Integrative taxonomy of crustacean y-larvae (Thecostraca: Facetotecta) using laboratory-rearing and molecular analyses of single specimens, with the description of a new vermiform species

JØRGEN OLESEN1,*, NIKLAS DREYER1–4, FERRAN PALERO5, DANNY EIBYE-JACOBSEN1, YOSHIHISA FUJITA6, BENNY K. K. CHAN2 and MARK J. GRYGIER7,8

1Natural History Museum of Denmark, University of Copenhagen, Denmark
2Biodiversity Research Center, Academia Sinica, Taipei, Taiwan
3Department of Life Science, National Taiwan Normal University, Taipei, Taiwan
4Biodiversity Program, Taiwan International Graduate Program, Academia Sinica, Taipei, Taiwan
5Institut Cavanilles de Biodiversitat i Biologia Evolutiva (ICBIBE), Valencia, Spain
6General Education Center, Okinawa Prefectural University of Arts, Naha, Okinawa, Japan
7Center of Excellence for the Oceans, National Taiwan Ocean University, Keelung, Taiwan
8National Museum of Marine Biology & Aquarium, Checheng, Pingtung, Taiwan

Received 5 August 2021; revised 17 January 2022; accepted for publication 16 February 2022

Facetotecta, the taxon established for ‘y-larvae’, is the last major crustacean group for which the adult stage remains unknown. With only 14 described nominal species, all in the genus Hansenocaris, their incompletely known life cycle, small size and dearth of molecular data have hampered assessments of their true species diversity. Based on field studies during which >11 000 y-larvae were sampled, a new integrative approach for studying the taxonomy of y-larvae is outlined. It focuses on last-stage nauplii and y-cyprids and includes methods for rearing lecithotrophic y-larvae for documenting the morphology of specimens with live photomicroscopy and scanning electron microscopy (SEM) and for obtaining molecular systematic data. This new and integrated approach, whereby each single specimen provides multiple kinds of information, was implemented to describe Hansenocaris demodex sp. nov., a unique y-larval form with semi-vermiform nauplii that occurs in the waters of Okinawa (southern Japan) and Taiwan. A preliminary Facetotecta phylogeny shows remarkable congruence between the morphology of all newly sequenced y-larvae and molecular data (18S rDNA). Four independent clades are formed by H. demodex and three other types/species of y-larvae, together being the sister-group to a smaller clade including H. itoi and unnamed species from GenBank.


INTRODUCTION

Planktonic crustaceans, such as copepods and krill, dominate the faunal biomass of the oceans of the world (Schminke, 2007; Atkinson et al., 2012; Bar-On & Milo, 2019). Larval plankton is no small part of this, particularly as dispersal stages in coastal waters. Life-history studies combined with sequencing technologies have uncovered the true identity of many planktonic larvae at several taxonomic levels (Palero et al., 2009; Bracken-Grissom et al., 2012; Torres et al., 2014; De Grave et al., 2015; Genis-Armero et al., 2020). One widely distributed group of crustaceans remains an enigma in marine biology: Facetotecta or y-larvae, which are still only known from their planktonic larval
Y-larvae are found in all major oceans, although mostly in small numbers, such as the 24 specimens collected in the Baltic Sea and Atlantic Ocean on which Hansen (1899) based the first significant report on y-larvae and the 29 specimens reported by McMurrich (1917) from Passamaquoddy Bay, Canada. The 103 specimens reported from Norway by Schram (1970b, 1972), 102 specimens from the Mediterranean reported by Belmonte (2005), 103 specimens from Sesoko Island in the Ryukyu Islands, Japan, examined with SEM by Grygier (1991a), 150 specimens collected around the Manazuru Peninsula in Sagami Bay, Japan, by Kikuchi et al. (1991) and Watanabe et al. (2000), hundreds of specimens reported from off Halley Bay, Antarctica, by Dahms et al. (1990) but not yet studied, and about 500 specimens collected from the White Sea, Russia, in four spring/summer seasons and reported by Kolbasov & Høeg (2003) and Kolbasov et al. (2021a) represent the large majority of the larvae reported to date. In many other cases, only a few specimens were caught (e.g. Bresciani, 1965; Swathi & Mohan, 2019) and even the groundbreaking taxonomic and morphological works of Itō (1984, 1985, 1986a, b, 1987a, b, 1989, 1990b, 1991) and Itō & Takenaka (1988) (see summaries by: Kikuchi et al., 1991; Watanabe et al., 2000; Kolbasov & Høeg, 2003; Kolbasov et al., 2007; Grygier et al., 2019) were based on perhaps as few as 35 individual larvae in total from Tanabe Bay on the Pacific coast of Honshu, Japan. Additionally, a number of oceanographic works without specimen counts have provided density data suggesting an abundance of planktonic y-larvae in various seas (e.g. Milelykovskiy, 1970; Böttger-Schnack, 1995; Gallego, 2014; Weydmann et al., 2014).

The current status of y-larva research is unsatisfactory, not least in light of the bulk of undescribed forms. Hansen (1899) considered ten to 12 species to be present in his limited material and suggested that more than 100 species of y-larvae may inhabit the world’s oceans. More than 20 undescribed species have been reported to occur in Tanabe Bay, Japan (Itō, 1990b) and more than 40 around Sesoko Island, Japan (Glenner et al., 2008).

Because of the scattered occurrence of y-larvae, their small size and incompletely understood life-cycle, knowledge of their diversity has been built up slowly based on tedious sorting of plankton samples, light-microscopy-based drawings and photography of single larval specimens, and attempts to combine plankton-caught larvae into developmental series. Starting with Hansen (1899), plankton-caught y-nauplii were numbered informally as distinct types, creating a parataxonomy [see summary by Grygier et al. (2019)];
types I–XII and subtypes VIII-a, -b and -c exist, with duplication of type VI by Steuer (1904) and Grygier (1987) and also ‘Manazuru Types I and II’ of Watanabe et al. (2000). Itô, Hansenocaris Itô, 1985 (type species: H. pacifica Itô, 1985), to accommodate three species based on y-cyprids described or mentioned in his previous papers (Itô, 1984; Itô & Ohtsuka, 1984). This could have provided a basis for a standardised treatment of y-larvae, but instead a complicated mixture of informal (e.g. Itô, 1986a, 1987a, b) and formal (binomial) nomenclature described or mentioned in his previous papers (Itô, 1985), to accommodate three species based on y-cyprids 1984; Itô & Ohtsuka, 1984). This could have provided a basis for a standardised treatment of y-larvae, but instead a complicated mixture of informal (e.g. Itô, 1986a, 1987a, b) and formal (binomial) nomenclature ensued. Among the 14 formally described species of y-larvae proposed by Itô and others (Table 1), six are based on nauplii alone, six on cyprids alone and only two, Hansenocaris furcifera Itô, 1989 and H. itoi Kolbasov & Høeg, 2003, comprise both cyprids and planktrophic nauplii. The designated type series of H. furcifera consisted only of y-cyprids, but nauplius y type IX of Itô (1987b) and successive instars are known to be conspecific with it (Itô, 1989, 1990b).

The usefulness of choosing wild-caught nauplii, representing one or few instars, as the basis for formal species descriptions, as was done for four species by Belmonte (2005) and one species by Swathi & Mohan (2019), is questionable, because it is likely to be troublesome to link these to the cyprid or other naupliar stages of the same species. Choosing the y-cyprid as the name-bearing instar (Itô, 1985, 1986b, 1989; Kolbasov & Høeg, 2003; Kolbasov et al., 2007; 2021b) has some practical value, as there is only one cyprid instar in the facetotectan life-cycle, facilitating homology-based comparative analyses between species. However, a superior strategy for studying the taxonomy of y-larvae would be to base species descriptions on a combination of nauplii and cyprids linked through direct evidence (i.e. individual moult sequences). Surprisingly, such efforts have been limited to Hansenocaris furcifera (Itô, 1989, 1990b: fig. 9) and one unnamed lecithotrophic species partly illustrated and briefly described by Itô (1991). The only other form of y-larvae for which both nauplii and the cyprid are known is H. itoi (Kolbasov et al., 2021a).

The current system for naming y-larvae, combining a formal taxonomy (with binomial names) that are often based on incomparable life-history stages with a parataxonomy based on wild-caught nauplii using Roman numerals as identifiers, is highly unsatisfactory. Nicknames for certain undescribed forms also exist (Grygier et al., 2019). This inconsistent approach, together with the scarcity of molecular data, has failed to reflect the true species diversity of y-larvae and has essentially resulted in parallel nomenclature systems for their taxonomy, which hinders efficient progress in exploring that diversity. To remedy this situation, a novel protocol is suggested herein with a focus on comparable (homologous) life stages among facetotectan taxa, namely the last-stage nauplius (LSN) and the cyprid. The protocol combines culturing techniques (with an emphasis on individual rearing of late larval stages), live photography and molecular techniques (DNA barcoding) based on individual larval specimens. This approach has proven to be particularly successful for y-larvae with lecithotrophic nauplii, which are able to moult without feeding under laboratory conditions as they pass through the various stages of development. It is recommended for future taxonomic work on facetotectans since it provides a basis for: (1) preparing species descriptions based on demonstrably conspecific nauplii and cyprids, thereby avoiding parallel taxonomies; (2) linking the resulting taxonomic names with adults, whenever these become known in the future; (3) identifying plankton-caught y-larvae based on hitherto unused morphological (e.g. colour patterns) or barcoding-type data; and (4) allowing comparisons between equivalent stages of different putative species.

Practical benefits and limitations of individual larval rearing were explored during fieldwork during 2017–19 at Green Island, Taiwan, and Sesoko Island, Okinawa, Japan (Fig. 1). Methodological details are outlined and new data are used to formally describe one of the most distinctive Facetotecta species from these two sampling sites. Integration of new culturing techniques, live photography, microscopical techniques (SEM and LM) and molecular analyses is essential to uncover the true species diversity of Facetotecta. Our integrated taxonomic approach is presented to provide a baseline for future descriptions of y-larvae with lecithotrophic nauplii.

**MATERIAL AND METHODS**

**OVERVIEW OF METHODOLOGY**

Y-larvae (nauplii or cyprids) captured alive off Sesoko Island are practically unidentifiable based on currently available knowledge due to their unanticipated diversity. For example, the catch of one morning (19 October 2018) resulted in 25 y-larvae that possibly represent > 15 different species (Fig. 2, live larvae: https://youtu.be/seo-63AK10E). To facilitate taxonomic work on such populations, a novel method is outlined for individual rearing of lecithotrophic nauplii to the cyprid stage that produces maximum information from different life-cycle stages of the same taxon. In many cases, this method provides both morphological and molecular data for the same individual at different points in development. The methodology involves: (1) sampling (Figs 1, 2); (2) individual rearing to last-stage nauplius (LSN) and cyprid (Fig. 3); (3) microscopy of live specimens (e.g. for documenting their colour patterns) (Figs 2, 3, 11); (4) subsequent fixation and storage of individual larvae.
<table>
<thead>
<tr>
<th>Species</th>
<th>Described life stage</th>
<th>Feeding strategy*</th>
<th>Type locality</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Hansenocaris hansenii</em> (Steuer, 1904)</td>
<td>Type IV nauplius</td>
<td>P</td>
<td>Gulf of Trieste, northern Adriatic Sea (Italy)</td>
</tr>
<tr>
<td><em>Hansenocaris corvinae</em> Belmonte, 2005</td>
<td>Nauplius: Kolbasov &amp; Høeg (2003); Kolbasov et al. (2021a)</td>
<td>P</td>
<td>Salento Peninsula (Italy)</td>
</tr>
<tr>
<td><em>Hansenocaris leucadea</em> Belmonte, 2005</td>
<td>Nauplius: Belmonte (2005); Swathi &amp; Mohan (2019)</td>
<td>P</td>
<td>Salento Peninsula (Italy)</td>
</tr>
<tr>
<td><em>Hansenocaris spiridonovi</em> Kolbasov et al., 2021b</td>
<td>Cyprid: Kolbasov et al. (2021b)</td>
<td>?</td>
<td>Azores Islands (Portugal)</td>
</tr>
<tr>
<td><em>Hansenocaris demodex</em> sp. nov.</td>
<td>Nauplius: This work</td>
<td>P</td>
<td>Sesoko Island, Okinawa (Japan)</td>
</tr>
<tr>
<td></td>
<td>Cyprid: This work</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*L, lecithotrophic; P, planktotrophic nauplii. As reported in literature or based on absence/presence of feeding spines as depicted in original illustrations (see more criteria in Materials and Methods). Cyprids of *H. pacifica* first linked by Itô to lecithotrophic nauplii of Type XI (Itô, 1986a), but this was later dismissed (Itô, 1987b). The real nauplii of *H. pacifica* have never been explicitly identified or described, but probably belong to one of Itô's many unpublished lab-reared forms, in which case they would be lecithotrophic. Swathi & Mohan (2019) identified some wild-caught planktotrophic nauplii from the Andaman Sea as conspecific with the Mediterranean *H. leucadea* and *H. corvinae*. However, due to the great distance between the localities and the large morphological diversity of planktotrophic nauplii (unpublished), these identifications are in need of confirmation with molecular methods.
for different purposes; (5) post-expedition sorting and digital handling of specimens (Fig. 3); (6) detailed microscopy (e.g. SEM); and (7) molecular sequencing of individual larval specimens. The setup for rearing was modified from Itô (1990a, b, 1991).

More than 11,000 y-larvae were collected and sorted from live plankton samples during several field trips to Sesoko Island, Okinawa, Japan and Green Island, Taiwan between 2017 and 2019 (Table 2). Twenty-seven specimens provide the basis for the present description of Hansenocaris demodex sp. nov., including 22 from Sesoko Island and five from Green Island (Fig. 3; Table 3), although four specimens from Sesoko that died soon after capture were discarded and not processed further. The last-stage nauplius (LSN) and the cyprid are described in detail, while only limited information is provided for earlier naupliar stages.

**Figure 1.** Facetotecta sampling sites in East Asia, 2017–20 and distribution of Hansenocaris demodex sp. nov. A, East China Sea showing distance (780 km) between the two main sampling sites, Sesoko Island (Okinawa, Japan) and Green Island (Taiwan). In 2020, several additional specimens of H. demodex were collected from Xiaoluqiu Island (MJG, unpublished). B, Sesoko Island (Japan, Okinawa) with indication of sampling site (Sesoko Station); C, Green Island (Taiwan) with indication of sampling site (Gonguan Fishing Harbour); D, pier at Sesoko Station; E, Gonguan Fishing Harbour.

