

Cryptic Diversity of the *Tetraclita squamosa* Complex (Crustacea: Cirripedia) in Asia: Description of a New Species from Singapore

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Abstract Benny K.K. Chan, L.M. Tsang, K.H. Chu (2007) Cryptic diversity of the *Tetraclita squamosa* complex (Crustacea, Cirripedia) in Asia: description of a new species from Singapore. *Zoological Studies* **46**(1): xxx-xxx. *Tetraclita squamosa* is a common intertidal barnacle of the Indo-Pacific. Taxonomic classification of *T. squamosa* has been confused by its high degree of morphological variation, and it is now considered to be a species complex. *Tetraclita squamosa* collected from Singapore exhibits diagnostic morphological and genetic differences compared to *T. squamosa* from the coast of southern China and *T. pacifica* in the northwestern Pacific (a recently described species formerly recognized as *T. squamosa*). *Tetraclita squamosa* from Singapore has a long tergal margin in the scutum and a very straight scutal margin in the tergum when compared to *T. pacifica* and *T. squamosa* from southern China. The setae on the cirri of *T. squamosa* from Singapore are similar to those of *T. squamosa* from southern China. Barnacles from Singapore and southern China differ from *T. pacifica* in lacking serrulate setae with 4 rows of setules on cirrus I. From a molecular analysis, *T. squamosa* from Singapore is differentiated from *T. squamosa* from southern China, and *T. pacifica* by more than 19%, 10%, and 11% divergences in the COI, 12S rRNA, and ITS1 sequences, respectively. These values are much higher than those within each taxon (< 1.5% in the most variable COI

sequence), revealing that they are genetically distinct. The clear genetic and morphological differences from both *T. squamosa* and *T. pacifica* indicate that *T. squamosa* from Singapore represents a new species of *Tetraclita*. *Tetraclita squamosa* in the Indo-West Pacific region is a cryptic species complex with high morphological and genetic diversity. Given the relatively restricted areas investigated to the present, it is likely that more species await discovery in Southeast Asia, Australia, and the Indian Ocean.

Key words : *Tetraclita squamosa*, *Tetraclita singaporensis*, cryptic species, species complex, Singapore.

Introduction

Acorn barnacles of the genus *Tetraclita* are common space occupiers in the intertidal zone of rocky shores in tropical and subtropical waters (Newman and Ross 1976). In spite of their ecological importance, there is taxonomic confusion about these barnacles because of their high levels of morphological variation. Darwin (1854) considered *T. squamosa* to constitute a species complex with 12 subspecies. Over the years, a number of his subspecies have been raised to specific rank, based on morphological and molecular analyses (e.g., Achituv and Mizrahi 1987, Hasegawa et al. 1996, Ross 1999). *Tetraclita squamosa* is characterized by having green parietes (Pilsbry 1916, Yamaguchi, 1987) and has a wide distribution throughout the Indo-Pacific (Newman and Ross 1976, Ren and Liu 1979). A recent study in East Asia revealed the presence of cryptic species within this taxon (Chan et al. in press). *Tetraclita squamosa* collected from the northwestern Pacific (Taiwan, and Okinawa and Honshu, Japan) is morphologically and genetically differentiated from those along the coast of southern China and has been described as a new species, *T. pacifica*. The allopatric distribution of the 2 taxa is believed to be associated with ocean current patterns and larval supply (Chan et al. in press).

The Indo-West Pacific region is a center of biodiversity, and the distribution and population structure of its marine taxa have been influenced by sea level fluctuations during glacial periods and ocean current patterns (see review in Benzie 1998, Barber et al. 2000 2002). Thus, it is likely that there are more unidentified species or genetic structuring in *T. squamosa* in Southeast Asia that remain unexplored. In a recent collection of *T. squamosa* from Singapore, we found that these barnacles appeared to differ in morphology from *T. squamosa* in southern China (also see Nilsson-Cantell 1934). In the present study, the morphology (opercular plate geometry, cirral setae, mouthparts, and tergo-scutal flaps) and DNA sequences of the mitochondrial 12S rRNA, cytochrome c oxidase I (COI), and the first internal transcribed spacer (ITS1) of nuclear rRNA of *T. squamosa* collected from Singapore were studied and compared with those of *T. squamosa* from southern China coast and *T. pacifica* from the northwestern Pacific to test for the presence of cryptic diversity in *T. squamosa* in Asia. The 3 genes have commonly been used in delimiting species of crustaceans (e.g., Tsoi et al. 2005, Chan et al. in press; Tsang et al. in press).

MATERIALS AND METHODS

Morphological analysis

Nineteen specimens of *T. squamosa* were collected from St. John's Island, Singapore Strait. The barnacles were dissected, and the pattern and color of the tergo-scutal flaps (see Southward and Crisp 1963) were recorded. The opercular plates (tergum and scutum) and soft bodies were removed from the parietes. The posterior side of the tergum and scutum was photographed under a stereomicroscope using a digital camera, and the geometry of the tergum and scutum (Chan et al. in press; Fig. 1B) was measured from the photographs using computer software (Sigma Scan Pro, SYSTAT). The cirri and mouthparts were dissected and dehydrated using graded ethanol, critical point-dried, coated with gold, and then observed under a scanning electron microscope

(SEM; FEI Quanta 200, USA). The terminology of setal classification follows Garm (2004).

