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Verification of the cryptic species *Penaeus pulchricaudatus* in the commercially important kuruma shrimp *P. japonicus* (Decapoda : Penaeidae) using molecular taxonomy

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Abstract. The kuruma shrimp *Penaeus japonicus* Bate, 1888 (Decapoda : Penaeidae) is economically important in the global shrimp market. It was regarded as the only species in the subgenus *Marsupenaeus*. However, our previous molecular analyses revealed two cryptic species (Forms I and II) in this species complex. In this study, we confirm the phylogenetic relatedness between the two cryptic species; revise their taxonomic status; and review their range distribution. The name *Penaeus pulchricaudatus* Stebbing, 1914 (with type-locality off the eastern coast of South Africa), previously considered as a junior synonym of *P. japonicus*, is fixed for Form II through a neotype selection. *P. japonicus* (Form I) is only confined to the East China Sea (including Japan, its type-locality) and the northern South China Sea. *P. pulchricaudatus* is widely distributed in the South China Sea, Australia, the Red Sea, the Mediterranean, and the western Indian Ocean. Phylogenetic analysis shows that *P. japonicus* is genetically homogeneous yet *P. pulchricaudatus* exhibits a strong phylogeographical structure. The Mediterranean stock of *P. pulchricaudatus* originated from the Red Sea population, supporting the Lessepsian migration hypothesis. The presence of two closely related cryptic species in the *P. japonicus* species complex provides important insights into fishery management and aquaculture development.

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Introduction

The kuruma shrimp *Penaeus japonicus* Bate, 1888 (Decapoda : Penaeidae) is widely distributed in Korea, Japan, the South China Sea, the Malay Archipelagoes, the northern coast of Queensland in Australia, the Red Sea and the western Indian Ocean to eastern South Africa (Pérez Farfante and Kensley 1997). It was first recorded in the Mediterranean in 1924 (Balss 1927) and this 'Lessepsian migrant' was believed to invade and spread across the Levantine Coast of the Mediterranean Sea via the Suez Canal (Balss 1927; Galil *et al*. 2002). This shrimp has subsequently been recorded on the Italian coast (Lumare and Casolino 1986), Aegean Sea (Kevrekidis and Kevrekidis 1996) and English Channel (Clark 1990). Two specimens were captured in 2007 from a depth of ~60 m in the Celtic Sea (Quigley *et al*. 2013) but the occurrence of a self-sustaining population at its northernmost limit is yet to be supported by current evidence (Quigley 2010). However its successful adaptation in the Mediterranean environment (Kevrekidis and Kevrekidis 1996) and rapid propagation (Gilberto and Héctor 2001) threaten the indigenous species (Galil 2006). For example, the native *P. kerathurus* (Forskal, 1775) has been eliminated by this invasive alien species from the easternmost areas of the Mediterranean (Galil and Zenetos 2002).

Penaeus japonicus is of economic importance in the global shrimp market and highly prized as the 'King of Seafood' in Japan (Shigueno 2001). As this shrimp tolerates handling and long-distance transportation (ASEAN 1978), the market price of living individuals could reach US$127 per kg (Tokyo Metropolitan Central Fish Market, 17 May 2014). Shrimp farms are the main market supplier of the shrimp. Global wild-capture of the shrimp is 2165 tonnes yet the aquaculture production has reached 51 178 tonnes with the net value of 303 million US dollars in 2012 (FAO 2014). It was the first penaeid species successfully cultured on a commercial scale (Hudinaga 1935, 1942). Shrimp farming techniques developed

in Japan have been well practiced in many areas ranging from the West Pacific (Liao and Chien 1994) to the Mediterranean (Galil and Zenetos 2002) and French Atlantic coast (Quero and Vayne 1998). However the industry has long been challenged by low egg quality and survival rate of the shrimp larvae (Okauchi *et al*. 1995; Chuntapa *et al*. 2003). In order to control production cost and to obtain high quality seeds for better survival and growth performance, most hatcheries still rely on the breeders collected from the wild stocks in nearby waters (Benzie 1998; Delmendo 1989; Treece 1999), including those in China (e. g. Wang 1997), Japan (Mock 2009), Australia (DPI 2013) and the Mediterranean (Scordella and Zecca 2002).

