SYMPOSIUM

Genetic and Morphological Differentiation of the Indo-West Pacific Intertidal Barnacle *Chthamalus malayensis*

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Synopsis *Chthamalus malayensis* is a common intertidal acorn barnacle widely distributed in the Indo-West Pacific. Analysis of sequences of mitochondrial cytochrome *c* oxidase subunit I reveals four genetically differentiated clades with almost allopatric distribution in this region. The four clades exhibit morphological differences in arthropodal characters, including the number of conical spines and number of setules of the basal guard setae on the cirri. These characters are, however, highly variable within each clade; such that the absolute range of the number of conical spines and setules overlaps between clades, and therefore, these are not diagnostic characters for taxonomic identification. The geographic distribution of the four clades displays a strong relationship between surface temperatures of the sea and ocean-current realms. The Indo-Malay (IM) clade is widespread in the tropical, equatorial region, including the Indian Ocean, Malay Peninsula, and North Borneo. The South China (SC) and Taiwan (TW) clades are found in tropical to subtropical regions, with the former distributed along the coasts of southern China, Vietnam, Thailand, and the western Philippines under the influence of the South China Warm Current. The TW clade is endemic to Taiwan, while the Christmas Island (CI) clade is confined to CI. There was weak or no population subdivision observed within these clades, suggesting high gene flow within the range of the clades. The clades demonstrate clear signatures of recent demographic expansion that predated the Last Glacial Maximum (LGM), but they have maintained a relatively stable effective population in the past 100,000 years. The persistence of intertidal fauna through the LGM may, therefore, be a common biogeographic pattern. The lack of genetic subdivision in the IM clade across the Indian and Pacific Oceans may be attributed to recent expansion of ranges and the fact that a mutation-drift equilibrium has not been reached, or the relaxed habitat requirements of *C. malayensis* that facilitates high concurrent gene flow. Further studies are needed to determine between these alternative hypotheses.

Introduction

The Indo-West Pacific (IWP) is famous for its extraordinary marine biodiversity (Hughes et al. 2002; Roberts et al. 2002; Tittensor et al. 2010). However, this "high" biodiversity is only the tip of the iceberg, as the rate of species discovery in the IWP, in particular, in the Coral Triangle, is high with new species continually being discovered (e.g., Bouchet et al. 2009; de Forges et al. 2009). The accurate description of species and their ranges provides critical baseline information for ecology and conservation, especially given concerns over increasing anthropogenic threats and global climatic change (Tittensor et al. 2010). Determination of species ranges and population connectivity is, therefore, becoming of increasing importance as climate warms and the seas become more acidic.

Elucidation of gene flow in the IWP is challenging due to the area's complex geological history and dynamic hydrology (Briggs 1999). There are many archipelagos and islands in the West Pacific and neighboring regions. Formation of land bridges and fluctuations in sea level were prevalent during repeated glacial cycles in the Pleistocene (Voris 2000; Lohman et al. 2011), and these promoted allopatric diversification of marine biota (e.g., Benzie 1999; Barber et al. 2000, 2006; Lourie et al. 2005). Gene flow between the Indian and Pacific Oceans was, for example, blocked due to lower sea levels during the Pleistocene glaciations, resulting in genetic differentiation of marine species between the two oceans (Gaither et al. 2010 and references therein). Contemporary ocean circulation patterns are believed to maintain and/or promote present-day genetic structuring and spatial distribution of taxa (Ito et al. 1995; Gordon and Fine 1996; Guan and Fang 2006; Yang et al. 2008). Fine-scale phylogeographic investigations in the IWP, especially those focusing on the South China (SC) Sea and the Coral Triangle, would help to elucidate the relative roles of these various factors in determining the biogeographic patterns observed in the IWP.

Chthamalus is a common genus of barnacles inhabiting the high shore of the rocky intertidal zone worldwide (Newman and Ross 1976). *Chthamalus* is often used as a model organism in intertidal studies because it is locally abundant and, being a high shore inhabitant, lives close to its physiological limit, and therefore may serve as a potential indicator for early signals of biotic responses to climatic change (Southward 1991; Southward et al. 1995; Herbert et al. 2003, 2007; Helmuth et al. 2006; Hawkins et al. 2008, 2009). Species of *Chthamalus* are, however, difficult to identify because of the great variation in their external morphology and high phenotypic plasticity (Southward 1983; Lively 1986; Southward et al. 1998; Southward and Newman 2003). Based on the arthropodal morphology, Dando and Southward (1980) and Southward et al. (1998) separated *Chthamalus* into four subgroups, including the *challengeri*, *fissus*, *stellatus*, and *malayensis* subgroups (Southward and Newman 2003). The *malayensis* subgroup is distributed in the IWP and is characterized by the presence of conical spines on the dorsal surface of the anterior ramus of cirri I and II (Fig. 1A–C). In addition, cirrus II of species in the *malayensis* subgroup has multicuspidate setae with basal guards (Fig. 1D; Southward et al. 1998; Southward and Newman 2003). *Chthamalus malayensis* Pilsbry 1916 is the key species of the *malayensis* subgroup, and an important species in terms of biogeographic comparisons as it is the dominant chthamalid in the IWP (Fig. 1A). Many early descriptions of this species are incomplete or erroneous, which has resulted in taxonomic confusion, as well as uncertainties of the species' true range (Southward and Newman 2003). Southward and Newman (2003) performed a detailed morphological re-examination of various museum collections when reviewing the distribution of *C. malayensis*, and confirmed that this species occurs widely in the Indian Ocean, Indo-Malay (IM) region, Indonesia, China, and tropical Australia, with little morphological differentiation over this broad spatial scale.

Our previous investigations of the genetic diversity of *C. malayensis* in the IWP, based on the mitochondrial cytochrome *c* oxidase subunit I (COI), revealed three divergent clades that have largely allopatric distributions (Tsang et al. 2008a). Some ecological differences, e.g., reproductive season (Koh et al. 2005; Yan et al. 2006) and tidal range (Tsang et al. 2008a), are evident among clades. Taxonomic identity of the three clades of *C. malayensis sensu lato*, however, remains obscure and further morphological examination is required to verify the taxonomic status of the different clades. The fine-scale distribution of these clades within the IWP is also largely unknown, as geographic coverage of sample sites, and the number of sequences used, were limited in the study by Tsang et al. (2008a). In this present study, we attempt to identify fine-scale spatial variation in morphology and population genetic structuring of *C. malayensis sensu lato* with an expanded geographic sampling effort, covering most of the locations where this species has been recorded in the IWP region. In order to disentangle the observed geographic/intraspecific variations from interspecific differentiation, and validate whether the molecular clades identified represent biological species, we examined morphological differences in the opercular plate morphology and the arthropodal characters of the various *C. malayensis* clades and populations. We also used DNA sequences of mitochondrial COI to infer the potential influences of historic geological events and contemporary oceanic current realms on the biogeographic patterns of marine species in the IWP.
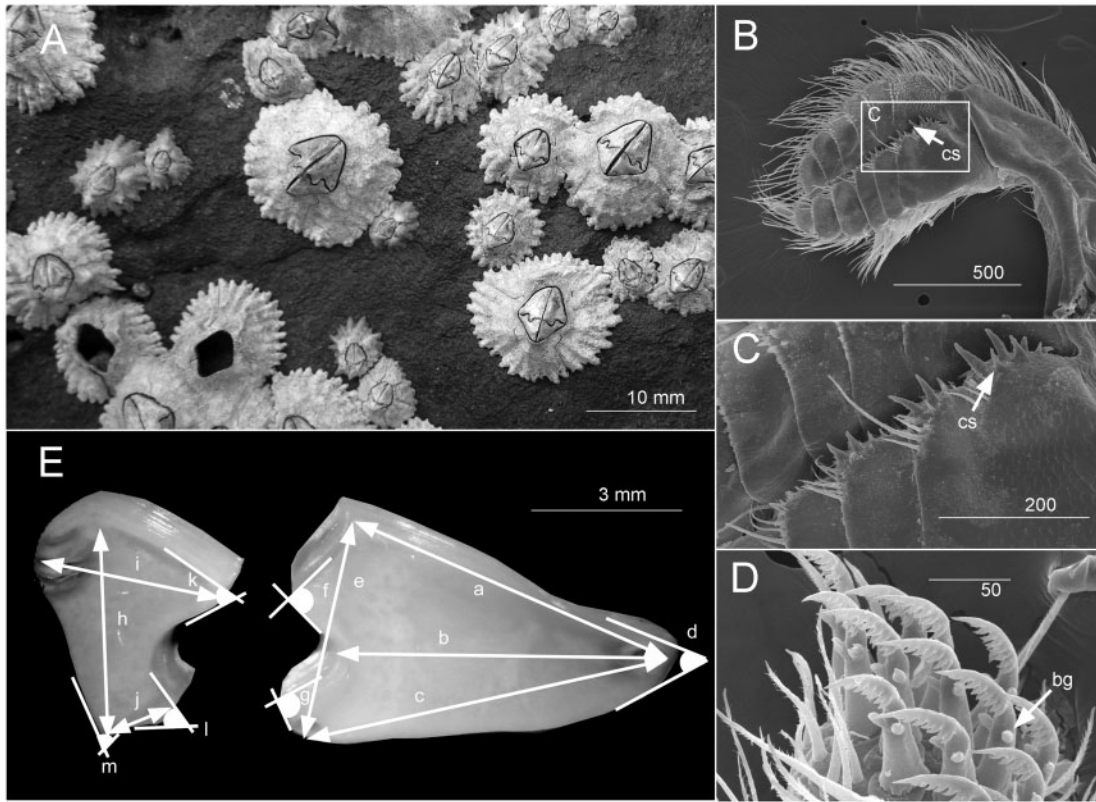
Fig. 1 (**A**) *Chthamalus malayensis* belongs to the *malayensis* subgroup of *Chthamalus* and is the major occupier of space on the IWP high shore. The key diagnostic characters of the species in the *malayensis* subgroup include conical spines on the dorsal surface of the anterior ramus of cirri I (**B** and **C**) and II and the presence of multicuspidate setae which have basal guards (bg) on cirrus II (**D**). (**E**) Length and angle parameters measured in tergum (left) and scutum (right) of *C. malayensis* for comparison of opercular geometry among different clades. *a* occludent margin, *b* length of scutum, *c* basal margin of scutum, *d* angle between occludent and basal margin, *f* angle 1 of the tergal margin, *g* angle 2 of the tergal margin, *h* tergum height, *i* tergum width, *j* basal margin of tergum, *k* angle between occludent margin and scutal margin, *l* basi-scutal angle, *m* spur angle. Scale bars in μm, except A, E which are in mm.