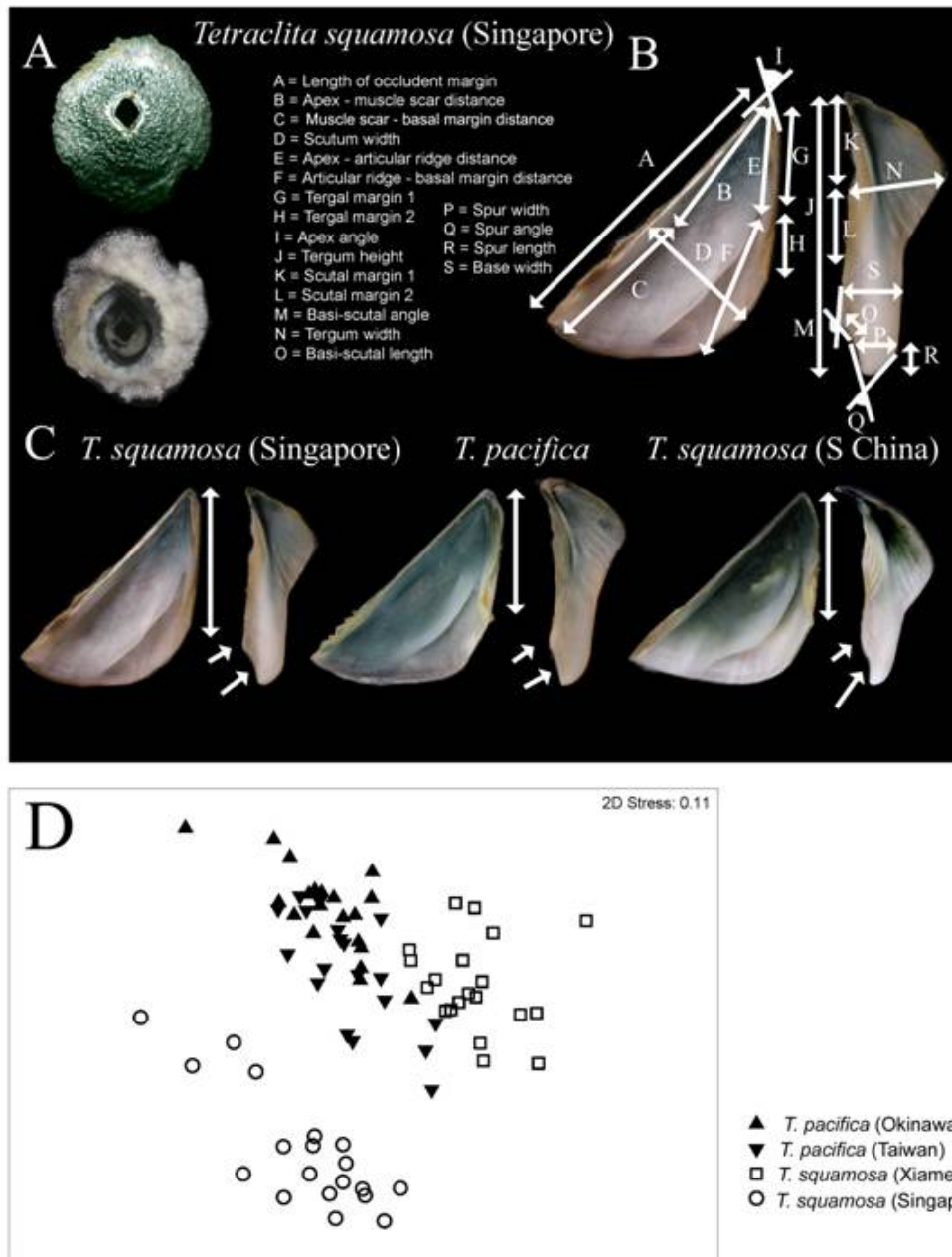


Fig. 1. A. Parietes of *Tetraclita squamosa* from Singapore viewed from the dorsal side and ventral sides. B. Parameters measured in the tergum and scutum for comparisons of the opercular plate geometry between *T. squamosa* from Singapore and Xiamen and

T. pacifica from Taiwan and Okinawa. C. Scutum and tergum of *T. squamosa* from Singapore and Xiamen and *T. pacifica*. White arrows indicate the diagnostic morphological differences. D. MDS ordination of the opercular plate morphology between *Tetraclita squamosa* from Singapore and Xiamen and *T. pacifica* from Taiwan and Okinawa. *Tetraclita squamosa* from Singapore is referred to as a new species, *T. singaporensis* sp. nov., in the present study.

