Incipient speciation within the *Namalycastis abiuma* (Annelida: Nereididae) species group from southern India revealed by combined morphological and molecular data

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Abstract

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Namalycastis abiuma (Grube, 1872), originally described from Brazil, comprises a species complex of morphologically similar forms occurring circumtropically, including India. Apart from the *Namalycastis abiuma* species group, four other *Namalycastis* species are presently known from India: *N. indica* Southern, 1921, *N. fauveli* Nageswara Rao, 1981, *N. glasbyi* Fernando & Rajasekaran, 2007, and *N. jaya* Magesh, Kvist & Glasby, 2012. Recent sampling along the southern Indian coast has uncovered new specimens of the *N. abiuma* species group. The present study uses combined morphological and molecular data (DNA barcoding) to explore species boundaries within the complex in southwest India and thereby resolve existing taxonomic confusion. In order to evaluate morphological variability within the *N. abiuma* species group, a total of 50 specimens were sampled from different geographical regions in southern India, and assessed using traditional methods. For 18 of the specimens, a 509 bp fragment of COI, the proposed DNA barcoding gene, was sequenced and subjected to tree reconstruction using both distance methods and maximum parsimony. Based on similarity alone, six different haplotypes were recognized within the dataset and these were also subsequently recovered as six distinct clades in the parsimony analysis. There is significant concordance between the morphotypes and the genetic haplotypes, suggesting that significant structural forces are acting on the specimens at a population level, and that these specimens may even be in an early stage of speciation.

Keywords

Nereididae; Namanereidinae; *Namalycastis abiuma*; Taxonomy; Phylogeny; Genetic variation; DNA barcoding.

Introduction

The subfamily Namanereidinae (Nereididae) consists of three genera (*Namalycastis* Hartman, 1959; *Namanereis* Chamberlin, 1919; and *Lycastoides* Johnson, 1903) and is most prominently known from the tropics and subtropics. Thirty-nine namanereidin species have been reported throughout the world and some of these pertain to larger complexes of problematic species, herein termed "species groups". The genus *Namalycastis* contains 22 species (Glasby 1999a, 1999b; Magesh et al. 2012; Conde-Vela 2013), four of which have so far been recorded on the Indian subcontinent. These include *N. indica* Southern, 1921, *N. fauveli* Nageswara Rao, 1981, *N. glasbyi* Fernando & Rajasekaran, 2007, and *N. jaya* Magesh,

Kvist & Glasby, 2012. In addition, there are several records of the *N. abiuma* species group in southern India (Glasby 1999a; Magesh et al. 2012).

The *N. abiuma* species group concept was introduced by Glasby (1999a) for a group of individuals that 'exhibit a greater amount of morphological variation over their range than is typical for a namanereidinae species' (Glasby 1999a: 115). It was expected that such species groups 'will probably be found to contain more than one species with further characterisation of reproductive mode and genetics' (P. 115); that is, species groups likely comprise morphologically cryptic species. Indeed the first cryptic species, *N. jaya,* was discovered recently (Magesh et al. 2012). Cryptic species are recognised

when the species group hypothesis of Glasby (1999) is falsified by independent data, which in the present study is the DNA barcode gene. Our concept of species most closely follows the Synapomorphic Species Concept as defined by Wilkins (2003: 635): 'A species is a lineage separated from other lineages by causal differences in synapomorphies'.

Recognition of the *Namalycastis abiuma* species group follows Glasby (1999a). It has a noticeably broad diagnosis, but may be distinguished from many other *Namalycastis* species by having brown epidermal pigment on the dorsal side of the body (including the pygidium), short antennae (not extending beyond the tip of the palpophore) and coarsely serrated spinigerous chaetae (but not falcigerous chaetae) in parapodia of the posterior part of the body. The concept includes several separately described species including *Lycastis meraukensis* Horst, 1918 (described from New Guinea), *L. nipae* Pflugfelder, 1933 (Sumatra), *L. vivax* Pflugfelder, 1933 (Sumatra), *Namalycastis rigida* Pillai 1965 (Philippines) and *N. meraukensis var. zeylanica* (Sri Lanka) (Glasby 1999). Although the association of these species with the *N. abiuma* species group in Glasby (1999a) was intended to represent formal synonymy, the fact that the ICZN rules do not apply to species groups, means that all of these names, with the exception of the variety *N. meraukensis var. zeylanica* (also not covered by ICZN), are currently valid and potentially available to newly discovered cryptic species.