Materials and methods

Collection of samples

Chthamalus malayensis were collected from 29 localities in the IWP, with a major focus on the SC Sea, either through our own site visits or by local collaborators between 2007 and 2011 (Table 1; Fig. 2). Whole individuals were removed from the rock surface and preserved in either 95 or 100% ethanol prior to laboratory analysis.

DNA extraction, polymerase chain reaction, and DNA sequencing

Genomic DNA was extracted from whole-soft tissues of individual barnacles using the commercial QIAamp Tissue Kit (QIAGEN). Partial sequences of mitochondrial COI were amplified using the universal primers LCO1490 and HCO2198 (Folmer et al.

1994). The amplifications were conducted in a reaction mix containing 1 μl of template DNA, 1 × PCR reaction buffer, 3 mM MgCl$_2$, 200 nM of each primer, 200 μM dNTPs, 1.5 units of *Taq* polymerase (Takara), and ddH$_2$O to a total volume of 25 μl. The PCR followed the standard profile with a 3-min initial denaturation at 94°C, followed by 35 cycles of 30 s at 94°C, 30 s at 47–51°C, 1 min at 72°C with a final extension for 5 min at 72°C. The successful amplicons were then either purified using the QIAquick Gel Purification Kit (QIAGEN) or Millipore Montage PCR96 Cleanup Kit. Sequences were generated using the forward primer and an Applied Biosystems (ABI) 3700 automated sequencer using the ABI Big-dye Ready-Reaction Mix Kit, following the standard cycle sequencing protocol. Sequencing reactions were also performed using the reverse primer if ambiguity of bases was observed in

Table 1 Sample localities, abbreviations, sample sizes (n), and genetic diversity of COI, including number of haplotypes (Na), haplotype diversity (h), nucleotide diversity (π), Tajima's D and Fu's F_S for each population

Population		Abbreviation	n	Na	h	π	D	F_S
Turtle Island (Gueishan Island), Taiwan	SC clade	TI	14	14	1.00	0.014	−1.347	−7.434**
	TW clade		15	11	0.95	0.005	−1.586	−6.15**
Gongliao, New Taipei City, Taiwan	SC clade	NE	13	13	1.00	0.012	−0.849	−7.264***
	TW clade		28	18	0.94	0.007	−1.403	−8.838**
Ping Long Qiao (NE coast, Keelung), Taiwan	SC clade	PL	16	16	1.00	0.013	−1.219	−9.897***
	TW clade		14	13	0.99	0.009	−1.407	−7.200***
Suao, Ilan, Taiwan	SC clade	SU	1	1	−	−	−	−
	TW clade		31	20	0.95	0.008	−1.339	−9.852**
Dasi, Ilan, Taiwan	TW clade	DA	27	21	0.97	0.008	−1.747*	−14.009***
Shi Ti Ping, Taitung, Taiwan	TW clade	ST	21	17	0.97	0.007	−1.705*	−11.129***
Penghu, Taiwan	SC clade	PH	36	33	0.99	0.012	−1.667*	−24.996***
Hong Kong, China	SC clade	HK	35	31	0.99	0.012	−1.757*	−24.01***
Hoi Ling Island, Guangdong, China	SC clade	HL	30	28	0.99	0.012	−1.743*	−22.226***
Sanya, Hainan, China	SC clade	SY	20	20	1.00	0.012	−1.393	−15.298***
Puerto Galera, Philippines	SC clade	PG	35	25	0.97	0.012	−1.473	−10.912**
Boracay Island, Philippines	SC clade	BC	10	9	0.98	0.010	−1.428	−3.040*
Da Nang, Vietnam	SC clade	DN	14	12	0.98	0.011	−1.197	−4.265*
Nha Trang, Vietnam	SC clade	NT	27	26	0.99	0.010	−1.743*	−24.281***
Muine, Vietnam	SC clade	MN	51	42	0.99	0.012	−1.842**	−25.008***
	IM clade		5	5	1.00	0.007	−0.895	−1.633
Phu Quoc Island, Vietnam	SC clade	PQ	66	55	0.99	0.011	−1.858**	−25.041***
Sri Chang Island, Thailand	SC clade	SC	7	7	1.00	0.013	−0.705	−2.016
Samui Island (Koh Samui), Thailand	SC clade	SM	30	30	1.00	0.013	−1.530*	−24.96***
Nexus Beach, Sabah, Malaysia	IM clade	NB	53	39	0.97	0.007	−1.982**	−25.882***
Labuan Island, N. Borneo, Malaysia	IM clade	BN	24	21	0.99	0.006	−1.737*	−19.127***
Labrador, Singapore	IM clade	SP	32	23	0.93	0.005	−1.962**	−21.846***
Mersing, Malaysia	IM clade	MS	28	22	0.98	0.005	-2.027**	−21.178***
Penang, Malaysia	IM clade	PN	32	19	0.83	0.004	−1.963*	−13.433***
Langkawi, Malaysia	IM clade	LK	18	13	0.93	0.004	−1.718*	−9.347***
Phuket, Thailand	IM clade	PK	18	12	0.90	0.004	−2.145**	−7.986***
Christmas Island, Australia	CI clade	CI	13	13	1.00	0.017	−0.086	−5.424**
Negombo, Sri Lanka	IM clade	SL	42	32	0.96	0.005	−2.248***	−26.265***
Tranquebar, India	IM clade	TQ	53	27	0.87	0.004	−2.023**	−24.746***
Mumbai, India	IM clade	MB	50	30	0.95	0.005	−2.004**	−26.041***

*$P<0.05$, **$P<0.01$, ***$P<0.001$

the chromatogram. COI sequences of other chthamalid species from the Pacific were also determined or obtained from GenBank as out-groups for comparison (Appendix 4).

Phylogenetic and population-genetic analyses

Nucleotide sequences were aligned using MUSCLE (Edgar 2004), with alignment being confirmed by translating into amino-acid sequences. Phylogenetic relationships between individuals were determined by neighbor-joining (NJ) based on analysis of Kimura 2-parameter (K2P) distance using PAUP* v4.0 b10 (Swofford 2000) and maximum likelihood (ML) as implemented in RAxML 7.0.3 (Stamatakis 2006). The model GTRGAMMAI was used for the ML analysis with α-shape parameters, and GTR rates and base
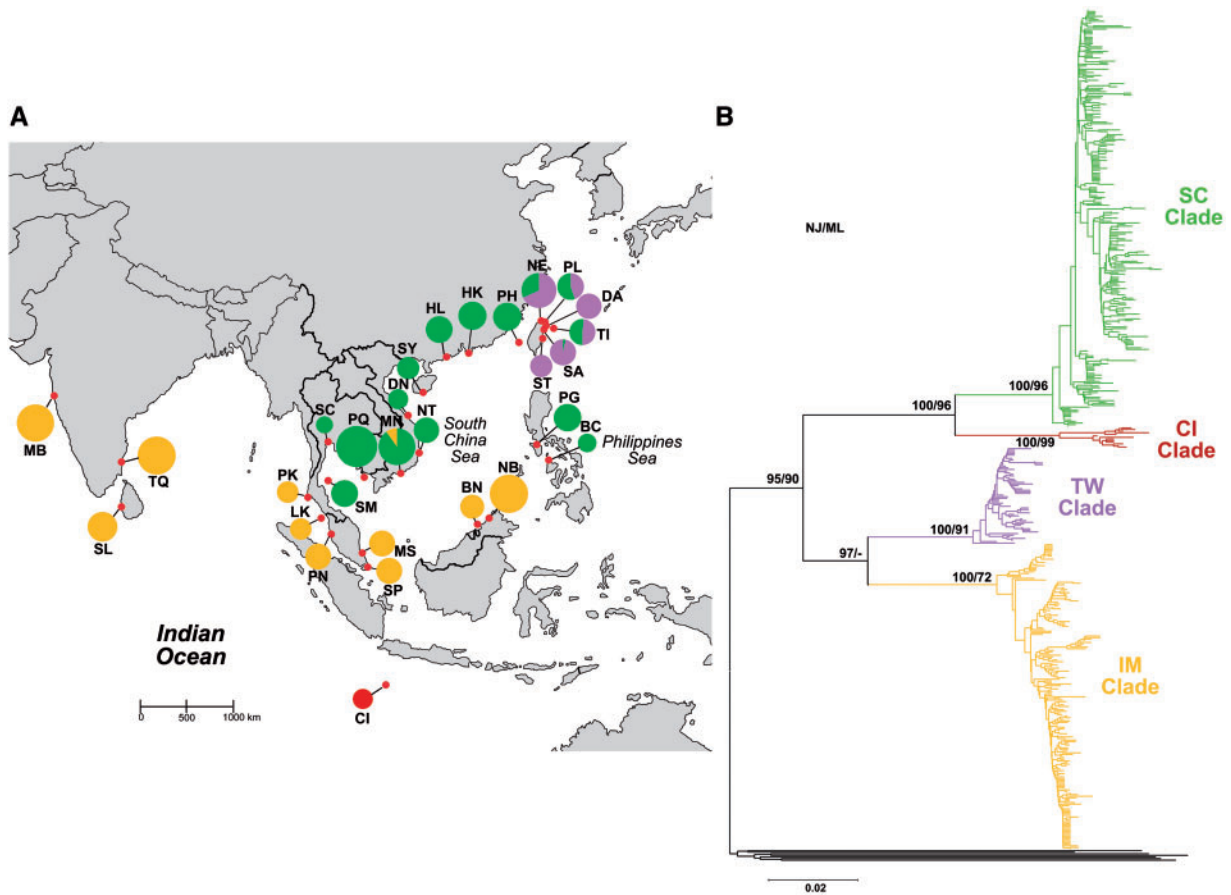
Fig. 2 (A) Sampling sites of *C. malayensis* in this study with pie charts showing the relative frequency of each clade. The area of the pie chart is proportional to the sample size and colors denoting different clades correspond to that in the NJ tree. (B) NJ tree of COI haplotypes. The percentage of bootstrap support for the NJ and ML analyses is shown for the major clades on the corresponding branch for all values ≥70. See Table 1 for abbreviations of different localities and Appendix 4 for identity of the out-groups.

