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A combined morphological and molecular approach in identifying barnacle cyprids from the Matang Mangrove Forest Reserve in Malaysia: essentials for larval ecology studies

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Abstract. Identification of larval mesoplankton is essential to the study of the supply-side ecology of marine benthic or sessile organisms, such as barnacles. Combined morphological and molecular identifications of wild-caught barnacle cyprids from Matang Mangrove Forest Reserve (MMFR), Malaysia were studied based on mitochondrial 12S-rRNA gene sequences of the unidentified larvae and identified adults. Six species of barnacle adults and cyprids had matched DNA sequences. These included *Fistulobalanus pattellaris*, *Fistulobalanus* sp., *Amphibalanus reticulatus*, *Amphibalanus variegatus*, *Amphibalanus amphitrite*, and *Euraphia withersi*. Morphological characters of the identified cyprids were described, and used to develop a morphology-based classification tree. Carapace sculpturing pattern on the cyprids was the most important morphological discriminator. Preliminary analysis of the diversity of barnacle cyprids in MMFR showed that the dominant species could be morphologically classified with high accuracy.

Key words. larval ecology, barnacle cyprids, classification, DNA barcoding, mangrove

INTRODUCTION

Thoracican barnacles are important filter feeders in the mangrove food web (Fry & Smith, 2002) and can play a role in the filtration function of mangroves (Soares-Gomes et al., 2010). They are common on the surface of roots, trunks, and leaves of mangrove plants, fallen propagules and plant debris, and shells of crustaceans and molluscs. In replanted mangrove system, barnacles are considered as pests because their settlement on the stems and leaves can result in mortality or reduced fitness of mangrove seedlings (Perry, 1988; Li & Chan, 2008; Li et al., 2009). In fact, barnacle infestation on newly replanted mangrove seedlings is recognised as one of the important problems in mangrove rehabilitation (Angsupanich & Havanona, 1996; Primavera & Esteban, 2008).

The life cycle of thoracican barnacles is composed of both planktonic larval and sessile adult stages. The planktonic larvae include six naupliar stages and a final cyprid stage prior to settlement. The distribution of the barnacle cyprids in the water column is patchy on spatial and temporal scales (Pineda, 2000) which can affect the subsequent recruitment dynamics of adults (Grosberg, 1982; Pineda et al., 2002), including those that inhabit the mangrove ecosystem (Ross & Underwood, 1997; Satumanatpan et al., 1999; Ross, 2001; Satumanatpan & Keough, 2001). In replanted mangroves at Ban Don Bay, Thailand (Angsupanich & Havanona, 1996), and Haji Dorani, Malaysia (Tan, 2013), the pulse recruitment of barnacle cyprids is often intense, resulting in rapid cover by barnacles on the replanted mangrove. The supply-side ecology of barnacle cyprids is, therefore, important to understanding the distribution and larval settlement processes of barnacles in mangroves. However, the remarkable similarity of cyprid morphology among species (Elfimov, 1995) and lack of detailed morphological descriptions of larvae of many species make identification difficult and pose a major obstacle to the study of barnacle supply-side ecology.

At present, descriptions of barnacle cyprids are mostly dependent upon laboratory-reared larvae. There are very few morphological keys for the identification of wild caught barnacle cyprids. Such keys are often limited in their usefulness. For instance, the guide developed by Standing (1981) pertains to only the cyprids of Oregon waters in U.S.A. A guide has yet to be developed for barnacle cyprids for any particular region in the tropics. Moreover, larval culture itself poses several challenges in terms of suitability of larval feed and rearing conditions to ensure sufficient larval survival. Molecular techniques which enable accurate species identifications could dispense with the need for larval culture. For example, DNA barcoding has been extensively used for species identification in recent years. By matching a chosen region of DNA fragments from the specimen with known reference specimens, identification can be achieved (Hebert et al., 2003). The method is very useful for the identification of species with different life stages, if the adult

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can be confidently identified by morphology but not for the young stages. Chen et al. (2013) have shown that DNA barcoding based on mitochondrial COI sequences is suitable for identifying wild-caught barnacle cyprids including those from possible invasive species. Other markers used to resolve barnacle taxonomic problems and biodiversity surveys include the 12S and 16S rRNA genes and nuclear ITS1 region (e.g., Chan et al., 2007a–c, 2009; Tsang et al., 2009; Chen et al., 2012; Cheang et al., 2012)

The Matang Mangrove Forest Reserve (MMFR) is the largest mangrove forest in the peninsular Malaysia and has attracted extensive ecological and scientific interest (Shaharuddin et al., 2007). The numerous creeks and channels with diverse water conditions, from the upper estuary to near shore waters, provide suitable habitats for barnacle colonisation on the fringing mangrove vegetation as well as on the numerous fish stakes, jetty pilings, and floating fish cages. Barnacle diversity in the MMFR waters has not been reported except for one biofouling study on floating fish cages, where *Balanus amphitrite* (=*Amphibalanus amphitrite*) was identified as the only species (Madin et al., 2009). The present study aimed to use a combined morphological and molecular approach to identify and describe the barnacle cyprids of MMFR, and to provide diagnostic morphological characters to identify barnacle cyprids in MMFR in Malaysia.

MATERIAL AND METHODS

Collection of barnacle cyprids and adults. Specimen collections were made from the upper estuary in the Matang Mangrove Forest Reserve (MMFR) as far as the coastal waters (< 12 km offshore) on two separate sampling occasions, one on 20–21 April 2011 and the other on 25–26 June 2012 (see Fig. 1 for the location of sampling sites). Adult barnacles were collected as species references, using a hammer and chisel to detach the animals from their substrates, including mangrove tree trunks and roots, and buoy for oyster culture. Multiple surface plankton samples were collected by a standard plankton net of 160 µm mesh size (45 cm mouth diameter) towed for either 5 or 10 min each. All collected specimens were immediately preserved in 95% ethanol for further analyses.

Morphological analyses. The adult barnacles were identified to species level based on their morphology and served as the adult reference collection for subsequent comparison. All barnacle cyprids were first sorted out from the plankton samples under a stereo microscope (Olympus SZX7). Approximately 250 cyprids that could represent the full range of observed morphological variations were selected for analysis. Photos of the lateral view of the selected set of cyprids were taken under normal bright field of a compound microscope (Zeiss Axio Scope A1) equipped with a camera (Panasonic Lumix G1). A series of photos at differential focus were taken for each larva and integrated into an extended-focus image using the iSolution Lite image processing software (i-Solution Inc., Vancouver, Canada) for optimal viewing and measurement. Morphometric measurements of the carapace of each cyprid were then taken from the extended-focus images using ImageJ (version 1.44; U.S. National Institute of Health, available at http://imagej.nih.gov/ij/). The measurements included carapace length (maximum distance between anterior and posterior margin), carapace height (maximum distance between dorsal and ventral margin), posterior carapace angle (angle formed by extension of dorsal and ventral margin), and calculated ratio of length-to-height (Fig. 2; also see Chen et al., 2013). Carapace sculpturing was examined, described, and recorded in addition to the morphometric measurements. Since not all the cyprids had their antennules and thoracic appendages extended, measurements were restricted to the carapace only.