Penaeus japonicus is featured with attractive brownish red bands and named as the kuruma shrimp because of its characteristic wheel-like banding pattern. Yet such body colouration pattern is similar to that of *P. canaliculatus* (Olivier, 1811) and *P. kerathurus*, and thus misidentifications have been occasionally reported. Morphological traits including the diagnostic pouch-like thelycum and characteristic features (such as its movable spines in the telson) are effective characters to identify the species. This species is currently described by two scientific names *P. japonicus* and *Marsupenaeus japonicus* due to the controversy of the status of the genus *Penaeus* Fabricius, 1798 (see Pérez Farfante and Kensley 1997; Lavery *et al*. 2004; Flegel 2007, 2008; McLaughlin *et al*. 2008; Ma *et al*. 2011). These names are independently used by various organizations and databases, e.g. FAO, World Register Marine Species (WoRM) and Delivering Alien Invasive Species Inventories for Europe (DAISIE). The present work follows the most updated results (i. e. Ma *et al*. 2011) for the definition of *Penaeus* and hence uses *Penaeus japonicus*. For convenient discussion, the subgenera of *Penaeus* described in Holthuis (1980) are also used.

Fig. 1. *Penaeus japonicus* Bate, 1888, a specimen from Taiwan, with the dark brown transverse bands extending to the lower half of the carapace (reaching the bottom).

Two morphologically similar colour forms of *Penaeus japonicus* have been identified in the South China Sea (Tsoi *et al*. 2005). These two forms, namely I and II, were characterised by diagnostic colour banding patterns on the carapace (see Figs 1, 2), which was generally regarded as a colour variant in previous taxonomic works (Yu and Chan 1986; Chan 1998). These two forms do not differ in other morphological traits or morphometric parameters (Tsoi *et al*. 2005), yet phylogenetic analyses using mitochondrial (mt) DNA and nuclear DNA markers concordantly reveal that the two forms are closely related (versus other *Penaeus* spp.) but genetically distinct (Tsoi *et al*. 2005). No shared mtDNA haplotype was observed among the specimens of these two forms, particularly in their sympatric zone. Hybridization was not detected from AFLP analysis, indicating that the sister taxa are reproductively isolated. All evidence supports the occurrence of cryptic species in the kuruma shrimp. Since the type locality of *P. japonicus* is Japan, Form I is recognised as the typical form. However, no proper scientific name has yet been given to Form II.

In a phylogeographical study of the species beyond the South China Sea, Tsoi *et al*. (2007) showed that Form I occurs only in Japan and China, including Taiwan, and dominates the populations in the northern South China Sea. This finding was also supported by Tzeng *et al*. (2004) and Shih *et al*. (2011), who showed that both forms were found along the Taiwan coast with Form I being dominant. Form II has a much wider range