frequencies estimated and optimized during the run. The support for individual nodes was evaluated by 1000 bootstrap replicates. Standard genetic diversity indices, including haplotype diversity (h), nucleotide diversity (π), Tajima's D (Tajima 1989) and Fu's F_S (Fu 1997) were estimated for each population and clade using ARLEQUIN version 3.0 (Excoffier et al. 2005). Pairwise Φ_{ST} values were calculated to estimate the level of gene flow among populations within each clade. Analysis of Molecular Variance (AMOVA) (Excoffier et al. 1992) was used to test for the hypothesis of reduced gene flow between putative phylogeographic breaks (e.g., between populations from the Indian and Pacific Oceans; see 'Results' section for detail of partitions). Analysis of AMOVA was implemented in the same program incorporating the K2P and 10,000 random permutations to test for statistical significance.

Coalescent model analyses of demographic history

Mismatch distribution analysis (Rogers and Harpending 1992) was conducted with ARLEQUIN. When a signature of rapid expansion (a unimodal distribution) was detected, the parameter τ was computed, which is used to estimate the approximate timing of population expansion (Rogers and Harpending 1992; Schneider and Excoffier 1999). A divergence rate of 3.1% per MY (million years) in the COI locus between lineages was calibrated for a trans-isthmian *Euraphia* sister-species pair (*Euraphia* is a closely related genus to *Chthamalus*; Wares 2001). The expansion time of different clades was calculated using $s = 2\mu t$, where s is the crest of mismatch distribution, μ is the substitution rate of nucleotides and t is the generation time, which was assumed to be 1 year.

A Bayesian skyline plot (BS; Drummond et al. 2006) was constructed using BEAST version 1.5.3 (Drummond and Rambaut 2007) to estimate the change in population size over time. Each data set was run for 50 million generations under a HKY + I + G nucleotide substitution model with individual parameters estimated from the data, a constant skyline model with 10 groups, and uniform priors. The chain was sampled every 1000 generations and the first 50,000 generations were discarded as burn-in. The analysis for each data set was run thrice to ensure consistency and the results from the three replicate runs were combined and re-sampled with LogCombiner v1.5.3 (Rambaut and Drummond 2007). Trace plots were inspected to assess convergence, mixing, and stationarity of the MCMC process in Tracer v1.5 (Rambaut and Drummond 2007) and the effective sample sizes were also checked and confirmed to be ≥ 200.

Morphological analysis among phylogenetic clades of *C. malayensis*

Preliminary DNA analysis of *Chthamalus* populations in the IWP region revealed four phylogenetic clades (see 'Results' section): IM clade, SC Sea clade, Taiwan (TW) clade, and Christmas Island (CI) clade. Morphological analysis of the opercular plates (scutum and tergum) and arthropodal characters (cirri I and II and morphology of the mandible) was conducted on barnacles with confirmed clade identity based on COI sequencing.

Geometry of the opercular plate

Individuals from the IM clade (20 from Tranquebar, India, and 20 from Labrador, Singapore, $\Sigma n = 40$), SC clade (33 from Hong Kong), and TW clade (17 from Shi Ti Ping, Taiwan) and CI clade (five from CI) were dissected. A total of nine characters in the scutum and tergum, including length and angle variables, was measured (Fig. 1E). Variation in the morphology of the scutum and tergum among clades was analyzed using multivariate analysis (PRIMER 6, Plymouth Routine in Multivariate Analysis; Clarke 1993). The shape of the parietes was not included in comparisons as it exhibits great intraspecific morphological variation and is therefore not a reliable character for taxonomic identification (Southward and Newman 2003). Data were square-root transformed prior to analysis and similarity matrices were calculated using the Euclidean distance index. Nonmetric Multidimensional Scaling (nMDS) was conducted to generate two-dimensional plots of scutum and tergum morphology between barnacle samples from different clades (Chan et al. 2007a, b). nMDS was used for morphological analysis in this study as the matrix was constructed based on the similarity of opercular variables between each pair of barnacle samples. As a result, samples with similar morphologies would cluster together in the nMDS plot, whereas as dissimilar individuals would be separated. Using nMDS to analyze scutum and tergum variables has proved useful in identifying cryptic species of *Tetraclita* (Chan et al. 2007a, b). Analysis of Similarity (ANOSIM) was conducted to test for differences in opercular variables among clades.

Arthropodal characters

The morphology of cirri I and II of individuals was examined from the IM clade (10 from Tranquebar, India, 10 from Labrador, Singapore; $\Sigma n = 20$), the SC clade (10 from Hong Kong, 10 from Hainan Island and 12 from Penghu, $\Sigma n = 32$), TW clade (18 from Shi Ti Ping, Taiwan), and CI clade (5 from CI). For each individual, the left cirri I and II were dissected and examined under the compound microscope at $400 \times$ magnification (Zeiss Scope A1 with Zeiss Plan APOChromat $40 \times / 0.95$ lens). A total of 13 variables was examined including the number of segments of the anterior and posterior rami of cirri I and II, the number of segments on the anterior ramus where conical spines were present in cirri I and II, the number of conical spines per segment in anterior ramus both cirri I and II (up to five segments), and the number of segments on the ventral side of the anterior and anterior rami which have basal guard setae (Fig. 3A and B). On the mandible, the number of major teeth (excluding the inferior angle) of the mandible, number of small teeth on the pecten of the mandible and the number of pectinations at the inferior angle were scored (Fig. 3C and D). Variation in the 13 arthropodal characters among clades was compared separately using one-way ANOVA (Factor: fixed, three levels, phylogenetic clades, software used: SigmaStat 3.5). Due to the small sample size of the CI clade, morphological data for this clade were not included in the ANOVA analysis but were presented in graphic form. Data that violated the assumption of homogeneity of variances (Levene median test) were transformed, prior to analysis.
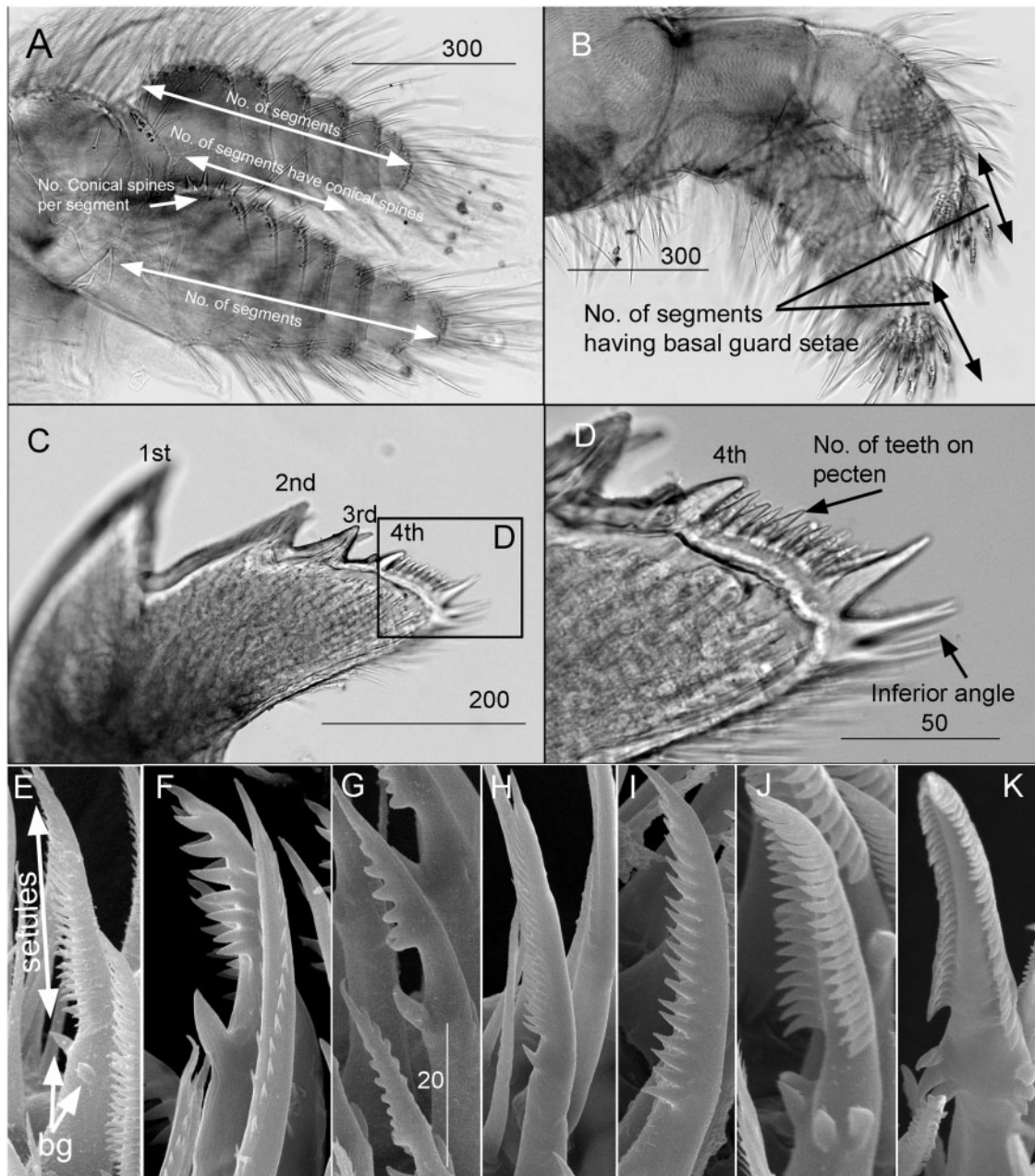
Fig. 3 Arthropodal characters of *C. malayensis* measured for comparison among clades. (**A**) Number of anterior and posterior rami on cirri I and II, number of segments having conical spines in the anterior ramus of cirri I and II, and number of conical spines per segment in the anterior ramus of cirri I and II. (**B**) Number of segments having the basal guard setae on the posterior side of the anterior and posterior rami of cirrus II. (**C**) Number of major teeth on the mandible. (**D**) Number of small teeth on the pecten of the mandibles and number of pectinations at the inferior angle of the mandible. (**E**) Number of setules and number of basal guards measured for the morphological comparison of basal guard setae. (**F–K**) Variation of the morphology of basal guard setae (F–K, same scale bar as G). (**F**) Short basal guard setae with sharp setules in the SC clade. (**G**) Short setae with blunt setules in the TW clade. (**H**) Basal guard setae with numerous setules in the SC clade. (**I**) Basal guard setae without basal guards are occasionally found in the IM clade. (**J**) Basal guard setae with two pairs of basal guards in the IM clade. (**K**) Basal guard setae with three pairs of basal guards in the IM clade. Scale bars in μm.