Variations in the morphology of the scutum and tergum among *T. squamosa* (collected from Xiamen, Fujian Province, representing southern Chinese populations), *T. pacifica* (collected from Taiwan and Okinawa, representing northwestern Pacific populations; data presented in Chan et al. in press), and barnacles in Singapore were analyzed using multivariate analysis (PRIMER 6, Plymouth Routine in Multivariate Analysis; Clarke 1993). The shape of the parietes was not included in the comparisons as they exhibit great intraspecific morphological variations and are therefore not a reliable character for species identification (Foster 1974, Chan 2001). Data were square root-transformed prior to analysis, and similarity matrices were calculated using the Euclidean distance index. Non-metric multidimensional scaling (nMDS) was conducted to generate 2-dimensional plots of the scutum and tergum geometry between barnacle samples from different geographical locations. Analysis of similarity (ANOSIM) was conducted to test for differences in opercular dimensions between species/varieties, and SIMPER (similarity percentage) analysis was used to detect significant discriminating opercular parameters (also see Chan et al. in press).

Determination and analyses of DNA sequences

Total genomic DNA was extracted from whole soft tissue of individual barnacles (with some individuals from the samples used for morphological analysis) using the QIAamp Tissue Kit (QIAGEN, Hilden, Germany). Partial sequences of the mitochondrial 12S rRNA and COI genes were amplified using the respective universal primers of 12Sai and 12Sbi (Simon et al. 1994), and LCO1490 and HCO2198 (Folmer et al. 1994). The complete ITS1 region was amplified using SP-1-5'138 and SP-1-3'

(Chu et al. 2001). The amplification of the 3 genes was conducted in a reaction mix containing 1 μ l of template DNA, 1X PCR reaction buffer, 2 mM MgCl₂, 200 nM of each primer, 200 μ M dNTPs, and 1.5 units of *Taq* polymerase (Amersham, Uppsala, Sweden), with double-distilled H₂O added to bring the total volume to 50 μ l. The PCR profile for 12S rRNA was as follows: 3 min at 94°C for initial denaturation, then 33 cycles of 30 s at 94°C, 30 s at 50°C, and 40 s at 72°C, with a final extension for 3 min at 72°C. A similar profile was employed for COI and ITS1 with respective annealing temperatures of 47 and 54°C. The PCR products were then purified using a QIAquick gel purification kit (QIAGEN). Sequences were generated using the same sets of primers and determined on an Applied Biosystems (ABI, Foster City, USA) 3100 automated sequencer using the ABI Big-dye Ready-Reaction mix kit, following the standard cycle sequencing protocol. Sequences from *T. squamosa* from Hong Kong and Xiamen, *T. pacifica* from Japan and Taiwan, and *T. japonica* and *T. formosana* determined in a previous study (Chan et al. in press) were included in the sequence analysis (Table 1).

Sequences were aligned using CLUSTAL W (Thompson et al. 1994) with default gap weighting parameters and adjustment by eye. Sequence data from the 12S rRNA, COI, and ITS1 gene segments were first separately analyzed. For the combined analysis, phylogenetic congruence among the 3 datasets was tested under parsimony criteria using the incongruent length difference (ILD) test (Farris et al. 1995) implemented in PAUP* v4.0 b10 as the partition-homogeneity test (Swofford 2000). A matrix of genetic distances was generated using Kimura's 2-parameter method (Kimura 1980) with MEGA v3.1 (Kumar et al. 2004).

Three methods of phylogenetic inference including maximum parsimony (MP), neighbor-joining (NJ), and maximum likelihood (ML) were performed using PAUP* to determine the phylogenetic relationships among individuals of *T. squamosa* (Xiamen and Hong Kong), *T. pacifica*, and *Tetraclita* from Singapore. Sequences from *T. japonica* and *T. formosana* were used as outgroups. The MP analyses were performed using heuristic search and tree-bisection-reconnection (TBR) with 10 random sequence addition replicates. Character states were unordered and equally weighted. Gaps were treated as missing data. Bootstrap support for the most parsimonious tree was

evaluated using 1000 replicates. Modeltest 3.7 (Posada and Crandall 1998) was used to select the best-fitting model of nucleotide substitutions for each dataset in the NJ and ML analyses. Support for individual clades was respectively obtained from 1000 and 100 bootstrap replicates found by heuristic searches in the NJ and ML analyses.

Table 1. *Tetraclita* included in the present study, with sampling localities, GenBank accession numbers of sequences determined, and data sources. *Tetraclita squamosa* from Singapore was identified as a new species, *T. singaporensis* sp. nov. in the present study.

