	A	T	T	C	C/A	T	T	C	T	–	A	
<i>F. gigantica</i>	3A	T	T	T	A	T	C	C	A	T	T	C	T	–	A	
<i>Fg/Fh</i>	Htz1	T/C	T/A	T/C	A/T	T/C	C/C	C/T	A	T/C	T/C	C	T	–	A/G	
<i>Fg/Fh</i>	Htz2	T/C	T	T/C	A/T	T	C	C	A	T	T	C	T	–	A/G	
<i>Fg/Fh</i>	Htz3	T	T	T	A	T	C	C/T	A	T/C	T/C	C	T	–	A	

^{*} Heterozygotic in position 874/288 not differentiating between *F. hepatica* and *F. gigantica* [Bargues et al., 2021 - see 31].

^{**} Heterozygotic in position 834/248 not differentiating between *F. hepatica* and *F. gigantica* [Bargues et al., 2021 - see 11].

haplotype HA proved to have the same sequence of 432 nucleotides and a 51.16% GC content in all specimens presenting the combined haplotype Fg-CH3A of the ITS region. The 5.8S gene was identical in all specimens, with 154 base pairs and 53.25% GC. The 364-bp-long and 48.35% GC ITS-2 was the only one exhibiting a mutation in position 210 of the ITS-2 alignment, that differentiates it from pure haplotypes of Fg-H1, Fg-H2 and Fg-H1/2. This polymorphic position does not differentiate between *F. hepatica* and *F. gigantica* (Table 6).

In the case of Fg/Fh hybrid haplotypes found, only two ITS-1 sequences presented heterozygotic positions, namely five positions in the Fg/Fh-Htz1 and three positions in Fg/Fh-Htz2 that differentiate between *F. hepatica* and *F. gigantica* (Table 6). The hybrid haplotype Fg/Fh-Htz3 presented an ITS-1 base-to-base coinciding with the sequence of "pure" *F. gigantica* (Table 6).

Regarding the ITS-2, all hybrid Fg/Fh haplotypes detected in Bangladesh presented heterozygotic positions differentiating between *F. hepatica* and *F. gigantica*, namely four positions in the Fg/Fh-Htz1 and three positions in the Fg/Fh-Htz3, whereas in only one position in the Fg/Fh-Htz2 (Table 6).

3.3. rDNA ITS1–5.8S-ITS2 region sequences obtained by cloning

A total of seven specimens from five localities were selected for cloning (Table 7), taking into account their peculiarities of double peak positions in their electropherograms obtained by direct sequencing, including one specimen used as control in whose sequence no double peaks were present. A total of 56 rDNA complete intergenic sequences were finally obtained after individually sequencing eight different colonies per each of the seven aforementioned samples.

Table 7

Intraindividual sequence heterogeneity of the ITS1–5.8S-ITS2 rDNA region found in the cloned samples showing double peaks in key positions of their electropherograms.

Sample No.	No. in map	Localities (Administrative Units)	Host species	Type of clones [*] (Number of sequences)
1	6	Rajshahi/Naogaon/Shapahar	Buffalo	Fh.a (1); Fg.b (3); Cl-b (1); Cl-c (1); Cl-j (1); Cl-k (1)
2	7	Rajshahi/Naogaon/Naogaon Sadar	Sheep	Fh.a (1); Fg.b (4); Cl-e (1); Cl-f (1); Cl-g (1)
3	7	Rajshahi/Naogaon/Naogaon Sadar	Sheep	Fh.a (1); Fg.a (1); Fg.b (5); Cl-d (1)
4	10	Rangpur/Lalmonirhat/Lalmonirhat Sadar	Goat	Fg.b (4); Cl-a (1), Cl-d (1), Cl-e (1), Cl-m (1)
5	10	Rangpur/Lalmonirhat/Lalmonirhat Sadar	Sheep	Fh.a (2); Fg.b (3); Cl-a (1); Cl-h (1); Cl-i (1)
6	13	Khulna/Jhenaidah/Shailkupa	Cattle	Fg.b (7); Cl-i (1)
7 ⁺	14	Khulna/Jhenaidah/Jhenaidah Sadar	Cattle	Fg.b (8)

^{*} Clone type nomenclature according to Table 8.

⁺ Positive control.

The 8 clones of the control proved to be "pure" *F. gigantica*, and among the other 48 clone sequences, 27 proved to be "pure" *F. gigantica* (56.25%), five were "pure" *F. hepatica* (10.42%), and the 16 remaining appeared to be Fg/Fh hybrids (33.33%). Polymorphic sites in the positions differentiating between *F. hepatica* and *F. gigantica* among the sequences of the 56 clones obtained are shown in Table 8.

The intra-individual analysis of the clone sequences within each of the six cloned specimens presenting double peaks evidenced a high heterogeneity of mixed sequences in the fluke specimens differing according to localities (Table 7):

- 6 different sequences (2 "pure" and 4 heterozygotic) in the fluke infecting buffalo from Shapahar;
- 5 different sequences (2 "pure" and 3 heterozygotic) in the fluke infecting a sheep from Naogaon Sadar;
- 4 different sequences (3 "pure" and 1 heterozygotic) in the fluke infecting another sheep from Naogaon Sadar;
- 5 different sequences (1 "pure" and 4 heterozygotic) in the fluke infecting a goat from Lalmonirhat Sadar;
- 5 different sequences (2 "pure" and 3 heterozygotic) in the fluke infecting a sheep from Lalmonirhat Sadar;
- 2 different sequences (1 "pure" and 1 heterozygotic) in the fluke infecting a cattle specimen from Shailkupa.

The eight clones of the control specimen infecting a cattle beast from Jhenaidah Sadar were all identical and proved to correspond to a "pure" *F. gigantica*, thus demonstrating the accuracy of the results of the cloning procedure.

Table 8

Haplotypes of the ITS1–5.8S–ITS-2 rDNA region found in the clones obtained in the seven cloned specimens, showing nucleotides in the positions differentiating between *Fasciola hepatica* and *F. gigantica*.

Haplotypes (pure and hybrid)	Clone code	Number of clones (n= 56*)	Polymorphic sites											
			Intergenic Region (ITS-1, 5.8S, ITS-2)											
			Polymorphic sites ITS-1						Polymorphic sites ITS-2					
			24	114	208	286	306	797	821	860	866	917	924	
<i>F. hepatica</i>	Cl-Fh.a	5	C	A	C	T	C	T	T	C	C	T	G	
<i>F. gigantica</i>	Cl-Fg.a	1	T	T	T	A	T	T	C	T	T	-	A	
<i>F. gigantica</i>	Cl-Fg.b	34*	T	T	T	A	T	T	C	C	T	-	A	
<i>F. g. / F. h.</i>	Cl-a	2	C	T	T	A	T	C	C	T	T	-	A	
<i>F. g. / F. h.</i>	Cl-b	1	C	A	T	A	T	C	C	T	T	-	A	
<i>F. g. / F. h.</i>	Cl-c	1	C	T	C	A	T	C	C	T	T	T	G	
<i>F. g. / F. h.</i>	Cl-d	2	C	A	C	T	C	C	C	T	T	T	G	
<i>F. g. / F. h.</i>	Cl-e	2	C	A	C	T	C	C	C	T	T	-	A	
<i>F. g. / F. h.</i>	Cl-f	1	C	A	C	T	C	T	T	T	T	-	A	
<i>F. g. / F. h.</i>	Cl-g	1	C	A	C	T	T	C	C	T	T	-	A	
<i>F. g. / F. h.</i>	Cl-h	1	T	A	C	T	C	C	C	T	T	-	A	
<i>F. g. / F. h.</i>	Cl-i	1	T	T	C	T	C	C	C	T	T	-	A	
<i>F. g. / F. h.</i>	Cl-j	1	T	T	C	T	C	T	T	C	C	T	G	
<i>F. g. / F. h.</i>	Cl-k	1	T	T	T	T	C	T	T	C	C	T	G	
<i>F. g. / F. h.</i>	Cl-l	1	T	T	T	A	T	T	T	C	C	-	A	
<i>F. g. / F. h.</i>	Cl-m	1	T	T	T	A	T	T	T	C	C	T	G	

Color in nucleotide positions: brown = nucleotides characterizing *F. hepatica*; blue = nucleotides characterizing *F. gigantica*; black = nucleotides of polymorphic position not differentiating between *F. hepatica* and *F. gigantica*.

* Including the 8 clones of the control.

4. Discussion

4.1. Fascioliasis and livestock husbandry practices in Bangladesh

Bangladesh is the world's eighth most densely populated country, with 84.4% of its population living in rural areas [37]. Livestock are an essential component of the rural economy and the livelihood of the household subsistence farmers. About 75% of the people rely on livestock to some extent for their livelihood, providing employment for the poorer segments of the population [38], mostly landless, marginal and small farmers. There is a rapid growth in demand for livestock products due to increase in income, rising population, and urban growth, which has led to a significant increase in small-scale dairy farming over the last few years. However, despite Bangladesh having one of the highest densities of livestock in the world, it has not been able to meet domestic demands for meat, milk, and leather. Increased disease loads and often-reported emerging and reemerging infectious diseases appear to underlie this situation. This is due to animal husbandry practices [39] and to climate-change impact. Indeed, Bangladesh is listed as one of the most vulnerable countries for global climate change due to its unique geographical location linked to sub-tropical monsoon climate [37].

In this country, there are currently (i) around 24.20 million cattle including an annually slaughtered total of 3.5 million [36], (ii) 26.60 million goats [40] with an annually slaughtered total of around 15 million [41], (iii) 3.40 million sheep [42], and (iv) 1.47 million buffaloes [43]. Most of these animals are raised by small-scale farmers who own 1–2 head of cattle and 2–3 head of goat/sheep. The management system is a subsistence farming type, combining both tethering and scavenging with few or no inputs for breeding, feeding and healthcare, and covering nearly 80% of the livestock farming environment [44].