Morphological variation of the basal guard setae on cirrus II in the IM clade (20 individuals from Tranquebar, India, 12 from Labrador, Singapore, $\Sigma n = 32$), the SC clade (27 from Hong Kong, 25 from Hainan Island, $\Sigma n = 52$), the TW clade (44 from Shi Ti Ping, Taiwan), and CI clade (5 from CI) was observed using Scanning Electron Microscopy (SEM methods for examination of setae followed Chan et al. 2008a). Under the SEM, the setules and basal guards of each seta can be observed clearly. The number of setules and number of basal guards on each basal guard seta in cirrus II were recorded. The number of setules and number of basal guards on all basal guard setae on cirrus II were averaged for each individual (Fig. 3E). The number of setules and number of basal guards on barnacles among clades were compared using one-way ANOVA (Factor, fixed, three levels, phylogenetic clades). CI clades were not included in the ANOVA analysis due to small sample size, but were presented in graphic form. SNK tests were performed to test for differences in arthropodal characters among phylogenetic clades for significant ANOVA tests.

Ecological survey of C. malayensis

The abundance of Chthamalus was recorded at different tidal levels at sites visited by BKKC (total = 26 sites; Table 1 for details of sites). At each site, a 20-m stretch of shoreline was chosen on a sloping rock platform. Three 20-m horizontal transects (named H, M, and L from high- to low-tidal levels) at 25-cm vertical intervals were established over the entire vertical distribution of C. malayensis. The highest transect (H) was located at the upper limit of C. malayensis and tidal levels were estimated from hourly tidal information (using WX tides, http://www.wxtide32.com/). Maximum tidal range varied between sites (see Appendix 3 for details), with most sites having semi-diurnal tides, except along the Vietnam coastline and Sabah in North Borneo where diurnal tidal patterns are predominant. Along each transect, 10, 15×15 cm quadrats were randomly located (the base of the quadrats was placed on the transect line, so that the 15×15 cm area above the transect line was recorded for each quadrat) and a digital photograph of each quadrat was taken. The percentage cover of C. malayensis in each quadrat was scored from the digital photograph (following Tsang et al. 2008a).

Sea-surface temperatures and patterns of oceanic currents

Average sea-surface temperatures (SST) for the IWP region in January and June 2009 were extracted from satellite remote-sensing data in the GIOVANNI Database, NASA, USA. Patterns of oceanic currents in the SC Sea were obtained from the model by Yang et al. (2008).

Results

Divergence of sequences and phylogenetic analysis

A total of 797 new sequences (GenBank accession nos. JQ754708–JQ755177) was collected and combined with 108 sequences from a previous study (Tsang et al. 2008a; GenBank accession nos. EU304363–EU304448). The final aligned COI data set contained 615 bp of 905 C. malayensis (sensu lato) individuals that consisted of 540 unique haplotypes (Table 1) and seven out-groups. No indels or stop codons were found. NJ and ML analyses revealed four strongly supported clades in C. malayensis (sensu lato) (Fig. 2B). The three clades formerly described by Tsang et al. (2008a), namely the SC Sea clade (hereafter referred to as SC clade) and TW and IM clades were confirmed and the distributional range of the clades were revealed in more detail with the expanded sampling (Fig. 2A). A fourth clade, restricted to CI (CI clade), was also discovered. The CI clade is more closely related to the SC clade, as opposed to the geographically closer IM clade. The IM clade is a sister taxon to the TW clade. The mean net sequence divergence (mean inter-clade divergence − the intra-clade divergence) among the four clades ranged from 5% between the closest taxa (SC and CI clades) to 10.5% between the most divergent clades (IM and CI clades; Table 2). These values were much larger than the greatest sequence divergences within each clade ($\leq 2.7\%$) or mean intra-clade divergence ($\leq 1.7\%$).

Haplotype diversity was very high for all four clades (Table 3). In contrast, the nucleotide diversity was only high in the SC and CI clades (>0.011 for both clades; Table 3) but much lower in the other two clades (0.005 in the IM clade and 0.007 in the TW clade; Table 3). In the SC clade, most of the haplotypes were sampled only once and no single haplotype could be found in all populations. One major haplotype occurred in 76 of the 356 individuals sequenced in the IM clade and this haplotype was shared by all populations of the IM clade, with

the exception of Mumbai. The majority of the remaining haplotypes were singletons, most of which differed from the major haplotype by 1–3 mutation steps. The haplotype distribution pattern of the TW clade was similar to that of the IM clade, with one major haplotype observed in 19 of the 136 sequences and many singletons. The sample size of CI was small, so that the results of population-genetic parameters were inconclusive.

Population genetic structure and demographic history of the clades

Most pairwise Φ_{ST} values between populations were small and nonsignificant within each of the IM, SC, and TW clades (Appendices 5, 6, 7), suggesting weak, or no, genetic subdivision within the range of the clades. In the IM clade, only the Mumbai (MB) population showed evidence of genetic differentiation from the other populations (Appendix 5). AMOVA, however, revealed a nonsignificant Φ_{CT} value when the populations were divided into the MB and other IM population groups (Appendix 8A). AMOVA also revealed no genetic differentiation between the Indian populations (MB, TG, and SL) and other sites (Appendix 8B), or between the Indian and Pacific Ocean samples (Appendix 8C). Genetic differentiation was, however, detected between the Philippine samples and the remaining locations along the continental coastline within the SC clade in the Pacific Ocean (Appendices 6 and 8D). A possible subdivision within the Philippines (between PG and BC) was also evident (Appendix 6) but the number of populations analyzed was insufficient to provide enough resolution of this pattern within the archipelago.

The mismatch distribution for the IM, SC, and TW clades (CI clade excluded in subsequent analysis due to its limited sample size) was unimodal (Appendix 1), suggesting a rapid demographic expansion. This is consistent with the negative and significant Tajima's D and Fu's F_S values (Table 3). τ, which reflects the location of the crest of mismatch distribution, was calculated to estimate the timing of expansion for the three clades (Table 3). Based on a mutation rate of 1.55% per million years within lineages (Wares 2001), the times of demographic expansion for the IM, SC, and TW clades were estimated as ~105,000, 320,000 and 277,000 years before present, respectively (Table 3). An exponential growth in effective population size shown in Bayesian skyline plots (BSP; Appendix 2) was consistent with the results of the mismatch distribution analysis, indicating demographic expansion in all three clades. The BSP, however, inferred slightly different timings of expansion from the mismatch analyses. The three clades shared a broadly similar timing of demographic growth, which began at ~200 kyr. None of the estimates, however, supports an expansion after the Last Glacial Maximum (LGM; ~20 kyr), suggesting that population expansion pre-dated the LGM and the barnacle persisted through the LGM in most of its current range. The IM and SC clades, which have a wide distribution, exhibited an effective population size almost 10 times that of the TW clade which is confined to Taiwan.

Table 2 Net sequence divergence (uncorrected p) of COI among different clades of *C. malayensis*

	CI	IM	SC	TW
CI clade	**0.017**			
IM clade	0.105	**0.007**		
SC clade	0.050	0.105	**0.013**	
TW clade	0.096	0.064	0.095	**0.009**

The mean within-clade divergences are shown **in bold** on the diagonal.