Scanning Electron Microscopy (SEM). Morphology and carapace sculpturing patterns of cyprids initially observed under light microscopy were further observed by scanning electron microscopy (SEM). Cyprids preserved in 95% ethanol were transferred into acetone, critical point dried, and coated with gold palladium before observation with a FEI Quanta 200 Scanning Electron Microscope (methods follows Chan & Leung, 2007). Measurements related to the carapace sculpturing pattern were made on SEM images. Maximum

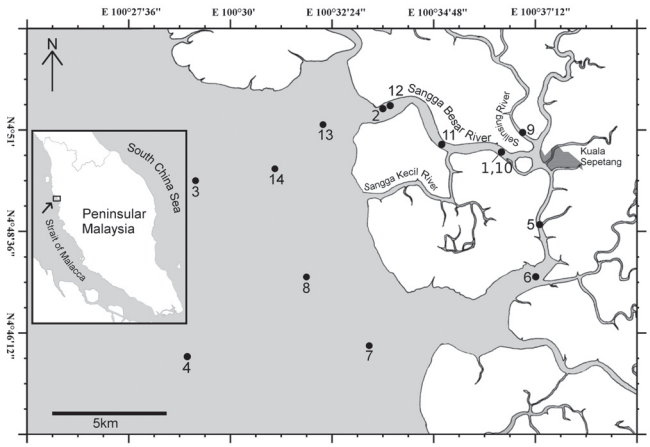

Fig. 1. Map of sampling locations at Matang Mangrove Forest Reserve (MMFR) in Perak, Malaysia. Sampling was carried out in April 2011 at sites 1–8 and in June 2012 at sites 9–14.

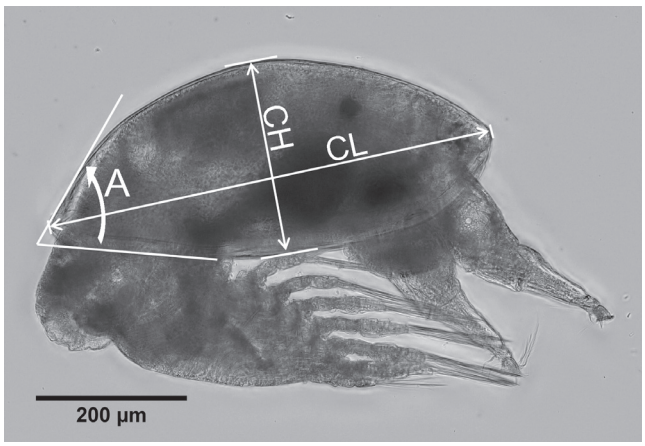

Fig. 2. Lateral view of cyris larvae of barnacle showing measurements used for morphometric analysis. CL: carapace length; CH: carapace height; A: posterior carapace angle. Ratio of CL/CH was also calculated.

feret diameter (largest distance between two parallel planes restricting an object) was used to measure the size of the ultrastructures if the use of diameter was not appropriate.

DNA extraction, PCR and sequencing. Total genomic DNA from adult and larval tissue was extracted using DNeasy blood and tissue extraction kit (Qiagen GmbH, Germany) after the cirripedes were identified and morphological measurements made. A faster alternative extraction method using extraction buffer containing 5% (w/v) Chelax®-100 resin (Bio-Rad, California, USA) was used only for cyprids DNA extraction (Walsh et al., 1991). For DNA extraction using the tissue extraction kit, soft tissue (~25 mg) of adult barnacle or whole barnacle cyprids were used for DNA extraction following the manufacturer's instructions. Polymerase chain reaction (PCR) was used to amplify a region of the mitochondrial 12S-rRNA gene from the DNA using forward primer 5'-GACCGTGCTAAGGTAGCATAATC-3' (Tsang et al., 2009) and reverse primer 5'-CCGGTCTGAACTCAAATCGTG-3'. Amplification was performed using reaction mixture containing 2 µL of template DNA, 12 µL Taq master mix (1.5 mM $MgCl_2$ type; Ampliqon, Denmark), 0.05 µM of each primer, and ddH_2O to a total volume of 20 µL. PCR conditions were set as follows: 2 min and 30 sec at 94°C for initial denaturation, then 30 cycles of 30 s at 95°C, 30 s at 48°C, and 1 min at 72°C, with final extension for 5 min at 72°C. Sequencing was performed using an ABI 3730 XL DNA analyser with BigDye terminator cycle sequencing reagents kit (Applied Biosystems, California, USA).

Sequence analyses. Cyprids were identified through comparison of their 12S-rDNA sequences with that of the identified adult barnacles. All sequences (including 207 cyprid sequences successfully obtained from the selected set, 16 adult sequences and three outgroup sequences from GenBank) were first aligned using MUSCLE (Edgar, 2004) using the default settings, and these were then manually inspected. The three outgroups used were *Verruca laevigata* (JX083933.1), *Metaverruca recta* (JX083931.1), and *Rostratoverruca krugeri* (JX083932.1). A neighbour-joining tree was constructed from the aligned sequences using MEGA 5.05 (Tamura et al., 2011), with a Kimura 2-parameter (K2P) model used to compute the genetic distances. Bootstrapping was conducted with 1000 replicates to estimate the reliability of the inferred tree. When the sequences of cyprids and adult references formed a "monophyletic" clade with high bootstrap support, it was considered to be the same species. Monophyletic groups that failed to cluster with any adult references were then considered as an operational taxonomy unit (OTU). To assess the strength of the current 12S sequence fragments for DNA barcoding purposes, the pair-wise genetic distances of all of the sequences (except outgroups) computed from the K2P model were also summarised to show the between—and among— clade genetic divergence.

Statistical analyses and construction of morphology-based classifier. Of 207 sequenced cyprids from the selected set, only 183 were used for the morphological analysis due to the exclusion of cyprids with low quality images. With species identity determined from the DNA barcoding analysis, morphology-based classification models were then constructed. The classification models would be used for quick preliminary classification and to show the effect of adding carapace sculpturing as a morphological variable to differentiate cyprids. The classification tree algorithm method was chosen over LDA (linear discriminant analysis) because it can handle mixed inputs of predictor variables (both quantitative and qualitative variables), and is easier to interpret (De'ath & Fabricius, 2000). Furthermore, classification trees are not limited by the number of samples used in each group (i.e., species), whereas LDA requires the number in each group to be not less than the number of variables. This is a problem for the present study as the specimen numbers of *A. amphitrite*, OTU 1 and OTU 2 were low in the training dataset. Two models of classification tree were constructed and compared, i.e., one with only quantitative morphological characters, and the other with both quantitative and qualitative (carapace sculpturing) morphological characters. The performance of the tree classifiers was evaluated using multiple runs of 5-fold cross validation. Within each run, the dataset of the selected cyprids was randomly partitioned into five subsets; four subsets were used as training sets and one subset was used as a validation set. This process was repeated until each subset had been used once as a validation set. The cross validation was then repeated for 100 runs and the misclassification rate of the classifier was estimated from the average over the 100 runs. All statistical analyses were conducted using R (version 2.13.0; R Development Core Team, 2011). R package 'tree' (Ripley, 2011) was used for classification trees.