Species	Locality	GenBank accession no.			Data source
		COI	12S rRNA	ITS1	
<i>T. squamosa</i>	Hong Kong	DQ363697	DQ363721	DQ363745	Chan et al. in press
	Xiamen, China	DQ363704	DQ363718	DQ363742	Chan et al. in press
<i>T. pacifica</i>	Badoutz, Taiwan	DQ363683	DQ363713	DQ363738	Chan et al. in press
	Shimoda, Japan	DQ363692	DQ363712	DQ363731	Chan et al. in press
<i>T. japonica</i>	Hong Kong	DQ363708	DQ363722	DQ363747	Chan et al. in press
<i>T. formosana</i>	Badoutz, Taiwan	DQ363709	DQ363724	DQ363748	Chan et al. in press
<i>T. squamosa</i>	Singapore	EF035162~EF035167	EF035160~EF035161	EF035168	present study

RESULTS

Morphological variations

Tetraclita squamosa collected from Singapore has green parietes and sheath (Fig. 1A, B), and the tergo-scutal flaps have 2 faint white spots on both the scutal and tergal margins. The inner surfaces of the scutum and tergum are dusky green (Fig. 1C). The tergum and scutum geometry exhibits diagnostic differences compared to *T. squamosa* from southern China (Xiamen) and *T. pacifica* from the northwestern Pacific (Taiwan and Okinawa; $R = 0.669$, $p < 0.05$). From the nMDS plots of the scutum and tergum morphology, *T. squamosa* from Singapore is clearly separated from *T. squamosa* from southern China and *T. pacifica* (Fig. 3). The length of the tergal margin in the scutum and the spur length and the basi-scutal angle contributed ~50% of the total difference between the barnacles from Singapore and southern China (SIMPER analysis, Fig. 1C, D). *Tetraclita squamosa* from southern China has a longer spur (spur length 1.5 ± 0.3 mm, $n = 20$), a larger basi-scutal angle (mean $153^\circ \pm 6.9^\circ$) and a shorter tergal margin (mean 2.6 ± 0.7 mm) than those from Singapore (mean spur length: 0.9 ± 0.14 mm; mean basi-scutal angle: $130^\circ \pm 7^\circ$, mean tergal margin: 4.8 ± 0.3 mm, $n = 20$; Table 2). *Tetraclita pacifica* has a shorter tergal margin than *T. squamosa* from Singapore (Table 2).

The mouthparts also differ between the barnacles from Singapore and southern China. The labrum of *T. squamosa* from Singapore has 4 or 5 large teeth on each side while *T. squamosa* from Xiamen has a few small fine teeth (Fig. 2A; Chan et al. in press). The labrum of *T. pacifica* has 3 or 4 large teeth (Chan et al. in press) on each side. The labrum, maxilla, and mandibular palps possess serrulate setae (Fig. 2B, C, D, I). The mandibles contain 4 teeth, of which the 2nd and 3rd teeth are double and the 1st teeth has 3-5 spines on the edge (Fig. 2G). There is an obvious double tooth at the lower angle (Fig. 2F, H).

The 1st and 2nd cirri of *T. squamosa* from Singapore possess feathery serrulate setae and plumose setae (Fig. 3A-F). Cirrus III has fine bidentate setae, in addition to the feathery plumose setae present on cirri I and II (Fig. 3G, H, I). On cirrus IV, there

are simple strong setae on the dorsal surface and also pectinate setae that consist of a single row of setules (Fig. 3J, H). The setal types of cirri V and VI are similar to those on cirrus IV.

Table 2. Morphological comparisons of the *Tetraclita squamosa* from Singapore and Xiamen and *T. pacifica* from the northwestern Pacific. *Tetraclita squamosa* from Singapore was identified as a new species, *T. singaporensis* sp. nov.

	<i>T. squamosa</i> (Singapore)	<i>T. squamosa</i> (southern China)	<i>T. pacifica</i> (northwestern Pacific)
Scutum	Very long tergal margin, short articular ridge-basal margin	Long articular ridge-basal margin	Short articular ridge-basal margin
Tergum	Straight scutal margin, not beaked, with wide spur	Most are beaked, with sharp and long spurs	Not beaked, with wide spur
Tergal-scutal flaps	Black with 2 white spots	Black with 2 white spots	Black with no pattern
Setae on cirrus I	Possessing plumose and bidentate serrulate setae	Possessing plumose and bidentate serrulate setae	Possessing plumose and bidentate serrulate setae, and an extra type of serrulate setae with four rows of setules and serrate setae