Table 3 Genetic diversity of the four clades of *C. malayensis*

Clade	N	Na	h	π	D	Fs	τ	Divergent time (years ago)
CI	13	13	1.00	0.017	−0.086 (ns)	−5.424**	1.588	83,000
IM	356	191	0.95	0.005	−2.480***	−25.805***	2.018	105,000
SC	400	261	0.99	0.012	−2.111***	−24.295***	6.115	320,000
TW	136	75	0.96	0.007	−2.118**	−25.603***	5.287	277,000

Number of sequences (*N*), number of haplotypes (*Na*), haplotype diversity (*h*), nucleotide diversity (*π*), Tajima's *D*, Fu's *F_S* the mismatch parameter *τ*, and the time of expansion, estimated from the mismatch distribution analysis for each clade, are shown.
ns = nonsignificant, *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

Morphological analysis

Opercular plate geometry

The ordinations based on opercular geometry of the IM, SC, TW, and CI clades clustered together in the nMDS plot and do not reveal distinct clusters for the clades (Fig. 4A), nor were any significant differences found between the opercular plate geometries among clades (ANOSIM, $P > 0.05$).

Arthropodal characters

From all the arthropodal characters measured, the number of segments having conical spines in cirrus I, the number of conical spines in the first segment of cirri I and II, and the number of setules of basal guard setae exhibited significant differences among clades (one-way ANOVA, Table 4; note that the CI clade was not included in this analysis due to its small sample size). The TW clades had the smallest number of segments with conical spines on cirrus I (average 2.7 segments), followed by the SC clade (average 3.4 segments). The IM clade had the largest number of segments with conical spines on cirrus I (average 3.8 segments; Fig. 4B; Table 4). The five specimens of the CI clade all had four segments in cirrus I with conical spines.

The SC clade had a greater number of conical spines on the first segment of cirri I (average 9 conical spines) and II (average 7 conical spines) than did the IM and the TW clades (Fig. 4C and D; Table 4). The TW clade had significantly fewer conical spines (average of 7 in cirrus I and 3.5 in cirrus II; Fig. 4C and D). On cirrus II, the IM clade had the largest number of setules in the basal guard setae (>10 setules) when compared with the SC (average of 9 setules) and TW (average of 5 setules) clades (Fig. 4E; Table 4). Variation in the number of setules and basal guards was, however, high within the same clade (Fig. 4E), with the absolute ranges of numbers of setules and basal guards overlapping among clades.

Distribution and zonation of *C. malayensis sensu lato* in the IWP

The four clades exhibited a parapatric distribution with different geographic ranges (Fig. 2A). The IM clade was the most widespread taxon, found in all the sampling locations in the Indian Ocean, as well as in western and eastern Malay Peninsula and in Borneo (Sabah and Labuan Island, Fig. 2A). The IM clade had similar zonation patterns among

sites, with dense numbers on the high shore (Fig. 5). The relative abundance of populations increased from the H tidal level to reach a maximum in the M tidal level and decreased to very low levels in transects located at the L tidal level (Fig. 5). The average percent cover of the IM clade was up to 80% at the M level where they were most abundant (Fig. 5) in India, Malaysia, and North Borneo, but this clade occurred in very low abundance in South Vietnam (MN) where it co-existed with the locally more abundant SC clade. The SC clade inhabited most of the coastline surrounding the SC Sea, including SC, Hainan Island, Vietnam, Thailand, and the Philippines and, based on the present data, its southern limit extends down to Samui Island, Thailand. The zonation pattern of the SC clade was similar to that of the IM clade, with an average cover of 80% on the shores of Vietnam and the Philippines. The vertical distribution in SC (HL and HK) was, however, more narrow when compared to other sites, with this clade only being abundant at the H tidal level. In northern Taiwan, the SC clade was found sympatrically with the TW clade, yet its abundance gradually decreased further south along the eastern coast of Taiwan to be finally replaced by the TW clade. The TW clade was only present in Taiwan, with a wide vertical coverage on the shore but low abundance (average percentage cover <10%). The CI clade was restricted to CI.

Discussion

Do the four clades of *C. malayensis* represent four species?

Accurate species delimitation and identification are an important first step in protecting biodiversity and environmental monitoring. Accordingly, alpha taxonomy and biogeography are critical pieces of baseline information for identifying the basic units of conservation. The four clades of *C. malayensis* differ by 5–10% in their net COI sequence divergence, which is comparable to that reported in the tropical, eastern-Pacific *Chthamalus* species (Wares 2001; Pitombo and Burton 2007) which has been attributed to recent radiation within the genus (Wares et al. 2009), but the high plasticity of chthamalid barnacles poses severe hurdles to field identifications.

The four clades did exhibit significant differentiation in arthropodal characters, such as the number of conical spines on cirrus I and the number of setules
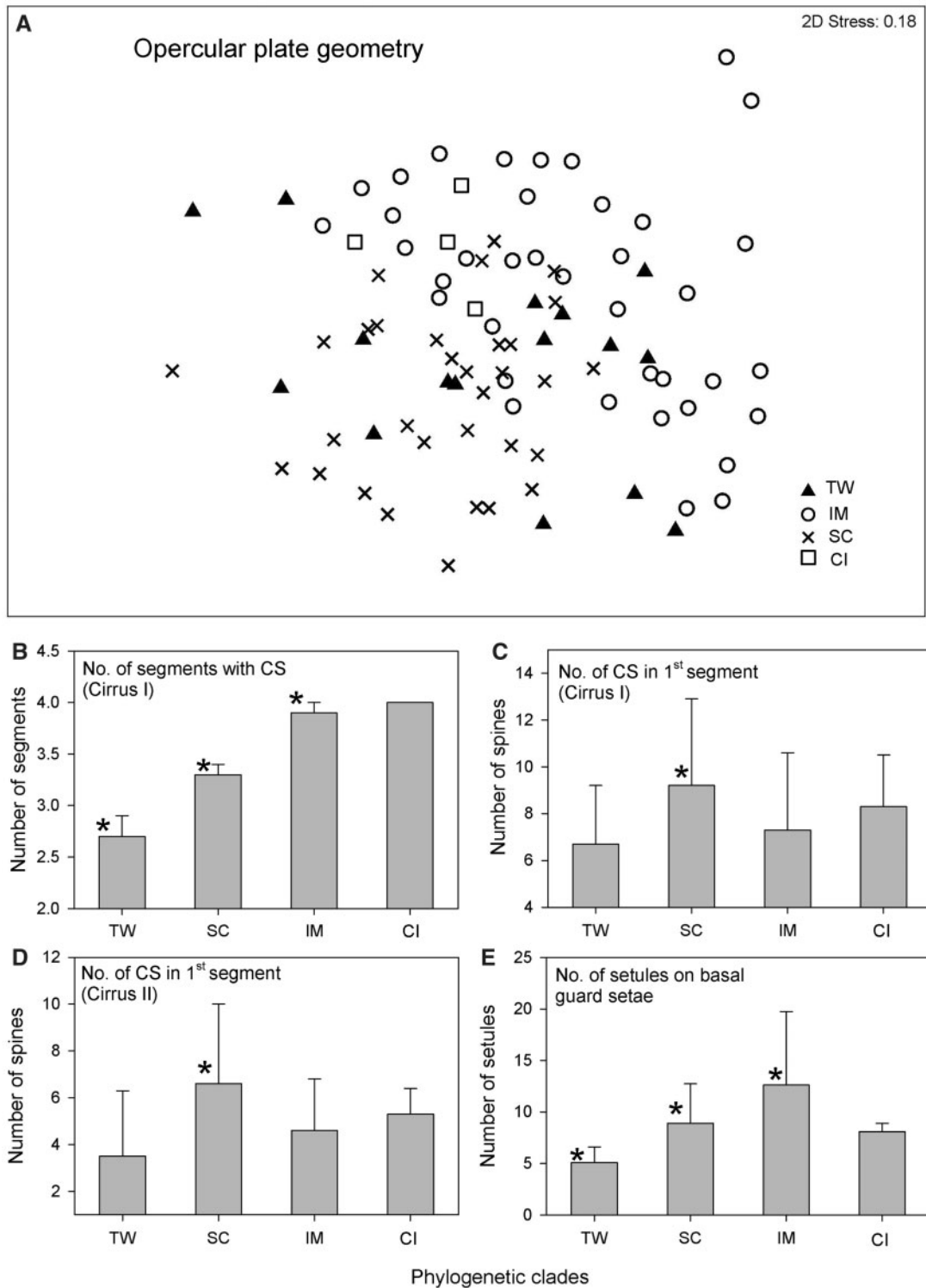
Fig. 4 (A) nMDS plot of the ordination of opercular plate geometry of the IM, SC, TW, and CI clades. (B) Variation in the average (+1 SD) number of segments with conical spines (CS) among IM, SC, TW, and CI clades. (C) Variation in the average (+1 SD) number of conical spines (CS) in the first segment of the anterior ramus of cirrus I. (D) Variation in the average (+1 SD) number of conical spines in the first segment of the anterior ramus of cirrus II. (E) Variation in the average (+1 SD) number of setules of basal guard setae among clades. Asterisks indicates significant difference (ANOVA) from other clades. Note that the CI clade was not included in the ANOVA analysis due to its small sample size.