**Sampling and Rough Sorting**

The larval material used in this work was collected near the Marine Science Research Station of Academia Sinica on Green Island, Taiwan, in 2017 (1 September to 1 November) and 2018 (24 August to 6 September) and the University of the Ryukyus Tropical Biosphere Research Center Sesoko Station on Sesoko Island, Okinawa Prefecture, Japan, in 2018 (16 October to 5 November) and 2019 (1 June to 23 June) (Fig. 1). Plankton samples were collected by handheld conical plankton nets (20 or 30 cm mouth opening, 65, 95 or 100 μm mesh size) deployed from wharfside at Gonguan Fishing Harbour on Green Island (22°40’33.1"N, 121°29’37.4"E) at 3–5 m depth or from the end of the laboratory pier at Sesoko Island (26°38’09.4"N, 127°51’55.2"E) in waters varying in depth from about 0.5 to 2.0 m, depending on the tide. Individual tows were typically 10–15 m long. Fresh samples were
Figure 2. Example of newly collected sample of still living y-larvae collected on 19 October 2018 at Sesoko Island off the pier shown in Figure 1D, representing a variety of developmental stages. A, 25 y-larvae likely representing > 15 different species, including 18 early lecithotrophic nauplii (*), five planktotrophic nauplii (†) and two cyprids (§). None of the depicted specimens can be assigned to already described species, but three of the planktotrophic nauplii are similar to Itô’s (1986a) Pacific type I and one is a cyprid of the type nicknamed ‘Big brown’, both types of which are sequenced as a part of this work and belong to the same clade as Hansenocaris demodex sp. nov. (see Fig. 15). B, close-up of selected y-larvae with same numbers assigned as in A. Live video of H. demodex and other y-larvae can be seen here: https://youtu.be/seo-63AK10E.
Figure 3. Overview of most specimens of *Hansenocaris demodex* sp. nov. collected at Sesoko Island during fieldwork in 2018 and 2019. A, 19 specimens, four of which reached the cyprid stage, collected in the plankton at the naupliar stage and reared individually, with length, dish number, sample number, and museum registration number indicated for each specimen as well as a colour code (red, blue, yellow) showing its ultimate treatment/fixation (respectively, molecular work, SEM or exuvium on slide); some larvae died before preservation and are only referred to by their dish number; B, overview of setup for individual rearing of larvae; top view of green tray containing 31 2.5-cm-wide dishes with tracking information written on lids, each containing one to five live larvae. Live video of *H. demodex* and other y-larvae can be seen here: https://youtu.be/seo-63AK10E.
brought to the lab for sorting of live y-larvae in either Petri dishes or Bogorov-type zooplankton counting chambers, using a Pasteur pipette under a dissecting microscope (Fig. 2). During fieldwork at Sesoko Island all larvae were counted and roughly categorized into larval type (Table 2).

**Rearing**

At Sesoko Island, stock seawater for cultures was prepared by passing pumped seawater from the laboratory through a 62-μm mesh hand-net, without sterilization. All y-larvae from a given sample were first placed in a small glass or plastic Petri dish. A second round of sorting separated cyprids, planktotrophic nauplii similar in body plan to Itô's (1986a) Pacific type I and supposedly lecithotrophic nauplii. The supposed lecithotrophic nauplii were usually divided into smaller groups of four to six individuals, representing either a single type, several distinctly different types to allow tracking of individuals or a random selection of the remaining nauplii in the sample, and maintained in 35 × 10 mm transparent lidded plastic dishes two-third-filled with stock sea water (Fig. 3B). Dishes at both sites were maintained on table tops in the laboratory at 25–26 °C, without agitation. The condition of all larvae in each dish was assessed and recorded generally once per day under a dissecting microscope. Dead and badly fouled specimens were discarded, and occasionally the surviving specimens were transferred to a fresh dish with fresh stock seawater. As soon as a last-stage nauplius (LSN) appeared – recognizable by the dark-pigmented compound eyes of the internally developing cyprid instar – it was placed in a separate dish (serially lettered) to await its final moult into a free-swimming y-cyprid. For example, the ten y-larvae taken at 13:00 on 14 June 2019 were split between the two dishes labelled first as ‘14-VI-19 13:00A’ and ‘14-VI-19 13:00B’ (maintaining their identity as parts of the same sample), and also as ‘dish 178’ and ‘dish 179’, respectively (their place in the entire survey). Each dish contained five supposedly lecithotrophic nauplii. One nauplius from dish 178 reached its final instar on the evening of 17 June, when it was separated out into dish 178A (moulted to cyprid two days later), and two nauplii from dish 179 reached their final instar in the afternoon of 19 June, when they were separated out into dishes 179A (moulted to cyprid three days later) and 179B (died). Survivorship to the LSN stage was about 10%, to the cyprid stage about 5%.

**Microscopy of live specimens during fieldwork**

To obtain objective and reproducible records of coloration and degree of transparency in situ and other features that are typically lost upon fixation, and to facilitate later grouping of larvae into types based on

### Table 2

<table>
<thead>
<tr>
<th>Year</th>
<th>Early nauplii, planktotrophs</th>
<th>Early nauplii, lecithotrophs</th>
<th>Last stage nauplius (LSN) mean number of larvae per tow</th>
<th>Max number of larvae in one tow</th>
<th>Total number of larvae</th>
</tr>
</thead>
<tbody>
<tr>
<td>2018</td>
<td>654</td>
<td>3481</td>
<td>1305</td>
<td>31.30</td>
<td>8876</td>
</tr>
<tr>
<td>2019</td>
<td>2868</td>
<td>3622</td>
<td>1323</td>
<td>31.30</td>
<td>7023</td>
</tr>
<tr>
<td>Totals</td>
<td>3542</td>
<td>5137</td>
<td>2628</td>
<td>31.30</td>
<td>15899</td>
</tr>
</tbody>
</table>

*Each tow covered a distance of 10–15 m. Includes 444 larvae that were not classified as planktotrophic or lecithotrophic.
as many features as possible, a large number of larvae were digitally photographed and/or videographed at various magnifications. Such observations were made with a Nikon ECLIPSE 80i compound microscope (Sesoko Island) equipped with Nomarsky (DIC) optics or an Olympus IX70 inverted compound microscope (Green Island), both fitted with a Canon EOS 5D Mark IV digital camera. Since last-stage y-nauplii (LSN) with a y-cyprid developing inside and the moulted y-cyprids themselves were considered to be the key stages for taxonomic work on facetotectans (see Introduction), all LSNs that became available, either directly from the plankton or by rearing from earlier stages, were digitally recorded whenever possible. Sometimes there was also time for photographic/videographic documentation of earlier instars, which, on account of the dish numbering system described above, could be matched with their later counterparts (see Fig. 3). About 1275 selected specimens out of 9621 specimens collected during our fieldwork in 2018 and 2019 at Sesoko Island were photographed/videographic this way.

Each nauplius or cyprid to be recorded was moved temporarily from its culture dish to a shallow depression slide and kept nearly immobile in a minimal amount of seawater without using a coverslip. Videos were taken in preference to still photographs. Increased depth of field for photographs was obtained by using the HD video 50 fps mode of the camera while focusing up and down a couple of times to produce sets of images amenable to combination into a single image by means of image-stacking software (ZERENE STACKER, v.1.04). After recording, the live specimens were either returned to their culture dishes in case of further moults (nauplii) or were single-fixed immediately for SEM or molecular processing (cyprids and moribund LSNs). In some cases, especially during the later phases of the fieldwork when there was no time to let the nauplii develop further, a significant number of lecithotrophic nauplii, regardless of instar, and even those from fresh samples, were digitally documented and subsequently fixed so as to cover as many types as possible. A large number of planktotrophic y-nauplii that did not moult while kept in culture, and supposed lecithotrophs that did not moult, were treated the same way. Any individual nauplius or cyprid that was preserved received its own sequential sample number of the form ‘TA-2018-131’ or ‘JA-2019-290’ (from Taiwan and Japan, respectively), and detailed entries were immediately made into an Excel file using these numbers, with cross-references to the culture dish numbers. Both sorts of numbers were used on labels prepared later for slides and specimen vials (see below). In cases where photographed larvae died before preservation, and sample numbers were therefore not assigned, their dish numbers are shown in the figures for reference.

FIXATION AND STORAGE OF MATERIAL

A large number of LSN exuviae were prepared on slides as semi-permanent glycerine jelly mounts shortly after moulting to the cyprid stage (normally within a day). After several hours in a drop of seawater-based formalin (to kill adhering bacteria and fungi), to which a similar amount of anhydrous glycerine was soon added, each specimen was transferred by a needle into a small droplet of molten glycerine jelly on a glass slide, which was then covered by a coverslip supported by four drops of dried nail varnish to avoid crushing the specimen. The same nail varnish was used later to seal the slide. Cyprids corresponding to the mounted LSN exuviae were either preserved in seawater-buffered 2–4% formaldehyde for scanning electron microscopy, or in 95–99% ethanol and kept in the freezer for subsequent molecular work. The resulting material included 272 microscopic slides with LSN exuviae of a large number of facetotectan types from the 2018 and 2019 expeditions to Sesoko Island, and 745 microvials with specimens fixed for either morphological or molecular work. The material is currently stored under the above-described sample number system(s) at the Natural History Museum of Denmark, but will gradually be registered with NHMD numbers as the taxonomic work progresses.

SORTING AND DIGITAL HANDLING OF SPECIMENS

Further sorting and grouping of y-larvae took place after fieldwork and was based on the photographic information obtained for a high number of selected live specimens (see above). In order to obtain comparable information for each specimen, selected frames from the HD video sequences were exported as a stack and blended in ZERENE STACKER v.1.04. These stacked images, together with their corresponding culture-dish and fixation data, were then sorted/grouped into large plates in CORELDRAW. Most of the data from H. demodex, which is one of the more distinct species, represented in 2018/2019 by 22 collected specimens (Sesoko), are combined in Figure 3, in which photos placed in horizontal rows represent different instars of the same organism. This overview served as a basis for deciding which specimens to process further for detailed morphological and molecular work on this new species, and also which to designate as the name-bearing type (holotype) in the description (see below).

NAUPLIAR DEVELOPMENT

For H. demodex, in addition to the nine LSN specimens obtained in 2017–19, either by rearing or by directly collecting them from the plankton, some information was obtained on early and mid-stage nauplii from Sesoko Island. This included
high-resolution photographs of live individuals or their exuviae at different stages of development (Figs 3, 11), as well as SEM images of several younger-stage specimens (Figs 12, 13). An incomplete outline of the naupliar development of this species can, therefore, be assembled. As the earliest post-hatching stage of the naupliar development is not known for H. demodex, the naupliar sequence was numbered from the LSN (last-stage nauplius) backwards, with the preceding stage called LSN – 1 (‘last-stage nauplius minus 1’), itself being preceded by the earlier stage LSN – 2, etc. This was inspired by the convention used for ostracods (e.g. Hiruta & Hiruta, 2014) and avoids the possible problems associated with Itô’s (1990b: 219) designation of the last five instars as ‘the first through fifth naupliar stages’ [= nauplius 1–5 of Kolbasov & Høeg (2003)] despite his suspicion that there may be another ‘true first stage’. If there are six naupliar stages altogether, or even seven as suggested by Kolbasov et al. (2021a) for H. itoi, the earliest would be called LSN – 5 or LSN – 6, respectively.

**ADVANCED MICROSCOPY**

Formalin-fixed specimens selected for SEM were prepared by rinsing in distilled water overnight, dehydration in a graded alcohol series to 100% ethanol and subsequent critical point drying. Specimens were mounted on metal stubs on carbon tape, coated with an alloy of palladium and platinum and observed/photographed in a JEOL JSM-6335-F (FE) scanning electron microscope at the Natural History Museum of Denmark, Copenhagen (Figs 4–10, 12, 13). Prior to treatment for SEM, cyprids were photographed using an inverted compound microscope (Olympus, IX83) using fully automated image-stacking techniques (Fig. 7A).

**TERMINOLOGY FOR MORPHOLOGICAL DESCRIPTION**

Morphological terminology partly follows Itô (1987b, 1990b). The naupliar cuticle consists of two main parts, the cephalic shield and the faciotruncal integment (faciotrunk) (yellow and blue overlays in Figs 4, 5, 12). When an early- or middle-stage nauplius moults, these two parts of the exuvium usually become separated, but the exuvium of a last-stage nauplius (LSN) typically remains entire. An incompletely moulted LSN specimen with two layers of unshed naupliar exuviae covering it displays the moulting zone between the cephalic shield and the faciotrunk (Fig. 13D, yellow stippled line). Figure 13 shows that the indivisible faciotrunk (Fig. 13D, E) consists of a wide, ventral faciomarginal area that surrounds the labrum and the three pairs of naupliar appendages anteriorly and laterally, but only extends a short distance posteriorly, and, posterior to this, the trunk (or hind body) per se, which includes dorsal, lateral and ventral cuticle. Although planktrotrophic y-nauplii have a well-defined labrum extending posteriorly from the level of the mouth opening (e.g. Itô, 1990b: Kolbasov & Høeg, 2003; Høeg et al., 2014; Kolbasov et al., 2021a), lecithotrophic y-nauplii lack this or have a median spine in its place. Nonetheless, the area anterior to the missing labral extension is usually swollen to various degrees and exists in different shapes (for H. demodex, see Figs 5K–M, 12E), with pores and a cuticular ridge pattern and sometimes ends posteriorly in a distinct declivity. Despite their differences in various types of y-nauplii, these structures have mostly been referred to as a labrum (e.g. Schram, 1970b, 1972; Itô, 1986a, 1987a, 1990b; Kolbasov & Høeg, 2003; Grygier et al., 2019), also in cases where a labral extension is missing. The term labrum is used in this study to denote the entire complex of generally homologous structures in the mouth region of y-nauplii, even though a labral extension overhanging the mouth opening is missing in H. demodex.

To identify the facets (plates) of the cephalic shield y-nauplii, a full set of detailed anterior, anterolateral, lateral, dorsal and, in case the lateral margins were inturned, also ventral views were obtained for all individuals examined with SEM. Nonetheless, plate identification was hindered by three factors: apparent absence of the instar upon which Itô’s (1987b) basic system of plate nomenclature was based (thus making the boundaries between ‘frontal’ plate F-1 and the ‘window’, and frontal plate F-4 and the ‘brim’, uncertain); absence in all available instars of clear plate delineations dorsally and dorsolaterally behind the ‘frontal’ plates and above the areas corresponding to the ‘marginal’ and ‘polygonal’ plates; and the lack of any full sets of naupliar exuviae of particular individuals, which prevented precise tracing of plate divisions. Itô’s (1990b) expanded system of plate nomenclature for later instars requires knowledge of the order of plate divisions; for example, the names of four plates derived from an earlier single plate by a meridional (anterior/posterior) division followed by a latitudinal (central/external) division will be different from those derived by division in the reverse order. Without such information, the ‘apostrophe’ system employed by Kolbasov et al. (2021a) can be employed if the cluster of plates corresponding to a larger plate of an earlier instar can be recognized. In the present case, with a minimum of ambiguity, it was possible to match the pattern of anterior and anterolateral plates of two specimens of H. demodex that appear to represent successive intermediate instars (NHMD-916635 and NHMD-916638; Fig. 13C, F) with that described by Itô (1990b: fig. 7) for the supposed third-stage nauplius of Hansenocaris furcifera. Based on
Figure 4. Hansenocaris demodex sp. nov., paratype, last-stage nauplius (LSN) from Sesoko Island photographed live before fixation (A) and in SEM after fixation and mounting (B–H). A, ventral view; B, ventral view; C, dorsal view; D, pair of ventral pores situated in front of labrum; E, close-up views of pores and sensilla of cephalic shield; F, caudal end in dorsal view; G, caudal end in ventral view; H, caudal end viewed from behind. Abbreviations: a1, first antenna; a2, second antenna; arthro membr, arthrodial membrane; ce, compound eye; dc sp, dorsocaudal spine; en, endopod; ex, exopod; fur sp, furcal spine; la, labrum; md, mandible; ne, nauplius eye; rud, rudimentary. Small Arabic numerals, many annotated with l or r for left and right, respectively, refer to cuticular structures (see Table 4). Yellow overlay, cephalic shield; blue overlay, faciotrunk; red overlay, anterior field of facets. Square with dotted outline: sample number, museum number and type status.