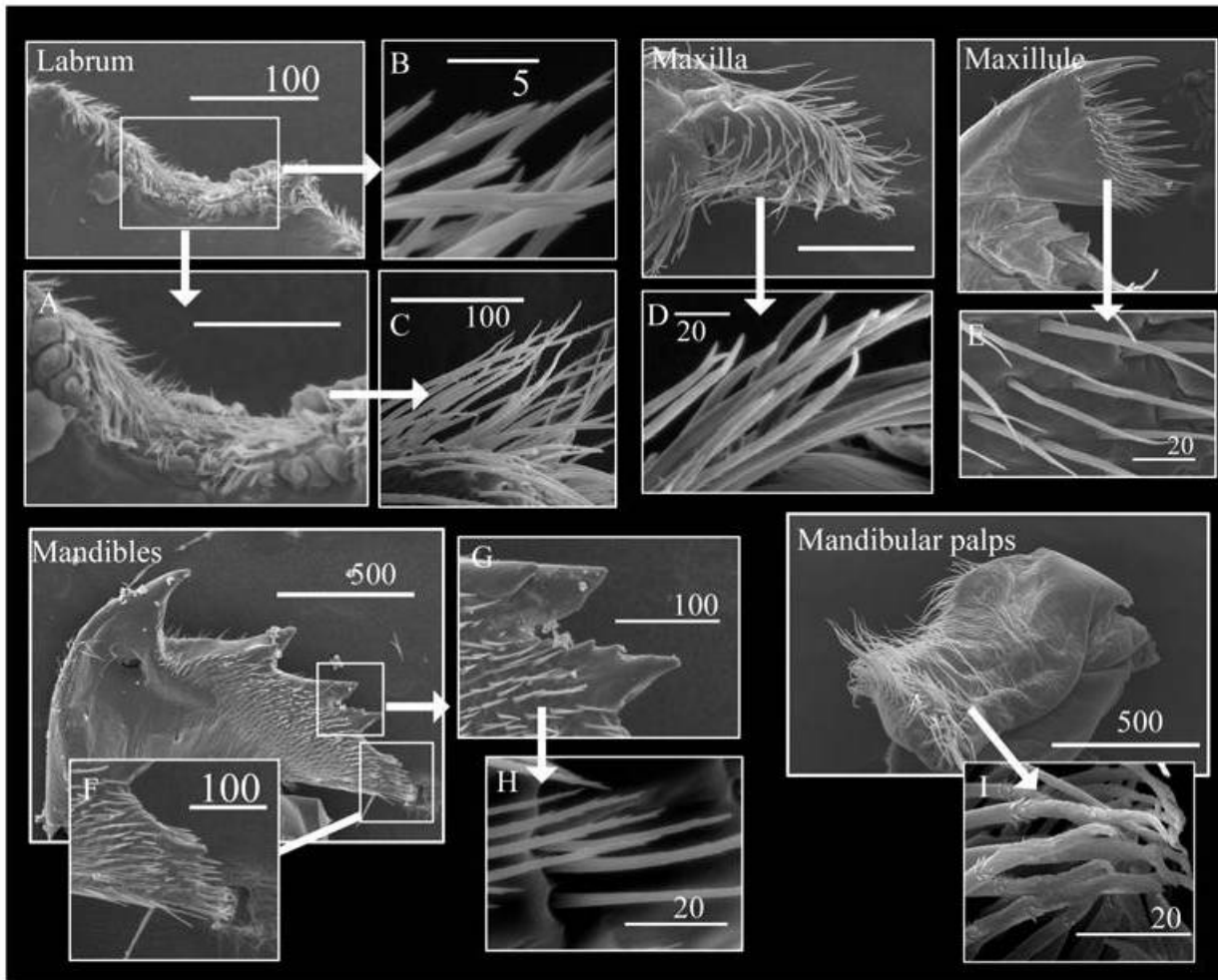


Fig. 2. SEM investigations of the mouthparts of *Tetracrita squamosa* from Singapore showing the setal types in the labrum (A-C), maxilla (D), maxillule, (E), mandibles (F-H), and mandibular palps (I). Scale bars in micrometers (μm). *Tetracrita squamosa* from Singapore is referred to as a new species, *T. singaporensis* sp. nov., in the present study.