The most widely reported *Namalycastis* species in India, *N. indica,* is very similar to the *N. abiuma* species group in external appearance, and unless chaetal types and distributions are examined carefully, the two species are very difficult to separate. Most descriptions of *N. indica,* in the taxonomic literature fail to give an adequate account of chaetal types and distributions and it is therefore quite possible that the two species have been extensively confused (Glasby 1999). Doubtful taxonomic references to *N. indica* include those of Ghosh (1963), Day (1967), and Sunder Raj & Raj (1987). Because of this potentially wide confusion of specimens pertaining to the *N. abiuma* species group and the morphological similarity with *N. indica,* the addition of molecular tools for separation of species is becoming increasingly pressing. Such tools, if applied correctly, would enable taxonomists to both evaluate synonymous taxa and to separate this species complex into distinct taxa. Thus, studying the genetic variations within these species groups is important for inferring solid species diagnoses and in identifying potentially novel species, as well as addressing the question of how many species (*sensu* Wilkins, 2003) are present within these species groups. Here, we shed some light on part of this issue by examining specimens that are morphologically compatible with *N. abiuma* from different regions across the Indian subcontinent, and use both molecular and morphological techniques to clarify taxonomic ambiguity.

Materials and methods

Specimen collection

Between January 2008 and December 2009, polychaete worms were collected from various localities, at varying salinities and depths, along the southern Indian coast; Kadinamkulam Lake (depth 2 m), Kayamkulam Kayal (2-3 m), Cochin (Kochi)

estuarine system (3 m), Thoothukudi mangroves (Tamilnadu; 1 m) and the Ariankuppam estuary of Puducherry (0.5 m) (Fig. 1). The sites were selected based on the habitat suitability and the presumed presence of *Namalycastis* spp. For the better part, specimens were collected from muddy sediments; at Kadinamkulam Lake, mud was commonly mixed with slightly rotting organic matter. All specimens have been deposited in the Zoological Reference Collection of the Zoological Survey of India, Kozhokode, Kerala, India.

Morphological examinations

Sampling strategies and identifications followed the method of Glasby (1999a) and Magesh et al. (2012). Descriptions are based on the same character set used by Glasby (1999a). After securing tissue for DNA extraction (see below), specimens were relaxed in isotonic $MgCl_2$, quickly submerged in 95% ethanol to evert the proboscis, fixed in 10% formalin and subsequently transferred to 70% ethanol. Fixed specimens were then dissected and the parapodia were mounted in polyvinyl lactophenol on microscope slides to enable microscopical examinations of the morphology. Tissues to be used for DNA sequencing were fixed in 95% ethanol and their further processing is described below.

DNA sequencing and analyses

A total of 18 specimens, identified as the *N. abiuma* species group were chosen for the molecular portion of this study and five specimens, including *N. jaya* and *Platynereis bicanaliculata* (Baird, 1863) were used as outgroup taxa; the trees were rooted with *P. bicanaliculata*. A complete list of specimens, sampling sites, and GenBank accession numbers can be found in Table 1.

Approximately 20-40 chaetigers of the posterior part of the worms (excluding the pygidium) were used for DNA extraction. Total genomic DNA was isolated from the specimens following the extraction protocol of Miller et al. (1988). Partial sequences of cytochrome *c* oxidase subunit I (COI) were PCR-amplified using the primers suggested by Ivanova et al. (2007) (i.e., FR1d [5'- TTCTCCACCAACCACAARGAYATYGG-3'] and FR1d_t1 [5'- CACCTCAGGGTGTCCGAARAAYCARAA-3']). The PCR used 30 cycles of the following protocol: an initial 5 minute denaturation step at 94°C for all samples, followed by 30 seconds denaturation at 94°C, 30 seconds annealing at 55°C, 2 minutes extension at 72°C and a final 5 minute extension step at 72°C for all samples. PCR products were subsequently checked on a 2% agarose gel and successful amplifications were gel eluted using a chromous gel extraction kit (Gel Extraction SPIN-50, Chromous Biotech, Bangalore, India) following the instructions given by the manufacturer. The DNA was then purified using a PureFast Genomic DNA purification kit (Helini Biomolecules, Chennai, India), cycle sequencing was carried out using the same primers as above, and ethanol precipitation prepared the DNA for sequencing. Nucleotide sequencing was then performed using an ABI 3500 XL Genetic Analyzer (Applied Biosystems, Foster City, CA). All nucleotide sequences are deposited at NCBI; accession numbers are presented in Table 1.