Application of morphology-based classifier. The decision tree classifier was then utilised to aid the classification of the remaining cyprid specimens to give a preliminary view of the species composition of cyprids at different locations in MMFR. To achieve this, the remaining collection was identified and counted under a compound microscope, and photos were taken as measurements which were needed before a decision on species identity could be made for individual species/OTU. The decision on species identity was assisted by the tree classifier.

RESULTS

Identification of adult barnacle. Six species of adult barnacle from MMFR were identified to species level, namely *Fistulobalanus patellaris*, *Fistulobalanus* sp. (an undescribed species), *Amphibalanus reticulatus*, *Amphibalanus variegatus*, *Amphibalanus amphitrite*, and *Euraphia withersi*.

Molecular analyses. Partial sequences of 12S-rRNA gene were successfully obtained from 207 individuals of cyprids and 16 individuals of barnacle adults. A neighbour-joining tree constructed from the sequences is shown in Fig. 3. Eight distinct clades were observed and six clades (including 195 cyprids sequences) had the sequences from the identified adult references. Two of the clades (comprising 12 of the cyprid sequences) with no matching adult sequence were

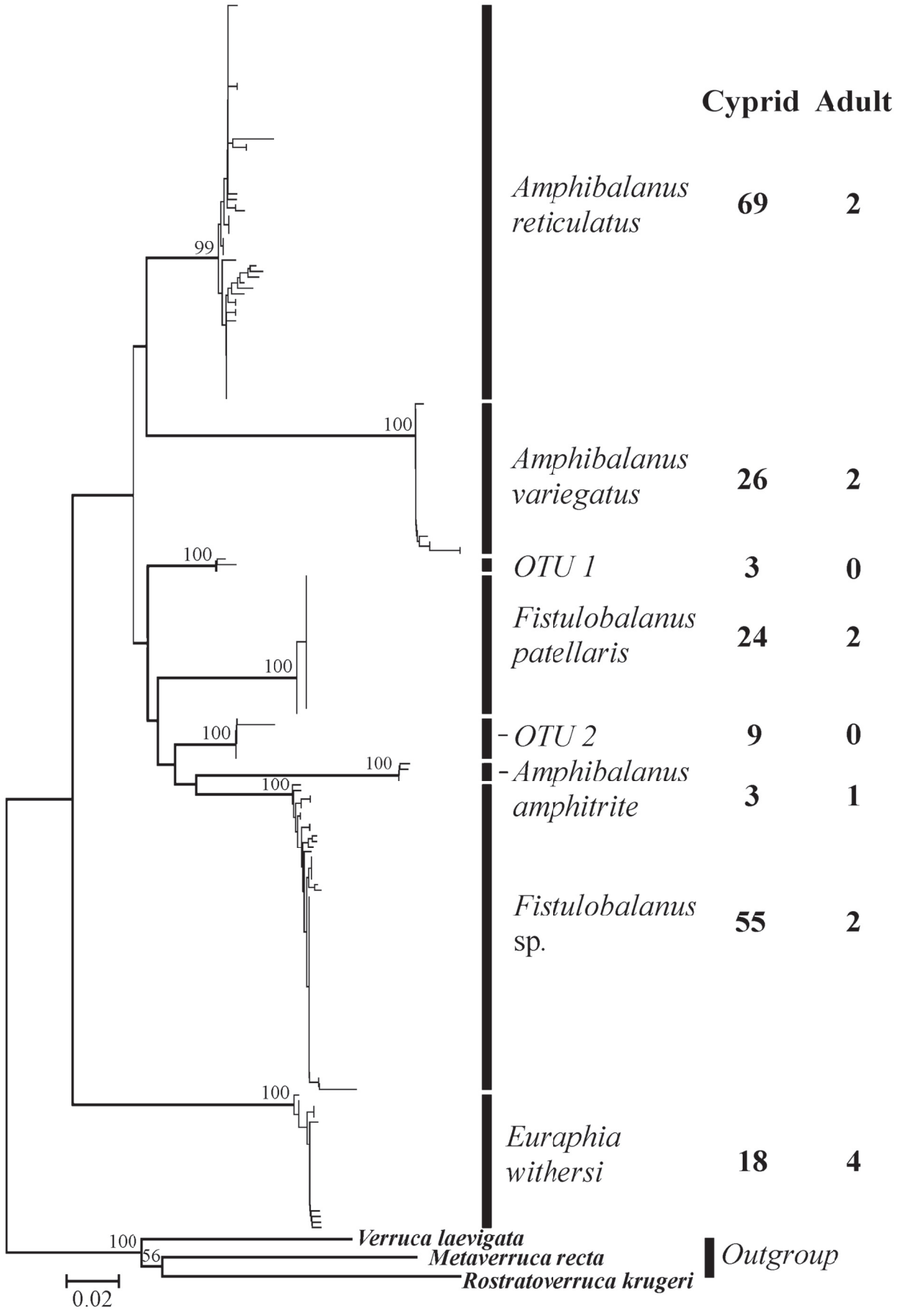

Fig. 3. Neighbour-joining tree contructed from partial 12S-rRNA gene fragment sequences of cyprids and adults of barnacle. The sequences were clustered into eight clades, and species name were labelled at the clades containing sequence(s) of identified adult of barnacle. Clades with no sequence of identified barnacle adult clustered within were designated as OTU (Operational Taxonomic Unit). Number of sequences in each clade were also shown. Scale bar denotes 0.02 base substituition per site.

designated as Operational Taxonomic Unit or OTU 1 and OTU 2. The mean within-species pairwise K2P distance was 0.6% (ranged from 0–3.5%) while the mean between-species distance was 13.5% (ranged from 5.4–25%). The non-overlapping ('barcode gap') of frequency distribution of pairwise K2P distance for within- and between-species suggests the suitability of the approach for barcoding purposes (Fig. 4).

Morphological analyses. In the present study, the range of carapace length from all cyprids collected was 439–685 μm, and the range of carapace height was 199–329 μm. The variations in the four quantitative morphometric characters of the carapace, namely length, height, angle and length-to-height ratio among species/ OTU are shown in Table 1. The carapace length and height data were also compared to those previously reported in the literature (Table 1). The sculpturing patterns were categorised into five types (details in Table 2). These carapace sculpturing patterns were not observable under a dissecting microscope and only at >100x magnification under a compound microscope (henceforth referred to as CM). SEM which provided morphological details of high resolution confirmed the pattern classification based on the type of sculpturing pattern (Fig. 5–7). Four barnacle species (*Fistulobalanus* sp., *Fistulobalanus patellaris*, *Euraphia withersi*, and *Ampibalanus variegatus*) showed diagnostic carapace sculptures (Table 2). However, four other taxa (*A. amphitrite*, *A. reticulatus*, OTU1 and OTU2) showed no sculpturing pattern (i.e., smooth carapace). The honeycomb pattern of type A (*Fistulobalunus* sp.; Fig. 5A–D) is readily identifiable under CM. Type B pattern (*Fistulobalunus patellaris*; Fig. 5E–J) was not very apparent under CM, but was revealed under SEM. Due to their larger size, lunular pores on the ventral side were easier to observe under CM (Fig. 5G) compared to the punctae on the dorsal side (Fig. 5F). For type C pattern (*Amphibalanus variegatus*, Fig. 6A–H), the punctate pattern was observed on the ventral aspect of the carapace (Fig. 6C) but was absent on its dorsal aspect. Differentiation between the punctae of type C and the lunules of type B on the ventral aspect could only be identified under SEM (Figs. 5C & 6C, respectively). However, under CM, type C can be differentiated from type B based on the presence of punctae on both the anterior and posterior ends of the carapace in type C, whereas punctae in type B are absent in both positions. Type D is featured by ridges or folds at the posterior end of the carapace of *Euraphia withersi* (Fig. 6J). These folds extend into the ventral aspect of the carapace (not shown). *Euraphia withersi* also has unique reddish pigmentation scattered around the ventral edge of carapace (Fig. 6I, highlighted by arrows) and a dark rounded pigmentation spot posterior to the cyprid eye (Fig. 6I, circled). The reddish pigmentation, however, faded after prolonged preservation in 95% ethanol. Four other species/ OTU (*Amphibalanus reticulatus*, *Amphibalanus amphitrite*, OTU 1 and OTU 2) do not have any carapace sculpturing and were named as type E (Fig. 7). Classification of these taxa depends on their carapace size and shape, where *A. reticulatus* and OTU 1 are longer than *A. amphitrite* and OTU 2, while OTU 2 has a higher posterior carapace angle than *A. amphitrite*. Discrimination between *A. reticulatus* and OTU 1 is difficult.