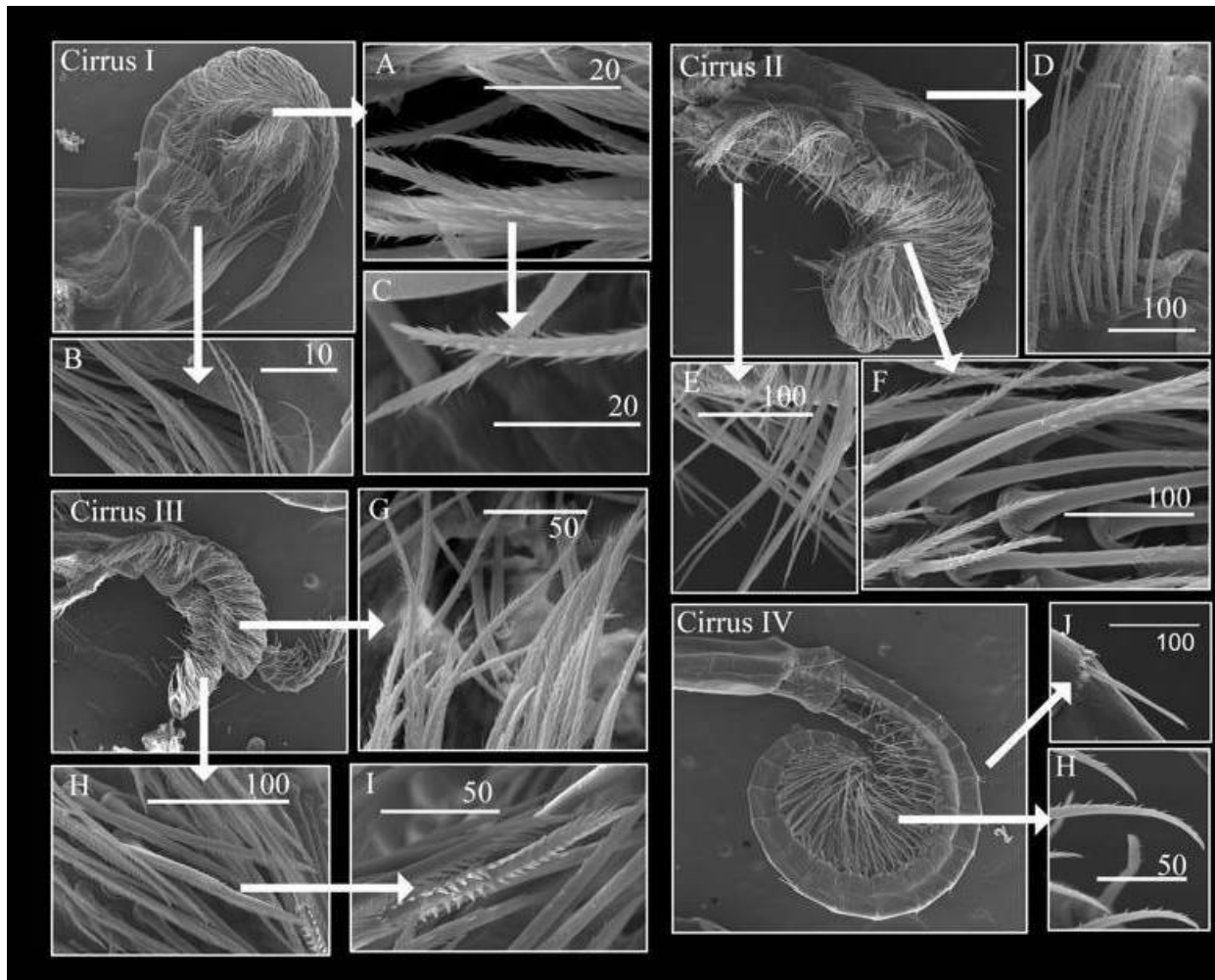


Fig. 3. SEM investigations in the cirral setae of *Tetracilita squamosa* from Singapore, showing the setal types on cirrus I (A-C), cirrus II (D-F), cirrus III (G, H), and cirrus IV (J-H). Scale bars in micrometers (μm). Cirri V and VI are not shown as they have similar morphology to cirrus IV. *Tetracilita squamosa* from Singapore is referred to as a new species, *T. singaporensis* sp. nov., in the present study.

Genetic divergence and phylogenetic relationships

COI (11 individuals), 12S rRNA (2 individuals), and ITS1 (5 individuals) sequences of *Tetraclita squamosa* from Singapore were analyzed, and all sequences were deposited in GenBank (accession nos.: EF035160-EF035168). The aligned COI fragments with other taxa (Table 1) consisted of 628 bp of which 182 sites were variable and 173 sites were parsimoniously informative. The aligned 12S rRNA sequences included 309 bp, with 41 out of 44 variable sites parsimony informative. The ITS1 sequences of barnacles from Singapore were 250 bp in length and differed from those of other *Tetraclita* spp. analyzed (258 bp for *T. pacifica*; 249 bp for *T. squamosa* of Hong Kong and Xiamen; and 258 bp for *T. japonica* and *T. formosana*). The aligned data set contained 262 sites, 59 of which were variable and 62 were parsimony informative.

The 11 COI sequences of *T. squamosa* from Singapore contained 6 haplotypes which differed from each other by < 1.5%. All 5 individuals analyzed for ITS1 shared the same genotype indicating little genetic differentiation within the population. Thus, only the 2 individuals with the 12S rRNA sequences determined were included in subsequent phylogenetic analyses. The ILD analysis revealed no significant incongruence among the 3 datasets ($p = 1.00$), and the results of separate analyses of the 3 gene segments and the combined data set were the same. Thus only the phylogenetic tree from the combined analysis is shown (Fig. 4).

The combined data set consisted of 1216 characters, 288 of which were variable and 273 were parsimony informative. The best-fitting model selected using Modeltest was GTR+I. All 3 phylogenetic inference methods revealed the same topology with 100% bootstrap support on all branches. Thus, only the ML tree is presented (Fig. 4). *Tetraclita squamosa* from Hong Kong and Xiamen formed a sister group to *T. pacifica* with 100% bootstrap support. Barnacles from Singapore were clustered together and clearly separated from the above 2 taxa. They were also differentiated from the outgroup taxa, *T. japonica* and *T. formosana*.

The individuals of *T. squamosa* from Singapore were differentiated from *T. squamosa* from Xiamen and Hong Kong, and *T. pacifica* by more than 19%, 10%, and

11% divergences in the COI, 12S rRNA, and ITS1 sequences, respectively. These values were much higher than those within each taxon (< 1.5% in the most variable COI sequence), showing that they are genetically distinct. The sequence divergences between barnacles from Singapore and the 2 outgroups *T. japonica* and *T. formosana*, were higher than 16%, 9%, and 17% for the COI, 12S rRNA, and ITS1 sequences, respectively.