© 2022 The Linnean Society of London, Zoological Journal of the Linnean Society, 2022, XX, 1–44
Figure 5. *Hansenocaris demodex* sp. nov., paratype, last-stage nauplius (LSN) from Sesoko Island in SEM. A, lateral view; B, cephalic shield, anterior view; C–J, close-up views of pores and sensilla of cephalic shield and faciotrunk; K–M, labrum and naupliar limbs (a1, a2, md). Abbreviations: a1, first antenna; a2, second antenna; md, mandible; la, labrum; scl, sclerite of a1. Small Arabic numerals, many annotated with l or r for left and right, respectively, refer to cuticular structures (see Table 4). Yellow overlay, cephalic shield; blue overlay, faciotrunk; red overlay, anterior field of facets. Square with dotted outline: sample number, museum number and type status.
that, corresponding regions in our earliest available instar of *H. demodex* (Fig. 12B, D) could be identified, as well as in its LSN (Fig. 5B).

Various terms are available for the limbs and caudal armature of y-nauplii. Terms adopted in this study include first antenna or a1, second antenna or a2,
Figure 7. *Hansenocaris demodex* sp. nov., holotype (A, B, D–I) and paratype (C), cyprids from Sesoko Island photographed before drying (A) and in SEM after drying and mounting (B–I). A, lateral view; B, lateral view; C, cephalic region, lateral view; D, cephalic region, ventral view; E, bifid appendix (frontal filaments?); F, labrum; G, pores and sensilla; H, close-up of hook of left first antenna; I, abdomen and telson, dorsal view. Abbreviations: a1, first antenna; a1 hk, first antenna hook; a2 rud, second antenna rudiment; ae, aesthetasc; en, endopod; ex, exopod; la, labrum; md rud, mandible rudiment; par occ pro, parocular process. Roman numerals I–VI – thoracic segments. Large Arabic numerals 1–3 – abdominal segments. Small Arabic numerals, many annotated with l or r for left and right, respectively, refer to cuticular structures (see Table 5). Square with dotted outline: sample numbers, museum numbers and type status.
mandible or md, furcal spines (one subterminal ventral pair) and dorsocaudal spine (terminal and unpaired). The latter spine is not always situated dorsally in y-larvae and, therefore, was termed a caudal spine by Grygier et al. (2019), but since it is homologous to the dorsocaudal spine of Cambrian Orsten crustacean larvae.
Figure 9. *Hansenocaris demodex* sp. nov., holotype (A, B, D, E) and paratype (C) cyprid from Sesoko Island in SEM. A, posterior thoracic segments and anterior abdominal segments, lateral view; B, thoracopods, ventral view; C, telson, dorsal view; D, telson, terminal view; E, right furcal ramus, posterior view. Abbreviations: fur ram, furcal ramus; ba, basis; co, coxa; en, endopod; ex, exopod; pro scle, proximal sclerites of thoracopods; thp 1, thoracopod 1. Roman numerals I–VI – thoracic segments. Large Arabic numerals 1–3 – abdominal segments 1–3. Small Arabic numerals, many annotated with l or r for left and right, respectively, refer to cuticular structures (see Table 5). Square with dotted outlines: sample numbers, museum numbers and type status.
and cirriped nauplii (see, e.g.: Walossek, 1993; Walossek et al., 1996; Chan et al., 2014), the term dorsocaudal spine is preferred, not to be confused with the so-called dorsocaudal organ, an organ of unknown function situated posterodorsally on the trunk in some y-nauplii (Elofsson, 1971; Schram, 1972; Høeg et al., 2014).

Figure 10. *Hansenocaris demodex* sp. nov., cyprid from Green Island in SEM. A, lateral view; B, cephalic shield, frontal view; C, putative lattice organ, close-up; D, thoracic limbs 1–6; E, thorax, abdomen and telson, dorsal view. Abbreviations: ba, basis; co, coxa; en, endopod; ex, exopod; la, labrum; pro sCLE, proximal sclerites of thoracopods; thp 1, thoracopod 1; thp 6, thoracopod 6. Small Arabic numerals, many annotated with l or r for left and right, respectively, refer to cuticular structures (see Table 5). Square with dotted outline: sample number and museum numbers. Dotted arrows in D point to long medial setae of thoracopodal endopods.
Figure 11. *Hansenocaris demodex* sp. nov., nauplii from Sesoko Island photographed alive (A–J) or as slide-mounted exuvium (K, L). A–E, different naupliar instars; F, penultimate naupliar instar with exuviae from two prior moults still attached posteriorly; G, cyprid with exuviae from three prior naupliar moults still attached posteriorly; H, last-stage nauplius in lateral view; I, posterior end of cyprid with three prior naupliar moults still attached; J, posterior end of cyprid with three prior naupliar moults still attached; K, L, exuvium of last-stage nauplius, ventral and lateral views. Abbreviations: a1, first antenna; a2, second antenna; ce, cypris eye; md, mandible; ne, nauplius eye; LSN, last-stage nauplius; thx, thorax. Squares with dotted outlines: dish numbers, sample numbers and museum numbers; some larvae died before preservation and are only referred to by their dish number. Type status in red.
Figure 12. *Hansenocaris demodex* sp. nov., early nauplius (LSN – 5 or LSN – 6?) from Sesoko Island in SEM. A, ventrolateral view; B, lateral view; C, dorsal view; D, frontal view; E, labrum and naupliar appendages, right side; F, naupliar appendages, right side, median view; G, first and second antennae, right side, lateral view, asterisk indicating rudimentary segment of a2 exopod; H, dorsocaudal spine and furcal spines, terminal view. Abbreviations: a1, first antenna; a2, second antenna; ba, basis; co, coxa; dc sp, dorsocaudal spine; en, endopod; ex, exopod; fur sp, furcal spine; F-1 to F-3, frontal plates 1–3; la, labrum complex; md, mandible; rud, rudimentary; W, window plate. Small Arabic numerals, many annotated with l or r for left and right, respectively, refer to those cuticular structures that could be matched with those of the LSN (see Table 4). Yellow overlay, cephalic shield; blue overlay, faciotrunk; red overlay, anterior field of facets. Square with dotted outline: museum number.

© 2022 The Linnean Society of London, *Zoological Journal of the Linnean Society*, 2022, **XX**, 1–44
Figure 13. *Hansenocaris demodex* sp. nov., paratypes from Sesoko Island in SEM: an early nauplius (LSN – 3?) (A–C) and a later nauplius (LSN – 2) with unmoulted later larval stages still inside. A, ventral view; B, lateral view; C, cephalic shield, frontal view; D, ventrolateral view; E, lateral view; F, cephalic shield, frontal view; G, cephalic shield, dorsal view. Abbreviations: a1, first antennae; a2, second antennae; dc sp, dorsocaudal spine; ex, exopod; fur sp, furcal spine; la, labrum; md, mandible. Small Arabic numerals, many annotated with l or r for left and right, respectively, refer to those surface structures that could be matched with those of the LSN (see Table 4). Green overlay, LSN; purple overlay, LSN – 1; red overlay, anterior field of facets. Squares with dotted outline: sample numbers, museum numbers and type status.
There is no existing terminology for the few setae and many pores on the body surface of nauplius y, although it has been suggested that those of the cephalic shield could be named after the facet (plate) in which they are first seen during the course of development (Kolbasov et al., 2021a). Here, as a stopgap measure, all have been mapped and arbitrarily numbered from #1 to #34, with the annotations ‘r’ and ‘l’ for the right and left sides, respectively, if the structure is paired (see detailed description below).

For y-cyprids, the established terminology that is widely applied to other crustaceans is used for body regions and appendages (e.g. thorax, abdomen, telson and thoracopods), but no terminology exists for most details of the complex cuticular ornamentation, such as pores and sensilla, and these are, therefore, here numbered arbitrarily (#1–#64) like those of the nauplius. Previously, the terms mouth cone, mouth parts of the piercing type (Bresciani, 1965), oral pyramid (e.g. Itô, 1985, 1986b) or sometimes just labrum (Kolbasov & Høeg, 2003; Kolbasov et al., 2007) have been used for the extended mouth region in y-cyprids. The term labrum is used here since parts of this structure can readily be homologized between the nauplii and the cyprid, based on the presence of a characteristic similar pore pattern (e.g. #30–#32 in nauplii, #62–#64 in the cyprid of H. demodex).

**MOLECULAR ANALYSES**

Individually fixed specimens were transported on ice in tightly sealed boxes from the collection sites to the molecular laboratory at Academia Sinica (Taipei, Taiwan). Fixed specimens were transferred in a 0.1–0.5-μL droplet of ethanol to 200-μL tubes (Gunster Biotech, New Taipei City, Taiwan) and incubated at 36 °C for 15 min with tube lids open to allow ethanol to evaporate. DNA extraction of Green Island specimens (N = 3) followed a modification of the protocol of Schizas et al. (1997): 1 μL of 10 x PCR buffer was diluted in 9 μL ddH₂O and added to the tubes containing individual y-larvae. Tubes were incubated at 94 °C for 2 min to denature the protein and then transferred to ice. After adding 1 μL protein kinase K (QIAGEN, Chatsworth, CA, USA) and vortexing the tubes, samples were incubated at 55 °C for 15 min, followed by 70 °C for 10 min. After returning the tubes to ice, 10 μL of GeneReleaser (BioVentures, Inc, TN, USA) was added to the tubes, which were then incubated in the following thermal cycle: 65 °C for 15 s, 8 °C for 15 s, 65 °C for 45 s, 97 °C for 90 s, 8 °C for 30 s, 65 °C for 90 s, 97 °C for 30 s, 65 °C for 30 s, 50 °C for 3 min and 4 °C for 10 min. Tubes were centrifuged for 1 min and c. 15 μL supernatant was transferred from each one to fresh 200-μL PCR tubes. Finally, 10 μL AE-buffer (QIAGEN, Chatsworth, CA, USA) was added to stabilize the DNA extract. Sesoko Island y-larvae (N = 19) were DNA-extracted by adding 40 μL AE-buffer and 4 μL protein kinase K (QIAGEN, Chatsworth, CA, USA) to 20-μL PCR tubes (Gunster Biotech, New Taipei City, Taiwan) containing single y-larvae. The tubes were then incubated at 56 °C for 1 h, and then at 72 °C for 15 min, modifying the protocol for extracting DNA from formalin-fixed specimens in Palero et al. (2010). This extraction method is preferred here over the modified Schizas protocol.

No Facetotecta-specific primers are available so far. To ensure polymerase chain reaction (PCR) amplification success, despite the small specimen size (which implies low quantities of available DNA) and potential degradation following heat exposure during transportation from the tropical sampling sites, new nuclear primers were designed to amplify a partial region of the 18S ribosomal RNA (rDNA) gene based on Facetotecta sequences available from GenBank. Primers spanning highly conserved sites and flanking hypervariable regions were designed to amplify short fragments (~300 bp) ensuring high amplification rates while simultaneously allowing species discrimination. Polymerase chain reactions, with total reaction volumes of 20 μL, contained ~3 μL genomic DNA, 0.4 μL (10 μmol/L) of each newly designed primer (18S Face1a: 5′-CTGGCAATGGGCTCATACATCGTCAT-3′ and 18S Face1b: 5′-GGTAGTCCAAATCACCCTACGAGCATGCT-G-3′), 4 μL Fast-Run Taq Master Mix (Protech Technology Enterprise, Taipei, Taiwan) and 12.2 μL ddH₂O (total reaction volume 20 μL). PCR reactions were carried out in a DNA Engine thermal cycler (Bio-Rad, Richmond, California, USA) including a denaturation step of 95 °C for 5 min and then 40 cycles of denaturation at 95 °C for 1 min, primer annealing at 60 °C for 30 s and extension at 72 °C for 30 s. The reaction was terminated with a 10-min extension at 72 °C and 20 min at 4 °C. PCR products were visualized using agarose (1.5%) gel electrophoresis. Chromatograms were generated with an ABI 3730XL Genetic Analyser by Genomics BioSci & Tech. Ltd (Taiwan). Sequences were edited, assembled and aligned using MAFFT with default parameters (see https://doi.org/10.6084/m9.figshare.17803628.v1). Our new 18S rDNA data (N = 22) and seven sequences from GenBank (Hansenocaris itoi from the White Sea: AF439393 and six unnamed, unphotographed specimens from Sesoko Island used in Pérez-Losada et al. (2009): FJ751877–751882) were used in subsequent phylogenetic analyses. ModelFinder as implemented in IQ-TREE was used to find the best nucleotide substitution model for the alignment (TNe+R2) and we subsequently inferred maximum likelihood trees with IQ-TREE (–allnni –B 1000 –m TNe+R2). Bootstrap support values were estimated using 1000 ultrafast bootstrap replicates.
RESULTS

**Sampling of y-larvae**

Sampling at Green Island (Taiwan) in 2017 and 2018 resulted in the handling of more than 1000 y-larvae, but rearing to cyprids was mostly unsuccessful and many individuals were fixed as late naupliar instars either for morphological or molecular work (see above). During field trips to Sesoko Island, Japan, in 2018 and 2019, 9621 y-larvae were collected (2018: 2710 specimens; 2019: 6,911 specimens) (Table 2). During sorting, the larvae were counted based on a rough division into the following four provisional categories (Fig. 2): planktotrophic early nauplii (specimens without yolk, with a flattened body and spine rows along the posterolateral margins); lecithotrophic early nauplii (specimens with yolk); mostly or entirely lecithotrophic last-stage nauplii (specimens with a pair of compound eyes in addition to the anteromedian nauplius eye common to all y-nauplii); and free-swimming cyprids. The most common type of larvae (53.3%) were putative lecithotrophic nauplii, of which the majority were early instars, followed by planktotrophic nauplii (37.6%) and considerably fewer but still numerous cyprids (5.1%) (Fig. 2; Table 2). The division of nauplii into lecithotrophs and planktotrophs was probably not entirely accurate, since some supposed lecithotrophs did not moult in culture, but the supposed planktotrophs could be further substantiated later by additional criteria: presence/absence of moulting in culture (planktotrophs do not moult), presence/absence of antennal and mandibular feeding spines and coxal endites (generally absent in lecithotrophs), and presence/absence of a wide, posteriorly protruding labral extension (generally non-protruding or spiniform in lecithotrophs). Table 2 also lists a few other general results from the collecting events, such as the average number of larvae collected per tow with the plankton net. A total of 29 specimens of *H. demodex* were included in the study: the 2018 and 2019 fieldwork at Sesoko Island resulted in 22 specimens, five of which died before they could be preserved; two specimens from Sesoko came from earlier (1991 and 2005) sampling; five specimens came from the 2017 sampling at Green Island (Taiwan). Live video of *H. demodex* and other y-larvae can be seen here: https://youtu.be/seo-63AK10E.

**Taxonomy**

**Pancrustacea** Zrzavy & Štys, 1997

**Thecostacea** Grivel, 1905

**Facetotecta** Grygier, 1985

No family-group taxon has formally been proposed.

---

**Genus Hansenocaris** Itô, 1985

**Hansenocaris demodex** Olesen, Dreyer, Palero & Grygier [sentence case caps]. sp. nov.

(Figs 3–13, 14A)


*Diagnosis:* Last-stage nauplius (LSN) with markedly elongate body tapering gradually towards bluntly rounded caudal end, with no distinct narrowing behind posterior end of cephalic shield in dorsal view. Dorsal region of cephalic shield posterior to poorly-defined ‘window’ plate smooth, wholly unfaceted, and devoid of ridges; surfaces between ridges on rest of shield also smooth. Plate I-1 of cephalic shield becoming subdivided by LSN – 3(?) instar. Anterior part of faciomarginal area with at least one obvious pair of pores. Labrum triangular or slightly bell-shaped, longer than wide, lacking any pattern of ridges; its posterior margin a shallowly indented declivity lacking any free posterior plate-like extension or labral spine. First antennae with four setae. Second antennae and mandibles devoid of feeding structures (lecithotrophic); natatory setal formulae of their exopods/endopods 1:1:1:2/0:1:1:2 and 2/2, respectively. Maxillules absent. Pair of reduced furcal spines situated ventrally, forward from base of short and blunt, terminal dorsocaudal spine. Dorso-caudal organ and lateral trunk spines absent.