Morphology-based classifier. The performance of morphology-based tree classifier increased dramatically when the carapace sculpturing character was added. The estimated misclassification rate for the tree classifiers decreased from 35.0 ± 11.1% (±SD) to 5.7 ± 5.0% (±SD) respectively for datasets without and with carapace sculpturing characters. This decrease is mainly due to the increased accuracy of classification of species that have unique carapace sculpturing in the latter dataset. Low accuracy was especially a problem for species/OTU present at low abundances in the training dataset (*A. amphitrite*, OTU 1, and OTU 2).

Assessment of cyprids distribution in MMFR. The morphological classification model which was obtained in preceeding steps (Fig. 8) was used to identify the cyprids collected at different stations in MMFR (Fig. 9). A total of 1124 and 736 cyprids were classified for the 2011 and 2012 collections, respectively. Marked differences in species composition were observed between 2011 and 2012 collections. The 2011 (April) collection was dominated by *E. withersi* and *A. reticulatus* while the 2012 (June) collection was dominated by *Fistulobalanus* sp. and *F. patellaris*. All species were found in both years except *E. withersi* which was not found in the 2012 samples. The within-year variations in species composition among stations were smaller compared to annual variability. However some differences were observed between stations, in particular the composition between the upper estuary and the rest of the stations.

DISCUSSION

The adult barnacle species identified in this study belong to three genera, namely *Amphibalanus*, *Fistulobalanus*, and *Euraphia*, which are commonly found in tropical and sub-tropical mangrove habitats (Rainbow et al., 1989; Prabowo & Yamaguchi, 2005; Crona et al., 2006; Marques-Silva et al., 2006; Li & Chan, 2008). This indicates the importance of mangrove habitat for these barnacle genera. However, two of the clades (OTU1 and OTU2) derived from the cyprid data did not match any of the identified adult barnacle sequences by barcoding analysis. This suggests that the larvae may either be advected cyprids from offshore adult species which are not resident in MMFR, or the cyprids came from adults not sampled in the MMFR. Hence, the identity of these two unknown species awaits further detailed surveys of adult barnacles within and outside the MMFR. Non-matched results are common in barcoding analyses especially for areas that are not sufficiently surveyed. Barber & Boyce (2006) used COI fragments to study the diversity of coral reef stomatopods. They reported 22 distinct OTUs that could not be matched with any adult stomatopod references. Chen et al. (2013) also reported 10 unidentified OTUs from wild collection of barnacle cyprids and suggested the possible invasion of cyprids from neighbouring regions. The presence of OTUs in the absence of their adults shows the apparent disconnectedness between the presence of

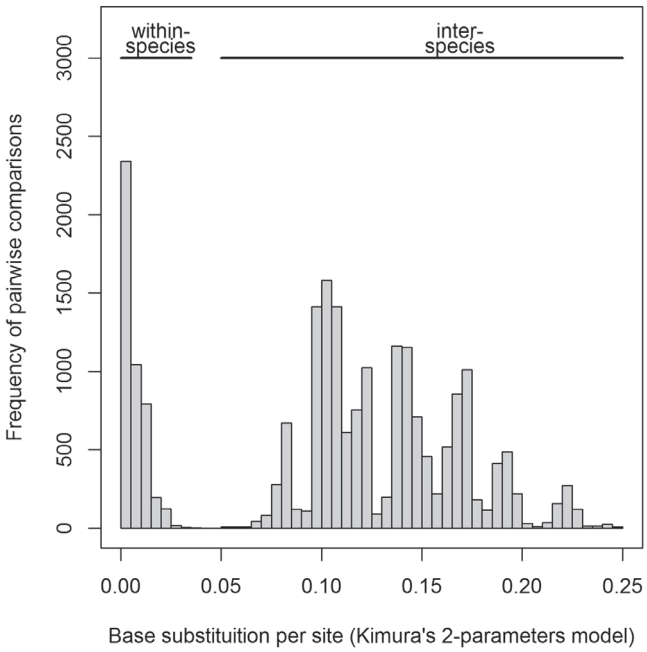

Fig. 4. Histogram showing variations of pair-wise genetic distances computed from 12S-rRNA gene fragment sequences using Kimura 2-parameter model. Note the distribution of within-species variations does not overlap with that of inter-species variation.

larval and their adults, which could be due to a reasonably long larval phase (10–45 days, Lohse & Raimondi, 2012) and hence, the potential to be widely dispersed by ocean current. Nonetheless, cyprids in the absence of settlement cues will not likely settle or survive on unsuitable substrates (Pawlik, 1992).

The 12S-rDNA region has proven to be successful and reliable for barnacle identification in this study. The 12S-rDNA fragments are shorter and relatively easier to amplify than COI fragments (unpublished data) and these are commonly used for species identification. Barnacle cyprids are usually small in size and their DNA can be easily degraded after a period of preservation. It is suggested that 12S-rDNA fragments can be obtained from small cyprids or cyprids that have been preserved for a prolonged period of time. However, the 12S-rDNA fragments have a smaller representation in online databases than COI fragments.