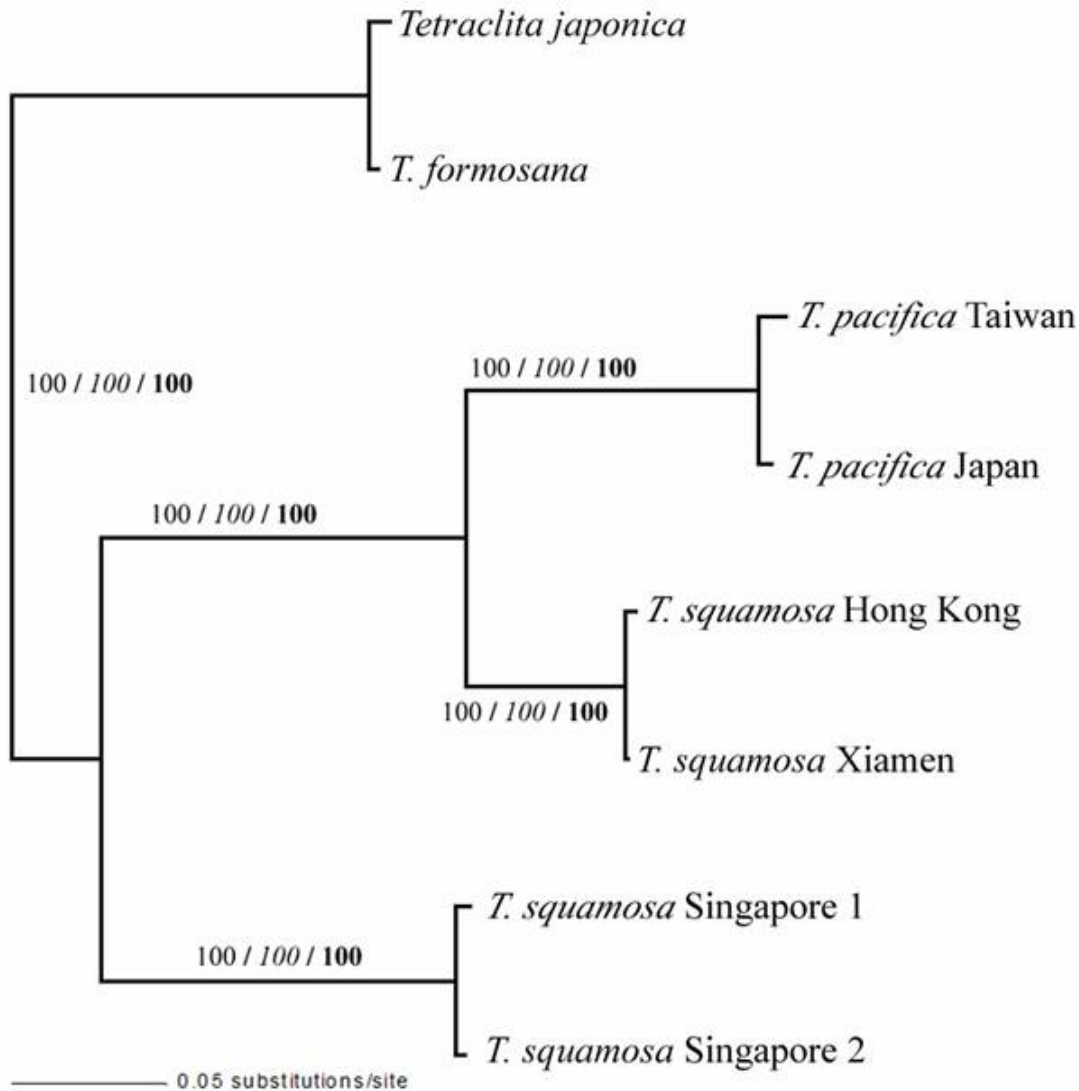


Fig. 4. ML phylogram constructed from the combined data of COI, 12S rRNA, and ITS1 gene sequences. The bootstrap values (ML, normal font; MP, italic; NJ, bold) are shown on the branches.

DISCUSSION

Morphological and molecular variations between *T. squamosa* from Singapore and the southern China coast

Tetraclita squamosa collected from Singapore exhibited diagnostic morphological and genetic differences from *T. pacifica* of the northwestern Pacific and *T. squamosa* of southern China. Although the external parietes of barnacles from Singapore, *T. pacifica*, and *T. squamosa* are similar by being green, the scutum and tergum differ among them. *Tetraclita squamosa* from Singapore has a long tergal margin of the scutum and a straight scutal margin of the tergum, and thus differs from *T. pacifica* and *T. squamosa* (southern China). The tergum of *T. squamosa* from southern China has a longer spur and large basi-scutal angle different from that of barnacles from Singapore and *T. pacifica* (Fig. 1C). The setal types on the cirri of barnacles from Singapore are similar to those of *T. squamosa* from southern China. However, barnacles from Singapore and southern China both differ from *T. pacifica* by lacking the serrulate setae with 4 rows of setules (Chan et al. in press).