Y-cyprid with long, fully faceted cephalic shield with nearly smooth surfaces between ridges. Small, bifid appendix in anterior midline between first antennae and anterior margin of cephalic shield. Labrum extended as linguiform process with multiple hooks (17 in holotype) on posterior side, arranged in three irregular rows. First antenna with gracile, curved hook. Second antennae and mandibles rudimentary. Thoracopods with unsegmented exopods. Tergites of thoracomeres V and VI with free pleural extensions, those of former with rounded ends, those of latter trapezoidal. Abdomen three-segmented, all segments short and lacking pleural extensions. Telson long and lacking serrate spines along postero-ventral margin, with about 19 pores, including two each on anterior-most plates of upper and lower lateral rows. Furcal rami short and cylindrical.

*Etymology:* The specific name, a Latin noun in apposition to the generic name, refers to the resemblance of the elongate body form of the naupliar instars of this species to that of mites of the genus *Demodex* Simon, 1842 (Chelicerata: Trombidiformes: Demodicidae), which are tiny parasites that live in or near the hair follicles of mammals. The name in turn is derived from Greek δημός (demos), fat, and δήξ (dex), a woodworm. The taxonomic description and supporting
molecular diagnosis of this new species were prepared by JO, ND, FP and MJG, who are thus responsible for making the specific name *demodex* available.

**Type locality:** Japan, Okinawa, Sesoko Island, pier at the University of the Ryukyus Tropical Biosphere Research Center, Sesoko Station, 26°38′09.3″N 127°51′55.2″E.

**Holotype:** Cyprid with exuvium of corresponding last-stage nauplius (LSN), considered as two separate parts of same individual specimen (cyprid: Figs 7A, B, D–I, 8B, C, 9A, B, D, E; LSN exuvium: Fig. 3A, top). Collected on 15 October 2018 (detailed sampling data in Table 3), fixed on 21 October 2018, following final moult after six days in culture. Cyprid stored in dried condition on SEM stub, LSN exuvium as glycerine jelly microscope slide, both at the Natural History Museum of Denmark (NHMD-916629).

**Paratypes:** Eight larvae from same locality as holotype but collected on different dates. Two cyprids mounted for SEM with corresponding LSN exuviae on glycerine jelly slides (NHMD-916630, NHMD-916632), exuvium of one LSN on glycerine jelly slide with corresponding cyprid preserved in ethanol for molecular work (NHMD-916631), exuvium of one LSN on glycerine jelly slide (NHMD-916639), one LSN mounted for SEM (NHMD-916636), one LSN (nested failed moltings, outermost cuticle is LSN – 2) mounted for SEM (NHMD-916638), two early nauplii mounted for SEM (NHMD-916633, NHMD-916635). Detailed sampling data in Table 3.


**Description**

**Last-stage nauplius (LSN)** (Figs 3A, 4–6, 11D, E, K, L, 14A): Mainly based on paratype NHMD-916636 (Figs 4, 5). Body markedly elongate, slightly depressed dorsoventrally. Total length (TL) 380 μm (alive) or 375 μm (after critical point drying), including dorsocaudal spine; lengths of other live specimens from Sesoko Island 352–390 μm (N = 8) (Fig. 3). Greatest width 140 μm and greatest dorsoventral thickness 100 μm. Cuticle transparent with nearly fully developed, yellowish/brownish cyprid clearly visible inside, with median nauplius eye and pair of compound eyes. In dorsal view, frontal margin evenly rounded, lateral margins tapering gradually towards bluntly rounded caudal end, with no sharp border between cephalic shield and trunk (slight indentation visible in most other specimens), ending in blunt dorsocaudal spine. In lateral view, body not entirely straight, but bent c. 35° ventrally behind naupliar appendages. Three pairs of naupliar appendages (a1, a2, md) arising close together on ventral side at about 25% of body length, flanking triangular, moderately bulbous labrum. Large parts of ventral side of body (posterior to labrum) and dorsal side of trunk ornamented with transverse cuticular ridges. Cephalic shield with ridge-bounded facets, except for smooth central area flanking dorsal (but not anterior) midline. Entire body displaying bilaterally symmetrical pattern of variety of diverse pores and sensilla, all fully mapped and numbered herein (see detailed description below). No ‘ghost-like’ image of part of the cyprid thorax, particularly the thoracopods and their setae, visible inside any LSN exuviae (as previously detected in many types of y-larvae; Grygier et al., 2019).

Anterior part of faciomarginal area almost featureless except for pair of closely-set pores with oval openings (#34, Fig. 4B, D) and pair of depressions (#33), latter resolved as pores with slit-like openings inside any LSN exuviae (as previously detected in many types of y-larvae; Grygier et al., 2019).

Large, triangular elevation (labrum) present between naupliar appendages (Figs 4B, 5K–M), slightly bell-shaped in mounted exuvium of paratype NHMD-916630 (Fig. 11K), with shallowly indented posterior margin. Labrum with five pores, including three unpaired pores in midline (#29* posteriorly, #30* and #31* near midlength), and one lateral pair positioned level with these latter two (#32), but no free posterior labral extension. Openings of pores #30* and #31* oriented obliquely, but in slightly different ways in this and the above-mentioned Green Island specimen (compare Figs 5M, 6C).

Naupliar appendages placed immediately adjacent to labrum in diagonal rows, with first antennae (a1) closest to midline, mandibles farthest from it (Figs 4B, 5K). Each limb arising from separate (a1) or partly continuous (a2 and md) outpocketings of cephalic cuticle, these probably serving to enhance appendage flexibility while swimming. First antennae short and digitiform (slightly shorter in above-mentioned Green Island specimen; Fig. 6C), consisting of two distinct segments and, embedded in cephalic outpocketing, sclerite possibly representing additional proximal segment (Fig. 5K). Distal segment twice as long as proximal segment, bearing two long apical setae, one apicolateral seta of intermediate length and one small (rudimentary) medial seta. Second antennae and
mandibles devoid of any feeding structures (including gnathobases or naupliar processes) (Figs 4B, 5K, L). Coxa and basis of second antenna both broad, ring-like, with rudimentary serration along distal margin; endopod narrowly inserted on medial part of end of basis, being small, cylindrical, and undivided with two distal setae (one long and one short); exopod more broadly inserted on lateral part of end of basis, being composed of five successively smaller annuli, the first bearing one small medial seta, the next three each carrying one long medial seta, and the small distal segment bearing two setae of intermediate length (setal formula 1:1:1:2); in Green Island specimen, a sixth rudimentary segment visible more proximally in exopod. Segmentation and setation of mandible similar to those of second antenna, except basis shaped differently and proximal annulus of exopod smaller and lacking any setae (setal formula 0:1:1:1:2).

Table 3. Material (24 specimens) of Hansenocaris demodex sp. nov. from Sesoko Island (Japan, Okinawa) and Green Island (Taiwan) used in the present study. ‘LSN’ refers to ‘last stage nauplius’ before metamorphosis to the y-cyprid. ‘LSN – 2’ and ‘LSN – 3’, respectively, refer to instars two and three molts earlier than the ‘LSN’; see Material & Methods, ‘Naupliar development’, for explanation. Some of the specimens from Sesoko Island shown in Figure 3 are not listed here as they died and were discarded without being preserved.

<table>
<thead>
<tr>
<th>Sample number</th>
<th>Museum number</th>
<th>Material</th>
<th>Collection data</th>
</tr>
</thead>
<tbody>
<tr>
<td>JA-2018-014</td>
<td>NHMD-916629</td>
<td>• LSN* (Fig. 3)</td>
<td>15-Oct-2018***</td>
</tr>
<tr>
<td></td>
<td></td>
<td>• Cyprid† (Figs 3, 7–9)</td>
<td></td>
</tr>
<tr>
<td>JA-2018-013</td>
<td>NHMD-916630</td>
<td>• LSN* (Figs 3, 13)</td>
<td>17-Oct-2018***</td>
</tr>
<tr>
<td></td>
<td></td>
<td>• Cyprid† (Figs 3, 7–9)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>• Cyprid† (Fig. 3)</td>
<td></td>
</tr>
<tr>
<td>JA-2018-111</td>
<td>No voucher</td>
<td>• Nauplius (failed moulting)* (Figs 3, 11)</td>
<td>22-Oct-2018***</td>
</tr>
<tr>
<td>JA-2018-248</td>
<td>NHMD-916632</td>
<td>• LSN* (Fig. 3)</td>
<td>3-Nov-2018***</td>
</tr>
<tr>
<td></td>
<td></td>
<td>• Cyprid† (Figs 3, 8)</td>
<td></td>
</tr>
<tr>
<td>JA-2018-274</td>
<td>NHMD-916633</td>
<td>• Nauplius† (Fig. 3)</td>
<td>3-Nov-2018***</td>
</tr>
<tr>
<td>JA-2019-001</td>
<td>No voucher</td>
<td>• Nauplius† (Fig. 3)</td>
<td>1-Jun-2019***</td>
</tr>
<tr>
<td>JA-2019-320</td>
<td>NHMD-916635</td>
<td>• Nauplius† (Figs 3, 11, 13)</td>
<td>22-Jun-2019***</td>
</tr>
<tr>
<td>JA-2019-321</td>
<td>No voucher</td>
<td>• Nauplius† (Fig. 3)</td>
<td>22-Jun-2019***</td>
</tr>
<tr>
<td>JA-2019-322</td>
<td>No voucher</td>
<td>• Nauplius† (Fig. 3)</td>
<td>22-Jun-2019***</td>
</tr>
<tr>
<td>JA-2019-165</td>
<td>No voucher</td>
<td>• LSN† (Fig. 3)</td>
<td>10-Jun-2019***</td>
</tr>
<tr>
<td>JA-2019-048</td>
<td>NHMD-916636</td>
<td>• LSN* (Figs 3–5)</td>
<td>10-Jun-2019***</td>
</tr>
<tr>
<td>JA-2019-099</td>
<td>NHMD-916638</td>
<td>• LSN (failed moulting, outermost cuticle is LSN – 2)* (Figs 3, 11, 13)</td>
<td>11-Jun-2019***</td>
</tr>
<tr>
<td>No number</td>
<td>NHMD-916639</td>
<td>• LSN*</td>
<td>2005‡‡</td>
</tr>
<tr>
<td>JA-2019-107</td>
<td>No voucher</td>
<td>• LSN (failed moulting)* (Figs 3, 11)</td>
<td>12-Jun-2019***</td>
</tr>
<tr>
<td>JA-2019-136</td>
<td>No voucher</td>
<td>• LSN (failed moulting)* (Figs 3, 11)</td>
<td>12-Jun-2019***</td>
</tr>
<tr>
<td>JA-2019-100</td>
<td>No voucher</td>
<td>• Nauplius† (Fig. 3)</td>
<td>14-Jun-2019***</td>
</tr>
<tr>
<td>JA-2019-378-0</td>
<td>No voucher</td>
<td>• Nauplius† (Fig. 3)</td>
<td>20-Jun-2019***</td>
</tr>
<tr>
<td>No number</td>
<td>NHMD-916640</td>
<td>• Early nauplius† (Fig. 12)</td>
<td>21 or 22-Sep-1991‡‡</td>
</tr>
<tr>
<td>TA-2018-112</td>
<td>NHMD-916641</td>
<td>• LSN† (Fig. 6)</td>
<td>4-Sep-2018***</td>
</tr>
<tr>
<td>TA-2018-152</td>
<td>NHMD-916642</td>
<td>• Cyprid† (Fig. 10)</td>
<td>6-Sep-2018***</td>
</tr>
</tbody>
</table>

*Japan: Okinawa, pier of Tropical Biosphere Research Center Sesoko Station.
†Taiwan, Green Island, Gongguan Harbour.
§Formalin-fixed exuvium, on glycerine jelly slide.
Formalin-fixed, on SEM stub.
Ethanol-fixed and processed for molecular work (resulting in lack of voucher).
*Collected and processed by DEJ, MJG, YF, ND, JO.
+Collected and processed by DEJ, MJG, YF, ND, FP, JO.
‡Collected and processed by MJG.
§§Collected and processed by ND, DEJ, JO.
Cephalic shield approximately 200 μm long, 140 μm wide, its posterior margin not clearly demarcated from trunk, but indicated indirectly by shift in cuticular ornamentation from cephalic shield’s longitudinal ridges to trunk’s transverse striations (Figs 4C, 5A). Lateral and frontal parts of shield bent ventrad and bearing cuticular reticulations (facets or plates) and lineations, while dorsal surface smooth, with weakly defined mid-dorsal ’window’. Ridge-defined reticulation most complicated anteriorly, with large diversity of small facets, some squarish and others oblong, while more lateral facets fewer and longer, transitioning into long, uninterrupted ridge-bounded belts reaching to shield’s posterior margin. Arrangement of ridges and facets roughly bilaterally symmetrical, but symmetry of size, shape and degree of subdivision of contralateral facets imprecise. Arrangement not described in detail, but full pattern of two LSN from Sesoko Island and Green Island as shown in Figures 4C, 5A, B and 6, respectively, similar in both specimens but with some differences: (1) arrangement of anterior facets more strictly symmetrical in Green Island specimen; (2) some oblong frontal facets of Green Island specimen, most notably those above central pore #1 (Fig. 6D), corresponding to pores of smaller facets in Sesoko specimen (Fig. 5B); and (3) all facet-bounding ridges less distinct in Green Island specimen, sometimes even absent (especially dorsally).

Cephalic shield ornamented with large number of other types of structures (pores, setae), these being fully mapped on paratype NHMD-916636 from Sesoko Island (Table 4; Figs 4, 5): 31 structures in total, mostly in pairs and concentrated in anterior third of shield. Most common type consisting of 23 relatively large (3–4 μm) pores, among which just one unpaired and situated on midline close to anterior margin (#1, Fig. 5B), remainder distributed anteriorly and laterally in pairs (Table 4; Figs 4, 5). A few other kinds of structures present dorsally: two widely separated pairs of anterior setae (#7 and #8; Fig. 4E), one mid-dorsal pair of small, circular pores (#12) and one pair of small sensilla near posterior margin (#16, Fig. 5F).

Pore/sensilla pattern of specimen NHMD-916641 from Green Island (Fig. 6) the same, except for minor differences in precise position of some structures (e.g. pore pair #19, located between different transverse lines in the two specimens).

Elongate trunk comprising about 45% of TL in dorsal view, 62% in ventral view. Behind slight indentation at posterior end of cephalic shield, trunk tapering smoothly with its lateral margins subtending angle of c. 20°, then somewhat rounding off at posterior end. Dorsocaudal spine robust, short, blunt in NHMD-916636 (Fig. 4), somewhat longer and more pointed in other LSN specimens (Fig. 6). Pair of reduced furcal spines situated ventrally, about 30 μm forward from base of dorsocaudal spine. Lateral flanks and posterodorsal part of trunk bearing about 25 paired or dorsally continuous, regularly spaced and annular cuticular ridges with posteriorly directed, serrate crests (Figs 4, 5). In specimen NHMD-916641 from Green Island, these ridges less distinct dorsally (Fig. 6B). In both specimens, nine pairs of large pores distributed mostly laterally and ventrally on trunk (#17–#25; Figs 4–6), including one pair (#25) flanking dorsocaudal spine. In addition, pore pair #13 of cephalic shield possibly actually belonging to outer border of faciomarginal area. Pore arrangement generally bilaterally symmetrical, but precise positions of many pores far from symmetrical. For example, in NHMD-916636 left member of pairs #17, #21 and #24 situated far more anteriorly than their right-side counterparts (most pronounced for most posterior dorsal pair, #24), and in both this and Green Island specimen, one member of pair #24 situated significantly more dorsally than its contralateral partner (Figs 4F, 6B).