In the present study, the quantitative characters (carapace length, height, posterior angle and length-to-height ratio) have a low discriminating power. This problem is exacerbated by closely related species or genera within this study. Carapace length and carapace height are two common morphological measurements used for cyprids in previous reports, and

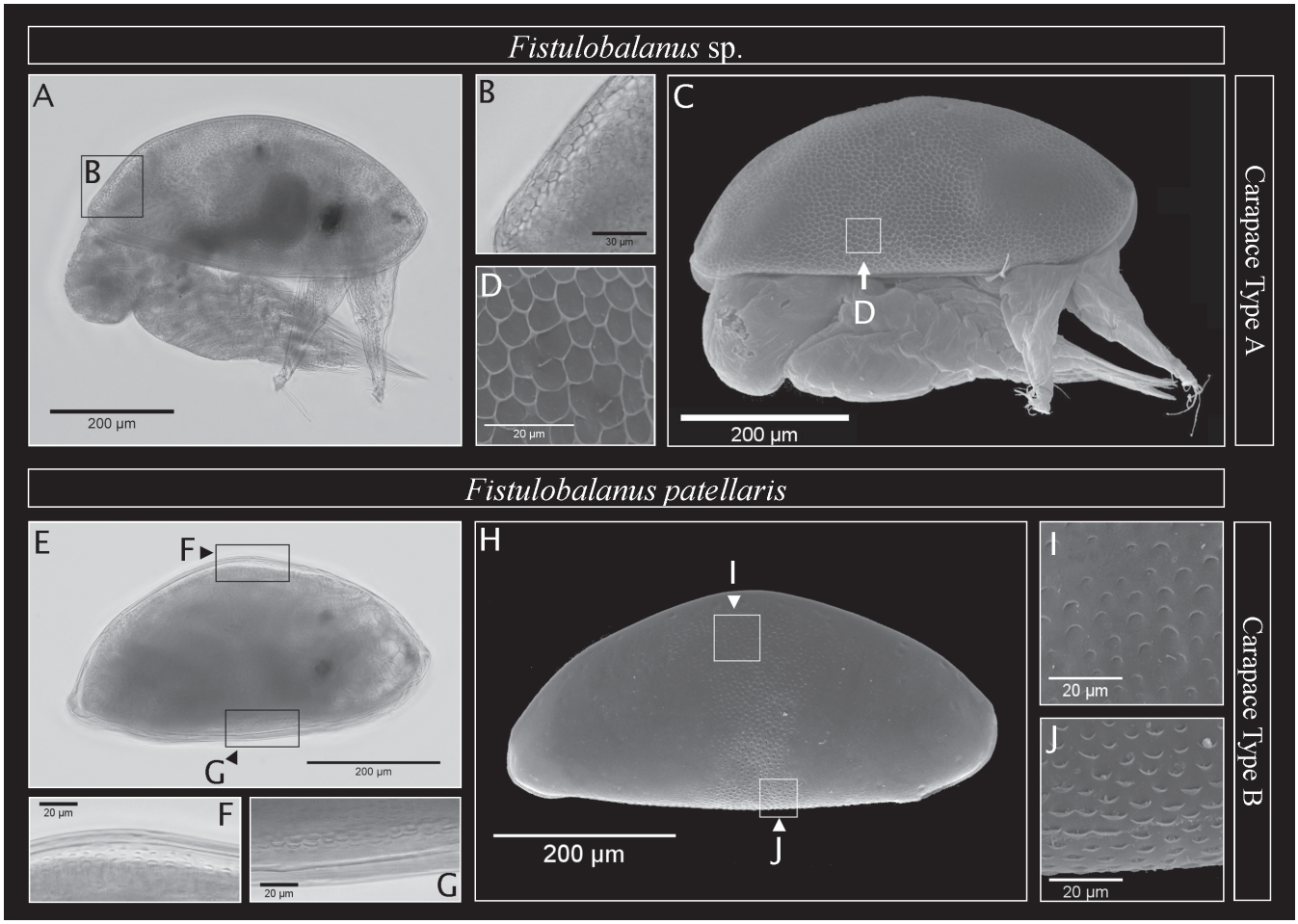

Fig. 5. Light and scanning electron micrograph of cyprids of: A–D, *Fistulobalanus* sp.; and E–J, *Fistulobalanus patellaris*. Details of specific carapace sculpturing patterns in each species are shown at higher magnification.

have been suggested for use in species discrimination. For instances, Burrows et al. (1999) suggested using carapace length to differentiate the cyprids of *Chthamalus stellatus* from *Chthamalus montagui* in British waters, and this was later verified by molecular evidence using mtDNA RFLP profiles (Power et al., 1999). Pineda et al. (2002) used carapace length and seasonal presence to select out the cyprids of *Semibalanus balanoides*. Nevertheless, the use of carapace length and height is only good enough to distinguish between a few species of cyprids which differ in size, and is of little use where many species are known to co-occur and similar in size, e.g., in the MMFR waters. Comparison of carapace length and carapace height of successfully identified species in this study (wild caught) to those obtained from previous reports (laboratory-reared) showed some discrepancy (Table 1). Discrepancy in carapace length and height was also found among the laboratory-reared cyprids from different studies of same species (Table 2). Thus, large within-species size variation may exist. Geographical origin or/and environmental conditions may be the cause(s) of size variation. O'Riordan et al. (2001) observed temporal and latitudinal variations in the length of cyprids collected from European localities. Desai et al. (2006) reported a significant effect of temperature and food concentration on the length of laboratory-reared barnacle cyprids. Thus, environmental and geographical factors may limit the usefulness of any cyprid identification key based on morphometrics to large geographical regions.

Carapace sculpturing is an important character for discriminating the dominant species of barnacle cyprids found in MMFR. For the purpose of classifying large numbers of cyprids, carapace features that are observable under CM are preferable as diagnostic features. Although SEM provided enlarged and much clearer details of the carapace sculpturing, these are important only for the purpose of description but not necessary for the classification model. In fact, it is impractical to use SEM for the purpose of identifying cyprids in large numbers. Egan & Anderson (1986) did not include carapace sculpturing for their description of *Amphibalanus variegatus* due to the absence of SEM evidence. The honeycomb or type A sculpturing that was found on unidentified *Fistulobalanus* sp. in the present study has also been previously reported for barnacle cyprids of *Chthamalus malayensis* (Yan & Chan, 2001), *Catomerus polymerus*, and *Chamaesipho tasmanica* (Egan & Anderson, 1989), and Cryptophialidae (Kolbasov & Høeg, 2007). Nevertheless, there could be some minor variations in the honeycomb sculpturing patterns of different species such as the size of the honeycomb unit, but the previous report did not describe its size and hence comparison is impossible. Lee et al. (1999) previously