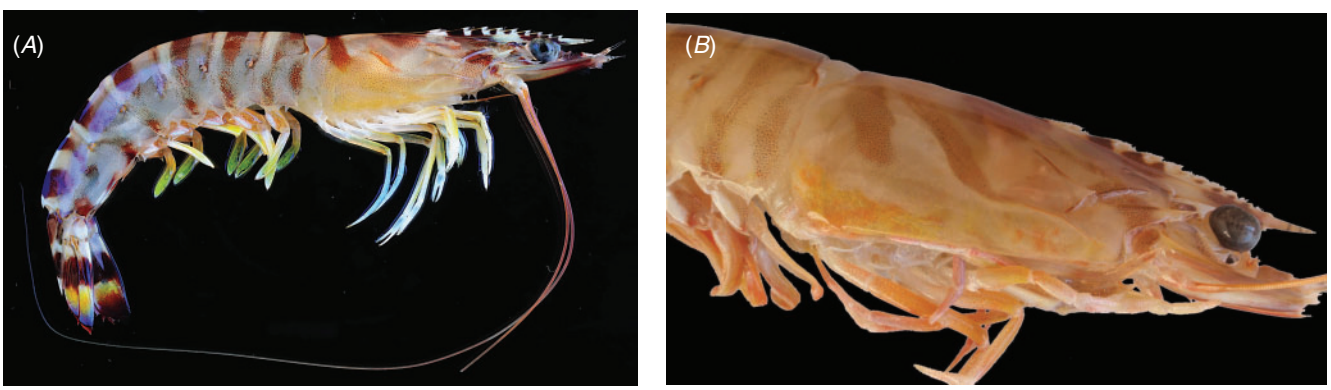

Fig. 2. *Penaeus pulchricaudatus* Stebbing, 1914 with the dark brown bands not extending to the lower half of the carapace (only reaching the middle part). (*A*) A specimen from Fort Dauphin on the south-east coast of Madagascar. (*B*) Neotype of *P. pulchricaudatus*, from Richards Bay off the eastern coast of South Africa.

including the South-east Asia, Australia, the western Indian Ocean, the Red Sea and the Mediterranean (Tsoi *et al.* 2007). Nevertheless, genetic information of specimens from the Red Sea and the western Indian Ocean is lacking in previous studies. The present study thus aims to (1) affirm the identity of Form II and the taxonomic status of the cryptic species in the species complex; (2) ascertain the comprehensive distribution pattern of the species complex and (3) elucidate the phylogenetic relationship of the stocks across its geographical range by extending our sampling effort.

Materials and methods

Sample collection and DNA extraction

A total of 131 individuals were collected from 16 localities (Fig. 3) including Oita of Japan (JP), Putai of Taiwan (TW), Xiamen (XM), Raoping (RP), Hong Kong (HK), Zhanjiang (ZJ) and Beihai (BH) in China, Negros of the Philippines (PH), Singapore (SP), Nha Trang of Vietnam (VN), Mackay of Australia (AU), Fort Dauphin of Madagascar (MD) and Richards Bay of South Africa (SA), Jizan (JZ) in the Red Sea, and Tel Aviv (TA) and Ashdod (AD) in the Mediterranean (Table 1). Specimens from BH, MD, JZ and SA were collected for the present study while those from the other localities were from our previous study (Tsoi *et al.* 2007). Specimens were collected from shrimp trawlers operating in the relevant areas, or from nearby fish markets. The shrimps were confirmed to be caught in coastal waters of the corresponding areas and were not imported or obtained from shrimp farms. The specimens were either preserved in 95% ethanol or kept frozen at –70°C before DNA extraction.

DNA extraction, PCR and sequencing

The total genomic DNA of each shrimp specimen was extracted from the muscle (10–15 mg) of its pleopod IV or V using QIAamp Tissue Kit (QIAGEN). The extracted DNA was kept at −20°C until analyses. The primers PCR−1R and 12S−2 (Chu *et al.* 2003) were adopted to amplify a 5′ segment of the mitochondrial control region (CR) (563 bp). PCR amplification was conducted in a reaction mixture containing 10 ng template DNA, 10 µL Mg^{2+} free PCR buffer, 2.5 mM $MgCl_2$, 0.12 µM of each primer, 400 µM of dNTPs, 1 unit of *Taq* polymerase (Promega, Madison, Wisconsin), and ddH_2O added up to 50 µL. The PCR thermal cycling profile was as follows: 93°C (3 min), 35 cycles of 93°C (30 s)/48°C (50 s)/72°C (50 s), and 72°C (5 min). The size and quality of amplified DNA fragments were assessed in 1% agarose gel electrophoresis. Prior to DNA sequencing, PCR products were purified using QIAquick gel purification kits (QIAGEN). Bi-directional sequencing was conducted using the corresponding primer sets and ABI PRISM dye-terminator sequencing kits (Applied Biosystems, Foster City, California) with an ABI 3100 automated DNA Sequencer.