Large, convex, medioventral region of trunk, reaching from point immediately behind labrum to point immediately anterior to base of dorsocaudal spine, this region being broadest anteriorly, reaching posterolateral margins of cephalic shield on both sides, then gradually tapering posteriorly to median pore (#26*) between furcal spines. In NHMD-916636 this region crossed by transverse cuticular ridges with posteriorly directed, serrate crests (Fig. 4B), these ridges being fewer and less distinct in Green Island specimen NHMD-916641 (Fig. 6C).

Cyprid (Figs 3, 7–10): Mainly based on holotype (NHMD-916629); minor variation found in a few other examined cyprids from Sesoko Island and Green Island (Taiwan) mentioned directly in description with indication of specimen identity (museum number). Body elongated, consisting of head with large but not all-enclosing cephalic shield (carapace), six-segmented thorax, three-segmented abdomen and telson. Three specimens measured in life 283–305 μm long, two measured after fixation in ethanol both 360 μm long and four measured after critical point drying 280–320 μm long. Total length of individual specimens greatly different when measured in life, in preservative (longer, perhaps due to relaxation of musculature) and after critical point drying (8–20% shrinkage).

Cephalic shield (or carapace) covering head anteriorly, dorsally and laterally and also covering anterior part of thorax dorsally and especially laterally. Small nauplius eye lying anterodorsally to pair of large compound eyes. Labrum and first antennae situated on ventral side of head, beneath compound eyes. Each of six thoracomeres bearing a pair of biramous thoracopods, unclear whether tergites of thoracomeres 1 and 2 separate dorsally.
(this area not visible by SEM). In live specimens, main body and anterior part of cephalic shield deep brown, lateral parts of cephalic shield largely transparent, non-functional gut orange (yolk?) and, in both fixed and living specimens, area beneath cephalic shield approximately at point of separation from main body with dorsolateral concentration of orange-coloured droplets (oil, yolk?) (Figs 7A, 8A).

Cephalic shield relatively long, univalved, only partially covering dorsal and lateral sides of main body of larva, with only thoracomeres V and VI being exposed dorsally and with lateral margins of shield reaching telson in live specimens (Fig. 8A). Shield resembling inverted boat with posterolateral parts much produced, altogether about 175 μm long along mid-dorsal line and 210 μm along lateral margins. Long longitudinal and short transverse and oblique cuticular ridges present, outlining plates (or facets) and longitudinal bands, these together occupying entire shield surface but being most distinct anteriorly and laterally; more anterior facets generally smaller. Arrangement of facets and bands not strictly bilaterally symmetrical; overall pattern in dorsal view almost so (see holotype, Fig. 8C), but significant asymmetry apparent in anterior view (Fig. 8B), with no clear left–right correspondence of facets around pore/sensilla pairs #9 and #10. In another paratype cyprid from Green Island (NHMD-916642; Fig. 10B), from Sesoko Island (NHMD-916630; not shown) and a sensilla pairs #9 and #10. In another paratype cyprid clear left–right correspondence of facets around pore/#1* on midline), these pores always paired, being concentrated anteriorly and laterally (Figs 7B, 8B, #1*). Oblique opening of pore #1* oriented differently from general cuticle by their situation 50–60 μm from anterior end of shield in four weak depressions surrounding most anterior of unpaired central pores (#14*). Their cuticle smooth and lacking any small pores (thus no pore field). First pair (#13) teardrop-like, about 10 μm long and 7 μm wide, strongly converging anteriorly and each narrowing posteriorly towards small terminal pore. Second pair (#15) elongate, 12 μm long and about 2.5 μm wide, slightly converging anteriorly and weakly narrowing towards tiny, posterior terminal pore. Third pair of lattice organs (#38) situated in front of posterior unpaired central pore (#39*), almost round with diameter of about 3 μm and with barely visible posterior terminal pore. Final two posterior pairs of lattice organs (#40 and #41) situated behind unpaired pore #39*, #41 in flattened posterior marginal area of shield. These two pairs, respectively, 10 μm long and about 1.5 μm wide and about 7.5 μm long and 2 μm wide, lack visible terminal pores. Lattice organs largely organized the same way in above-mentioned Green Island specimen (NHMD-916642), but additional rudimentary pair possibly present anterior to counterparts of above-described two anterior pairs (Fig. 10B, C).

Proximal parts of first antennae not visible in SEM preparations. Segmentation of distal parts also unclear, but distal armament consisting of conspicuous curved hook (claw), large aesthetasc and, between these structures, one short seta with scattered setules and one smaller simple seta (Fig. 7B–D, H). Claw remarkably gracile, semicircular. Small, bifid, thin-walled appendix, possibly homologous to frontal filaments in nauplii, present in anterior midline between first antennae and anterior margin of cephalic shield (Fig. 7E). Labrum extended as linguiform process with 17 hooks on posterior side, arranged in three irregular rows of five, six and six hooks (Fig. 7C, D, F). Posterobasal part of labrum with five pores: one lateral pair with slit-like openings (#64) and three in midline, among which two with oblique slit-like openings (#62, #63) and one partly hidden proximally (#61; vestigial mouth opening?). Vestiges of second antennae and mandibles present lateral to labrum, showing remains of exopods and endopods in one specimen (NHMD-916630, Fig. 7C), but small, rounded and wrinkled in another (NHMD-916629, Fig. 7B). Small pair of so-called bifurcate paraocular processes present anterior to these, with anterior and slightly thicker posterior branches both 15 μm long and situated parallel and adjacent to lateral margin of cephalic shield (Fig. 7B–D). No pair of postocular filamentary tufts seen in these preparations.

Among thoracomeres I–VI (Figs 7B, 9A, 10A, E), posterodorsal margins of tergites V and VI serrate (Figs XX, 1–44).
Tergite VI also equipped with two or three other transverse, serrat cutting ridges and bearing widely spaced pair of setae close to posterior margin (#45; Fig. 7I). Cuticular ridges less distinct on Green Island specimen (Fig. 10E). Tergites V and VI with free pleural extensions, those of former with rounded ends, those of latter trapezoidal with sharp posterolateral ends (less trapezoidal in Green Island specimen) (Figs 9A, 10A).

Each thoracomere bearing pair of biramous thoracopods (Figs 9A, B, 10A, D). Each thoracopod consisting of lateral basal array of sclerites, coxa (distinct only in thoracopods 1–5), basis and pair of rami (exopod and endopod). Proximal sclerites not described further as this flexing zone appears variable between specimens. Two-segmented endopod of first thoracopod with short proximal segment and elongate (three times longer than wide) distal segment with two long apical setae; endopod slightly shorter in Green Island specimen (Fig. 10D). Unsegmented exopod slightly shorter than endopod, significantly wider proximally than distally, and bearing two apical setae: short outer one and long inner one. Thoracopods 2–5 composed of the same elements as thoracopod 1, but with distal segment of endopod generally longer and carrying long medial seta (dotted arrows on Figs 9B, 10D) in addition to two terminal setae; and with exopod larger and bearing three terminal setae: short outer one and two long inner ones. Thoracopod 6 generally similar to preceding thoracopods but shorter, and with unsegmented protopod (coxa and basis fused?).

Abdomen consisting of three short segments, all lacking pleural extensions but possessing serrate transverse cutting ridges and posterior margins; first segment with dorsolateral pair of setae (#46), third segment shortest, tapering ventrally and sometimes strongly intercalated between second segment and telson (7B, I, 9A, 10A, E). Telson long with dimensions varying somewhat among specimens, 1.5–1.6 times as long as greatest width in a specimen from Sesoko Island (Figs 7E, 9C), but 1.3 times as long as wide in one from Green Island (Fig. 10E). Telson with dense reticulation of serrate ridges roughly outlining two dorsal longitudinal rows of broad plates (Figs 7I, 10C) (anterior two or three pairs only weakly or not divided at midline), two lateral rows on each side and five ventral rows (Figs 7B, 9B–D, 10A). Number of plates in each dorsal row approximately 13 (11 in Green Island specimen), 11 in each lateral row and ten in each ventral row, with those of mid-ventral row set off half a step from those of other rows.

Total of 19 pores and setae present on telson (Table 5). Four pairs (#48–#51) placed in characteristic pattern anterolaterally (Figs 9A, 10A). Pair of pores with slit-like opening (#52) present in third plate from front in upper row of lateral plates (Figs 7B, 9C, 10A, E); similar pore (#53) in fourth plate from rear in lower row of lateral plates (Figs 7B, 9C, D, 10A). Another similar pair of pores and pair of setae in pits (#54 and #55, respectively) situated either in same contralateral pair of posterior dorsal plates (Fig. 7I) or with pores and setae in successive pairs of plates (Fig. 9C, D) or, in Green Island specimen, pore pair #54 absent (Fig. 10E). Terminally, one pair of dorsal pores (#56) and one ventral central pore (#57*). Three pairs of ventral pores (Figs 9B, D) located far anteriorly in outer row of ventral plates (#60) and in two adjacent posterior plates in same row (#58 and #59). Furcal rami short and cylindrical, perhaps even disc-like, with three setae each: two unequal lanceolate setae with serrate margins, and one irregularly denticulate short seta (Fig. 9D, E).

Earlier naupliar stages

Number of naupliar stages: An outline of the naupliar development of *H. demodex* is provided herein based on a combination of high-resolution photographs in life of different instars of the same individual (Figs 3, 11) and SEM images (Figs 12, 13) of several specimens representing three distinct instars younger than the last-stage nauplius (LSN). Four nauplii developed to cyprids while in culture (NHMD-916629, NHMD-916630, NHMD-916631, NHMD-916632), transitioning 3–6 days after the date of sampling (Table 6). Of particular importance are four specimens – three of them LSN as shown by presence of the developing cyprid’s compound eyes within – that failed to moult properly, resulting in a nested set of unshed exuviae with the most recent stage innermost (Fig. 11F, G, I, J). These specimens provide direct evidence of the last four stages of naupliar development, LSN, LSN−1, LSN−2 and LSN−3. In each series, the dorsocaudal spine generally becomes thinner and less blunt as development progresses, with the thinnest spines being found in the LSN. There appear to be even earlier instars in our material. One of these, NHMD-916635 (Fig. 13A, B), has a dorsocaudal spine that is blunter than that of LSN−3 (cf. Fig. 11F); it most likely belongs to an earlier instar, perhaps LSN−4. Another specimen (Fig. 12), the earliest-stage specimen examined during this study, has much weaker developed cuticular reticulation and other cuticular armature than the specimen mentioned above and appears to be one, or more likely two, instars earlier than it, i.e. LSN−5 or LSN−6. The naupliar development of *H. demodex* thus comprises at least five to six instars, more if instars have been missed (see Discussion).

Colour, yolk and cyprid morphogenesis during the naupliar phase: Coloration clearly shown and yolk boundaries distinct in our photographs of living nauplii at various moult stages (Figs 3, 11). Anterior and posterior parts of body brownish in earliest stages (Fig. 27).
Table 4. Overview of distribution of 63 cuticular surface structures (pores, sensilla, etc.) on different body regions of last-stage nauplius (LSN) of *Hansenocaris demodex* sp. nov., paratype (NHMD-916636) from Sesoko Island (Japan: Okinawa). Numbers refer to individual structures as indicated in Figures 4 and 5 and in other depicted nauplii. All structures paired except asterisks (*) indicate unpaired structures found only in midline.

<table>
<thead>
<tr>
<th>Region</th>
<th>Large slit-like pore (3–4 μm diam.)</th>
<th>Pore with large seta</th>
<th>Small circular pore (1 μm diam.)</th>
<th>Pore with small sensillum</th>
<th>Intermediate-sized or small slit-like pore</th>
<th>Distinct cuticular depression</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cephalic shield</td>
<td>1*–6, 9–11, 13–15</td>
<td></td>
<td>12</td>
<td>16</td>
<td></td>
<td></td>
<td>31</td>
</tr>
<tr>
<td>Trunk, dorsal and lateral</td>
<td>17–25</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>18</td>
</tr>
<tr>
<td>Trunk, medial ventral region</td>
<td>26*</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>3</td>
</tr>
<tr>
<td>Cephalic part of faciotrunk, ventral</td>
<td>28, 31*, 32</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>11</td>
</tr>
<tr>
<td>Total</td>
<td>47</td>
<td>4</td>
<td>4</td>
<td>2</td>
<td>4</td>
<td>2</td>
<td>63</td>
</tr>
</tbody>
</table>

11A, B). Bright-coloured cylindrical region – orange in specimens from Sesoko Island but yellow in those from Green Island – extending from behind nauplius eye to dorsocaudal spine, partly subdivided into droplets or granules and assumed to be at least partly made of yolk. Yolky region dividing into two parts early in naupliar development, these corresponding to future thorax and abdomen of the cyprid (Fig. 11B). Cyprid achieving its final form within last-stage nauplius (LSN) and capable of movement (e.g. thoracopodal beating, abdominal dorsoventral flexion), while still inside naupliar cuticle. Before final moult, abdomen of cyprid becoming gradually thinner and yolk becoming concentrated in dorsal midline, as also seen in free-swimming newly moulted cyprids (orange area in Figs 7A, 8A).

Naupliar stage LSN – 5 or LSN – 6? (Fig. 12): Earliest stage among all nauplii of *H. demodex* examined here with SEM. Body short (345 μm long), dorsocaudal spine blunt. Overall body shape much like that of LSN, but markedly more compact, less elongate and distinctly inflated (due to greater quantity of yolk?). Large facets (plates) on anterior and lateral parts of cephalic shield few in number and separated by faint ridges. Anterior field of large facets marked with red overlay in Fig. 12A–C in order to trace their fate in following instars. Assuming only one row of ‘brim’ plates anteriorly, red overlay encompassing all axial ‘frontal’ plates (F-1 to F-4; definitive boundaries uncertain, so labelled as F*) in addition to small parts of ‘elongate’ plate pair E-1 (i.e. E-1*) in upper corners and both members of ‘intercalary’ plate pair I-1 and ‘polygonal’ plate pair P-1 in lateral areas, none of these being yet delineated from their adjoining ‘frontal’ plates (cf. fully delineated state in later instars). Overlay area flanked on each side from top to bottom by four plates identifiable under Itô’s (1990b) and Kolbasov et al.’s (2021a) expanded systems as ‘intercalary’, ‘elongate’, ‘polygonal’ and ‘marginal’ plates E-1* + I-3(a), I-2, P-2 (bordering pore #4 posteriorly) and M-1. ‘Marginal’ plate M-2 and ‘superlateral’ plate S, adjoining M-1 posteriorly and posteroventrally, with S bordering pore #2 anteriorly and adjoining plate M-3(e) posteriorly. Other plates, especially dorsally, too poorly delineated to identify with confidence except for M-2 + M-3(c) behind M-1 and lateral band behind S consisting of M-3(e) and combined M-4 to M-7.