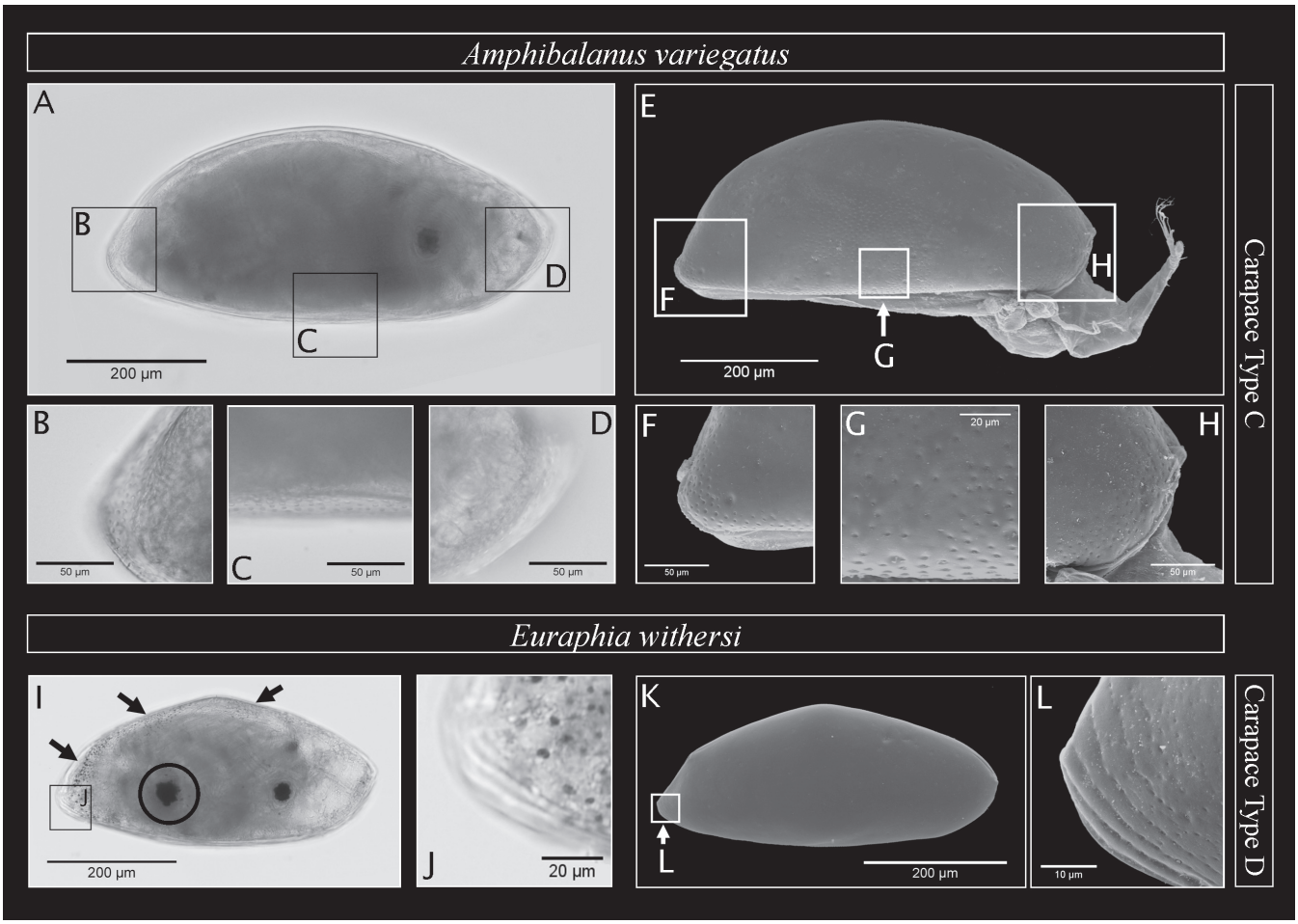

Fig. 6. Light and scanning electron micrograph of cyprids of: A–H, *Amphibalanus variegatus*; and I–L, *Euraphia withersi*. Details of specific carapace sculpturing patterns in each species are shown at higher magnification. 6I, *E. withersi* has reddish pigments around the carapace (arrows) and a dark rounded pigmentation spot (circled).

Table 1. Quantitative morphological characters and comparison to other studies

Species	Carapace Length (µm)	Carapace Height (µm)	Posterior Carapace Angle (°)	Length-to-Height Ratio	Remarks	Reference
Amphibalanus amphitrite	480.75±38.04	227.79±18.03	65.72±1.13	2.12±0.27	Field collected; mean± SD	This study
	510	N/A	N/A	N/A	lab-reared; single measurement	Karande (1974)
	450±20	N/A	N/A	N/A	lab-reared; mean± 95% CI	Egan & Anderson (1986)
	550	250	N/A	N/A	lab-reared; single measurement	Glenner & Høeg (1995)
	421–480	211–230	N/A	N/A	lab-reared; range	Anil et al. (2001)
Amphibalanus reticulatus	605±21.13	275.72±16.67	60.95±6.56	2.20±0.14	Field collected; mean± SD	This study
	547±26.3	250±25.5	N/A	N/A	lab-reared; mean ± unspecified bar	Thiyagarajan et al. (1997)
	613±21	245±17	N/A	N/A	lab-reared; mean± SD	Lee et al. (1999)
Amphibalanus variegatus	636.46± 24.91	284.17± 17.41	65.16±6.79	2.25±0.14	Field collected; mean± SD	This study
	520±40	N/A	N/A	N/A	lab-reared; mean± 95% CI	Egan & Anderson (1986)
	615	275	N/A	N/A	lab-reared; single measurement	Karande (1974)
Fistulobalanus sp.	555.44±13.49	270.78±14.25	67.79±5.12	2.06±0.11	Field collected; mean± SD	This study
Fistulobalanus patellaris	554.31±19.45	281.19±14.41	71.69±6.56	1.97±0.09	Field collected; mean± SD	This study
Euraphia withersi	482.42±14.88	230.77±7.17	72.57±4.40	2.09±0.09	Field collected; mean± SD	This study
OTU 1	598.01±17.03	256.15±11.32	57.48±0.49	2.34±0.17	Field collected; mean± SD	This study
OTU 2	522.86±20.18	226.95±16.22	54.71±10.94	2.31±0.19	Field collected; mean± SD	This study

Table 2. Types of carapace sculpturing patterns

Type	Description	Species/ OTU
Type A	'Honeycomb' pattern of raised pentagonal and hexagonal units. Maximum feret diameter of the combs is 7.6±1.2 μm (mean±SD, n=67)	*Fistulobalanus* sp.
Type B	Sculpturing spans through dorso-ventral axis, with punctae on the dorsal aspects and lunular pores on the ventral aspects. Maximum feret diameter of the punctae is 2.4±0.5 μm (mean±SD, n=65), and 5 ± 0.9 μm (mean±SD, n=70) for the pores.	*Fistulobalanus patellaris*
Type C	Rounded punctae on ventral side, anterior and posterior ends. Diameter of the pits is 2.8±0.7 μm (mean±SD, n=65)	*Amphibalanus variegatus*
Type D	3–4 distinct ridges or folds at posterior end	*Euraphia withersi*
Type E	No sculpturing of carapace	*Amphibalanus reticulatus, A. amphitrite,* OTU 1 and OTU 2

reported that the carapace of *Amphibalanus reticulatus* is covered with numerous small denticles but this was not supported by SEM evidence in the present study. Neither LM nor SEM in the present study showed any denticles. Instead, the species, whose identification was confirmed by molecular analysis has a smooth carapace. Thiyagarajan et al. (1997) also did not observe any denticles for *Amphibalanus reticulatus.* Such variation in the denticles on the carapace may be due to the presence of cryptic species. Although the sculpturing pattern appears to be species-specific for the cyprids in Matang mangrove waters, the type of pattern shows no generic affinity. This supports the findings of Standing (1981), where he described carapace sculpturing in *Pollicipes polymerus*, *Balanus improvisus*, and *Balanus glandula*, but none in *Chthamalus dalli*, *Balanus crenatus*, *Balanus nubilus*, and *Semibalanus cariosus*. The function and evolutionary history of carapace sculpturing in cyprids is presently unknown.

The combination of quantitative characters with carapace sculpturing characters gave better classification accuracy. This suggests that a combination of both qualitative and quantitative characters in classification problems should be considered especially when few characters are available. The use of classification trees is suitable for combined characters, and a good alternative to LDA (Feldesman, 2002). The other advantage is variable selection. This is automatically performed by the classification tree algorithm, because the variables that are not useful in reducing the misclassification errors are not used. This could remove the variable selection step, and simplify the models for quick classification. The classification tree based on the complete data with carapace sculpturing (Fig. 8) did not use carapace length and length-to-height ratio as predictors, which is simpler than using all of the variables. It has to be noted that the selected variables may differ when a different statistical package is used to compute the classification tree. Classification trees have previously been used in the taxonomic identification of fish (Guisande et al., 2010) and pollen grains (Lindbladh et al., 2002).