Table 4 One-way ANOVA showing the variation of arthropodal characters among the IM, SC, and TW clades

	DF	MS	F	P-value
Number of segments with conical spines (cirrus I)				
Clades	2	5.97	12.45	<0.001
Residual	67	0.48		
Total	69			
SNK tests:	IM > SC > TW			
Number of conical spines in first segment (cirrus I)				
Clades	2	53.89	5.11	<0.001
Residual	67	10.54		
Total	69			
SNK tests:	SC > IM = TW			
Number of conical spines in first segment (cirrus II)				
Clades	2	62.03	6.94	<0.001
Residual	67	8.93		
Total	69			
SNK tests:	SC > IM = TW			
Number of setules in basal guard setae (cirrus II)				
Clades	2	423.7	20.18	<0.001
Residual	67	21.02		
Total	69			
SNK tests:	IM > SC > TW			

Bonferroni corrections have been conducted on all 15 (13 arthropodal characters + 2 basal guard setae characters) ANOVA tests and significant P-values were adjusted to 0.003.

of the basal guard setae on cirrus II. The absolute range of these characters, however, overlaps among clades and they are continuous, making these characters unsuitable for diagnostic purposes. Fernando (2006) claimed that the number of teeth on the pecten of the mandibles may exhibit geographic variation, but this was not the case in this study. The great variability of the arthropodal characters explains why previous efforts (Southward and Newman 2003), without the benefit of molecular data, could not disentangle the differences among clades from natural geographic variation, as the characters are highly variable even within a clade (Southward and Newman 2003). In addition to adult morphological differences, Yan and Chan (2001) noticed critical morphological differences among larvae collected from Hong Kong (presumably belonging to the SC clade based on our present data) and those described in India (IM clade; Karande and Thomas 1976). The larval developmental period for the SC clade (Hong Kong; Yan and Chan 2001) was also longer (~21 days) than for the IM clade (7–12 days; Karande and Thomas 1976). Integrating the independent lines of evidence from larval biology, arthropodal characters, and genetic divergence, we believe that the four clades are four distinct biological species. As the morphological differentiation is continuous, and no single diagnostic morphological character can separate the four species with confidence at the present stage, it does not seem appropriate to invoke a formal species description until reproductive isolation is confirmed by nuclear DNA markers. Many common and ecologically/economically important marine species that were once thought to be widespread in the IWP have recently been shown to be cryptic species (e.g., kuruma shrimp *Penaeus japonicus* (Tsoi et al. 2005), blue swimming crab *Portunus pelagicus* (Lai et al. 2010), littorinids *Echinolittorina malaccana* (as *E. trochoides*; Reid et al. 2006), barnacles *Tetraclita squamosa* (Chan et al. 2007)). These data and our results underscore the need for accurate delimitation of species as conservation units in the biologically diverse IWP.

Biogeographic pattern in the IWP related to oceanography

The interaction between gradients in SST, hydrographic barriers and oceanic currents are important factors that may account for the spatial distribution of barnacle species (Pannacciulli et al. 1997; Wares 2001; Herbert et al. 2003, 2007; Chan et al. 2007, 2008b; Keith et al. 2011). The distinct distribution of the four clades appears to have a strong linkage with the physical environmental conditions in the IWP. The SC clade is mainly distributed along coastlines of southern China, Vietnam, and eastern Thailand, which are all under the influence of the South China Sea Warm Current (SCSWC; Guan and Fang 2006; Yang et al. 2008; Fig. 6C). This current has a year-round northward flow along the continental shelf and slope of the northern SC Sea (Yang et al. 2008); and enters the Taiwan Strait and flows into the East China Sea (Yang et al. 2008; Jan et al. 2010). The presence of *C. malayensis* populations which belong to the SC clade on Penghu Island in the Taiwan Strait, suggests that this clade is probably transported by the SCSWC along the coast of China to the Taiwan Strait. The SC clade is further transported by the SCSWC to northern Taiwan, where a mixing of the SC and TW clades occurs. The SC
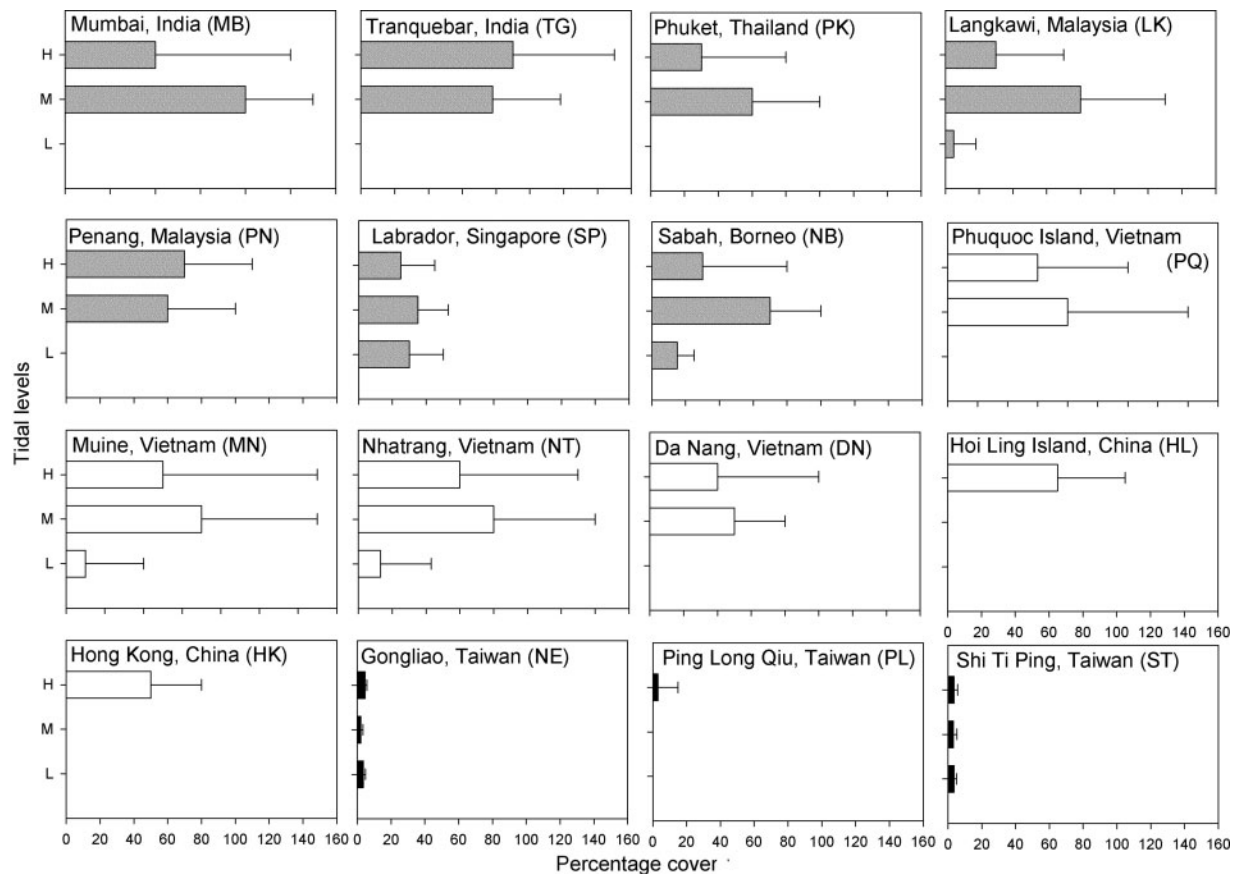
Fig. 5 Zonation pattern of different clades of *C. malayensis*. For clarity, the tidal levels of transects are abbreviated as H − upper limit, M − abundant zone, and L − lower limit of barnacles. The vertical intervals of height between H, M, and L were 25 cm. Grey bars indicate IM clade, white bars indicate SC clade, and black bars indicate TW clade. In Muine, although SC and IM clades were both recorded, the IM clade was in very low abundance. As it is impossible to identify the clades from external morphology, all barnacles in Muine were regarded as belonging to the SC clade in the zonation graph. In PL and NE in Taiwan, the SC clade is present at lower abundance than the TW clade. The SC and TW clades cannot be distinguished by morphology in the field, therefore all individuals in these two sites were regarded as TW clade.

clade is, however, absent from eastern and southeastern Taiwan, as the hydrography of these areas is affected by the Kuroshio Current. In addition to the SCSWC, there is the SC Sea Branch of the Kuroshio Current (SCSKB; Hu et al. 2000; Hsueh and Zhong 2004; Yang et al. 2008) which is a basin-wide cyclonic gyre that enters from the Luzon Strait and flows into the SC Sea. This current hits the eastern coast of Vietnam, and mixes with the Southeastern Vietnamese Offshore Current, which originates from the southern SC Sea (Cai et al. 2007). The mixing of these two currents at the southeastern Vietnamese coast may explain the mixing of the SC and IM clades in Muine (southeastern Vietnam). A separate population of the SC clade is found in

the western Philippines, where the effect of the SCSWC is very limited (Fig. 6C). It is also possible that this population colonized the western Philippines during the demographic expansion in the Pleistocene period, and the lack of present larval exchange results in reduced gene flow and consequent genetic differentiation.