Assembly of forward and reverse strand sequences was carried out using BioEdit ver. 7.0.5.2 (Hall 1999), and reconciled sequences were aligned using MAFFT ver. 7 (Katoh & Standley
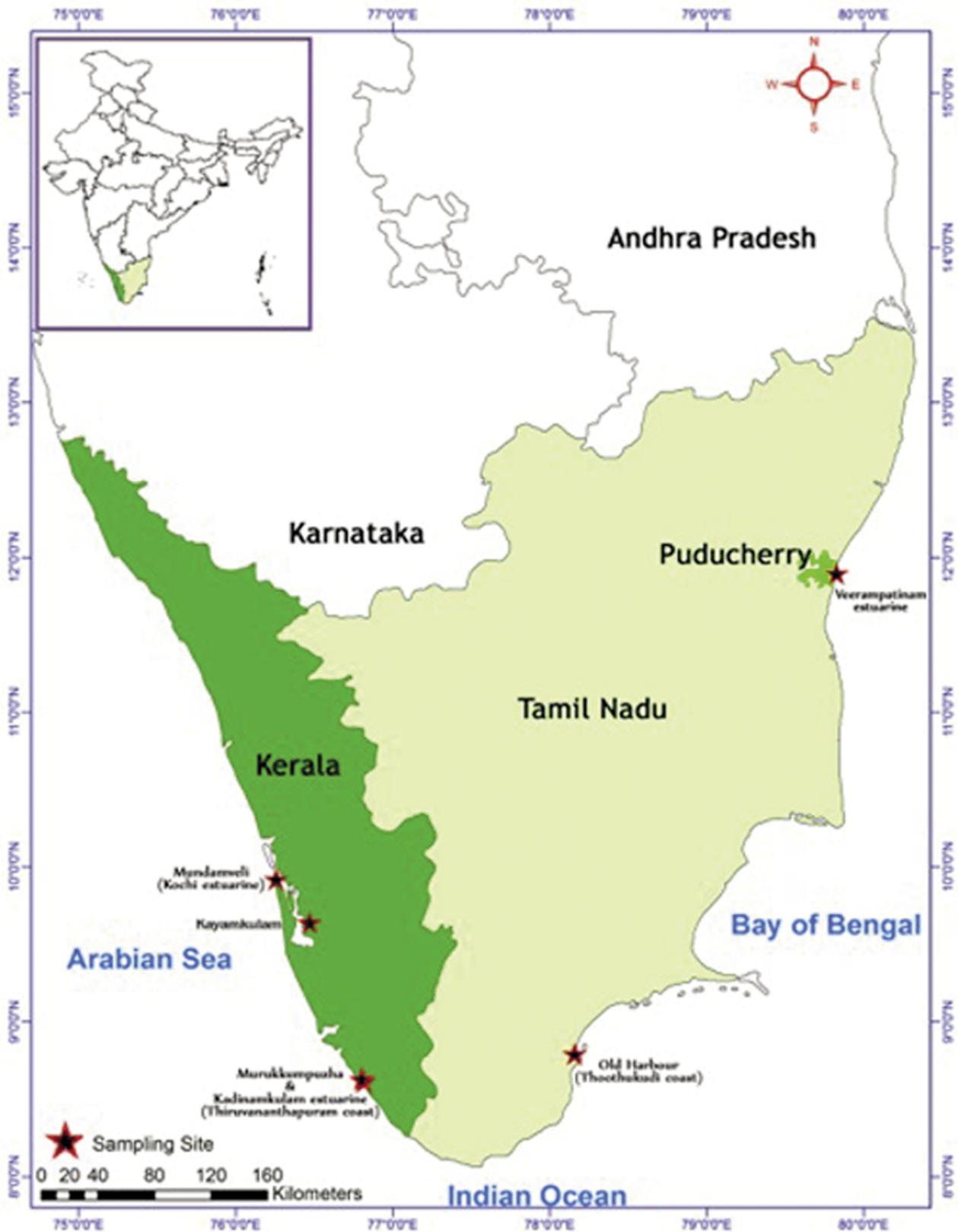
Figure 1. Map of the collection localities for the specimens of the *Namalycastis abiuma* species group.