Thus, goats and cattle are the most important livestock species in Bangladesh by far. Goats may be considered the main resource of this country. The production and marketing of goats is one of the oldest and most widespread livestock enterprises in this country, with small-holder farmers as the main actors in goat rearing. Approximately 65% of households are connected with goat farming either as a primary or secondary occupation. An analysis of the goat supply chain shows that goats from the local areas are distributed throughout the country [41]. The native Black Bengal goat is a well-adapted, highly prolific breed that can be easily raised on low-quality feed and with little investment.

Infectious diseases are, however, an important threat to productivity, causing a mortality of nearly 45% [40,41]. This could lead local farmers to import goats. Imported exotic breeds and/or commercial strains have resulted in crossbreeds by crossing with native types/varieties [44]. Such crossbred animals are today evident in Bangladesh [45]. Unfortunately, information on goat importation into Bangladesh is neither available in the literature nor directly from farmers who keep this under strict confidentiality [41].

Regarding cattle, Bangladesh has one of the highest cattle densities: 145 animals/km² compared with 90 for India, 30 for Ethiopia, and 20 for Brazil [37]. This number is still insufficient for the national internal requirements and, consequently, many bulls and oxen are smuggled from India to Bangladesh regularly. Around 40% of the annually slaughtered cattle are imported through cross-border trade. The informal supply chain between India and Bangladesh for cross-border trading of cattle accounts for 1.5 million head every year (around 20,000 to 30,000 cattle daily). Traders from both India and Bangladesh bring thousands of cattle to Puthkhal market which is very close to the border and only five km from Benapole Port Station, along the route from Kolkata to Dhaka [37]. From 2019, however, this trade has decreased due to increased internal production in ruminant husbandry.

Unfortunately, animal quarantine measures are not applied to informal importation practices, nor to officially registered imports because of insufficient manpower and funds to enforce the Animal Quarantine Act (Act no-VI of 2005) enacted by the Parliament of Bangladesh [37]. Moreover, liver fluke infection is not looked for in the freely released imported animals, nor are those animals preventatively treated against fascioliasis before being added to local herds.

The results of our livestock surveys demonstrate that fascioliasis is distributed throughout the whole country of Bangladesh and in all agro-ecological zones analyzed. While mentioning is that there is no locality in which uninfected animals could be found, which indicates a high infection risk of fascioliasis everywhere. Taking into account that buffaloes, cattle, goats and sheep are well-known liver-fluke reservoir hosts [46–48], it may be concluded that all livestock species participate in the diffusion and maintenance of the fasciolid life cycle in this country.

The prevalences found in the four livestock species prove to be very high and thus confirm the present fascioliasis problem in Bangladesh. The prevalence of 35.4% found in goats appears to be the highest recorded in this animal species in Bangladesh so far, when compared to

the range of 3.8–32% noted in a previous review [49]. The same may be concluded for the prevalence of 55.5% we found in buffaloes, higher than the previous range of 19–51% reported for this animal in the country [49]. Our prevalences of 29.3% in cattle and 26.8% in sheep are also very high, despite lying within the respective ranges of 15–66% and 8.4–31% previously reported for these two livestock species in Bangladesh [49].

These prevalences also appear to be very high when compared to lowland plain areas of similar latitudes in the neighboring India. Prevalences of 13.90%, 10.79%, 2.78% and 2.35% in buffaloes, cattle, sheep and goats, respectively, were found in epidemiological studies conducted in different geo-climatic zones (hills, tarai and plains) in six states of northern India. Even the highest prevalences found during the seasonal peaks in these zones (11.84% cattle, 15.57% buffaloes, 4.60% sheep, 2.71% goats) [50] were markedly lower than the prevalences in Bangladesh. In the nearby Indian state of Uttar Pradesh, mean and monthly peak prevalences of 2.02% and 13.3% in goats and 1.69% and 5.1% in sheep, respectively [51], were also very low compared to Bangladesh. Prevalences were also similarly low in the Indian state of Rajasthan, including 12.68% in buffaloes followed by 9.70% in cows and 7.5% in goats [52].

In the case of buffaloes, however, the behavioral tendency of this species to congregate in freshwater bodies underlie very high prevalences in some localities, such as 31.14% in northern Indian states [50], 37.5% in Uttar Pradesh [53], and even up to a local 94% in the north-eastern part of Uttar Pradesh [54], which fits a similarly impressively very high local prevalence of 72.0% found in the lymnaeid vector *Radix acuminata* in one transmission focus in the easternmost area of Gorakhpur of this Indian state, the so-far highest worldwide record of fasciolid infection in a lymnaeid population [55].

An analysis of prevalences and intensities from the ecological and geographical points of view shows that the highest infection rates are found in the floodplains and in the western part of the country. Floodplains are evidently appropriate habitats for the characteristics of the fascioliasis transmission foci, given the requirements for surface freshwater of the aquatic/amphibious lymnaeid snail vectors. Moreover, these results fit well with the predicted geographical distribution of animal fascioliasis in Bangladesh according to a risk map based on three-year data [49]. Interestingly, the high prevalences and intensities appear in western Bangladesh where cross-border importation of animals from the state of West Bengal of India occur.

4.2. rDNA ITS1–5.8S-ITS2 region sequence variation

4.2.1. Original arrival of *Fasciola gigantica*

The geographical location underlies the role as a crossroad played by Bangladesh during the great human-guided domesticated animal movements over thousands of years in southern Asia (Fig. 2). In the West, the use of cattle, equines and dromedaries as pack animals and goats and sheep as livestock for subsistence purposes, along the Grand Trunk Road from the Near East and Afghanistan through Pakistan and the northern plains of India up to Bangladesh allowed for the arrival and colonization of the latter country by the warm-lowland-preferring fasciolid species *F. gigantica* [8]. In the East, Bangladesh traded with southern China and further with South East Asia by connecting with the Tea-Horse Road via a northern route through Kamrup in Indian Assam and a southern route via eastern Indian Manipur, both traversing the mountainous regions of upper Burma (present-day Myanmar) and ending in Bengal. Equines and cattle, but also yaks at high altitudes, were used as pack animals on the Tea-Horse Road [8]. The mule was the key animal species, because it is a powerful pack animal very useful in long distance movements along difficult rugged mountainous routes [56], such as those the Tea-Horse Road traversed. Mules have recently proved to be efficient reservoirs in the spread of fascioliasis [57]. This might explain the combined haplotype Fg-CH3A found widely distributed in all livestock species throughout Bangladesh and which proved to

be identical to the haplotype recently reported from Vietnam (GenBank Acc. No. MN970009) [58].

4.2.2. Arguments against an old arrival of *Fasciola hepatica*

The detection of combined haplotypes of “pure” *F. hepatica* in the sequences of the clones obtained needs further analysis.

The only way for the arrival of “pure” *F. hepatica* in the past could have been from the North by the Tea-Horse Road through the Tibetan capital Lhasa and subsequently via the Indian mountain pass at the Indian Sikkim state located in between Nepal and Bhutan (Fig. 2). Indeed, the Tea-Horse Road connected with the eastern routes of the northern Silk Road where the cooler temperatures facilitated the spread of *F. hepatica* [8]. Moreover, the main lymnaeid species vector of *F. hepatica*, *Galba truncatula*, is known to be today present in both Nepal and Bhutan, transhumant agropastoralism has traditionally been practiced in western Bhutan since ancient times, and there was a secondary south-north road which started at the Jaldhaka river in India, just northward from present-day Bangladesh [8].

However, the absence of *Galba/Fossaria* snail species in the lowlands of Bangladesh and surrounding India and the high temperatures of these lowlands not fitting within the range of the development temperature thresholds of *F. hepatica* make it hard to envisage such an entry in the past.

4.2.3. Arguments indicating a very recent introduction of *Fasciola hepatica*

The detection of these “pure” *F. hepatica* sequences only in clones obtained from specimens showing heterozygotic positions indicate hybridization phenomena occurred evolutionarily very recently. In other words, results suggest that native “pure” *F. gigantica* to have crossbred very recently with introduced *F. hepatica* specimens, so that concerted evolution did not yet have sufficient time to homogenize the rDNA operon. The obtaining of different sequences in the clones from several individual fasciolid specimens indicates the same phenomenon.

Although the time needed for the rDNA operon to homogenize different formerly coexisting sequences remains unknown, recent studies suggest that it may need at least several generations over a number of years when only lymnaeid vector species specific for a given *Fasciola* species are present [31]. In Bangladesh, only two lymnaeid species belonging to the *Radix* group are present, namely *R. acuminata* and *R. luteola*, both involved in the transmission of *F. gigantica* [8]. This means that the evolutionary vector filter will act favoring the *F. gigantica* genotype [31].

Moreover, the detection of different sequences coexisting in the rDNA operon of the cloned specimens is clear evidence not only of recent hybridizations, but also crossbreeding between parental specimens which were already hybrids, indicating repeated, superimposed and rapidly evolving hybridization events.

The aforementioned, both official and informal procedures of livestock importation in recent years in Bangladesh due to the non-stop increasing intranational demand explain well the wide variation of hybrid fasciolid specimens found and agree with the previous description of fasciolid intermediate forms reported from this country [27]. Regarding official livestock importation during the 1973–2014 period, information obtained about cattle imported from other countries is illustrated in Table 9, in which exporting countries presenting only *F. hepatica* as Australia and Spain are noted.