The TW clade was only recorded in Taiwan, indicating that this clade is endemic. No *C. malayensis* could be collected at locations further north than Taiwan, suggesting northern Taiwan to be the northern limit of *C. malayensis*. In eastern and southeastern Taiwan, the entire population belongs to the TW clade, suggesting that the clade is distributed by the Kuroshio Current along the coast from SE to NE
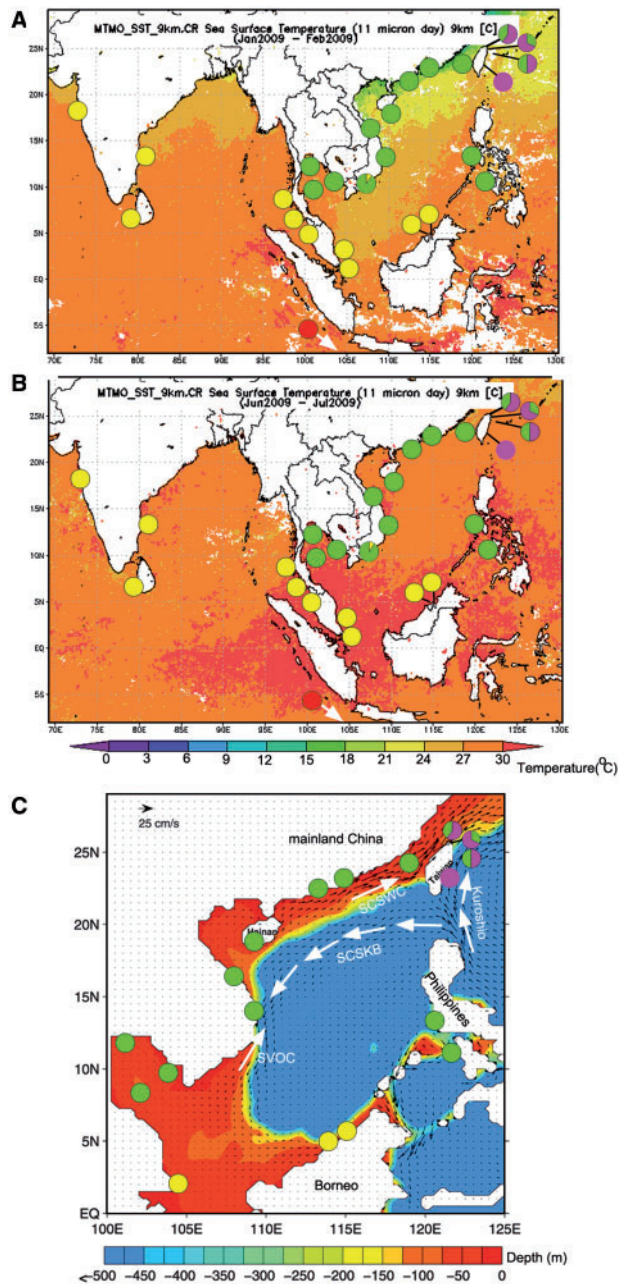
Fig. 6 (**A**) Distribution pattern of the IM (yellow), SC (green), TW (purple), and CI (red, slightly out of range in the map, approximate location shown by white arrow) clades (relative abundance of clades is shown as pie charts for each site) in relation to SSTs from a satellite image of the IWP in January–Febraury 2009. (**B**) June–July 2009 (Analyses and visualizations used in A–B was produced with the Giovanni online data system, developed and maintained by the NASA GES DISC). (**C**) Distribution of IM, SC, and TW clades (CI clade is not shown as it is out of the geographical range) in the SC Sea, in relation to different oceanic currents. The map was retrieved from Yang et al. (2008) which simulated the model bathymetry and velocity from the standard run in the SC Sea, showing the SCSWC along the northern SC Sea flowing northwards and entering the Taiwan

Taiwan. There is a gradient of increasing frequency of TW-clade individuals when moving from the northern tip down the eastern coast of Taiwan, suggesting that larvae of the SC clade carried by the SC Sea Warm Current cannot reach this area. This spatial structuring can be attributed to variation in larval supply driven by distinct oceanic current systems, as has been proposed for other barnacles such as *Tetraclita* species, where the eastern Taiwan and SC coasts are occupied by different species (Chan et al. 2007, 2008b; Tsang et al. 2008b, 2011).

The IM clade is found along the equatorial region of the Arabian Sea and Bay of Bengal in the Indian Ocean and is also present in the Malay Peninsula, where the SST is generally higher than in the areas inhabited by the SC and TW clades (Fig. 6A, B). CI in the northeastern Indian Ocean is only occupied by the CI clade, since the transfer of water from the West Pacific to the Indian Ocean through the Indonesian Archipelago is very limited and was almost completely blocked during the periods of lowered sea level in the Pleistocene (Gordon and Fine 1996; Voris 2000). There is, however, a large sampling gap with no samples from Indonesia. As a result, the fine-scale transition and the distributional patterns of the IM and CI clades in this region cannot be determined.

Under the effect of global climatic change, intertidal species are experiencing rapid range shifts in the Atlantic Ocean (reviewed by Southward et al. 2005; Helmuth et al. 2006; Hawkins et al. 2008, 2009), including barnacles (Southward 1991; Southward et al. 1995; Herbert et al. 2003; Poloczanska et al. 2008) and gastropods (Lima et al. 2006, 2007a, b; Mieszkowska et al. 2006; Firth et al. 2009). Shifts in the ranges of intertidal species in the IWP are poorly documented, primarily because of species misidentifications and limited knowledge of geographic ranges. The distribution of the four clades of *C. malayensis* suggest this taxon may be a good indicator for monitoring biotic responses to climatic change, especially as they live in the high shore and so are living close to their physiological limits and

Strait. The model also illustrates the intrusion of the SCSKB from the Luzon Strait into the SC Sea basin, where it impinges upon the SE Vietnam coast and mixes with the Southeast Vietnam Offshore Current (SVOC; Cai et al. 2007). Oceanographic map reproduced from Figure 3 in Yang et al. (2008). Copyright (2008). American Geophysical Union, Reproduced/modified by permission of American Geophysical Union.

are likely to be affected by rising temperatures (Wethey 2002). The four clades exhibit parapatric distributions and may differ in thermal tolerances; the IM clade, for example appears to be associated with higher SSTs, while the SC and TW clades are distributed in subtropical zones with lower water temperatures. When sea temperature rises, the geographical ranges of the IM, SC, and TW clades may shift accordingly. It may be, however, that the distributions of the clades are limited by nonclimatic factors, e.g., competitive exclusion with other species, or slow range expansion after vicariance events. Further studies focusing on the species' biological characteristics, e.g., the variation in thermal tolerance of different clades of *C. malayensis,* would help to uncover the factors governing the observed distributions.

Phylogeography of the *C. malayensis* complex in the IWP

The separated coalescent analyses suggest that the clades experienced exponential increases in effective population size. This is not surprising given that lowering of sea level during the Pleistocene glacial periods led to changes in temperature and oceanic current patterns, as well as in habitat loss, that would dramatically affect the survival and distribution of marine organisms (Hewitt 1999, 2000). The Sunda Shelf, where the majority of the clades currently occur, was exposed and formation of land bridges was prevalent in the region (Voris 2000; Lohman et al. 2011) which caused severe fragmentation and contraction in species' ranges. Demographic and range expansion of marine organisms following the retreat of glaciers (Hewitt 1999, 2000) are thought to account for the population expansions commonly reported in other genetic analyses (e.g., Chenoweth and Hughes 2003; Crandall et al. 2008).

The timings of demographic expansion in three of the clades were broadly similar, and were initiated at ~200,000 years before present. Accordingly, the onset of demographic expansion significantly pre-dates the LGM (~20 kyr before present) and the clades have maintained a relatively stable effective population since 100 kyr ago. Marko et al. (2010) presented similar findings for species from northeastern Pacific rocky shores and proposed that regional persistence through the LGM and long-term stability in effective population size are common in the region. Data for *Chthamalus* suggest that persistence through the LGM may be a common biogeographic

pattern, not only in the northeastern Pacific, but also for the IWP intertidal biota.

It is, however, surprising that a phylogeographic break between the Indian and Pacific Ocean populations, documented in many taxa (Lavery et al. 1996; Benzie 1999; Benzie et al. 2002), was not detected in the widely distributed IM clade in this region. One possible reason is that the break is not evident for taxa that exhibit a high level of gene flow across the oceans as has been observed in some fish species (e.g., Craig et al. 2007; Gaither et al. 2011). The IM clade of *C. malayensis* is believed to display a cosmopolitan habitat requirement as it is very abundant locally and can live on many different substrata with various degrees of wave exposure (Fernando 2006). Survival on a wide range of substrata and microhabitats may account for the lack of genetic subdivision in intertidal fauna, including barnacles (Ayre et al. 2009; Tsang et al. 2011).

An alternative explanation for the lack of genetic differentiation between populations from the Indian and Pacific Oceans is that it is a result of recent demographic expansion, such that the population did not have sufficient time to attain a mutation-drift-equilibrium, thereby resulting in a seeming lack of genetic difference, in spite of restricted contemporary gene flow (Slatkin 1993; Hellberg 2009; McGovern et al. 2010). Contraction and expansion of ranges following the retreat and advance of glaciers are globally widespread (Hewitt 2000; Hellberg 2009) and recent demographic growth is postulated to be a major explanation for genetic homogeneity across tentative phylogeographic breaks in marine systems (e.g., Atlantic-Mediterranean (Patarnello et al. 2007), northeastern Pacific (McGovern et al. 2010), including the IWP (e.g., Gopurenko et al. 1999; Lessios et al. 2003)). As larval development of the Indian *C. malayensis* takes only 7–12 days, long-distance dispersal seems unlikely. Recent demographic expansion of the IM clade appears to be a more parsimonious explanation as compared to high gene flow, although additional data are needed to validate this hypothesis. Analysis using the recently developed isolation-with-migration approach (Hey and Nielsen 2004) supplemented with multiple, independent nuclear loci could allow us to determine historic and present levels of gene flow and disentangle these two alternative hypotheses (McGovern et al. 2010; Marko and Hart 2011).