Morphological characters besides those described in the current study may be used to discriminate species that do not have any sculpturing. Chen et al. (2013) showed that the antennular morphology provides higher inter-species variations than carapace morphology, which would appear very useful for species identification, but is beset by the problem that not all preserved cyprids showed extended antennules. Kamiya et al. (2012) proposed a promising auto-fluorescence pattern approach to identify cyprids, but the method works only with fresh and unpreserved samples. Recent advances in image acquisition and processing have shown good promise in the development of large scale automated classification of planktons (Culverhouse et al., 2006), and such tools could be adapted to the specific purpose of cyprid classification in the future.

Field samples showed that cyprid composition, dominated by four species, varied spatially (between stations) and temporally (between sampling years), indicating the dynamic nature of their supply in MMFR waters. Cirripede nauplii were observed to be most abundant in the inshore waters of MMFR (<15 km off shore) compared to estuarine and offshore waters, being consistently found throughout the year but with peak abundance in May and October during the intermonsoon months (Chew, 2012). Thus, the difference in composition of cyprid samples in the present study is likely a result of temporal variability. Nonetheless, the present field study is preliminary and future studies requiring more exhaustive sampling over larger spatial and temporal scales are necessary to elucidate the supply-side ecology of barnacle larvae in the estuary.

In summary, the molecular approach used in this study, i.e., 12S-rDNA sequence-matching of larval and adult barnacles, has successfully identified most of the sequenced cyprids (195 out of 207 sequences, six species out of eight clades). A morphology-based classifier has been developed with good classification accuracy for the dominant species of barnacle cyprids (*Fistulobalanus.* sp., *F. patellaris, A. reticulatus, E. withersi*) in the MMFR. However, the identification of

cyprids/OTU classified with lower accuracy still requires molecular tools. At present, there is still no single approach that can provide identification of barnacle cyprids with high accuracy, high speed, and low cost at the same time. The selection of the best approach will largely depend on the research question. The approach used in the current study achieves a balance of these three criteria. Future global or regional-scale cyprid identification keys are likely to use an automated integrated approach combining the usage of carapace sculpturing features, geometric morphometrics and cyprid appendicular features.

KEY TO THE COMMON BARNACLE CYPRIDS IN MATANG MANGROVE FOREST RESERVE (MMFR)

1. Carapace sculpturing absent, i.e., smooth (carapace Type E).2
– Carapace sculpturing present. 3
2. Carapace length less than 550 µm...*Amphibalanus amphitrite*
– Carapace length more than 550 µm.*Amphibalanus reticulatus*
3. Carapace punctate at anterior, posterior and ventral aspects (carapace Type C) and large in size (carapace length more than 600 µm). ..... *Amphibalanus variegatus*
– Carapace not punctate with carapace length less than 600 µm.....4
4. Carapace with honeycomb sculpturing pattern (carapace Type A). .....*Fistulobalanus* sp.

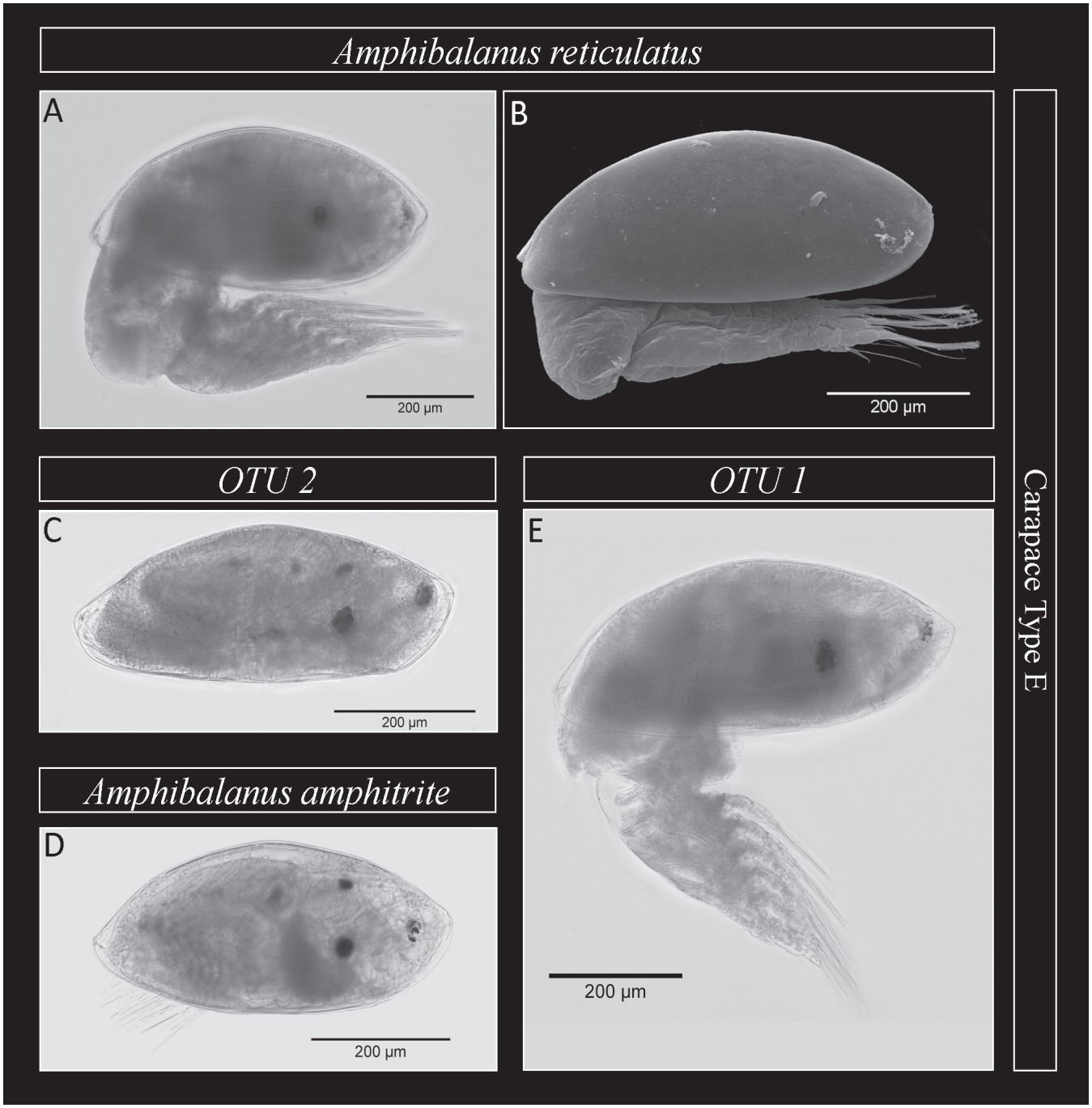

Fig. 7. Light and scanning electron micrograph of cyprids of: A, B, *Amphibalanus reticulatus*; C, OTU 2; D, *Amphibalanus amphitrite*; and E, OTU 1. Carapace sculpturing were absent in this group of cyprids.