2013) applying the L-INS-i strategy and default settings; note that the final alignment was devoid of gaps such that the sequences can be treated as pre-aligned. Intraspecific and interspecific variations were then calculated using MEGA ver. 5.2.2 (Tamura et al. 2011) with the following settings: uncorrected-p distances, pairwise deletion of gaps and using 1st, 2nd and 3rd codon positions. A Neighbour-Joining tree was constructed in PAUP* ver. 2.0b10 (Swofford 2002) using uncorrected-p distances. Also, a phylogenetic tree under the criterion of maximum parsimony was constructed using PAUP, where a heuristic search was performed employing 1000 initial addition sequences and TBR branch swapping. Support values for the nodes were estimated through bootstrap resampling using 100 random addition sequences and the same settings as above.

Results

Morphological analyses

In total, 50 specimens were collected that were identified to the *Namalycastis abiuma* species group. These specimens all fit within the range of the known intraspecific variation of the species, as follows:

Diagnosis. Brown epidermal pigment dorsally and on pygidium; prostomium with shallowly cleft anteriorly, antennae extending short of tip of palpophore; jaws with a single robust terminal tooth , 4-5 subterminal teeth, 3-5 teeth ensheathed proximally; notochaetae present or absent; neurochaetae arrangement Type A sensu Glasby (1999a); subneuroacicular falcigers in parapodia of chaetiger 10 with blades 4.3-5.7× longer than width of shaft head and having 4-15 fine to moderate sized teeth; subneuroacicular falcigers in parapodia of chaetiger 10 with blades 3.7-7.2 x longer than width of shaft head, up to 18 teeth; subneuroacicular spinigers in parapodia of posterior body with blades coarsely serrated proximally; heterogomph chaetae with boss not prolonged; pygidium with multi-incised rim (modified slightly after Glasby 1999a).

Preliminary morphological investigations suggested that the specimens can be further subdivided into two morphologically distinct lineages (subgroup 1 and 2). The morphological and some ecological characteristics of the subgroups are further discussed below. Within subgroup 1, a total of four morphotypes could be identified and within subgroup 2, an additional two morphotypes were found for a total of six morphotypes among the 50 specimens (Table 1).

Subgroup 1

Thirty-seven out of the 50 specimens were categorized as subgroup 1 on the basis of morphological characters.

Diagnosis: as for the *N. abiuma* species group except body uniform in width anteriorly, tapering gradually posteriorly. Eyes, 2 pairs, black, arranged obliquely, unequal in size, posterior pair larger than anterior pair. Posterodorsal tentacular cirri short and extending posteriorly to end of first chaetiger. Jaws with, 6 or 7 subterminal teeth and 4 teeth unsheathed proximally. One or two notochaetae per notopodium. Notopodial sesquigomph spinigers observed from chaetigers 5-11 until mid body; spinigers absent in posterior part of body. Supra-

neuroacicular sesquigomph spinigers in chaetiger 10. Supra-neuroacicular falcigers in chaetiger 10 moderately serrated and teeth non-uniform in length. Sub-neuroacicular spinigers in chaetiger 10 with medium or finely serrated blades; blades with coarse serrations proximally posteriorly from chaetiger 30-120.

Intraspecific variation among morphotypes

M1. Specimens AQPA1- AQPA 10: Notochaetae present from chaetiger 8 to mid body, one or two per notopodium, in no particular order. Jaws with 11 teeth (Fig. 2A).

M2. Specimens AQMM1- AQMM10; AQMM51 – AQMM55: From fifth chaetiger to mid body, notochaetae single (rarely two; e.g. AQMM51) or absent in many mid-body parapodia. Jaws with 9 teeth (Fig. 2C). Number of homogomph spinigers usually greater than number of heterogomph chaetae; only subneuroacicular spinigers present in some chaetigers (e.g. chaetiger 18).

M3.Specimens AQMM82 & AQMM92: Notochaetae single or absent in posterior podia. Jaws with 10 teeth (Fig. 2B).

M4. Specimens AQMM6 and AQMM61-AQMM65: Parapodium and dorsal cirrus very wide in middle and posterior chaetigers (Fig. 2K). Dorsal cirri increase in length posteriorly (Fig. 2J).

Subgroup 2

Thirteen out of the 50 specimens were categorized as species group 2 on the basis of morphological characters.

Diagnosis: as for *N. abiuma* species group except, entire body with width tapering posteriorly. Antennae extending to tip of palpophore. Eyes equal or unequal in size (with posterior pair smaller); sometimes faded or absent.