Different banding patterns obtained by PCR restriction fragment length polymorphism (PCR-RFLP) applied to the ITS-1 of fasciolids of livestock from Bangladesh also suggested the existence of sequences of both *F. gigantica* and *Fasciola* sp. within individual flukes, although this technique did not allow for the obtaining of the sequences and their nucleotide position analyses, whereas a 535-bp-long fragment of the mtDNA *nad1* gene proved to represent that of *F. gigantica* close to those known in other Asian countries, i.e. no introgression indicating hybridization was found [59]. Interestingly, a combination of an *F. gigantica* haplotype and an *F. hepatica* haplotype suggesting

Table 9

Information on official importation of cattle into Bangladesh, after respective government approval processes, in the 1973–2014 period.

Year	Country of origin	Breed	Description of cattle		Total no. of head	Remarks	Information source ^a
			Cow/Heifer	Bull/Bullcalf			
1973	Australia	Friesian	62	2	64	Gift	1
1973	Australia	Jersey	58	3	61	Gift	2
1987	Pakistan	Sahiwal	95	5	100	Import	3
1989	Spain	Friesian	69	3	72	Gift	4
1990	Pakistan	Sahiwal	80	20	100	Import	5
1992	Pakistan	Sahiwal	96	–	96	Import	6
1993	Australia	AFS ^{***}	150	–	150	Import	7
1995	Australia	Friesian	250	4	254	Import	8
2006	Australia	Friesian	25 (Pregnant)	–	25	Import	9
2012	India ^{***}	–	–	–	1,287,800	Influx [*]	
2013	India ^{***}	–	–	–	1,796,904	Influx [*]	
2013	Australia	Jersey	–	7	7	Import	10
2013	Australia	Friesian	–	17	17	Import	11
2014	Australia	Friesian	–	2	2	Import	12
2014	India ^{***}	–	–	–	1,746,514	Influx [*]	

^{*} influx: local term used in Bangladesh to refer to an indiscriminately, randomly importation (lacking records and carried out without following standard importation procedures).

^a 1–7 and 9: Central Cattle Breeding Station, Department of Livestock Services, Bangladesh; 8: Personal communication with Maj. (Rt.) Aktaruzzaman, owner of the Gochhata Dairy & Fish Farm; 10–12: Department of Livestock Services, Bangladesh.

^{***} AFS: Australian Friesian.

^{***} Information from National Board of Revenue, Segunbaagicha, Dhaka, earned influx revenue but details data not available.

hybridization was found in the nuclear intron 4 of domain 2 of the taurocyamine kinase gene (TkD2Int4) when cloning and sequencing a fasciolid specimen infecting cattle from Bangladesh [60].

The finding of the heterozygotic sequences concentrated in the localities at the western part of Bangladesh (with the exception of the two central localities surrounding of Mymensingh, easily understandable due to the demands of such a large city) (compare Table 4 and Fig. 2), further indicate a role of the livestock importation from neighboring India. The analysis of the distribution of heterozygotic hybrid combined haplotypes according to host species additionally indicates that goats may play a crucial role in the diffusion of hybrid specimens, mainly through the uncontrolled small-scale household farming management and the goat supply chain which distributes animals throughout the country.

5. Concluding remarks

The high infection rates by fasciolids in buffaloes, cattle, goats and sheep indicate a high human infection risk throughout Bangladesh, especially given that 84% of its population lives in rural areas and 75% of the people rely on livestock. This suggests that human fascioliasis may be underestimated in this country and local physicians should be made aware of the cause and diagnosis of this disease.

The high proportion of hybrid fasciolids found in the livestock analyzed moreover highlights a potential increasing infection trend in animals, when taking into account the usually higher spreading power of hybrid forms in cases in which they prove to be viable, i.e. transmissible by the locally available lymnaeid snail vector species. Consequently, human infection risk may also increase, similarly as it has recently occurred in Vietnam [16].

Finally, the recent increase in hybridization events emphasizes the need to put suitable quarantine measures in place, to include liver fluke diagnosis and treatments for imported animals, and to make efforts to help rural people control the disease in their farming management, mainly concerning goats. The capacity of fasciolid metacercariae to infect different host species independently of the *Fasciola* host species isolate of origin [61–63], indicates that each of the four host species studied may act as a reservoir for the other host species.

The results of this study highlight the importance of livestock management regarding the transmission, epidemiology, spread and control of fascioliasis within a One Health strategy against human and animal

fascioliasis, as it has been already observed in a human fascioliasis hyperendemic area [64]. Moreover, the molecular assessment demonstrates the appropriateness of DNA marker sequencing of the fasciolid causal agents within such a One Health strategy when dealing on a country in which both *Fasciola* species may be involved, as known throughout the continents of Asia and Africa where the two fasciolids overlap in many regions [65]. The sequences of the rDNA ITS-1 and ITS-2 markers prove to be highly useful for the detection of recent hybrid fasciolids. This study shows the high degree of variation that ITS-1 and ITS-2 may reach in the species differing positions in these hermaphroditic flukes able to crossbreed, when:

- (i) one *Fasciola* species is introduced by livestock importation into an area where there was originally only the other *Fasciola* species, and
- (ii) high infection prevalence and intensity rates facilitate the occurrence of many and repeated crossbreeding encounters of different fluke species within a biliary canal or gallbladder of a host individual.

It should be highlighted that ITS-1 and ITS-2 are extremely conserved in *Fasciola* species [11,28]. This markedly differs from ITSs in other invertebrates in which accumulation of mutations, insertions such as microsatellite and minisatellite repeats [66], and sometimes even duplications such as pseudogenes [67] occur. In *Fasciola*, microsatellites, minisatellites or pseudogenes have never been reported, and mutations are very rare outside of the few positions allowing for the differentiation of the two species. In the species *F. nyanzae* specific to the hippopotamus, the very few specific mutations in the ITSs concern the same positions differing between *F. hepatica* and *F. gigantica* [11]. This indicates that, in *Fasciola*, the function of ITS-1 and ITS-2 in the production of precursor molecules and mature rRNA products may be more important than in other organisms, i.e. including nucleotide positions surrounding the processing sites subject to functional constraints similar to those acting on the rRNA genes. Mutations in other positions potentially able to disrupt the secondary structures in the ITS regions, which may reduce or eliminate the production of precursor molecules and mature rRNA products, do not appear. Indeed, an analysis shows that none of the differences between the three *Fasciola* species in this region would affect the secondary structure [68].

In its turn, these hybridization phenomena between the two *Fasciola*

species found in Bangladesh also suggest that crossbreeding events should have been frequent inside the same *Fasciola* species infecting domestic animal species regularly used for goods transport along very long distances in round trips in several world zones during thousands of years in the past, when situations of concentration of high numbers of animals facilitating high infection rates should have occurred and no anti-fasciolid treatments were available. These monospecific *Fasciola* crossbreeding events should have undoubtedly underlaid the peculiar characteristics observed at present in the nuclear genome of these hermaphroditic fasciolid flukes throughout [8], in aspects such as the extreme length of the genome, gene duplication, polymorphisms and very high repeat content [69,70].

Ethics statement

All research dealing on animals was performed with the approval of the Evaluation of Projects concerning Animal Research at University of Valencia (Órgano Habilitado para la Evaluación de Proyectos de Experimentación Animal de la Universidad de Valencia) (A1263 915,389,140), strictly following the institution's guidelines based on Directive 2010/63/EU. Permission for animal research was additionally obtained from the Servicio de Sanidad y Bienestar Animal, Dirección General de Producción Agraria y Ganadería, Consellería de Presidencia y Agricultura, Pesca, Alimentación y Agua, Generalitat Valenciana, Valencia, Spain (No. 2015/VSC/PEA/00001 tipo 2). Fasciolid collection was carried out by taking advantage of livestock slaughtering for other purposes. Animal ethics guidelines regarding animal care were strictly adhered.

Consent for publication

All authors read and approved the final version for publication consideration.

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Author contributions

Study concept and design: SMC, MDB; participated in experimental work: SAA, ADEE, PA; participated in field work: SAA, MZA, MMHM, EHC; local coordination, protocols, and logistics: SMC, SAA, MZA, MMHM, EHC; analysis and interpretation of data: SMC, DB, MDB; drafting of the manuscript: SMC, MDB; critical revision of the manuscript for important intellectual content and for final approval: SMC, DB, MDB; obtained project funding: SMC, MDB; principal investigator: SMC.

Author statement

On behalf of all the authors, I can confirm that everyone has read and approved this review.

Declaration of Competing Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Data availability

All data are presented in the article. The sequence data have been submitted to GenBank and have been included under the following accession Nos: OQ064778- OQ064781.