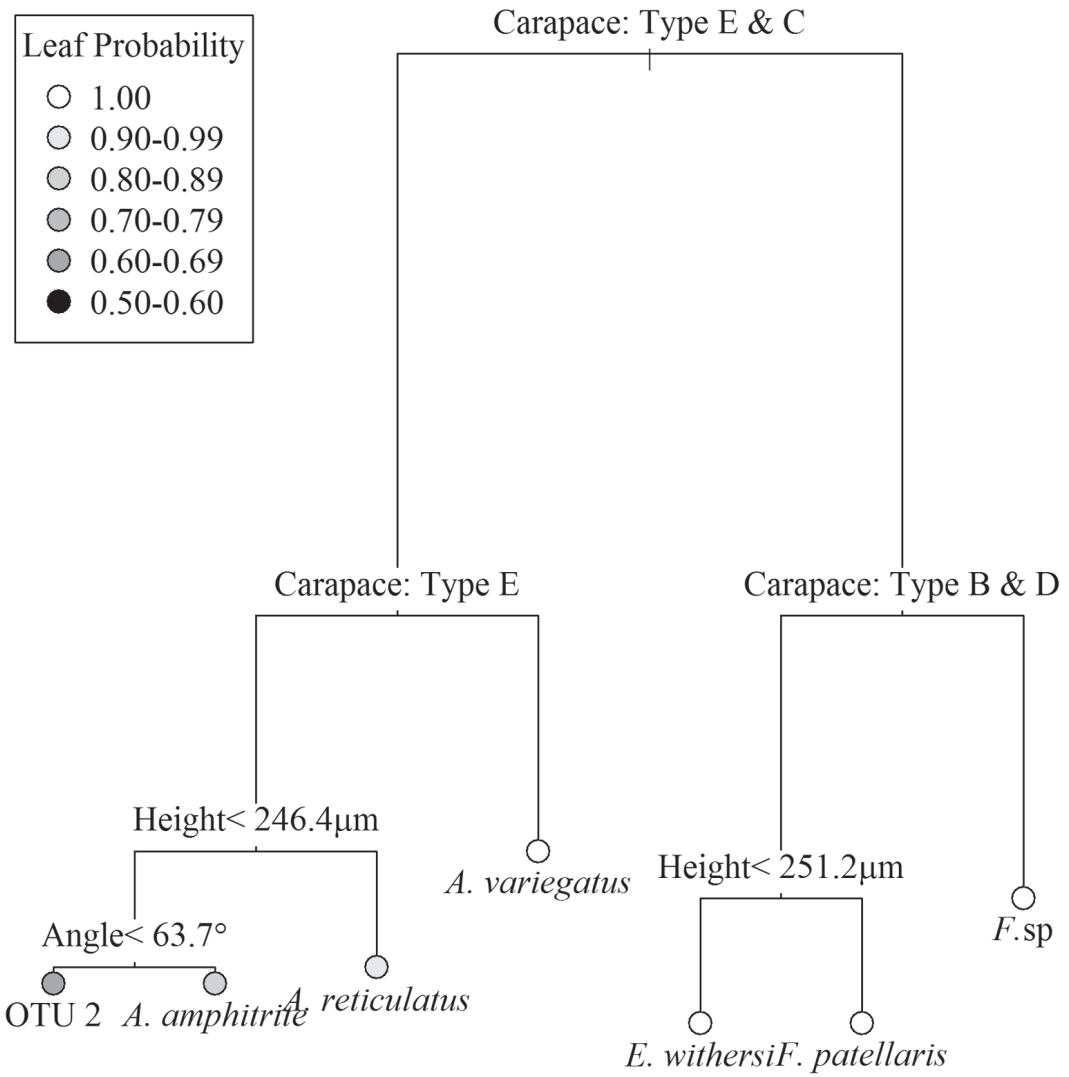

Fig. 8. Classification tree model computed from the morphometric characters of complete specimen data (with carapace sculpturing). A binary decision is made at each node, where 'true' for the node description lead to branch at left and 'false' to right. Probability of correct prediction ('recall') at each terminal node ('leaf') is also shown.

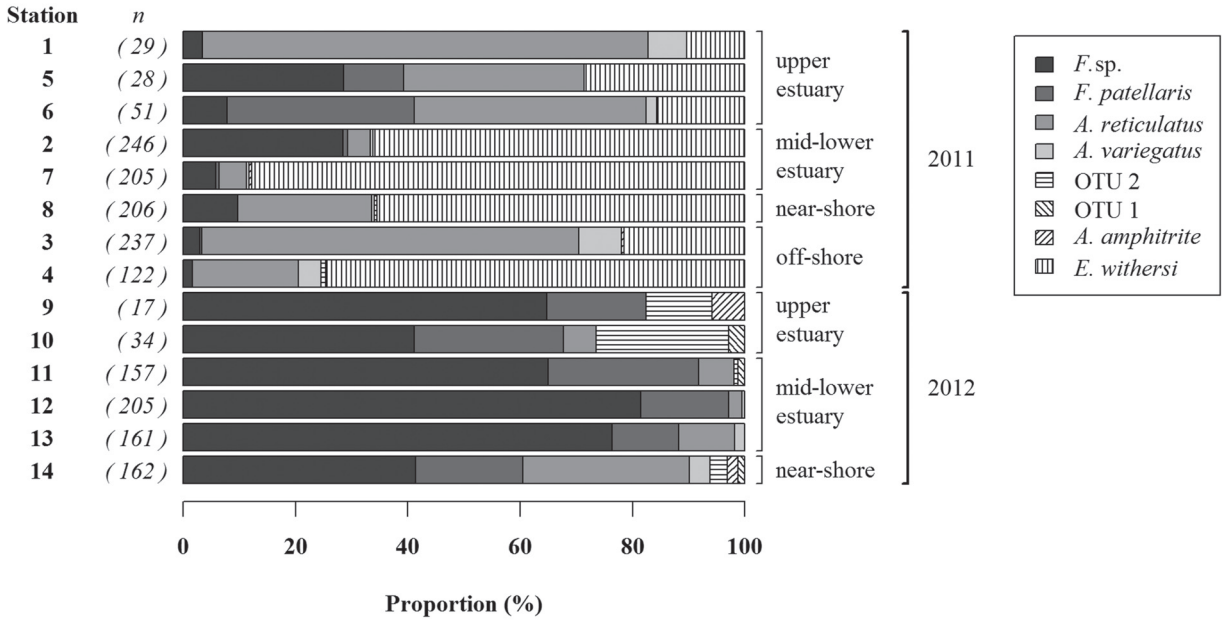

Fig. 9. Composition of barnacle cyprid diversity at different stations and different year of collection.

– Carapace without honeycomb sculpturing pattern. 5
5. Carapace with dark rounded pigmentation spot posterior to cyprid eye, ridged sculpturing on the posterior end (carapace Type D), height less than 250 µm. ..... *Euraphia withersi*
– Carapace with rounded and lunular sculpturing at dorsal and ventral aspects, respectively (carapace Type B), height more than 250 µm. ..... *Fistulobalanus patellaris*

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