Posterodorsal tentacular cirri long and extending posteriorly up to chaetiger 4 or 5. Jaws with 8 teeth (Fig. 2D). Notochaetae present from chaetiger 10-12. Supra-neuroacicular falcigers in chaetiger 10 with blades moderately or coarsely serrated, about 11 teeth. Sub-neuroacicular falcigers in chaetiger 12 with 14 teeth. Sub-neuroacicular spinigers in anterior body with blades finely serrated and sub-neuroacicular spinigers in posterior body with coarsely serrated blades.

Intraspecific variation among morphotypes

M5. Specimens K1-K10; K51-K55: Eyes are faded in a few specimens (e.g. K1, K3 and K53), absent in a few specimens (e.g. K5-10), and merged in a few specimens (Figs. 2I, 2E and 2F, respectively).

M6. Specimens K24 and K242: Three eyes present and about equal in size (Fig. 2G, H). Three acicula (rather than the usual two) present in chaetiger 10; Figs. 2L and 2M).

DNA barcoding and Neighbour-Joining

A total of 18 specimens were successfully sequenced for a 509 bp region of COI, representing all of the six morphotypes specified above (M1-M4 in species group 1, and M1-M2 in species group 2). In addition, COI sequences from *Namalycastis jaya* (HQ456363 and JN790065-67) and *Platynereis*
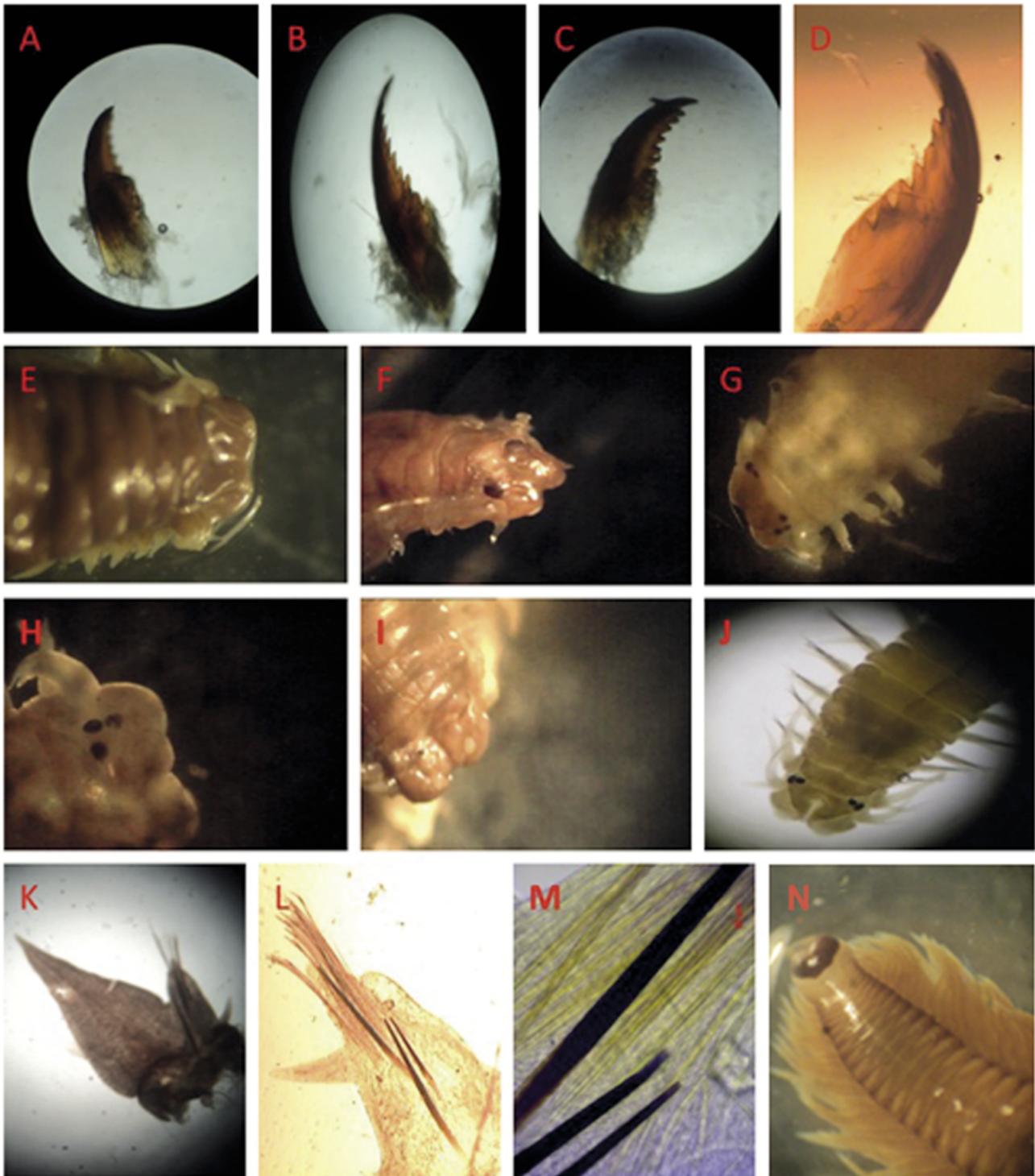
Figure 2. Selected morphological characters of the various *N. abiuma* species group morphotypes (M1-M6). A, M1, jaws with 11 teeth; B, M3, jaws with 10 teeth; C, M2, jaws with 9 teeth; D, M5 and M6 jaws with 8 teeth; E, M5, specimen with eyes absent; F, specimen with merged eyes; G-H, M5, specimens with three eyes; I, M5, specimen with faded eyes; J, M4, specimen with the longer tentacular cirri, indicative of species group 2 (see text); K, M4, specimen with relatively wider parapodium; L-M, M6, specimens showing three acicula; N, multi-incised pygidium.