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References

- [1] A.D. Hayward, P.J. Skuce, T.N. McNeill, The influence of liver fluke infection on production in sheep and cattle: a meta-analysis, *Int. J. Parasitol.* 51 (2021) 913–924.
- [2] World Health Organization, Sustaining the Drive to Overcome the Global Impact of Neglected Tropical Diseases, Department of Control of Neglected Tropical Diseases, World Health Organization, WHO Headquarters, Geneva, 2013, pp. 1–128.
- [3] World Health Organization, Ending the Neglect to Attain the Sustainable Development Goals. A Road Map for Neglected Tropical Diseases 2021–2030, World Health Organization, Geneva, 2020, pp. 1–47. Available online, http://www.who.int/neglected_diseases/Ending-the-neglect-to-attain-the-SDGs-NTD-Roadmap.pdf (accessed on 23 July 2020).
- [4] N.V. De, T.H. Le, V.H. Agramunt, S. Mas-Coma, Early postnatal and preschool age infection by *Fasciola* spp.: report of five cases from Vietnam and worldwide review, *Am. J. Trop. Med. Hyg.* 103 (2020) 1578–1589.
- [5] S. Mas-Coma, V.H. Agramunt, M.A. Valero, Neurological and ocular fascioliasis in humans, *Adv. Parasitol.* 84 (2014) 27–149.
- [6] J. Gonzalez-Miguel, M.A. Valero, M. Reguera-Gomez, C. Mas-Bargues, M. D. Bargas, F. Simon-Martin, S. Mas-Coma, Numerous *Fasciola* plasminogen-binding proteins may underlie blood-brain barrier leakage and explain neurological disorder complexity and heterogeneity in the acute and chronic phases of human fascioliasis, *Parasitology* 146 (2019) 284–298.
- [7] S. Mas-Coma, Human fascioliasis emergence risks in developed countries: from individual patients and small epidemics to climate and global change impacts, *Enferm. Infecc. Microbiol. Clin.* 38 (2020) 253–256.
- [8] S. Mas-Coma, M.A. Valero, M.D. Bargas, Human and animal fascioliasis: origins and worldwide evolving scenario, *Clin. Microbiol. Rev.* 35 (2022), e000819.
- [9] K. Afshan, C.A. Fortes-Lima, P. Artigas, M.A. Valero, M. Quayum, S. Mas-Coma, Impact of climate change and man-made irrigation systems on the transmission risk, long-term trend and seasonality of human and animal fascioliasis in Pakistan, *Geospat. Health* 8 (2014) 317–334.

- [10] M.D. Bargues, P. Artigas, R. Angles, D. Osca, P. Duran, P. Buchon, R.K. Gonzales-Pomar, J. Pinto-Mendieta, S. Mas-Coma, Genetic uniformity, geographical spread and anthropogenic habitat modifications of lymphatic vectors found in a One Health initiative in the highest human fascioliasis hyperendemic of the Bolivian Altiplano, *Parasit. Vectors* 13 (2020) 171.
- [11] M.D. Bargues, A. Halajian, P. Artigas, W.J. Luus-Powell, M.A. Valero, S. Mas-Coma, Paleobiogeographical origins of *Fasciola hepatica* and *F. gigantica* in light of new DNA sequence characteristics of *F. rypanae* from hippopotamus, *Front. Vet. Sci.* 9 (2022), 990872.
- [12] M.A. Valero, M.D. Bargues, M. Khoubbane, P. Artigas, C. Quesada, L. Berinde, F. M. Ubeira, M. Mero, J.L. Hernandez, V.H. Agramunt, S. Mas-Coma, Higher physiopathogenicity by *Fasciola hepatica* than by the genetically close *F. hepatica*: experimental long-term follow-up of biochemical markers, *Trans. Roy. Soc. Trop. Med. Hyg.* 110 (1, Special Issue) (2016) 55–66.
- [13] A.S. Lukambagire, D.N. Mchale, M. Nyindo, Diagnosis of human fascioliasis in Arusha region, northern Tanzania by microscopy and clinical manifestations in patients, *BMC Infect. Dis.* 15 (2015) 578.
- [14] M.V. Periago, M.A. Valero, P. Artigas, V.H. Agramunt, M.D. Bargues, F. Curtale, S. Mas-Coma, Very high fascioliasis intensities in schoolchildren of Nile Delta governorates: the Old World highest burdens found in lowlands, *Pathogens* 10 (2021) 1210.
- [15] T. Bekana, N. Berhe, T. Eguale, M. Amero, G. Medhin, B. Tulu, Y. Ghiwot, S. Liang, W. Hu, B. Erko, Prevalence and factors associated with intestinal schistosomiasis and human fascioliasis among school children in Amhara Regional State, Ethiopia, *Trop. Med. Health* 49 (2021) 25.
- [16] T.B.N. Nguyen, N.V. De, T.K.L. Nguyen, H.H. Quang, H.T.T. Doan, T. Agatsuma, T. H. Le, Distribution status of hybrid types in large liver flukes, *Fasciola* species (Digenea: Fasciolidae), from ruminants and humans in Vietnam, *Kor. J. Parasitol.* 56 (2018) 453–461.
- [17] J.M. Behar, J.S. Winston, R. Borgstein, Hepatic fascioliasis at a London hospital - the importance of recognizing typical radiological features to avoid a delay in diagnosis, *Brit. J. Radiol.* 82 (981) (2009) e189–e192.
- [18] B.C. Das, A.S. Khan, M.S. Uddin, M.M. Chowdhury, M.Z. Rahman Khan, Fascioliasis - an uncommon cause of recurrent cholangitis, *J. Surg. Sci.* 19 (2015) 35–38.
- [19] J. Ramachandran, S. Ajampur, A. Chandramohan, G.M. Varghese, Cases of human fascioliasis in India: tip of the iceberg, *J. Postgrad. Med.* 58 (2012) 150–152.
- [20] R. Madhumitha, S. Gohel, L. Latha Vishwanathan, R. Gopalakrishnan, Liver lesions, fever and eosinophilia caused by *Fasciola hepatica* in a 15-year-old girl, *Indian J. Ped.* 82 (2015) 56–62.
- [21] K. Narain, D. Biswas, S.K. Rajguru, J. Mahanta, Human distomatosis due to *Fasciola hepatica* infection in Assam, India, *J. Comm. Dis.* 29 (1997) 161–165.
- [22] S.V. Pareek, S.N. Oak, A. Maydeo, B.V. Sanghvi, P.B. Joshi, N. Chaubal, R.T. Patil, S.K. Sahoo, P.J. Lal, N. Sampath, A. Koticha, Biliary fascioliasis: management in a child using endoscopic retrograde cholangiopancreatography, *J. Indian Ass. Ped. Surg.* 18 (2013) 23–24.
- [23] R. Sah, S. Khadka, M. Khadka, D. Gurubacharya, J.B. Sherchand, K. Parajuli, N. P. Shah, H.P. Kattel, B.M. Pokharel, B. Rijal, Human fascioliasis by *Fasciola hepatica*: the first case report in Nepal, *BMC Res. Notes* 10 (2017) 439.
- [24] R. Sah, S. Khadka, P.J. Lakhey, S. Pradhan, N.P. Shah, V.P. Singh, S. Mas-Coma, Human case of *Fasciola gigantica*-like infection, review of human fascioliasis reports in Nepal, and epidemiological analysis within the south Central Asia, *Acta Parasitol.* 63 (2018) 435–443.
- [25] T. Maeda, H. Yamada, N. Akao, M. Iga, T. Endo, T. Koibuchi, T. Nakamura, T. Odawara, A. Iwamoto, T. Fujii, Unusual radiological findings of *Fasciola hepatica* infection with huge cystic and multicellular lesions, *Inter. Med.* 47 (2008) 449–452.
- [26] World Health Organization, Ending the Neglect to Attain the Sustainable Development Goals. One Health Companion Document to the Neglected Tropical Diseases Road Map 2021–2030. Draft for Public Consultation (updated 19 October 2021), World Health Organization, Geneva, 2021, pp. 1–23. Available online, <https://cdn.who.int/media/docs/default-source/ntds/rabies/online-public-consultation-one-health-companion-document/draft-for-public-consultation-one-health-companion-document-for-ntd-road-map.pdf> (accessed 15 December 2021).
- [27] S.A. Ahasan, M.A. Valero, E.H. Chowdhury, M.T. Islam, M.R. Islam, M.M. H. Mondal, R.V. Peixoto, L. Berinde, M. Panova, S. Mas-Coma, CIAS detection of *Fasciola hepatica* / *F. gigantica* intermediate forms in bovines from Bangladesh, *Acta Parasitol.* 61 (2016) 267–277.
- [28] S. Mas-Coma, M.A. Valero, M.D. Bargues, *Fasciola* lymphatic and human fascioliasis, with a global overview on disease transmission, epidemiology, evolutionary genetics, molecular epidemiology and control, *Adv. Parasitol.* 69 (2009) 41–146.
- [29] S. Mas-Coma, R. Funatsu, M.D. Bargues, *Fasciola hepatica* and lymphatic snails occurring at very high altitude in South America, *Parasitology* 123 (2001) S115–S127.
- [30] M.D. Bargues, V. Gayo, J. Sanchis, P. Artigas, M. Khoubbane, S. Birriel, S. Mas-Coma, DNA multigene characterization of *Fasciola hepatica* and *Lymanaea neotropic* and its fascioliasis transmission capacity in Uruguay, with historical correlation, human report review and infection risk analysis, *PLoS Negl. Trop. Dis.* 11 (2017), e0005352.
- [31] M.D. Bargues, M.A. Valero, G.A. Trueba, M. Fornasini, A.F. Villavicencio, R. Guaman, A. De Elias-Escribano, I. Perez-Crespo, P. Artigas, S. Mas-Coma, DNA multi-marker genotyping and CIAS morphometric phenotyping of *Fasciola gigantica*-sized flukes from Ecuador, with an analysis of the *Radix* absence in the New World and the evolutionary lymphatic snail vector filter, *Animals* 11 (2021) 2495.
- [32] L. Chougar, S. Mas-Coma, P. Artigas, K. Harhoura, M. Aissi, V.H. Agramunt, M. D. Bargues, Genetically “pure” *Fasciola gigantica* discovered in Algeria: DNA multimarker characterization, trans-Saharan introduction from a Sahel origin and spreading risk into northwestern Maghreb countries, *Transb. Emerg. Dis.* 67 (2020) 2190–2205.
- [33] M.A. Valero, M.D. Bargues, L. Calderon, P. Artigas, S. Mas-Coma, First phenotypic and genotypic description of *Fasciola hepatica* infecting highland cattle in the state of Mexico, Mexico, *Infect. Genet. Evol.* 64 (2018) 231–240.
- [34] S. Kumar, G. Stecher, M. Li, C. Knyaz, K. Tamura, MEGA X: molecular evolutionary genetics analysis across computing platforms, *Mol. Biol. Evol.* 35 (2018) 1547–1549.
- [35] D. Glez-Peña, D. Gómez-Blanco, M. Reboiro-Jato, F. Fdez-Riverola, D. Posada, ALTER: program-oriented conversion of DNA and protein alignments, *Nucleic Acids Res.* 38 (Web Server issue) (2010), <https://doi.org/10.1093/nar/gkq321>. W14–8.
- [36] M.D. Bargues, S. Mas-Coma, Reviewing lymphatic vectors of fascioliasis by ribosomal DNA sequence analyses, *J. Helminthol.* 7 (2005) 257–267.
- [37] M.Z. Ali, G. Carlle, M. Giassuddin, Impact of global climate change on livestock health: Bangladesh perspective, *Open Vet. J.* 10 (2020) 178–188.
- [38] Anonymous, National Livestock Development Policy, Monogram. Government of the People's Republic of Bangladesh, Ministry of Fisheries and Livestock. Dhaka, Bangladesh, 2007, pp. 1–40.
- [39] A.A. Roess, P.J. Winch, N.A. Ali, A. Akhter, D. Afroz, S. El Arifeen, G.L. Darmstadt, A.H. Baqui for the Bangladesh PROAHNMO Study Group, Animal husbandry practices in rural Bangladesh: potential risk factors for antimicrobial drug resistance and emerging diseases, *Am. J. Trop. Med. Hyg.* 89 (2013) 965–970.
- [40] J.H. Rahman, M.O. Faruque, M.Z. Ali, M.Z. Hossain, Incidence of diseases in goats in Bangladesh, *The Bangladesh Veterinarian* 37 (1–2) (2020) 14–20.
- [41] S.D. Chowdhury, M.M. Hossain, K.M.M. Rahman, M.K. Saha, S. Hossain, Study on Goat Value Chain in Bangladesh, Final report, Heifer International Bangladesh, Dhaka, Bangladesh, 2015, pp. 1–64.
- [42] S.S. Islam, M.S. Hasan, N. Ghosh, M.S. Islam, M.M. Islam, Prospects and problems of indigenous sheep production in south-western coastal regions of Bangladesh, *J. Agric. Sci. Sri Lanka J* (2021) 54–66.
- [43] M.R. Habib, M.N. Haque, A. Rahman, M. Afabuzzaman, M.M. Ali, M. Shahjahan, Dairy buffalo production scenario in Bangladesh: a review, *Asian J. Med. Biol. Res.* 3 (2017) 305–316.
- [44] A.K. Fazul Haque Bhuiyan, Farm Animal Genetic Diversity Country Report – Bangladesh, Department of Animal Breeding and Genetics, Bangladesh Agricultural University, Mymensingh 2202, Bangladesh, 2013, pp. 1–74.
- [45] S. Mas-Coma, P. Buchon, L.R. Funatsu, R. Angles, C. Mas-Bargues, P. Artigas, M. A. Valero, M.D. Bargues, Donkey fascioliasis within a One Health control action: transmission capacity, field epidemiology, and reservoir role in a human hyperendemic area, *Front. Vet. Sci.* 7 (2020), 591384.
- [46] S. Mas-Coma, P. Buchon, L.R. Funatsu, R. Angles, P. Artigas, M.A. Valero, M. D. Bargues, Sheep and cattle reservoirs in the highest human fascioliasis hyperendemic area: experimental transmission capacity, field epidemiology, and control within a One Health initiative in Bolivia, *Front. Vet. Sci.* 7 (2020), 583204.
- [47] S. Mas-Coma, P. Buchon, L.R. Funatsu, R. Angles, C. Mas-Bargues, P. Artigas, M. A. Valero, M.D. Bargues, Donkey fascioliasis within a One Health control action: transmission capacity, field epidemiology, and reservoir role in a human hyperendemic area, *Front. Vet. Sci.* 7 (2020), 591384.
- [48] S. Mas-Coma, L.R. Funatsu, R. Angles, P. Buchon, P. Mas-Bargues, M.A. Artigas, M. D. Bargues Valero, Domestic pig prioritized in one health action against fascioliasis in human endemic areas: experimental assessment of transmission capacity and epidemiological evidence of reservoir role, *One Health* 13 (2021), 100249.
- [49] A.K.M.A. Rahman, S.K.S. Islam, M.H. Talukder, M.K. Hassan, N.K. Dhand, M. P. Ward, Fascioliasis risk factors and space-time clusters in domestic ruminants in Bangladesh, *Parasit. Vectors* 10 (2017) 228.
- [50] R. Garg, C.L. Yadav, R.R. Kumar, P.S. Banerjee, S. Vatsya, R. Godara, The epidemiology of fasciolosis in ruminants in different geo-climatic regions of North India, *Trop. Anim. Health Prod.* 41 (2009) 1695–1700.
- [51] C.L. Yadav, S. Vatsya, R. Garg, R.R. Kumar, P.S. Banerjee, G. Rajesh, Seasonal prevalence of *Fasciola gigantica* infection in sheep and goats in western Uttar Pradesh, *J. Parasit. Dis.* 31 (2007) 141–144.
- [52] G. Swarnakar, B. Sanger, Epidemiological study of liver fluke (Trematoda: Digenea) in domestic ruminants of Udaipur District, *Int. J. Curr. Microbiol. App. Sci.* 3 (2014) 632–640.
- [53] S.C. Gupta, S. Ghosh, O.K. Raina, D. Joseph, P. Rawat, B.P. Singh, A.K. Mishra, D. Chandra, S. Samanta, Studies and prevalence of fasciolosis in cattle and buffaloes in different agro-climatic zones of Uttar Pradesh, India, *J. Vet. Parasitol.* 22 (2008) 59–63.
- [54] O. Singh, R.A. Agarwal, Toxicity of certain pesticides to two economic species of snail in Northern India, *J. Econ. Entomol.* 74 (1981) 568–571.
- [55] K. Sunita, S. Mas-Coma, M.D. Bargues, M.A. Sadaf, M. Khan, S. Habib, S.A. Husain Mustafa, Buffalo infection by *Fasciola gigantica* transmitted by *Radix acuminata* in Uttar Pradesh, India: a molecular tool to improve snail vector epidemiology assessments and control surveillance, *Acta Parasitol.* 66 (2021) 1395–1405.
- [56] P. Mitchell, The Donkey in Human History. An Archaeological Perspective, Oxford University Press, Oxford, 2018, p. 306.
- [57] R. Mera y Sierra, G. Neira, M.D. Bargues, P.F. Cuervo, P. Artigas, L. Logarzo, G. Cortinas, D.E.J. Ibaceta, G.A. Lopez, I.E. Bisutti, S. Mas-Coma, Equines as reservoirs of human fascioliasis: transmission capacity, epidemiology and pathogenicity in *Fasciola hepatica*-infected mules, *J. Helminthol.* 94 (2020), e189.
- [58] T.H. Le, K.L.T. Pham, H.T.T. Doan, T.K.L. Le, K.T. Nguyen, S.P. Lawton, Description and phylogenetic analyses of ribosomal transcription units from species of Fasciolidae (Platyhelminthes: Digenea), *J. Helminthol.* 94 (e136) (2020) 1–6.