Tetraclita squamosa from Singapore also exhibits high genetic differentiation from *T. squamosa* of southern China and *T. pacifica*. This is consistent with the results from an unpublished study by YX Cai (http://www.pims.ust.hk/fellowship/fellowship_report.html) who showed that *T. squamosa* collected from Singapore is genetically distinct based on 3 mitochondrial gene segments (12S rRNA, 16S rRNA, and COI) as compared to other *Tetraclita* spp. In the present study, we analyzed 3 gene segments (12S rRNA, COI, and ITS1) from both the mitochondrial and nuclear genomes. The high sequence divergences determined are comparable to intraspecific divergences reported among other *Tetraclita* species (Appelbaum et al. 2002; Chan et al. in press) as well as those in other barnacles genera (e.g., *Chthamalus* and *Euraphia*, Wares 2001; *Cantellius* and *Savignium*, Mokady et al. 1999). The high divergence in the nuclear marker, ITS1, provides strong evidence for reproductive isolation among barnacles from the northwestern Pacific, southern China, and Singapore. The clear genetic and

morphological differences of barnacles from both *T. squamosa* and *T. pacifica* indicate that the barnacle from Singapore represents a new species. *Tetraclita squamosa* from southern China represents the true taxonomic identity of *T. squamosa*, because Pilsbry (1916) redescribed *T. squamosa* on the basis of samples collected in southern China.

Description of new species

Sub-family Tetraclitinae Gruvel, 1903

Genus *Tetraclita* Schumacher, 1817

Tetraclita singaporensis sp. nov. (Figs. 1-3)

Holotype. Pulau Tekukor, St. John's I., Singapore Strait, Singapore, 12 Apr. 2006, Coll. KS Tan (Raffles Museum of Biodiversity Research ZRC 2006.0153).

Paratype. Same data as for holotype (Research Museum, Research Center for Biodiversity, Academia Sinica, Taipei, Taiwan, ASIZCR000198; The Swire Institute of Marine Science Museum, Department of Ecology and Biodiversity, The Univ. of Hong Kong, Hong Kong, SWIMS-CRU-2006-03).

Etymology. The name of this species denotes its discovery in Singapore.

Diagnosis. Parietes green and tergal-scutal flaps black with faint white spots. Inner surfaces of scutum and tergum dusky-green. Tergum usually without beaks, with a very straight scutal margin, spur wide and short (Fig. 1C). Tergal margin of scutum long. Mandibles containing 4 teeth (Fig. 2), 2nd and 3rd teeth consisting of double teeth, 1st tooth with 4 or 5 small spines. Cirri I and II possessing serrulate and pappose setae (Fig. 3). Cirrus III possessing serrulate, pappose, and fine bidentate setae, without cuspidate setae (Fig. 3).

Description (holotype). Basal tergal-scutal length 26.28 mm and height 15.96 mm, orifice diameter 4.53 mm.

Implications for the taxonomy of the *T. squamosa* species complex

The taxonomy of the *T. squamosa* complex has long been problematic as a result of the high degree of morphological variation. The green parietes of *T. squamosa* were considered to be a diagnostic feature separating it from the other *Tetraclita* species (as *T. squamosa viridis* in Darwin 1854, Pilsbry 1916, Newman and Ross 1976, Ren and Liu 1979). However, morphological and molecular studies of green barnacles in Asia have uncovered at least 2 new species previously considered to be *T. squamosa* (Chan et al. in press; present study). This indicates that the barnacles recognized as *T. squamosa* in the Indo-West Pacific region are a cryptic species complex with unexpected high morphological and genetic diversities. Given the relatively restricted area which has been investigated to the present, it is very likely that more species will be identified in Southeast Asia, Australia, and the Indian Ocean. This will require further detailed studies based on samples throughout the geographical range of '*T. squamosa*'. Elucidating the taxonomic status of this species complex and the geographic distribution of each species in this complex not only will provide a solid taxonomic framework for future biological and ecological studies on this important intertidal organism, but will also enhance our understanding of the phylogeography and evolutionary history of the intertidal fauna of the Indo-West Pacific.

Acknowledgments: The authors would like to thank Wolfie C.-H. Lai (HKU) and K.S. Tan (National Univ. of Singapore) for collection of *Tetraclita* from Singapore and P.-C. Tsai, C.-H. Hsu and T.-L. Lin (Academia Sinica) for their support in the SEM investigations. The work described in this paper was substantially supported by a grant from the Research Grants Council, Hong Kong (project no. HKU7597/05M), and a grant from the National Science Council, Taiwan (NSC95-2621-B-001-014). The authors also thank the Agriculture, Fisheries and Conservation Department of the Hong Kong SAR Government for permission to collect *Tetraclita* in the Cape d'Aguilar Marine Reserve. The experiments complied with the current laws of Hong Kong SAR and Taiwan.

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