bicanaliculata (GU362685) were downloaded from NCBI to allow for a wider range of interspecific comparisons, as well as phylogenetic analysis. The final alignment was devoid of inserted gaps, such that the sequences could be treated as pre-aligned. The genetic divergence comparisons between haplotypes are presented in Table 2. Genetic variation within the total dataset of '*N. abiuma*' specimens averaged 0.69% ± 0.21 and ranged between 0 (in several comparisons) and 0.99% (for specimens AQMM6, AQMM62-63 [haplotype H2] when compared against K5, K52-53 [haplotype H3]). As a reference, the average genetic variation between the '*N. abiuma*' haplotypes and *N. jaya,* and *P. bicanaliculata* was 1.42% ± 0.33, and 24.61% ± 0.19, respectively.

As a complement to the genetic distances, the resulting Neighbour-Joining (NJ) tree is presented in Fig. 3. The tree includes five main clades, four of which include representatives of the *N. abiuma* species group – specimens of *N. jaya* represent the remaining clade. These clades correspond to the haplotypes in the genetic variation analysis. This finding suggests some level of population structure and possibly incipient speciation within the specimens of the *N. abiuma* species complex treated in the present study. However, at the same time, the clades recovered in the NJ tree do not entirely reflect the morphotype separation. Specifically, the clade comprising specimens of haplotype H1 includes specimens displaying three different morphotypes (M1-M3; Fig. 3). The remaining clades each include only a single morphotype such that both morphology and molecules corroborate the separation of members of these clades.

Phylogeny

Nineteen out of the 509 aligned positions in the final COI dataset were parsimony informative. The heuristic search resulted in two equally parsimonious trees and these are presented in Figs. 4a and b, respectively. The resulting topologies are largely congruent with that of the NJ tree, as expected. Bootstrap support (BS) is low across the tree, most likely owing to the numerous identical haplotypes present in the dataset, in combination with the low number of parsimony informative characters. Notably, specimens of the *N. abiuma* species group do not form a monophyletic group, since specimens of *N. jaya* nest within the clade. In both of the most parsimonious trees, haplotype H2 (AQMM6, AQMM62 and AQMM63) is recovered as the sister group to the remaining taxa with bootstrap support (BS) of 61% (Figs. 4a and b). However, the trees disagree on the sister group of specimens pertaining to haplotype H1 (AQPA3-5, AQMM5, AQMM7-9, AQMM52 and AQMM92): in one of the trees, *N. jaya* is recovered as the sister group (BS <50%), whereas a clade containing haplotypes H3 and H4 (K24, K242, K5, K52 and K53) is recovered as the sister group of haplotype H1 in the remaining tree (BS 56%).