- [59] U.K. Mohanta, M. Ichikawa-Seki, T. Shoriki, K. Katakura, T. Itagaki, Characteristics and molecular phylogeny of *Fasciola* flukes from Bangladesh, determined based on spermatogenesis and nuclear and mitochondrial DNA analyses, *Parasitol. Res.* 113 (2014) 2493–2501.
- [60] W. Saijuntha, C. Tantrawatpan, T. Agatsuma, C. Wang, P.M. Intapan, W. Maleewong, T.N. Petney, Revealing genetic hybridization and DNA recombination of *Fasciola hepatica* and *Fasciola gigantica* in nuclear introns of the hybrid *Fasciola* flukes, *Mol. Biochem. Parasitol.* 223 (2018) 31–36.
- [61] M.A. Valero, S. Mas-Coma, Comparative infectivity of *Fasciola hepatica* metacercariae from isolates of the main and secondary reservoir animal host species in the Bolivian Altiplano high human endemic region, *Folia Parasitol.* 47 (2000) 17–22.
- [62] M.A. Valero, N.A. Darce, M. Panova, S. Mas-Coma, Relationships between host species and morphometric patterns in *Fasciola hepatica* adults and eggs from the Northern Bolivian Altiplano hyperendemic region, *Vet. Parasitol.* 102 (2001) 85–100.
- [63] M.A. Valero, M. Panova, A.M. Comes, R. Fons, S. Mas-Coma, Patterns in size and shedding of *Fasciola hepatica* eggs by naturally and experimentally infected murid rodents, *J. Parasitol.* 88 (2002) 308–313.
- [64] R. Angles, P. Buchon, M.A. Valero, M.D. Bargues, S. Mas-Coma, One Health action against human fascioliasis in the Bolivian Altiplano: food, water, housing, behavioural traditions, social aspects, and livestock management linked to disease transmission and infection sources, *Int. J. Environ. Res. Publ. Health* 19 (2022) 1120.
- [65] S. Mas-Coma, M.A. Valero, M.D. Bargues, One Health for fascioliasis control in human endemic areas, *Trends Parasitol.* 38 (2023) 650–667.
- [66] S. Mas-Coma, M.D. Bargues, Populations, hybrids and the systematic concepts of species and subspecies in Chagas disease triatomine vectors inferred from nuclear ribosomal and mitochondrial DNA, *Acta Trop.* 110 (2009) 112–136.
- [67] M.D. Bargues, M.A. Zuriaga, S. Mas-Coma, Nuclear rDNA pseudogenes in Chagas disease vectors: evolutionary implications of a new 5.8S+ITS-2 paralogous sequence marker in triatomines of North, Central and northern South America, *Infect. Genet. Evol.* 21 (2014) 134–156.
- [68] J.A. Morgan, D. Blair, Trematode and monogenean rRNA ITS2 secondary structures support a four-domain model, *J. Mol. Evol.* 47 (1998) 406–419.
- [69] K. Cwiklinski, J.P. Dalton, P.J. Dufresne, J. La Course, D.J.L. Williams, J. Hodgkinson, S. Paterson, The *Fasciola hepatica* genome: gene duplication and polymorphism reveals adaptation to the host environment and the capacity for rapid evolution, *Genome Biol.* 16 (2015) 71.
- [70] S.N. McNulty, J.P. Tort, G. Rinaldi, K. Fischer, B.A. Rosa, P. Smircich, S. Fontenla, Y.J. Choi, R. Tyagi, K. Hallsworth-Pepin, V.H. Mann, L. Kamilli, P.S. Latham, N. Dell'Oca, F. Dominguez, C. Carmona, P.U. Fischer, P.J. Brindley, M. Mitreva, Genomes of *Fasciola hepatica* from the Americas reveal colonization with *Neorickettsia* endobacteria related to the agents of Potomac horse and human Sennetsu fevers, *PLoS Genet.* 13 (2017), e1006537.