Labrum similar to that of LSN in general form and pores, but preceded by distinct median elevation reaching to pore pair #34, and pore #29 relatively larger than in LSN and positioned significantly farther forward, away from posterior margin. Naupliar appendages differing from those of LSN in minor ways: all limbs relatively shorter and more robust than in LSN; exopod of second antenna six-segmented ways: all limbs relatively shorter and more robust than in LSN; exopod of second antenna six-segmented. Other appendages differing from LSN in minor ways: all limbs relatively shorter and more robust than in LSN; exopod of second antenna six-segmented. Other appendages differing from LSN in minor ways: all limbs relatively shorter and more robust than in LSN; exopod of second antenna six-segmented.
TAXONOMY OF CRUSTACEAN Y-LARVAE

Figure 14. Morphology of four types of y-larvae from Sesoko Island used for molecular analyses (see overview in Table 6), all photographed in life. A, *Hansenocaris demodex* sp. nov., lecithotrophic LSN (last-stage nauplius) with cyprid inside. B, ‘Bumblebee’ type, lecithotrophic LSN with cyprid inside. C, ‘Big brown’ type, lecithotrophic LSN with cyprid inside. D, planktotrophic nauplius, identified as Itô’s (1986a) Pacific type I. Squares with dotted outline: sample numbers.

plate W), and pore pair #9 present dorsally posterior to midlength of shield. On trunk, dorsolateral pore pairs #17, #19, #21 and #24 more-or-less identifiable, but interpretations complicated by left/right asymmetry in pore distribution. Pore pair #25 flanking dorsocaudal spine at posterior end of body and single mesial pore #26 situated between blunt, rudimentary furcal spines, these spines being closer to terminal end than in LSN. Anterior central pore of cephalic shield (#1) and pore pair #3 flanking it not yet apparent. Posteriorly on...
shield, seta pair #16 and pore pairs #11, #12 and either #14 or #15 not yet present. Ventrolateral pore pair #13 also not seen.

**Naupliar stage LSN − 3 (Figs 11B, 13A−C):** This stage at least one and probably two instars later than preceding specimen and probably one instar earlier than LSN − 2 specimen described below. Facet arrangement of cephalic shield more complicated than in preceding specimen, with greater number of longitudinal bands and division of anterior region into smaller facets. For example, anterior face of shield (Fig. 13C, red overlay) now subdivided into three rows of clearly delineated, ridge-bounded facets in three rows: axial row extending from anterior margin of shield to approximately pore pair #6, comprising part of ‘brim’ and all derivatives of ‘frontal’ (F-1 to F-4) plates, and pair of shorter, eight-facet rows flanking it to left and right, consist of derivatives of certain ‘polygonal’ (P-1), ‘intercalary’ (I-1) and ‘elongate’ (E-1) plates. Unpaired far-anterior pore (#1) and a few other pores (e.g. pair #3 on anterior face of shield and pair #23 on ventral side of trunk) now present. In general, pores more distinctly delineated and with more pronounced slit-like openings than in above-described earlier stage. Segmentation and setation of naupliar appendages identical to those of that specimen, but trunk with greater number of more distinct vertical and transverse cuticular ridges, including narrow row of such ridges along ventral midline (Fig. 13A).

**Naupliar stage LSN − 2 (Figs 11G, 13D−G):** Incompletely moulted LSN/cyprid specimen with cephalic shields and faciotrunk integuments of two previous instars still attached in nested fashion (LSN − 1, yellow overlay, and LSN, green overlay). Moulting zone of both LSN − 1 and LSN − 2 cuticles running along dorsal transverse seam between rear margin of shield and trunk dorsum, then continuing ventrally around border between faciomarginal area and lateral and anterior margins of cephalic shield. Mainly outermost LSN − 2 stage observed by SEM (Fig. 13D−F), but unmoulted cyprid y observed and photographed in life within three surrounding layers of naupliar cuticle (Fig. 11G). LSN − 2 differing from stage described above (LSN − 3?) in having about twice as many and narrower elongate, band-like facets posterolaterally on cephalic shield and generally more complex facet arrangement anteriorly. For example, brim plates crossing and flanking anterior midline of shield margin in previous larva (Fig. 13C, arrow), and there comprising three facets in total, now comprising two transverse rows of four smaller facets each (Fig. 13D, F, arrows). Pores and setae of this nested-cuticle specimen not examined in detail, but evidently not appreciably different from those of LSN. Segmentation and setation of naupliar appendages apparently identical to those of earlier stages. Trunk more slender than in previously described stages, weakly tapering terminally with longer, thinner dorsocaudal spine and with less distinct vertical and transverse cuticular ridges.

**Molecular analyses**

Twenty-two larval specimens were sequenced, together representing one feeding (planktotrophic) and three non-feeding (lecithotrophic) types of nauplii from Sesoko Island (N = 19 specimens) and Green Island (N = 3 specimens) (Fig. 14; Table 6). New 18S sequences for the 22 specimens in the phylogenetic analyses have been uploaded to GenBank (Accession codes: OM135272–OM135293). Images of all sequenced specimens (except some *H. demodex*), photographed while still alive, were mapped to the phylogeny (Fig. 15).

Because most Facetotecta nucleotide sequences previously registered in GenBank are based on larvae with no morphology recorded, the true nature of those larvae can only be inferred. Congruence between molecular (18S rDNA) and morphological data is nonetheless evident in Figure 15, with similar specimens grouped together into two main clades (A and B in Fig. 15). Clade A contains *Hansenocaris itoi* (AF439393) and two unnamed taxa from GenBank (FJ751879, FJ751881). Clade B is larger and includes four subclades, each represented by several specimens with mutually identical haplotypes. The first subclade is composed of four specimens of Itô's (1986a) Pacific type I (planktotrophic) and three unnamed taxa from GenBank (FJ751877, FJ751878, FJ751882). The second subclade is composed of nine *H. demodex* specimens. The third subclade is composed of two specimens of a type nicknamed 'Big brown' and one unnamed taxon from GenBank (FJ751880). Finally, the fourth subclade is composed of seven specimens of a type nicknamed 'Bumblebee'.

**DISCUSSION**

**Y-larvae: individual rearing for species descriptions**

The challenge of matching wild marine invertebrate larvae with their adult counterparts dates back to the 19th century (Thomson, 1830; Müller, 1864; Garstang, 1951). In the modern era, efficient matching of different life-stages in invertebrate life-cycles has been greatly facilitated by the advancement of culturing procedures, more recently supplemented by molecular methods (Pardo et al., 2009; Tang et al., 2010; Feller et al., 2013; Carreton et al., 2019). Both methods were applied here.
to the Facetotecta, the last major invertebrate group for which the adult stage remains unknown. Some evidence suggests that the adults are endoparasites in undiscovered host organisms. Most significantly, Glenner et al. (2008) found a new life-stage in the facetotectan life-cycle, the ypsigon, a slug-shaped stage that emerged from y-cyprids that had been exposed to a moultng hormone in the laboratory. Similarities of the ypsigon to the vermigon, the infective stage of certain parasitic barnacles (Rhizocephala), led Glenner et al. (2008) to infer endoparasitism of the unknown y-adults.

As a source of individual y-larvae for laboratory culturing, the present study primarily relied on a shallow-water site at Sesoko Island (Japan: Okinawa) that has repeatedly been suggested to show a remarkable abundance and diversity of y-larvae (Grygier, 1991a; Glenner et al., 2008; Grygier et al., 2019) (Fig. 2). The 9621 y-larvae collected and handled during more than six weeks of intensive fieldwork there in 2018 and 2019 amount to more than 25 times the greatest number of y-larvae collected in any single previous study, viz., the 350 or so specimens of Hansenocaris itoi from the White Sea reported by Kolbasov & Høeg (2003). On average, more than one y-larva was collected in each of the 7021 plankton tows made during these Sesoko surveys. In a few cases, more than 30 y-larvae were present in a single 10-15 m tow (Table 2) and, on these occasions, y-larvae were among the dominant members of the smaller-sized crustacean plankton. No other studies at the same scale are found in the literature, so it remains to be seen how widespread the mass occurrence of y-larvae actually is, either along Japanese coastlines or elsewhere. The full implications of this dataset, including the results from bi-hourly monitoring of y-larva occurrence, will be presented elsewhere.

None of these almost 10 000 wild-caught y-larvae could be convincingly identified to any of the currently described species, but a few seemed to conform to previously reported larval types such as Itô’s (1986a) Pacific type I, which can readily be identified (Fig. 2). Even a quick glance at a fraction of a given sample (Fig. 2) suggests that numerous types/species are involved. Y-larva taxonomy is in a highly unsatisfactory state, when considering the high local abundance of these larval types and their likely ecological importance. To remedy this situation, a new approach to species descriptions is required. For the sake of standardization, it is here suggested that future species descriptions of y-larvae focus on a combination of last-stage nauplii and cyprids, since each of these stages are homologous to their counterparts across y-larvae and can be readily identified. For y-larvae with lecithotrophic nauplii, building on previous results (Itô & Takenaka, 1988; Glenner et al., 2008; Grygier et al., 2019), a novel and larger scale integrated taxonomic protocol has been outlined and employed herein to describe Hansenocaris demodex, a unique form of y-larva with semi-vermiform nauplii that occurs in the waters of Japan and Taiwan.

**Hansenocaris demodex – a rare but distinctive y-larva from Japan and Taiwan**

Hansenocaris demodex is a fairly rare species in our samples, with only 22 specimens (0.2%) out of 9621 y-larvae collected at Sesoko (Okinawa) in 2018 and 2019. Sampling at Green Island (Taiwan) was more haphazard, with considerably less quantitative recording of the take than was done later at Sesoko Island, but the species is clearly rare at both sites. Hansenocaris demodex is the first species of Facetotecta for which several stages of lecithotrophic nauplii, as well as the cyprid, have been described in full detail, something not accomplished by Itô (1991), and with the nauplii and cyprid confirmed to be conspecific based on individual moulting sequences and molecular data. Its nauplius and cyprid stages all present unique characteristics. The extraordinarily elongate, semi-vermiform shape of the nauplii of all known stages and the distinctive, multihamate labrum of the cyprids make this species easily recognizable among all y-larvae described so far, and also among all other y-larvae handled during the present fieldwork at Sesoko and Green Island.

In this paper, *H. demodex* is documented from Green Island, Taiwan, and Sesoko Island, Japan, which are separated by c. 780 km (Fig. 1). 18S rDNA nucleotide sequences unequivocally demonstrate that the populations at the two sites are conspecific (Fig. 15), which is in accordance with the morphological results, except for coloration, with specimens from Green Island being distinctly yellower (not shown in the figures). The distribution of this species is probably linked to the Kuroshio Current, a warm oceanic current that originates east of the Philippines and flows in a north-eastward direction past Taiwan, Okinawa and Japan at a velocity of up to 4 m/s (345 km/day) (Jayne et al., 2009). Intertidal communities of free-living and parasitic marine organisms are known to experience a large gene flow along the Kuroshio, resulting in a lack of genetic differentiation between populations, even over long distances (Chan et al., 2007; Chang et al., 2017; Jung et al., 2019; Ma et al., 2019). This probably explains the great molecular similarity between the Green Island and Sesoko Island populations of *H. demodex*. Indeed, this and other species of y-larvae may be distributed throughout the entire Kuroshio region, something that can only be confirmed by further sampling. One of us (MJG, unpublished) collected several additional specimens of...
H. demodex in 2020 at the island of Xiaoliuqiu off the south-west coast of Taiwan. Water masses move from Taiwan to Okinawa in 2–3 days, which is fast enough to transport young nauplii of H. demodex between these two places before they moult to the cyprid, a transition that our data showed to require 3–6 days.

Figure 15. Molecular phylogeny of y-larvae (Facetotecta) based on 18S data combining sequences of nine specimens of Hansenocaris demodex sp. nov. and 13 other specimens from Sesoko Island with sequences of Hansenocaris itoi and other limited information from GenBank. For more information on specimens and types, see Table 6 and Figure 14.
in the lab. However, since the majority of the sampled nauplii of *H. demodex* at Sesoko Island, as well as the other forms of lecithotrophic nauplii collected there (Fig. 2; unpublished data), were young instars, nearby sources (i.e. spawning y-adults) appear necessary to sustain the large populations there, regardless of any Kuroshio-mediated long-distance transport that may occur.

**Naupliar morphology of *Hansenocaris demodex* compared to other y-nauplii**

The nauplii of *H. demodex* are lecithotrophic, as shown by the large amount of internal yolk and the lack of antennal and mandibular feeding structures. Many types (likely species) of lecithotrophic y-nauplii are known to exist at Sesoko Island (Glenner *et al.*, 2008; Grygier *et al.*, 2019; unpublished data) and elsewhere in Japan (Itô, 1990b; Watanabe *et al.*, 2000), but limited detailed information is available for comparison. At least 15 different types (putative separate species) of lecithotrophic y-nauplii have been reported, all of which are different from the present specimens. Among the three types of y-nauplii described by Itô (1986a), two lacked endites of the second antennae and were, therefore, the first lecithotrophs reported in the literature (but Itô did not use the term ‘lecithotrophic’). Besides lacking feeding armature, these two naupliar types have a swollen body like *H. demodex* but in other ways are clearly different from it, being robust and having long, pointed dorsocaudal spines. Later, Itô (1987a) described three forms of large, disc-shaped, dorsoventrally flattened y-nauplii (‘type VIII’) that lacked the well-developed feeding armature of the second antennae and mandibles and were, therefore, also lecithotrophic, but different from the nauplii of *H. demodex*. Itô (1991) illustrated and briefly described (but did not name) yet another lecithotrophic form of y-larvae with a large dorsocaudal spine, based on a possibly complete larval series obtained by laboratory-rearing, the first of its kind. Belmonte (2005) based his description of *H. mediterranea* Belmonte, 2005 on a few dozen lecithotrophic y-nauplii (see also Høeg *et al.*, 2014: fig. 18.1H, I). Also, Grygier *et al.* (2019) presented photographs, but no descriptions, of eight clearly mutually distinct forms of lecithotrophic last-stage nauplii, all of which are different in general body form from *H. demodex*.

Y-larvae are well-known for their signature feature, a complex arrangement of cuticular facets (or ‘plates’) on the surface of the cephalic shield of both nauplii and cyprids, the function of which is unknown (Schram, 1972; Itô 1987b, 1990b; Kolbasov & Høeg, 2003; Kolbasov *et al.*, 2021a). Most information on y-naupliar facet patterns and their development comes from planktotrophic y-nauplii, while almost nothing has been published on lecithotrophs. Schram (1972) proposed a facet nomenclature, which he based on an early-stage planktotrophic nauplius with a simple and symmetrical facet arrangement. Itô (1987b) proposed a modification of Schram’s (1972) system to encompass different types of early-stage y-nauplii, including one lecithotroph (type VII). Efforts were later made, with only partial success, to extend Itô’s nomenclature system to later naupliar stages of *H. furcifera* (see: Itô, 1990b) and, with modification, also to *H. itoi* (see: Kolbasov *et al.*, 2021a), both of which have planktotrophic nauplii. The present paper includes the first SEM study of the cephalic shield (and other body parts) of a lecithotrophic species of y-nauplius, at a level of detail surpassing the few available SEM habitus views.