Discussion

In combination with the NJ and parsimony trees, the intraspecific versus interspecific variation within the dataset used here conclusively shows that the *N. abiuma* species group
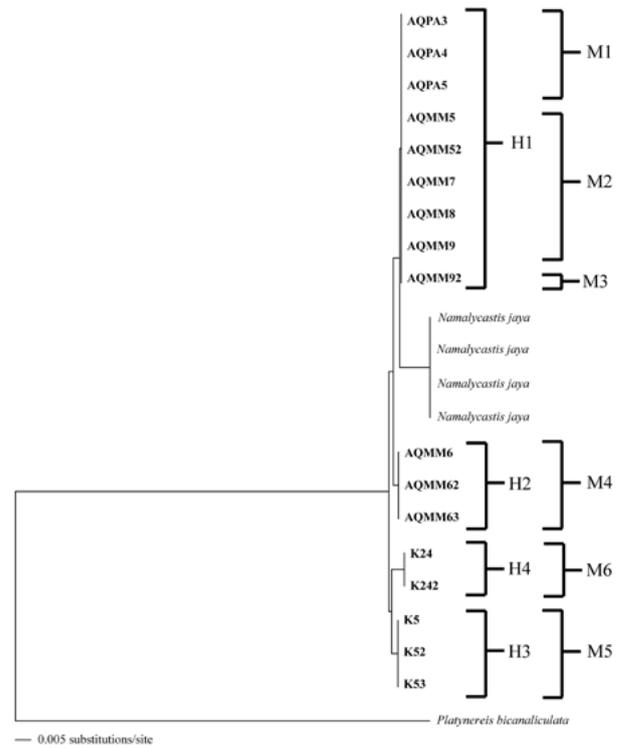

Figure 3. Neighbour joining tree derived from the COI dataset. Specimens pertaining to the *N. abiuma* species group are indicated in bold font, and morphotype (M1-M6) and haplotype (H1-H4) numbers, as referred to in text, are denoted by the brackets

harbours more intraspecific diversity than previously noted. By and large, there is high congruence between the separation of morphotypes and haplotypes in the dataset. There is some evidence towards a separation, so far only at a population level, between haplotype H2 (specimens AQMM6, AQMM62 and AQMM63; also corresponding to morphotype M4) and the remaining haplotypes. This is supported both by morphology (specimens within morphotype M4 possess a wider parapodium and dorsal cirrus in middle and posterior chaetigers than other specimens), genetic distances (0.88% average distance when compared to remaining *N. abiuma* haplotypes) and phylogenetics (haplotype H2 constitutes a separate clade, as sister to the remaining specimens). Comparable patterns of congruence between morphology and molecules, when focusing on the separation of populations, were also recovered for haplotypes H3 (corresponding to morphotype M6) and H4 (corresponding to morphotype M7).

Both of the most parsimonious trees recover specimens of *N. jaya* nested within the major clade of the *N. abiuma* species group (albeit with negligible support). As a result, the detailed topology of the trees (Figs. 4a and 4b) further suggests the separation of haplotype H2 from the remaining taxa. This may indicate that haplotype H2 may be in a later stage of speciation than the remaining haplotypes, based on its phylogenetic
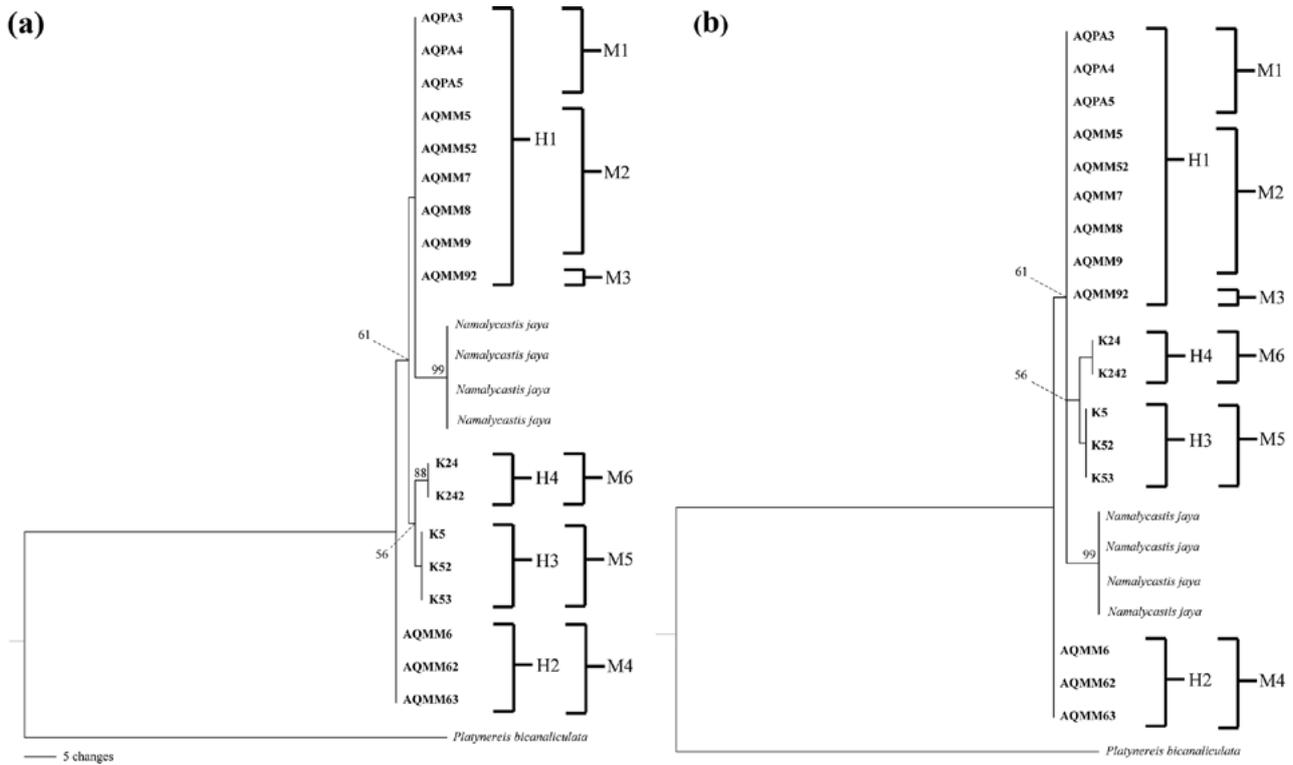
Figure 4. A-B shows the two equally parsimonious trees recovered from the phylogenetic analysis based on the COI dataset (length: 153, CI: 0.896, RI: 0.935). Specimens pertaining to the *N. abiuma* species group are indicated in bold font, and morphotype (M1-M6) and haplotype (H1-H4) numbers, as referred to in text, are denoted. Bootstrap support values above 50% are shown at each node. Branch lengths are drawn proportional to change.