Phylogenetic and population genetic analyses

Sequences were aligned using MUSCLE (Edgar 2004) implemented in MEGA v5. 0 (Tamura *et al.* 2011) with manual adjustments. Phylogenetic relationships among the individuals were analysed using Bayesian inference (BI) carried out in a BEAST v1. 7. 4 (Drummond *et al.* 2012) substitution model selected by Modeltest2 based on BIC (Darriba *et al.* 2012). A run of 50 million MCMC generations,

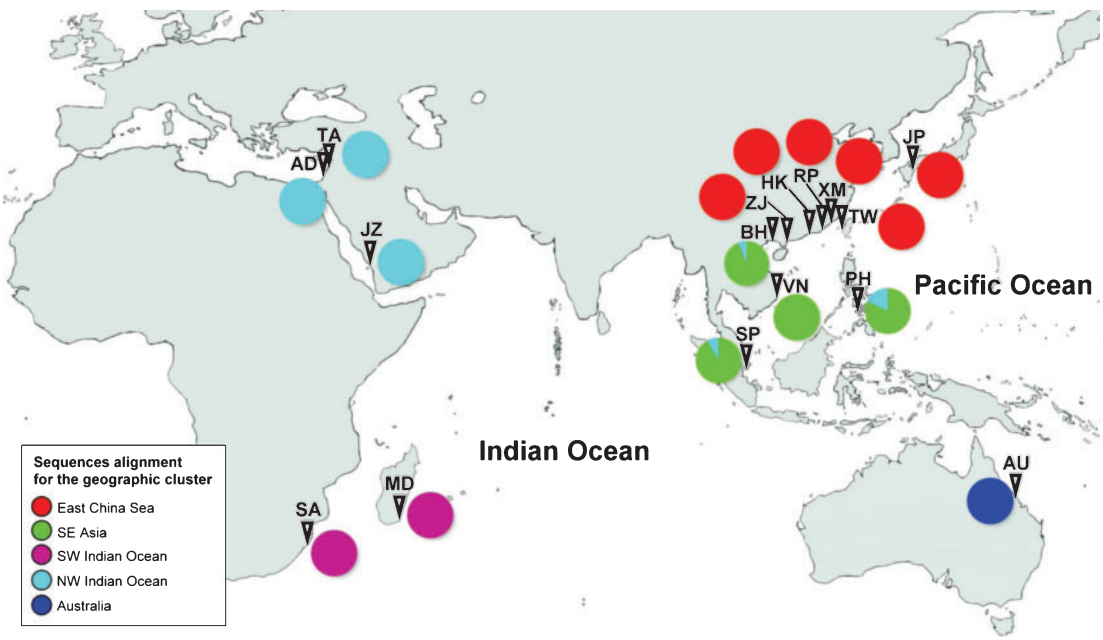

Fig. 3. Geographical locations of the sampling sites (refer to Table 1) and relative frequency of the clades established by the BI analysis of CR sequence data from each locality (refer to Fig. 4). Red colour represents the sequences aligned for the geographic cluster of East China Sea (N = 20); green for SE Asia (N = 52); purple for SW Indian Ocean (N = 30); light blue for NW Indian Ocean (N = 24); and deep blue for Australia (N = 5).

Table 1. Geographical locations of the sampling localities and GenBank accession numbers of the sequences determined

Populations	Location	Abbreviations	Latitude	Longitude	GenBank accession nos.[A]
East China Sea	Oita, Japan	JP	33.15°N	131.36°E	AY853462–853466 (5)
and	Xiamen, China	XM	24.26°N	118.07°E	AY853456–853458 (3)
Northern South China	Putai, Taiwan	TW	23.22°N	120.10°E	AY853467–853469 (3)
Sea	Raoping, China	RP	23.18°N	116.40°E	AY853453–853455 (3)
	Hong Kong, China	HK	22.11°N	114.54°E	AY853459–853461 (3)
	Zhanjiang, China	ZJ	21.10°N	110.20°E	AY853470–853472 (3)
SE Asia	Beihai, China	BH	21.49°N	109.12°E	KJ765731–765746 (16)
	Nha Trang, Vietnam	VN	12.15°N	109.10°E	AY853489–853493 (5)
					KJ765814–765821 (8)
	Negros, The Philippines	PH	10.00°N	123.00°E	AY853479–853483 (5)
					KJ765787–765792 (6)
	Singapore	SP	1.15°N	104.00°E	AY853484–853488 (5)
					KJ765804–765810 (7)
NW Indian Ocean	Jizan, Red Sea	JZ	16.89°N	42.55°E	KJ765769–765786 (18)
		AD	31.48°N	34.38°E	AY853476–853478 (3)
		TA	32.05°N	34.46°E	AY853473–853475 (3)
SW Indian Ocean	Fort Dauphin, Madagascar	MD	25.02°S	46.98°E	KJ765747–765768 (22)
	Richards Bay, South Africa	SA	28.78°S	32.03°E	KJ765796–765803 (8)
Australia	Mackay, Australia	AU	21.08°S	149.11°E	AY853494–853498 (5)