Article

Mating Interactions between *Schistosoma bovis* and *S. mansoni* and Compatibility of Their F1 Progeny with *Biomphalaria glabrata* and *Bulinus truncatus*

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Abstract: Contrary to the majority of other Trematoda, *Schistosoma* species are gonochoric. Consequently, in endemic areas where several schistosome species overlap and can co-infect the same definitive host, there may be frequent opportunities for interspecific pairing. Our experimental study provides novel insight on the pairing behavior between *Schistosoma bovis* and *S. mansoni* in mixed infections in mice. We used six mate choice experiments to assess mating interactions between the two schistosome species. We show that mating between the two *Schistosoma* species is not random and that *S. mansoni* exhibits greater mate recognition compared to *S. bovis*. We also performed reciprocal crosses (male *S. mansoni* × female *S. bovis*) and (female *S. mansoni* × male *S. bovis*) that produce active swimming miracidia. These miracidia were genotyped by ITS2 sequencing and proposed for mollusc infection. Molecular analyses show that all the miracidia are parthenogenetically produced (i.e., their harbor the mother ITS2 genotype) and as a consequence can only infect the mollusc of the maternal species. Offspring produced by male *S. mansoni* × female *S. bovis* pairing can only infect *Bulinus truncatus* whereas offspring produced by female *S. mansoni* × male *S. bovis* can only infect *Biomphalaria glabrata* snails. Evolutionary and epidemiological consequences are discussed.

Keywords: *Schistosoma bovis*; *Schistosoma mansoni*; mating interactions; F1 progeny; compatibility; *Bulinus truncatus*; *Biomphalaria glabrata*

1. Introduction

The class Trematoda is a diversified phylum of worms characterized by their parasitic way of life. Trematoda are usually hermaphroditic; however, the hundred species belonging to the family of Schistosomatidae are exceptional because they are gonochoric [1,2]. This family contains some species of considerable medical and veterinary importance [3,4]. Among them, the *Schistosoma* genus is the most important in terms of human health and social-economic impacts. Human schistosomiasis affects about 250 million people in about 78 countries worldwide, with the largest disease burden throughout sub-Saharan Africa [5]. The *Schistosoma* genus is composed of about 23 recognized species with at least 19 species that infect livestock and wild animals [6]. Although only five species are of veterinary importance to domestic animals, at least six species infect humans and are of medical interest [6]. Because several of these species are co-endemic and can share the

same definitive host, interspecific crossing opportunities may occur. Several studies, both in the field and in the lab have addressed the potentiality of different *Schistosoma* species to encounter, to mate and to interbreed.

In the field, numerous studies have reported the existence of several potential interspecific crosses between different species of schistosomes [7]. Early reports were mainly based on the physical appearance of the eggs [8]. However, the viability of these eggs was not evaluated and these early physical observations have often been considered as misdiagnosis [7]. The use of molecular techniques has later confirmed hetero-specific pairing or resulting hybrid progeny between different schistosome species, when adult worms or miracidia were genotyped, respectively [7]. Natural interspecific hybridization may occur between human *Schistosoma* species (*S. haematobium* × *S. guineensis* or *S. mansoni*), animal schistosomes (*S. bovis* × *S. curassoni*) or between human and animal schistosomes (*S. haematobium* × *S. bovis* or *matthei* or *curassoni* or *S. mansoni* × *S. rodhaini*). Interspecific pairing may induce some important outcomes for the parasites' transmission in the field. For example, in Cameroon, hybridization between *S. haematobium* and *S. guineensis* has led to the local extinction of the latter species and the establishments of the former and their hybrid offspring [9,10]. Another major outcome is the zoonotic potential of some crosses as it has been observed in *S. haematobium* × *S. bovis* crosses [11,12].

In parallel to field identification, experimental approaches allowed to analyze interspecific interactions and to infer some biological aspects, such as mate choice, mate competition or the genetic outcome of the crosses, that cannot be address as easily in the field. Several interbreeding experiments in the laboratory have confirmed that *Schistosoma* species can successfully hybridize for several generations [13]. These experimental studies have evidenced either random mating or a preponderance of homo-specific pairing according to the phylogenetic distance of the interacting species [13]. When closely related species interact such as *S. haematobium* × *S. bovis* [14], *S. bovis* × *S. curassoni* [15], *S. haematobium* × *S. intercalatum* [16] or *S. intercalatum* × *S. guineensis* [17] the pairing is random. All these latter species are included in the same monophyletic *S. haematobium* group [3]. At the opposite when the species belong to two different evolutionary lineages mate preference is observed as evidenced for *S. mansoni* × *S. intercalatum* crosses [18]. The genetic background of the resulting progeny may also depend on the phylogenetic distance between the interacting species, ranging from parthenogenetic individuals [19] to substantial genomic introgression [14,20,21]. One visible consequence of such genetic signatures may be observed at the parasites' life history trait level, and in particular for the parasite-mollusc compatibility. When the progeny is partheno-genetically produced, the mollusc host spectrum is limited to the host spectrum of the maternal schistosome species [19]. On the contrary,, for closely related species, hybridization can enlarge the host spectrum as evidenced in *S. haematobium* × *S. intercalatum* crosses [22].

The present study addresses inter-specific interactions between *S. mansoni* and *S. bovis* in the laboratory. These species belong to two different evolutionary lineages of schistosomes; the *S. mansoni* and the *S. haematobium* groups, respectively. *S. mansoni* is known to infect Humans, Non-Human Primates or rodents, while *S. bovis* is known to infect livestock and rodents [6]. In the field, both parasite species can share the same rodent host, as has been found in *Mastomys huberti* and *Arvicanthis niloticus* in Senegal [23]. This last study also evidenced interspecific pairing between an *S. mansoni* male and a *S. haematobium* × *S. bovis* hybrid female in *Mastomys huberti* [23]. A single study has experimentally exposed rodents to mixed infections between male *S. bovis* and female *S. mansoni* [24]. This last author has observed eggs with typical maternal species shape, but few contained viable miracidia and the work failed to infect *Bulinus* snail. In the current context of schistosomes' potential zoonotic transmission, this study proposes to experimentally analyze *S. mansoni* and *S. bovis* interactions through: (i) mate interactions thanks to mate choice experiments; (ii) the compatibility between the progeny and the parental mollusc species (*Biomphalaria glabrata* and *Bulinus truncatus*) after forced reciprocal crosses; and (iii) the nuclear genetic background of the progeny.

2. Materials and Methods

2.1. The Ethics Statement

This research was carried out according to national ethical standards established in the write of 1st February 2013 (NOR: AGRG1238753A), setting the conditions for approval, planning and operation of establishments, breeders and suppliers of animals used for scientific purposes and controls. The experiments carried out for this study were approved and provided a permit A66040 for animal experimentation by the French Ministry of Agriculture and Fishery (Ministere de l'Agriculture et de la Pêche), and the French Ministry for Higher Education, Research and Technology (Ministere de l'Éducation Nationale de la Recherche et de la Technologie). The investigator has the official certificate for animal experimentation, obtained from both ministries (Decret n° 87/848 du 19 octobre 1987; number of authorization 007083).

2.2. Origin and Maintenance of Schistosome Strains

Schistosoma bovis and *S. mansoni* were maintained in the laboratory using *Bulinus truncatus* (Spanish strain) and *Biomphalaria glabrata* (Brazilian strain), respectively. The definitive hosts used were Swiss OF1 mice (Charles River Laboratories L'Abresle, Saint-Germain-Nuelles, France). The parasite strains *S. bovis* and *S. mansoni* originated from Yegua-Salamanca (Spain) and Recife (Brazil), respectively. The *S. bovis* strain isolated in the early 1980s originates from Villar de la Yegua-Salamanca, and was provided by Ana Oleaga from the Spanish laboratory of parasitology of the Institute of Natural Resources and Agrobiology in Salamanca [25]. The *S. mansoni* strain isolated in 1975 originates from Recife (Brazil) and was provided by Pr. Y. Golvan from the Faculty of Medicine St Antoine, Paris (France). Compatibility of the F1 progeny was tested on four mollusc strains: two *B. truncatus* (from Spain and from Morocco) and two *B. glabrata* (from Brazil and from Guadeloupe).