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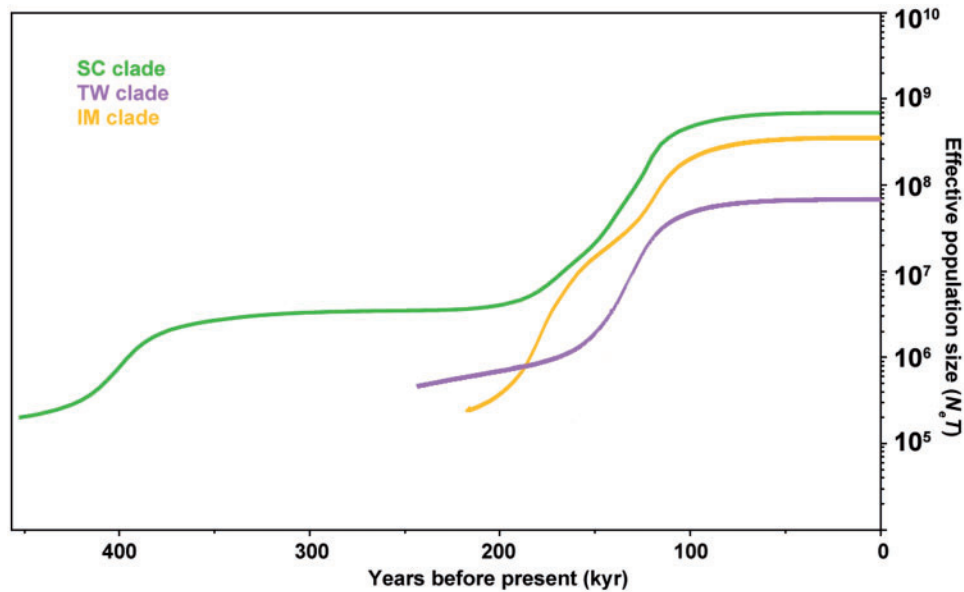## Appendix 1

The observed mismatch distribution (bars), and the expected mismatch distribution under the model of sudden demographic expansion (solid line) for the COI haplotypes: (A) IM clade; (B) SC clade; (C) TW clade. Harpending's raggedness indices are as follows: $r = 0.022$ ($P = 0.998$) for the IM clade; $r = 0.005$ ($P = 1.00$) for the SC clade; $r = 0.013$ ($P = 0.68$) for the TW clade.
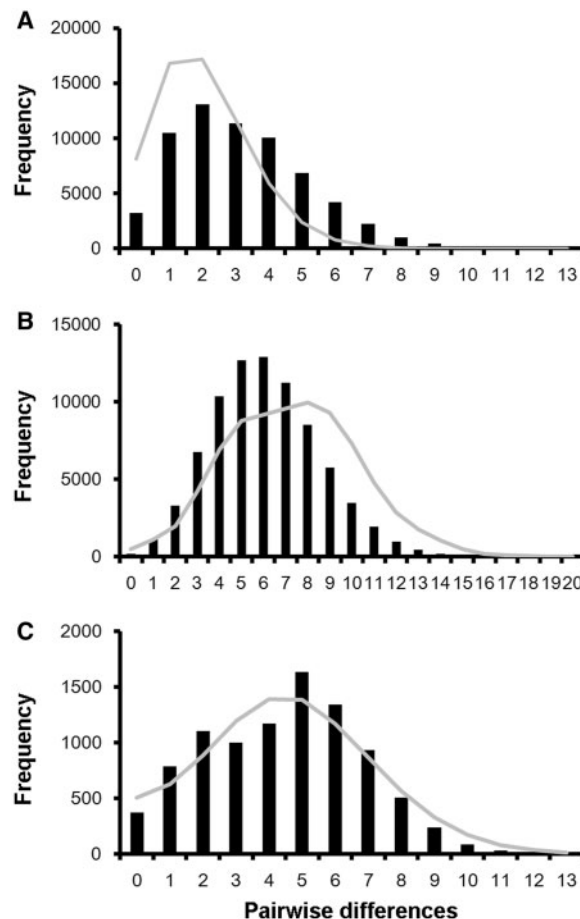
Appendix 2

Bayesian skyline plots for the demographic reconstruction based on the mtDNA COI data. N_eT, where N_e = effective population size and T = generation time.

Appendix 3

Maximum tidal range of spring tides (based on 2009; estimated from the world tidal program, WX Tides) of the study sites.

Appendix 4

List of taxa with their collection locality or source of information, and GenBank accession numbers for the chthamalid barnacles used as out-groups in phylogenetic analyses.

Site	Maximum spring tidal range (2009)
Mumbai, India	4.7 m
Tranquebar, India	1.2 m, data from Chennai
Phuket, Thailand	2.8 m
Langkawi, Malaysia	3 m, data from Lumut
Penang, Malaysia	2.3 m
Labrador, Singapore	3 m
Sabah, Malaysia	2 m
Da Nang, Vietnam	1.7 m
Nha Trang, Vietnam	2.2 m
Muine, Vietnam	2.1 m, data from Mui Dinh
Phu Quoc, Vietnam	1.1 m, data from Ha Tien
Hoi Ling Island, China	3 m, data from Hoi Ling Shan
Hong Kong, China	2.5 m
NE Taiwan	1.8 m
E Taiwan	2 m

Species	Collection locality	Source	GenBank Accession no
Chthamalus challengeri	Wakayama, Japan	Tsang et al. (2008a)	EU304447
Chthamalus fragilis	Woods Hole, MA, USA	This study	JQ755179
Chthamalus moro	Hualien, Taiwan	Tsang et al. (2008a)	EU304448
Chthamalus neglectus	Hong Kong, China	This study	JQ755180
Chthamalus proteus	Honolulu, Hawaii, USA	This study	JQ755181
Chinochthamalus scuteliformis	Hong Kong, China	This study	JQ755182
Tetrachthamalus sinensis	Sanya, Hainan, China	This study	JQ755178

Appendix 5

Pairwise Φ_{ST} values for populations of the IM clade based on COI. The comparisons with significant values ($P<0.05$) are in bold. Table 1 for the abbreviation of the sites.

	SB	BN	MN	NB	MS	PN	LK	PK	SL	TG
BN	**0.024**	—								
MN	0.013	−0.017	—							
NB	**0.026**	−0.002	0.001	—						
MS	**0.032**	0.004	0.012	0.001	—					
PN	**0.028**	0.001	0.003	−0.004	−0.007	—				
LK	0.020	−0.001	−0.030	0.004	0.000	−0.002	—			
PK	0.011	0.001	0.044	−0.001	0.002	0.013	0.015	—		
SL	**0.027**	0.010	0.009	**0.019**	**0.016**	**0.017**	0.004	0.000	—	
TG	**0.043**	0.006	0.013	−0.005	0.002	−0.009	−0.001	0.027	**0.024**	—
MB	**0.068**	**0.035**	0.074	**0.077**	**0.093**	**0.097**	**0.071**	**0.066**	**0.073**	**0.102**

Appendix 6

Pairwise Φ_{ST} values for populations of the SC clade based on COI. The comparisons with significant values ($P<0.05$) are in bold. Table 1 for the abbreviation of the sites.

	TI	NE	PL	PH	HK	HL	SY	PG	BC	DN	NT	MN	PQ
NE	−0.011	—											
PL	−0.017	−0.025	—										
PH	0.002	−0.026	−0.010	—									
HK	0.006	−0.030	−0.003	−0.010	—								
HL	−0.007	−0.012	−0.002	0.000	0.004	—							
SY	0.001	−0.012	−0.010	−0.009	−0.005	0.006	—						
PG	0.036	**0.055**	0.005	**0.046**	**0.062**	**0.054**	**0.046**	—					
BC	**0.063**	**0.142**	**0.074**	**0.157**	**0.151**	**0.150**	**0.161**	**0.112**	—				
DN	0.002	−0.020	0.000	−0.017	−0.004	−0.018	−0.007	0.036	**0.185**	—			
NT	0.026	0.007	0.020	0.001	0.004	−0.008	0.007	**0.054**	**0.218**	−0.016	—		
MN	0.003	−0.015	−0.016	−0.011	0.004	−0.001	−0.006	**0.031**	**0.151**	−0.014	0.001	—	
PQ	−0.001	−0.025	−0.009	−0.008	−0.003	−0.006	0.002	**0.054**	**0.135**	−0.013	0.008	−0.004	—
SM	0.001	−0.006	−0.014	0.001	0.008	−0.008	−0.004	**0.044**	**0.123**	−0.014	0.009	−0.003	−0.005

Appendix 7

Pairwise Φ_{ST} values for populations of the TW clade based on COI. The comparisons with significant values ($P<0.05$) are in bold. Table 1 for the abbreviation of the sites.

	DA	NE	PL	SU	ST
NE	−0.003	—			
PL	−0.010	−0.022	—		
SU	0.011	0.025	0.007	—	
ST	−0.017	−0.011	−0.015	0.025	—
TI	0.015	0.039	0.050	**0.060**	−0.003

Appendix 8

Results of hierarchical analysis of molecular variance (AMOVA).

Source of variation	d.f.	Sum of square	Variance component	% variation	Φ	P value
(A) AMOVA for partition among MB and the populations in the IM clade						
Among groups	1	12.313	0.112	6.59	$\Phi_{CT}=0.066$	0.085
Among populations within groups	9	20.259	0.023	1.34	$\Phi_{SC}=0.014$	<0.001
Within populations	345	540.471	1.567	92.06	$\Phi_{ST}=0.079$	<0.001
(B) AMOVA for partition among Indian and other populations of the IM clade						
Among groups	1	3.798	−0.001	−0.01	$\Phi_{CT}=-0.001$	0.521
Among populations within groups	9	28.774	0.054	3.30	$\Phi_{SC}=0.033$	<0.001
Within populations	345	540.471	1.567	96.71	$\Phi_{ST}=0.033$	<0.001
(C) AMOVA for partition among Indian and Pacific populations of the IM clade						
Among groups	1	2.654	−0.006	−0.40	$\Phi_{CT}=-0.040.$	0.959
Among populations within groups	9	29.918	0.057	3.52	$\Phi_{SC}=0.031.$	<0.001
Within populations	345	540.471	1.567	96.88	$\Phi_{ST}=0.035$	<0.001
(D) AMOVA for partition among the Philippines and other SC clade populations						
Among groups	1	19.924	0.201	5.13	$\Phi_{CT}=0.051$	0.025
Among populations within groups	14	53.598	0.005	0.12	$\Phi_{SC}=0.001$	0.363
Within populations	384	1427.784	3.718	94.76	$\Phi_{ST}=0.052$	0.007