A major difference between *H. demodex* and *H. furcifera* is that, while facets are found on the entire cephalic shield (and even on the trunk dorsally) in all stages of the latter, the entire dorsomedian part of the shield is smooth in all known stages of *H. demodex*. This makes a full comparison between the facet patterns in these two species difficult, but from what can be seen, it appears that the development of the facets in the two species follows the same overall pattern. This includes the presence of essentially only one row of central longitudinal facets (Fig. 12, red overlay) and a few rows of long rectangular facets laterally. In both *H. demodex* and *H. furcifera* the facet pattern gradually gets more complicated in later stages, typically by further subdivision of already existing facets, the details of which could not be followed in the new species due to missing instars. However, one important difference is evident: in *H. furcifera* and also *H. itoi* (see: Kolbasov *et al.*, 2021a) up through LSN, and in Itô’s (1991) undescribed species at least up through LSN–1, facet I-1 never divides. In contrast, in *H. demodex* facet I-1, while initially joined with some of the F-facets, becomes discrete and subdivided into four subfacets no later than LSN – 3? (Fig. 13C; divided differently on the left and right sides). This subdivision is tentatively regarded as a diagnostic feature of the species herein.

Despite the role of the facets in defining Facetotecta, and the occasional use of their arrangement to distinguish various types of y-nauplii (e.g. Watanabe *et al.*, 2000), not enough comparative information is available to apply such data to larger questions. It would be fascinating to know whether a general pattern of facets is to be found in all y-nauplii, besides the early-instar (N-V or N-VI) pattern recognized by, for example, Itô (1987b). If not, what sort of evolutionary divergence among facet patterns has taken place? Are the facets on the cephalic shield of certain ascothoracidan nauplii, for example, *Sessillogoga captiva* Kolbasov & Grygier, 2020 (Kolbasov *et al.*, 2020), homologous to those of Facetotecta, as assumed by Chan *et al.* (2021)?
It is conceivable that heterochrony may have played a role in the evolution of the facet patterns of y-larvae. Even within the single species *H. demodex*, the facet pattern of the frontal part of the cephalic shield of the Green Island LSN specimen (Fig. 6D) is distinctly more symmetrical than that of an LSN specimen from Sesoko Island (Fig. 5B), and in this respect is more similar to earlier stages from Sesoko Island (Fig. 13C, F).

The pattern of cuticular surface structures (pores and setae) has been fully mapped for the LSN of *H. demodex* and partly for some selected earlier stages. Because this is the first such study for any lecithotrophic y-larva, these data provide a solid basis for comparison with other described species and forms. In total, the LSN has 63 cuticular pores and setae (Figs 4, 5; Table 4), most of which are paired while five pores on the midline are unpaired, including a frontal pore on the cephalic shield (#1*), a pore between the rudimentary furcal spines (#26*) and three pores of differing morphology on the labrum (#29*, #30*, #31*). The only species for which comparable data are available are the planktotrophic *H. furiçera* (see: Itô, 1989) and *H. itoi* (see: Kolbasov et al., 2021a). The pattern of two pairs of large setae (Fig. 4C, E) flanking the poorly defined ‘window’ on the cephalic shield in *H. demodex* is also present in *H. furiçera*. While not explored in detail here, it seems possible to homologize a number of the pore pairs on the cephalic shield of *H. demodex* with those of *H. furiçera*, while the pattern depicted for *H. itoi* seems more remote. *Hansenocaris furiçera* appears to lack many pores that were described for *H. demodex*, not least on the trunk. For example, *H. demodex* has a characteristically arranged row of four lateral pores on each side of its long trunk (total of eight: Fig. 5A, #17, #19, #21 and #24) while *H. furiçera* has just two pairs of pores (total of four) in a comparable position on its short trunk. While it is not possible to say whether or how these sets of pores actually correspond, serial duplication of such pores as an autapomorphy of *H. demodex* related to the extreme elongation of its trunk region might be an explanation.

Among the ventral cuticular surface structures, the anterior ones are generally easier to homologize between *H. demodex, H. furiçera* and *H. itoi*, while this is more difficult for the posterior structures. For example, equivalents of the frontal filaments in *H. demodex* (Fig. 4D, #34) also appear to be present in *H. furiçera* and *H. itoi*. The two lateral pores of the labrum (Fig. 5K, M, #32) are also present in all three species. The homologues of the three characteristic central pores of the labrum in *H. demodex* (#29*, #30*, #31*) are more uncertain. Itô (1989) drew only one central pore in *H. furiçera*, but its position relative to the lateral pores suggests that it is homologous to either pore #30* or #31* in *H. demodex*, while the equivalent to pore #29* (vestigial mouth opening?) in *H. demodex* may be hidden under the labral extension in *H. furiçera*, as is clearly shown in *H. itoi*. A comparison with our unpublished SEM photos of a suite of y-nauplii from Sesoko Island, representing a diversity of different species (both with planktotrophic and lecithotrophic nauplii), showed that the presence of two central pores (#30 and #31) in *H. demodex* may be unique to this species. The posteroventral pore pattern of the trunk in *H. demodex* is difficult to homologize precisely with that of *H. furiçera* and *H. itoi*. Itô (1990b) drew three pore pairs in this region in later stages of *H. furiçera*, and the same number is seen in our earliest stage of *H. demodex* (Fig. 12A, B, #18, #20, #22), which may indicate homology, but twice this number of ventral pores is seen in the LSN (Fig. 4B).

In *H. demodex*, the medial pore (#26*), possibly the vestigial anus, between the rudimentary furcal spines is not represented by any comparable structure in *H. furiçera* or *H. itoi*.

Pore patterns in crustaceans often provide information that is either species-specific or important at higher taxonomic levels (Mauchline, 1988; Olesen, 1996; Ozawa, 2013; Karanovic & Kim, 2014), but in *H. demodex*, the number of pores and their arrangement change as naupliar development progresses. Therefore, it may be misleading to compare non-equivalent stages across taxa. With this in mind, in Facetotecta it will be most useful to choose the last-stage nauplius (LSN) for comparison, as this stage is certainly homologous between species (see above). Supplementary data from earlier stages should be added whenever possible (as in this study) to check the degree of stability of pore patterns during development.

The naupliar appendages in *H. demodex*, including second antennae and mandibles that lack feeding armature on the coxa and basis and have an unsegmented endopod, are simple in form and, consequently, unlikely to display any unique features. However, although the proximal segment of the second antennal exopod is either lacking or rudimentary in the LSN (Fig. 5K), it is clearly present in the earliest examined stage (Fig. 12G). Practically the same morphology is observed in other lecithotrophic y-nauplii, such as types VII and VIII (Itô, 1986a, 1987a), and in a variety of other lecithotrophic y-nauplii from Sesoko and Green Islands studied with SEM (unpublished). In contrast, the coxa and basis of the naupliar second antennae and mandibles of *H. furiçera* and *H. itoi* bear median spines, presumably used in feeding, and both limbs have a two-segmented endopod (Itô, 1990b; Kolbasov et al., 2021a). The antennal and mandibular morphology of nauplii of *H. demodex* and other lecithotrophs is probably derived (assuming the loss of planktotrophy), but...
Table 5. Overview of distribution of 120 cuticular surface structures (pores, sensilla, lattice organs, etc.) on different body parts of cyprid y of *Hansenocaris demodex* sp. nov., all numbered as indicated in Figures 7–9 and based primarily on the holotype (NHMD-916629) from Sesoko Island (Japan: Okinawa). Most structures paired, but asterisks (*) indicate unpaired structures in midline.

<table>
<thead>
<tr>
<th>Structure</th>
<th>Large pore with slit-like opening (3–4 μm)</th>
<th>Pore with seta</th>
<th>Circular pore (0.5–1 μm diam.)</th>
<th>Group of micro-pores (each 0.5 μm)</th>
<th>Lattice organs</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cephalic shield (carapace)</td>
<td>1*, 2, 3, 17, 18, 23, 26, 28, 29(?), 37</td>
<td>4, 6, 8, 10, 12, 16, 21, 25, 30, 35, 36, 42–44</td>
<td>11, 14*, 19, 20*, 22, 27(?), 31–34, 39*</td>
<td>5, 7, 9, 24</td>
<td>13, 15, 38, 40, 41</td>
<td>84</td>
</tr>
<tr>
<td>Thoracomeres</td>
<td>Absent</td>
<td>Absent</td>
<td>Absent</td>
<td>Absent</td>
<td>Absent</td>
<td>0</td>
</tr>
<tr>
<td>Abdominal segments 1–3</td>
<td>45, 46</td>
<td>47</td>
<td>47</td>
<td>47</td>
<td>47</td>
<td>6</td>
</tr>
<tr>
<td>Telson</td>
<td>48–54, 56, 57*, 58, 60</td>
<td>55</td>
<td>59</td>
<td>61*</td>
<td>25</td>
<td>5</td>
</tr>
<tr>
<td>Cephalic region, ventral</td>
<td>62*, 63*, 64</td>
<td>38</td>
<td>40</td>
<td>8</td>
<td>10</td>
<td>120</td>
</tr>
</tbody>
</table>

**Hansenocaris demodex** is the first lecithotrophic species of y-larvae for which a suite of naupliar stages has been examined in detail. As in Itô’s (1990b) study, early larvae were sampled in the plankton and reared in the lab. Y-larvae cannot be kept in reproducing cultures as no y-adults are known, so it is uncertain whether the most frequently caught, early-looking y-nauplii represent the true nauplius 1 (the hatching stage) or a later stage. Itô (1990b) discussed this point as it applies to *H. furcifera*, but nonetheless referred to the stage with the simplest ‘turtle-shell’ ornamentation of the cephalic shield as nauplius stage 1 because no earlier stage could be demonstrated. No final conclusion can be reached concerning the total number of naupliar stages in the development of *H. demodex*, again due to the limited material, but our data suggest at least five or six naupliar instars. Largely because our SEM material does not include a stage with the same (or even a truncated) clearly defined pattern of ridges corresponding to that of Itô’s stage 1, the earliest specimen examined by us (Fig. 12) is inferred to be earlier than that. It possibly represents an LSN − 5 or even an LSN − 6, assuming the seven-instar sequence inferred by Kolbasov et al. (2021a) for *H. itoi* is correct and common to other forms of y-larvae. It might be the ephemeral ‘true instar 1’ first hypothesized by Itô (1990b), with the ‘standard’ plate pattern not yet established, but the fully developed limb armature with apparently functional natatory setae tends to argue against this.

Fouling might have been reduced, and survivorship higher, if the rearing dishes had been constantly agitated. The same may be true if the protocol recommended by Itô (1990a) had been fully adopted. Based on his experience of rearing about 20 putative species of lecithotrophic y-nauplii to the cyprid stage,
he reported that survivorship of up to 90% or more (compared to about 5% in the present study) can be achieved if nauplii are transferred twice daily to fresh filtered seawater in sterilized dishes. This protocol can be used in future efforts to obtain complete moult series based on fairly small numbers of individuals of selected target species, but the intensive labour involved made it impractical while maintaining several hundred specimens simultaneously, as was typical in the present study.

CYPRID MORPHOLOGY OF **Hansenocaris demodex** COMPARED TO OTHER Y-CYPRIDS

*Hansenocaris demodex* is the first formally named species of *y*-larva with lecithotrophic nauplius for which nauplii and the cyprid have been described simultaneously. It is also only the third form of *y*-cyprid to have been extensively described using SEM, after *H. papillata* Kolbasov & Grygier in Kolbasov *et al.*, 2007 and *H. spiridonovi* Kolbasov *et al.*, 2021b (see: Kolbasov *et al.*, 2007, 2021b). Among *y*-larvae in general (Table 1), matching nauplii and cyprids has been done with confidence only for *H. furcifera* and *H. itoi*, both of which have planktotrophic nauplii. Itô (1986a) identified an early nauplius as *H. pacifica* and noted certain similarities, but more differences, between it and nauplius type IV from European seas. Itô (1987b) then recanted this identification and designated the nauplius in question as ‘type XI’, while noting that its cyprid, although resembling that of *H. pacifica*, was smaller (see also: Itô & Takenaka, 1988). Kolbasov *et al.* (2007) summarized morphological information and provided a key to the seven nominal species of *Hansenocaris* that have described cyprids. They suggested informal groupings of those with a long cephalic shield and those with a short shield, supported by other characters. However, they admitted that these groupings are of hardly any taxonomic value, while a cladistic analysis employing a larger set of characters would be premature due the limited number of species.

The cyprid of *H. demodex* is in some respects different from all formerly described *y*-cyprids. All of these, except for that of *H. acutifrons* (see Itô, 1985), have a large, protruding process on the labrum that usually bears about five hooks (typically one apical and two subapical pairs posteriorly), which may be indicative of parasitism (attachment to host). In *H. demodex* this process is particularly extended and carries multiple rows of hooked spines that little resemble the arrangement in other species unless each cluster of five distal and three more proximal hooks corresponds to a single usual hook. The pair of small appendices in the midline anterior to the first antennae (Fig. 7E), interpreted here as the frontal filaments, have as far as is known not been documented in any other *y*-cyprid. The cephalic shield, as well as the entire body, especially the telson, of *H. demodex* is significantly more elongated than those of most other species, except for the even more elongated cephalic shield of *H. itoi* (Kolbasov & Høeg, 2003; Kolbasov *et al.*, 2021a). Among the described species, the cephalic shield of *H. demodex* most resembles that of *H. furcifera* in overall form (see: Itô, 1985), but the similarity is too general to be an indication of close relationship. The paraocular processes are small in the new species, but similar in size to those of, for example, *H. itoi*. Clear vestiges of the naupliar second antennae and mandibles are present in the examined cyprids (Fig. 7B–D) with an indication of biramosity, but such structures have also been reported in *H. furcifera* (Itô, 1989) and *H. itoi* (Kolbasov & Høeg, 2003), as well as in certain ascothoracidan ‘cyprids’ (e.g. Grygier, 1988, 1991b).

The thoracopodal protopod of *H. demodex* follows the general pattern for *y*-cyprids in being two-segmented (coxa and basis) in thoracopods 1–5. Based on our data, the unsegmented protopod of thoracopod 6 in all known *y*-cyprids originated by fusion of the coxa and basis (Fig. 10D). Uniquely among described *y*-cyprids, the exopods of all thoracopods in *H. demodex* are also unsegmented, or, if a rudimentary proximal segment is present, it is so tiny that it is concealed in the articulation zone between the basis and exopod (Figs 9A, B, 10D). In other *y*-cyprids the thoracopodal exopods are all two- or three-segmented. Endopodal segmentation of *y*-cyprids broadly defines two groups and may be of phylogenetic significance: either two-segmented in all limbs, with a small proximal and a long distal segment, or three-segmented in thoracopods 2–6 but two-segmented in thoracopod 1. Besides *H. demodex*, the first pattern, which must be a derived state arising from the fusion of the two distal segments in the second pattern, is displayed by *H. acutifrons* Itô, 1985, *H. pacifica* Itô, 1985, *H. rostrata* Itô, 1985, *H. spiridonovi* and, except for an unsegmented thoracopod 1, also by *H. tentaculata* Itô, 1986. In these species, segmental fusion in these endopods is indicated not only by a comparison of segment length, but also by the presence of an inner seta midway along the distal segment (Figs 9B, 10D, white arrows). In accordance with Schram’s (1970a) interpretation, this armature element is positionally homologous to the seta in the remaining three species, *H. furcifera*, *H. itoi* and *H. papillata*, that is located between the discrete and fully articulated second and third endopodal segments. Our data are limited, but a tentative pattern has emerged. The cyprids of at least some facetotectan species with planktotrophic nauplii (*H. furcifera* and *H. itoi*) have three-segmented thoracopedal exopods (thoracopods 2–6), while those of species with lecithotrophic nauplii (*H. demodex*) have only two segments. If this distinction applies generally,
then the currently unknown nauplii of *H. acutifrons, H. rostrata, H. spiridonovi* and *H. tentaculata* ought to be lecithotrophic, since their cyprids have two-segmented thoracopodal exopods.