2.3. Experimental Protocol

2.3.1. Snail Infection and Obtention of Unisexual Clonal Population of Schistosome

Methods for miracidium recovery follow the previously published procedure [26,27]. Seventy-two *Biomphalaria glabrata* snails were exposed individually overnight to a single miracidium of *S. mansoni* each and 96 *Bulinus truncatus* snails were individually exposed overnight to a single miracidium of *S. bovis* each so that each infected snail would produce single-sex cercarial population. The molluscs were separated into two breeding tanks according to species and fed *ad libitum* for a duration of 35 days for miracidium to develop to cercariae. Molluscs placed in 24 well-plates according to species were stimulated under light for cercariae shedding. After 2–3 h, the 24 well-plates were examined under a binocular microscope for the presence of cercariae and any snail found to emit cercariae was assigned with an identity number. Three cercariae from each infected snail were individually captured for molecular sexing and each snail was separated into a plastic cup and fed *ad libitum*. Molecular sexing of *S. mansoni* and *S. bovis* cercariae was performed according to [14,28] respectively (see Supplementary Materials for detailed molecular biology protocols [28,29]). The snails were finally separated into four distinct tanks according to sex and species of cercariae.

2.3.2. Mice Infection, Parasite Recovery and Species Identification

Mice were infected using the paddling method and worms were recovered thanks to hepatic perfusion technique. Details for mice infection and parasite recovery follow previously published procedure [26,27]. The sex, species and number of cercariae combination used for each mouse exposure are shown in Table 1.

Table 1. Number of cercariae used for each experiment according to sex and species of parasite.

Experiment Number	<i>S. bovis</i>		<i>S. mansoni</i>		Number of Mice
	Males	Females	Males	Females	
Limited choice experiments					
1. limiting sex: female <i>S. bovis</i>	60	60	60	–	8
2. limiting sex: female <i>S. mansoni</i>	60	–	60	60	8
3. limiting sex: male <i>S. bovis</i>	60	60	–	60	8
4. limiting sex: male <i>S. mansoni</i>	–	60	60	60	8
Full choice experiment					
5. no limiting sex	45	45	45	45	8
Forced reciprocal mating experiment					
6. F1 progeny	–	100	100	–	4
6. F1' progeny	100	–	–	100	4

The experimental design to quantify the frequency of homo- and hetero-specific coupling between *S. bovis* and *S. mansoni* is composed of five experiments (n°1–5—Table 1). Experiments n°1–4 aimed to test the individual choice of each species and sex. In experiments n°1 and n°2, we tested the female choice for *S. mansoni* and *S. bovis*. In experiments n°3 and n°4, we tested the male choice for *S. mansoni* and *S. bovis*. Experiments n°1–4 served as a restricted choice of mate where excess of one sex of the two species competing for pairing will ensure that all individuals of the other sex (that had the choice for homo- or hetero-specific mating) will be paired. Experiment n°5 served as full choice of mate. Mice were infected with the same number of cercariae of both sexes and species so that we could evaluate all pairing combinations simultaneously. Experiment n°6 consists in producing F1 and F1' progeny through forced reciprocal mating experiments. These last crosses were designed to obtain a first generation of miracidia to know whether these progenies are compatible with the snail intermediate host.

After cercarial exposure, the mice were euthanized at two months and adult worms recovered by hepatic perfusion. We used a magnifier lens and a small paintbrush to separate the dimorphic worms according to their sex (male or female). Each worm, whether mated or unmated, was placed in a 1.5 micro tube and labeled appropriately. Tubes containing worms were stored in the freezer at -20°C for genetic analysis. The species of all worms (mated and unmated) were identified after DNA extraction using amplification methods (see Supplementary Materials for detailed molecular biology protocols).

2.3.3. Mollusc Exposition with F1 and F1' Miracidia

Albino mice exposed to male *S. mansoni* $\times$ female *S. bovis* cercariae and vice versa (F1 & F1', see Experiments n°6, Table 1), were euthanized at two months post-cercarial exposure and eggs from the livers were hatched to recover first generation miracidia [26,27]. Forty miracidia of each cross were stored on Whatman FTA cards [30]. The sex of these miracidia was determined by PCR and a part of the ITS2 gene was sequenced for 66 specimens (see molecular biology methods Supplementary Materials for details). Sequences obtained were compared to reference sequences from Genbank database (AF531314.1 for *S. mansoni* and FJ588862.1 for *S. bovis*). We used 24 well-plates to expose 48 *Biomphalaria glabrata* snails each of Brazil and Guadeloupe strains individually overnight with 10 F1 or F1' miracidia. The same procedure was used to expose 48 *Bulinus truncatus* (Morocco and Spain strains). The protocol for mollusc infection with F1 and F1' miracidia is shown in Table 2 below. The molluscs were separated into eight breeding tanks according to their infected miracidia (F1 or F1'), snail species (*B. glabrata* or *B. truncatus*) and snail strain (Brazil, Guadeloupe, Morocco, or Spain). Snails were fed ad libitum for a duration of 60 days for miracidia to de-

velop into cercariae. Molluscs placed in 24 well-plates according to group were stimulated under light to emit cercariae.

Table 2. Snail infection with F1/F1' miracidia for compatibility testing. Sb: *S. bovis*, Sm: *S. mansoni*.

Exp	Intermediate Snail Host (Strain)	Number of Snails Exposed	Progeny
A	<i>Biomphalaria glabrata</i> (Brazil)	48	♂Sb × ♀Sm (F1')
B	<i>Biomphalaria glabrata</i> (Guadeloupe)	48	♂Sb × ♀Sm (F1')
C	<i>Biomphalaria glabrata</i> (Brazil)	48	♀Sb × ♂Sm (F1)
D	<i>Biomphalaria glabrata</i> (Guadeloupe)	48	♀Sb × ♂Sm (F1)
E	<i>Bulinus truncatus</i> (Morocco)	48	♂Sb × ♀Sm (F1')
F	<i>Bulinus truncatus</i> (Spain)	48	♂Sb × ♀Sm (F1')
G	<i>Bulinus truncatus</i> (Morocco)	48	♀Sb × ♂Sm (F1)
H	<i>Bulinus truncatus</i> (Spain)	48	♀Sb × ♂Sm (F1)

2.4. Statistical Analysis

The total number of adult worms recovered for each schistosome species was counted, e.g., homo-specific pairs, hetero-specific pairs, and single worms. We used the null hypothesis of random pairing to calculate the expected number of single and paired worms, e.g., in experiment 1 the expected number of homo-specific paired *S. bovis* females equals the total number of *S. bovis* females, times the total number of *S. bovis* males over the total number of males. We used the Chi-square tests with Yates correction for continuity to compare the expected and observed numbers of homo- and hetero-specific pairs. The p-value was adjusted for multiple comparisons using the Benjamini & Hochberg method. Statistical analysis was done using R Studio v1.4.1106.

3. Results

For each experiment, Table 3 shows the sex and species of the choosing partner. Random pairing is observed when *S. bovis* is the species that can choose whether male ($\chi^2 = 2.73$; $p = 0.8$ after Benjamini & Hochberg correction) or female ($\chi^2 = 0.29$; $p = 1.00$ after Benjamini & Hochberg correction). Homo-specific pairs are more numerous than heterospecific pairs when *S. mansoni* is the species that can choose whether male ($\chi^2 = 9.85$; $p = 0.016$ after Benjamini & Hochberg correction) or female ($\chi^2 = 7.66$; $p = 0.048$ after Benjamini & Hochberg correction). Table 4 shows the number of homo-, hetero-specific pairs and single worms when mice are exposed to equal number of cercariae whatever their sex and their species. The number of homo-specific pairs is bigger than expected under the hypothesis of random association ($\chi^2 = 31.86$; $p < 0.001$ after Benjamini & Hochberg correction). However, no ♂Sb × ♀Sm pairs were observed because all *S. mansoni* female were monopolized by male *S. mansoni*.

Table 5 shows compatibility of F1 (female *S. bovis* × male *S. mansoni*) and F1' (male *S. bovis* × female *S. mansoni*) miracidia with the intermediate snail hosts of both schistosomes' parental species. F1' miracidia readily infect *B. glabrata* from Brazil (50%) and from Guadeloupe (20.8%). F1 miracidia readily infect *B. truncatus* from Spain (10.6%) and from Morocco (3.3%). Loss of compatibility was noted in F1 miracidia for *Biomphalaria* and in F1' miracidia for *Bulinus* snail. Both sexes were identified in the F1 and F1' progenies. Among the F1 miracidia, 9 and 24 were female and male, respectively. Among the F1' miracidia, 15 and 18 were female and male, respectively. 505 base pairs of the ITS2 nuclear gene have been sequenced for 66 miracidia. On the 33 sequences of the F1 miracidia all exhibit a *S. bovis* gene profile (no heterozygous profile) and on the 33 sequences of the F1' miracidia all exhibit a *S. mansoni* gene profile (no heterozygous profile).

Table 3. Summarized information (limited choice experiments: exp. 1–4), to show numbers of paired (homo- and hetero-specific) and unpaired worms recovered from mice exposed to a limited combination of cercariae. Expected numbers of pairs under random mating is shown in brackets. Sb: *S. bovis*, Sm: *S. mansoni*.