position in the parsimony trees. However, the average COI genetic distance between haplotype H2 and the remaining specimens of the *N. abiuma* species group is lower than normal estimations of interspecific divergence (e.g., Hebert et al. 2003a, 2003b; Smith et al., 2005; Ratnasingham & Hebert, 2007). It thus seems premature to formally separate haplotype H2 from the remaining *N. abiuma* specimens, but our results suggest that these specimens may be in early stages of speciation.

The material included in the original description of *Namalycastis indica* was collected both from Calcutta and Cochin Backwater (Southern 1921) and specimens from the different locations differed slightly in their morphology (e.g. antennae shape, teeth count on jaw and size of tentacular cirri; Glasby 1999). The present study seems to include both of these variants as some specimens differed in their possession of shorter (Kadinamkulam and Cochin) or longer (Kayankulam) tentacular cirri. However, a number of globally distributed *Namalyastis* species have previously been incorrectly described as distinct separate species, and several of these were later assigned to the *N. abiuma* species group (although not formally synonymised; see Introduction) by Glasby (1999). These include *Lycastis meraukensis* (Horst, 1918; Fauvel, 1932), *Lycastis indica* (Horst, 1924; Fauvel, 1932; Aziz, 1938; Ghosh, 1963), *Namalycastis* cf. *abiuma* (Hutchings & Glasby, 1985),

Lycastis nipae (Pflugfelder, 1933), *Lycastis vivax* (Pflugfelder, 1933), *Lycastis senegalensis* (Monro, 1939), *Lycastris* [sic] *indica* (Day, 1951), *Namalycastis rigida* (Pillai, 1965) and *Namalycastis meraukensis zeylanica* (Silva, 1961) (see Glasby (1999) for a full account of synonyms). Therefore, it would be premature to conclude that some specimens of the present study indeed represent both variants found by Southern (1921).

One of the strangest findings of the present study is the occurrence of three eyes (in one side) and three acicula (10th chaetiger) in morphotype M2. This is the first record of a *Namalycastis* species possessing such a set of characteristics. However, a more rigorous and taxon-inclusive morphological assessment is needed prior to drawing any conclusions. For example, it is still possible that the polluted environment of the Kayamkulam collection area is the cause of this oddity. This is particularly plausible, seeing as the genus already presents several adaptations of the eyes to low-salinity or semi-terrestrial habitats (Sadasivan Tampi 1949, Storch & Welsch 1972).

In conclusion, the *N. abiuma* species group does seem to possess a higher degree of diversity than currently reflected in the taxonomy, as was suggested by Glasby (1999). Because of the somewhat confusing morphology of these species, it is important that future studies also include molecular information.

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