Among the other distinctive features of *H. demodex* are the rounded, free pleural extensions of thoracic segment V (Fig. 9A), which are pointed in cyprids of most other species and quadrato in *H. spiridonovi*, and the unusually small, short segments of the abdomen (Figs 7I, 9A, 10A, E). The telson appears relatively longer than in other species and lacks the serrate spines along the posteroventral margin displayed by all of them, except for *H. tentaculata*. Finally, the furcal rami in *H. demodex* are shorter (perhaps even disc-like) than in any other species.

All cuticular surface structures (pores, setae, lattice organs, etc.) of the cyprid of *H. demodex* have been mapped using SEM (Table 5), the second time this has been attempted for any y-cyprid and the first with complete labelling, thus being able to serve as a baseline for future studies of y-cyprids. In total, 120 surface structures have been traced and are numbered in Figures 7–10. The pore patterns of y-cyprids may prove useful for the taxonomy of y-larvae at the species level, as demonstrated for the LSN mentioned above, but the full extent of this is as yet uncertain. For example, 56 pores and seta-bearing pits, mostly arranged in pairs, are present on the cephalic shield of *H. demodex*, excluding lattice organs and micropore fields. This is somewhat fewer than the 74 or more such structures that are reportedly present on the cephalic shield of *H. spiridonovi* (Kolbasov et al., 2021b); unfortunately, the latter structures were not fully mapped, precluding a detailed comparison. Due to the large quantity of pores and the lack of established landmarks on the cephalic shield, it is also practically impossible to homologize pores of the cephalic shield with any degree of certainty between the present cyprids of *H. demodex* and those of *H. furcifera* or *H. itoi*, two of the best-known species described by light microscopy.

For taxonomy, it may be more useful to compare ‘nose-prints’ based on SEM photos of the anterior face of the shield (e.g. Fig. 8B) or to search for characteristic pore patterns of smaller body parts other than the shield. An example of the latter may be the telson, which in *H. spiridonovi* has an unpaired posterior dorsal pore, two anterior dorsolateral pairs and two posterior ventrolateral pairs (Kolbasov et al., 2021b: fig. 5). In contrast, on the same surfaces of the telson, *H. demodex* has an unpaired anterior dorsal pore, two dorsal pairs along the dorsal-dorsolateral boundary ridge, three pairs in the upper row of lateral plates and four pairs in the lower row of lateral plates (Figs 9C, E, 10A, E). In particular, on each side of the telson the double-dyad anterior arrangement of four dorso- and ventrolateral pores (#48–#51) appears unique to the new species (Fig. 9A).

**Molecular diversity, taxonomy, phylogeny and the future of y-larvae systematics**

DNA barcoding and integrative taxonomic approaches have been applied in crustacean studies and larval systematics for over a decade (e.g. Palero et al., 2009, 2014; Tang et al., 2010; Raupach & Radulovici, 2015; Jäkel et al., 2020), but there have been practically no prior attempts to address the diversity or systematics of y-larvae from a molecular perspective. Pérez-Losada et al. (2009) sequenced three nuclear genes (Histone-3, 18S and 28S rDNA) of six unnamed taxa from Sésoko Island and of *Hansenocaris itoi* from the White Sea, but this was part of an effort to demonstrate the monophyly of Facetotecta and its position in the Thecostraca, not alpha-taxonomy. In fact, except for *H. itoi*, the precise taxonomic identity of the specimens they used remains unknown. Sampling of molecular data for y-larvae has been significantly increased by the present study, adding nucleotide sequences (partial 18S rDNA) for 22 y-larval specimens, mainly from Sésoko Island. These data grouped the specimens into four clades, thereby supporting the monophyly of *Hansenocaris demodex*, Itô’s (1986a) ‘Pacific type I’ and two undescribed types/species of y-larvae nicknamed by us as ‘Big brown’ and ‘Bumblebee’ (Figs 14, 15). 18S rDNA thus appears to provide a useful supplement to morphological characterization, although it must be emphasized that Facetotecta-specific primers targeting mitochondrial markers are greatly needed. In several invertebrate taxa, hypervariable regions of the 18S rDNA gene have been used to distinguish between genera and species (Wu et al., 2015), even though this gene is traditionally used to infer higher level phylogenies (Pérez-Losada et al. 2009; Wilson, 2009; Jäkel, 2004).

Some of the unnamed specimens sequenced by Pérez-Losada et al. (2009) appear to belong to the same type as some of our specimens photographed in life. Their ‘Facetotecta sp. 4’ (FJ751880) has the same nucleotide sequence as the above-mentioned ‘Big brown’ and probably represents the same species. Three of their taxa, ‘Facetotecta sp. 1, 2 and 6’ (FJ751877, FJ751878, FJ751882), were almost identical (> 99.8%), thus representing the same species, and are conspecific with four newly sequenced planktotrophic y-nauplii that were identified in this study as Itô’s (1986a) ‘Pacific type I’. However, two of Pérez-Losada et al.’s (2009) specimens, ‘Facetotecta sp. 3’ (FJ751879) and ‘Facetotecta sp. 5’ (FJ751881), did not match any of the newly sequenced specimens, which highlights the need to associate molecular data with voucher specimens or photos when sequences are deposited in GenBank.
Table 6. Overview of naupliar characteristics of four types of y-larvae from Sesoko Island (Japan) and Green Island (Taiwan) sequenced for molecular analysis as a part of this paper (see Figs 14, 15)

<table>
<thead>
<tr>
<th>Name and number of sequenced specimens</th>
<th>Sample numbers and collection data</th>
<th>Size</th>
<th>General morphology</th>
<th>Feeding strategy**</th>
<th>Naupliar developmental time††</th>
<th>Previous reports</th>
</tr>
</thead>
</table>

*Collected and processed by DEJ, MJG, YF, ND, JO.
*Collected and processed by ND, DEJ, JO.
*Length without posterior spines. Width at broadest point.
*Based on last-stage nauplius if known, otherwise on earlier nauplii. See Figure 14. LSN, last-stage nauplius; DC, dorso-caudal.
*L, lecithotrophic; P, planktrophic nauplii (based on absence/presence of feeding spines and labral extension, see more criteria in Results).
*Naupliar developmental time after sampling until moulting to cyprid. Hansenocaris demodex sp. nov. based on four specimens, ‘Big brown’ based on five specimens, ‘Bumblebee’ based on 20 specimens. The true developmental time from hatching (which has never been observed for y-larvae) until appearance of the cyprid may be longer.
The new phylogeny of Facetotecta presented here (Fig. 15) is the most comprehensive to date, but it is still provisional as it is based on only partial 18S rDNA data and includes less than 10% of the form variation sampled at Okinawa during this work. Nevertheless, the strong congruence with general larval morphology justifies a brief discussion of the phylogenetic implications. In the presented phylogeny, Facetotecta is split basally into two clades. Clade A is represented only by Hansenocaris itoi and two unnamed taxa from GenBank. Clade B is represented by H. demodex, two types of y-larvae with lecithotrophic nauplii (‘Bumblebee’ and ‘Big brown’) and one type with planktotrophic nauplii (‘Pacific type I’). These four types appear to be closely related, but the morphological disparity among the members of Clade B is significant. The nauplii of H. demodex, ‘Bumblebee’ and ‘Big brown’ are all elongate, cylindrical, posteriorly tapered larvae with no clear demarcation between the cephalic shield and the rest of the body, in contrast to many other lecithotrophic y-nauplii that have a rounded ‘belly’, a distinct and often upturned dorsocaudal spine, and a clearer delineation between the cephalic region and the trunk as seen in dorsal view (e.g. Itô, 1991; Høeg et al., 2014). More particularly, owing to the reduction of the dorsocaudal and furcal spines, the nauplii of H. demodex and ‘Bumblebee’ both display a blunt terminal end of the trunk (Fig. 14A, B). Confirmation of a close relationship among the lecithotrophic species in clade B will require detailed studies of both nauplii and cyprids, which are pending.

Among the four types of y-larvae in clade B, the one we have identified as Itô’s (1986a) Pacific type I deviates the most. The morphological differences between the lecithotrophic nauplii of H. demodex, ‘Big brown’ and ‘Bumblebee’, and the nauplii of their tiny planktotrophic relative, Pacific Type I (Fig. 14; Table 4), are remarkable compared to, for example, barnacle larvae. In the Cirripedia, nauplii of closely related species mostly resemble each other, irrespective of the different habitats inhabited by adult barnacles (Chan et al., 2014). Planktotrophy in marine larvae is often, but not universally, considered plesiomorphic, and lecithotrophy derived (Rouse, 2000; Nielsen, 2007; Collin & Moran, 2018). In y-larvae, plesiomorphic planktotrophy of nauplii is congruent with the phylogeny in Figure 15, as the planktotrophic Pacific type I is the sister-group to the three lecithotrophic types and another planktotroph, H. itoi, appears in clade A. The evolutionary polarity of planktotrophic vs. lecithotrophic feeding in y-nauplii, as well as the apparently huge morphological diversity of y-larva (c.f. Figs 2, 14, 15; Grygier et al., 2019), clearly need to be assessed in a sequence-based multilocus phylogeny based on broader (more taxa) and more robust (more genes) data.

**CONCLUSIONS**

- **Y-larvae** occur locally in large quantities and with considerable diversity; y-larvae (both nauplii and cyprids) caught inshore at, e.g. Sesoko Island (Japan), are practically unidentifiable, mostly not corresponding either to previously described nominal species or to currently recognized ‘types’.
- The current taxonomic approach involves parallel systems of nomenclature, with formally described nominal species being based on incomparable life-history stages and many naupliar types being included in a Roman-numeral-based paratymology; it fails to reflect the true diversity of y-larvae.
- An integrated taxonomical approach is presented that combines rearing through several moult stages, live photography, detailed microscopy of selected specimens and molecular techniques (DNA barcoding), in order to establish a reliable standard for future species descriptions (at least for lecithotrophs). Future species descriptions of y-larvae with lecithotrophic nauplii should be based on a combination of last-stage nauplii and cyprids.
- A more complete assessment of y-larval diversity in any given region is needed in order to: (1) provide identification keys; (2) match y-nauplii and y-cyprids of the same species; and (3) not least, assign via molecular data already available species names for larvae to the corresponding y-adults once the nature of these (parasitic?) becomes known.
- During fieldwork at Green Island (Taiwan) and Sesoko Island (Japan) in 2017–19, about 11 000 y-larvae were sampled and handled, more than 25 times the number reported in any previous study. Extensive sampling and sorting were fundamental to the development of the novel methodology presented in this paper.
- To demonstrate the proposed methodology, a morphologically unique form of y-larva, Hansenocaris demodex, is described based on material from Sesoko Island (Japan) and Green Island (Taiwan). Its life cycle displays a naupliar phase with elongate, yellow/orange-coloured stages and a cyprid bearing an unprecedentedly large number of hooks in a particular pattern on its labrum.
- Specimens of H. demodex from both localities exhibit only minor differences, for example, in the size of the cyprid’s telson (relatively shorter at Green Island).
- Hansenocaris demodex is the first formally described y-larva with lecithotrophic nauplii for which both nauplii and the cyprid are known. All cuticular surface structures (pores and setae) for both the last-stage nauplius (LSN) (63 structures

© 2022 The Linnean Society of London, Zoological Journal of the Linnean Society, 2022, **XX**, 1–44
over the entire body) and the cyprid (120 structures) are fully mapped. This is done for the first time for y-larvae to provide a baseline for exploring the importance of pore/setae patterns in classifying species of this group.

- *Hansenocaris demodex* is the first formally described lecithotrophic y-larva for which more than one stage in the naupliar development has been studied. The naupliar phase consists of at least five to six instars, approaching the seven instars currently inferred for the best-studied planktotrophic species, *H. itoi*.
- The largest molecular diversity dataset for Facetotecta compiled so far is presented here, with 22 individual y-larvae sequenced anew, representing four different types (including *H. demodex*).
- Our preliminary phylogenetic tree, based on partial 18S rDNA sequences, shows significant congruence with larval morphology, supporting the utility of hypervariable regions of this marker as a barcoding tool for y-larvae.
- Based on 18S rDNA sequences of specimens identified from photographs, four out of six unnamed ‘species’ uploaded to GenBank in a previous work (*Pérez-Losada et al.*, 2009) could be identified as belonging to either Itô’s (1986a) ‘Pacific type I’ or to a form nicknamed ‘Big brown’.

ACKNOWLEDGEMENTS

Fieldwork at the Sesoko Station in 2018 and 2019 could not have been carried out without the helpful support of Yoshikatsu Nakano and other members of the staff at the station, who kindly provided excellent lab facilities. We are also grateful for the support from Ming-Jay Ho at the Marine Science Thematic Center, Academia Sinica at Green Island, Taiwan. Special thanks go to Vanessa Pei-Chen Tsai for tireless support of all aspects of the work at both Green Island and Academia Sinica in Taipei. This work has been generously supported by a VILLUM Experiment grant (project no. 17467) to JO from the Velux Foundations. ND was jointly supported by a double-degree graduate grant from the Biodiversity Research Center, Academia Sinica, and the Natural History Museum of Denmark. MJG’s work was enabled by support to the Center of Excellence for the Oceans at National Taiwan Ocean University from the Featured Areas Research Center Program of the Taiwan Ministry of Education’s Higher Education Sprout Project and by a grant from Taiwan’s Ministry of Science and Technology (MOST 108-2611-M-019-002-). FP acknowledges the projects ‘CIDEGENT/2019/028 – BIOdiversity PATterns of Crustacea from Karstic Systems (BIOPACKS): molecular, morphological, and functional adaptations’ funded by the Conselleria d’Innovació, Universitats, Ciència i Societat Digital and ‘PRO2020-S02-PALERO – Fauna aquàtica en coves anquihalines del País Valencià: un mon encara per descriure’ funded by the Institut d’Estudis Catalans. Joachim Haug (Munich) and Rony Huys (London) are thanked for valuable comments on a late version of the manuscript. JO and ND dedicate this paper to mentor, former advisor and friend Jens Thorvald Høeg for his great contributions to barnacle larval studies and for providing invaluable inspiration and support at the onset of the study.

AUTHOR CONTRIBUTIONS

JO, ND and MJG conceived the project and designed the experimental approach. JO, ND, FP, DEJ, YF and MJG performed experiments and carried out fieldwork. JO took photographs/videos and prepared figures. ND carried out molecular lab work and sequence analyses. FP designed oligonucleotide primers and supervised molecular work. BKKC contributed reagents, molecular lab facilities, and supervised molecular work. JO secured funding for fieldwork. BKKC and JO secured funding for molecular work. JO wrote the first draft. ND, FP, DEJ, BKKC and MJG edited the first and subsequent drafts. All authors read and approved the final version of the manuscript.

CONFLICT OF INTEREST

All authors declare that they have no conflicts of interest.

DATA AVAILABILITY

Most morphological data supporting the taxa described here are available directly as figures in the paper. A short video showing live larvae from the paper can be seen here: https://youtu.be/seo-63AK10E. More images/videos are available from the corresponding author, JO, upon reasonable request. Sequences used to estimate phylogenies are deposited in GenBank under accession numbers OM135272–OM135293. The code, alignment and IQ-TREE log files are stored at https://doi.org/10.6084/m9.figshare.17803628.v1.

REFERENCES


 Thomon JV. 1830. On the cirripedes or barnacles; demonstrating their deceptive character; the extraordinary metamorphosis they undergo, and the class of animals to which they undisputably belong. *Zoological Researches, and Illustrations; or Natural History of Nondescript or Imperfectly Known Animals Vol. 1, Pt. 1, Mem 4*: 69–82.