Exp	Host	Choosing Partner	Homo-Specific Pairs	Hetero-Specific Pairs	Single Worms	
1.	Mouse	♀Sb	♀Sb × ♂Sb	♀Sb × ♂Sm	♂Sm	♂Sb
	1		2	0	5	4
	2		8	4	7	4
	3		1	0	5	6
	4		5	3	5	3
	5		6	6	5	5
	6		4	3	4	5
	7		1	12	0	1
	8		1	1	1	0
Total			28 (30)	29 (27)	32	24
2.		♀Sm	♀Sm × ♂Sm	♀Sm × ♂Sb	♂Sm	♂Sb
	1		5	1	7	8
	2		13	4	0	1
	3		8	1	6	4
	4		12	0	6	13
	5		4	3	0	0
	6		8	2	0	3
	7		9	1	0	1
	8		7	6	0	0
Total			66 (54)	18 (30)	19	30
3.		♂Sb	♂Sb × ♀Sb	♂Sb × ♀Sm	♀Sm	♀Sb
	1		4	2	2	3
	2		1	3	6	4
	3		1	7	2	2
	4		3	6	4	8
	5		0	5	4	4
	6		0	3	2	1
	7		0	1	7	4
	8		2	2	2	4
Total			11 (16)	29 (24)	29	30
4.		♂Sm	♂Sm × ♀Sm	♂Sm × ♀Sb	♀Sm	♀Sb
	1		15	1	11	0
	2		8	0	1	4
	3		4	0	5	4
	4		5	0	0	0
	5		9	1	0	2
	6		8	2	0	9
	7		6	2	0	1
	8		12	3	0	3
Total			67 (55)	9 (21)	17	23

Table 4. Summarized information of homo-specific pairs, hetero-specific pairs and unpaired worms recovered from mice exposed to simultaneous infections to full combinations of cercariae. Expected number of pairs under random mating is shown in brackets. Sb: *S. bovis*, Sm: *S. mansoni*.

Exp 5.	Homo-Specific	Homo-Specific	Hetero-Specific	Hetero-Specific	Single Worms			
Mouse	♂Sm × ♀Sm	♂Sb × ♀Sb	♂Sm × ♀Sb	♂Sb × ♀Sm	♂Sm	♂Sb	♀Sm	♀Sb
1	6	3	1	0	3	4	0	0
2	4	2	3	0	1	1	0	0
3	5	3	6	0	3	3	0	1
4	5	5	3	0	0	1	0	0

Table 4. Cont.

Exp 5.	Homo-Specific	Homo-Specific	Hetero-Specific	Hetero-Specific	Single Worms			
5	9	4	4	0	4	2	0	1
6	6	2	2	0	1	2	0	0
7	5	3	5	0	0	0	0	2
8	6	5	1	0	0	1	0	0
Total	46 (30)	27 (18)	25 (36)	0 (15)	12	14	0	4

Table 5. Compatibility of F1 progeny of *S. bovis* (male) × *S. mansoni* (female) and *S. mansoni* (male) × *S. bovis* (female) in *Biomphalaria glabrata* and *Bulinus truncatus*. Each snail was exposed to 12 miracidia. Sb: *S. bovis*, Sm: *S. mansoni*.

Exp	Snail Species	Progeny	Snails Exposed	Snails Surviving	Snails Infected	% of Snails Infected
A	<i>B. glabrata</i> (Brazil)	♂Sb × ♀Sm (F1')	48	46	23	50
B	<i>B. glabrata</i> (Guadeloupe)	♂Sb × ♀Sm (F1')	48	48	10	20.8
C	<i>B. glabrata</i> (Brazil)	♀Sb × ♂Sm (F1)	48	48	0	0
D	<i>B. glabrata</i> (Guadeloupe)	♀Sb × ♂Sm (F1)	48	48	0	0
E	<i>B. truncatus</i> (Morocco)	♂Sb × ♀Sm (F1')	48	29	0	0
F	<i>B. truncatus</i> (Spain)	♂Sb × ♀Sm (F1')	48	45	0	0
G	<i>B. truncatus</i> (Morocco)	♀Sb × ♂Sm ♂ (F1)	48	30	1	3.3
H	<i>B. truncatus</i> (Spain)	♀Sb × ♂Sm ♂ (F1)	48	47	5	10.6

4. Discussion

Several studies have shown that, in experimental mixed infections, there are no physiological barriers preventing encounters and mating of schistosomes of different species, even species belonging to a different genus in the definitive host [19]. Our overall findings on the experimental combinations between *S. mansoni* males' × *S. bovis* females and vice versa demonstrated that (i) mating between the two *Schistosoma* species is not random; (ii) *S. mansoni* exhibits greater mate recognition compared to *S. bovis*; (iii) the progeny is parthenogenetic; and, as a consequence, (iv) the mollusc host spectrum of the F1 progeny is limited to the maternal schistosome species host spectrum.

Random pairing has been observed in crosses between *S. haematobium* × *S. bovis* [31], *S. bovis* × *S. curassoni* [15], *S. haematobium* × *S. intercalatum* [16] and *S. intercalatum* × *S. guineensis* [17]. Our study shows mate choice recognition, and is in tandem with results obtained in mixed infections of *S. haematobium* × *S. matthei* [32] or *S. mansoni* × *S. intercalatum* [18]. Mate recognition seems to be dependent on the genetic proximity of the interacting species: *S. bovis*, *S. curassoni*, *S. intercalatum*, *S. haematobium* and *S. guineensis* are more related among each other compared to *S. haematobium* and *S. matthei*, and even less so between *S. mansoni* and *S. bovis* or *S. intercalatum*.

Our study also shows that *S. mansoni* exhibited greater specific mate preference than *S. bovis* and this indicates that intra-*S. mansoni* recognition is stronger than intra-*S. bovis*; evidence of *S. mansoni* exhibiting greater mate recognition than *S. intercalatum* [18] and *S. haematobium* exhibiting greater mate recognition than *S. matthei* [32] has been reported and our results further buttress the existence of mechanisms in *Schistosoma* species favoring the pairing of homo-specific partners. In addition, it has been reported that, whatever their genotype, *S. mansoni* males show a stronger competitiveness at coupling with females than *S. intercalatum* males [33,34]. It has been demonstrated that the former will change partner to mate with conspecific females in preference to hetero-specific females whenever the opportunity arises [33,34]. It has also been evidenced that, in the absence of *S. mansoni* female worms, unpaired *S. mansoni* male worms that arrive in a pre-established *S. intercalatum* infection are more competitive and can pull away female *S. intercalatum* from male *S. intercalatum* [33,34].

Depending on the evolutionary lineage of the species, the interspecies sexual interactions in schistosomes may lead to either hybrids or parthenogenetic offspring [19,24]. Our experimental design on mating interactions between *S. mansoni* males' $\times$ *S. bovis* females (two species of schistosomes that belong to two different evolutionary lineages) and vice versa shows that the F1 progenies are parthenogenetically produced. Within the family of schistosomatidae, parthenogenesis has been reported in some species [13,19]. Apart from female *Schistosomatium douthitti*, which regularly mature and produce numerous viable eggs in unisexual infections [35], the induction of parthenogenesis in other female schistosomes has been evidenced in crosses with males from a different species group. Induced parthenogenesis has been observed in female *S. mansoni* stimulated by either *S. japonicum*, *S. intercalatum* or *S. douthitti* male, and in female *S. japonicum* or female *S. matthei* stimulated by male *S. mansoni* [19]. Cytogenetic studies on the progeny of these last crosses have shown that all type of parthenogenesis can be observed: haploid, apomictic diploid or automictic diploid; the haploid parthenogenesis seems to be the most frequent mechanism [19]. Because the parthenogenetic status of the *S. mansoni* $\times$ *S. bovis* F1 progeny was assessed by sequencing a nuclear gene, we cannot infer the ploidy of this progeny. Finally, because F1 progeny only harbor the mother genotype, it can only infect the snail corresponding to the mother species (*B. truncatus* for *S. bovis* and *B. glabrata* for *S. mansoni*). A similar result has been observed in *S. japonicum* $\times$ *S. mansoni* crosses, where female *S. japonicum* $\times$ male *S. mansoni* can only infect *Oncomelania hupensis* snail while where female *S. mansoni* $\times$ male *S. japonicum* can only infect *B. glabrata* [36].

5. Conclusions

The production of hybrid offspring in laboratory experiments is a useful approach to determine levels of zoonotic potential in schistosome species. This can also help predict if hybrid offspring could be evidenced in the field. Our study demonstrated that pairing is possible between *S. mansoni* and *S. bovis* with the production of viable parthenogenetic offspring, but with a limitation in their ability to infect both parental mollusc hosts. Theoretically, F1 *S. mansoni* $\times$ *S. bovis* miracidia could be evidenced in the field at least in rodent host, where both parasites' species can be found [23]. The barrier between schistosomes infecting human or animal has recently been challenged with the discovery of widespread *S. haematobium* $\times$ *S. bovis* hybrids in several West African countries [11,12,37–39]. Even if *S. mansoni* are not closely related to *S. bovis*, contrary to *S. haematobium* they have the same tropism for the mesenteric vein system and *S. mansoni* $\times$ *S. bovis* could therefore also be evidenced in human feces. Without molecular biology, a female *S. mansoni* $\times$ male *S. bovis* F1 progeny not be identified because it harbors the expected *S. mansoni* egg shape and infects the host attributed to *S. mansoni* transmission. However, *S. bovis* egg shape in human feces might attract attention. The presence of *S. bovis* egg shape in human feces has already been evidenced but attributed to contamination after the ingestion of cows infected by *S. bovis* parasite. However, this type of observation is very rare and the fact that the F1 individuals produced are parthenogenetic should strongly limit the spread of this hybrid. Interestingly our results may also have implications for schistosomiasis transmission and thus its control. They suggest that co-infection and the formation of heterospecific pairs in reservoir hosts such as rodents, although not leading to hybridization, is sufficient to allow female of each species to perpetuate the parasites life cycles even in the absence of conspecific mates. This supports the fact that focusing on reducing human schistosomiasis alone may not be sufficient for sustainable control, especially if animal-infecting species can stimulate the transmission of single-sex human-infecting species.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/microorganisms10061251/s1>. Molecular Biology Protocols. References [40,41] cited in Supplementary Materials.

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