[A]No. of specimens in parentheses.

sampled every 5000 generations, was conducted using the Yule process tree prior and strict clock model. Tracer v1.5 was used to evaluate convergence of runs (Rambaut and Drummond 2007) and the trees were annotated by using Tree Annotator v1.7.4 (Drummond *et al*. 2012). Analysis of molecular variance (AMOVA) in Arlequin 3. 11 (Excoffier *et al*. 2005) was adopted to assess the geographic division among the populations and the significance was determined by 1000 random permutations of sequences. Minimum spanning networks (MSNs) (Rohlf 1973) were constructed. The MSNs of haplotypes were computed in Arlequin 3. 1 (Excoffier *et al*. 2005). Using the same package, pairwise population F_{ST} values (Nei 1977) with the significance of genetic distances tested by 10 000 permutations were calculated. A neutrality test was performed to calculate the Tajima's *D*, and Fu & Li's *D* values for detecting the occurrence of bottlenecks (or population expansion) and allelic deficiency.

Results

Genetic analyses

Nucleotide composition The aligned partial sequences of CR were deposited with GenBank (Table 1). On average 508 bp of CR fragments were unambiguously determined. The aligned partial CR segment revealed 238 variable and 206 phylogenetically informative sites. The base frequencies were A: 37. 1; %, T: 44. 3%, C: 10.6% and G: 7. 9%. Sequences of CR showed strong AT bias (81. 4%).

Phylogenetic analyses

The best-fit model for the CR was GTR+I+G. The *P. japonicus* haplotypes are separated into two lineages corresponding to Form I and Form II with very strong support (Fig. 4). Form I was found only in the East China Sea and the northern South China Sea, including specimens from Japan (JP), Xiamen (XM), Taiwan (TW), Hong Kong (HK), Raoping (RP), and Zhanjiang (ZJ). They were collectively defined as the East China Sea clade. Four clades were defined in Form II. The South-east (SE) Asian clade included specimens from Beihai (BH), Vietnam (VN), the Philippines (PH) and Singapore (SP). Yet four specimens from the SE Asia (2 PH, 1 BH and 1 SP) were found to be more closely related to the North-west (NW) Indian Ocean clade, which contained all specimens from the Mediterranean Sea and the Red Sea with the two being reciprocally monophyletic. The South-west (SW) Indian Ocean clade (including the Madagascar and South African populations) exhibited a close relationship with another genetically distinct Australian clade (only comprising the Mackay specimens).

Genetic distances and population differentiation

The relative frequency distribution of the clades in each sampling site based on BI analysis of the CR sequence data is presented in Fig. 3. The Kimura 2-parameter distance matrices (K2P) of CR sequence data are shown in Table 2. Mean genetic distance of the CR ranged from 0.057 to 0.235. High genetic divergence supported the genetic differentiation of the two forms (ranging between 0.164 and 0.244), as well as the clades within the Form II (0.047, 0.171). The high and significant F_{ST} (0.793, 0.906, $P < 0.00001$) (Table 3), AMOVA and Φ_{CT} (0.659, $P < 0.05$) (Table 4) also confirmed that the East China Sea clade (Form I) was genetically distinct from the populations of other regions. This clade was genetically homogeneous with low and insignificant F_{ST} (<0.139, $P > 0.05$), while genetic differentiation was detected among Form II populations, as revealed in F_{ST} and AMOVA (Tables 3, 4). The Australian clade was most genetically diverged

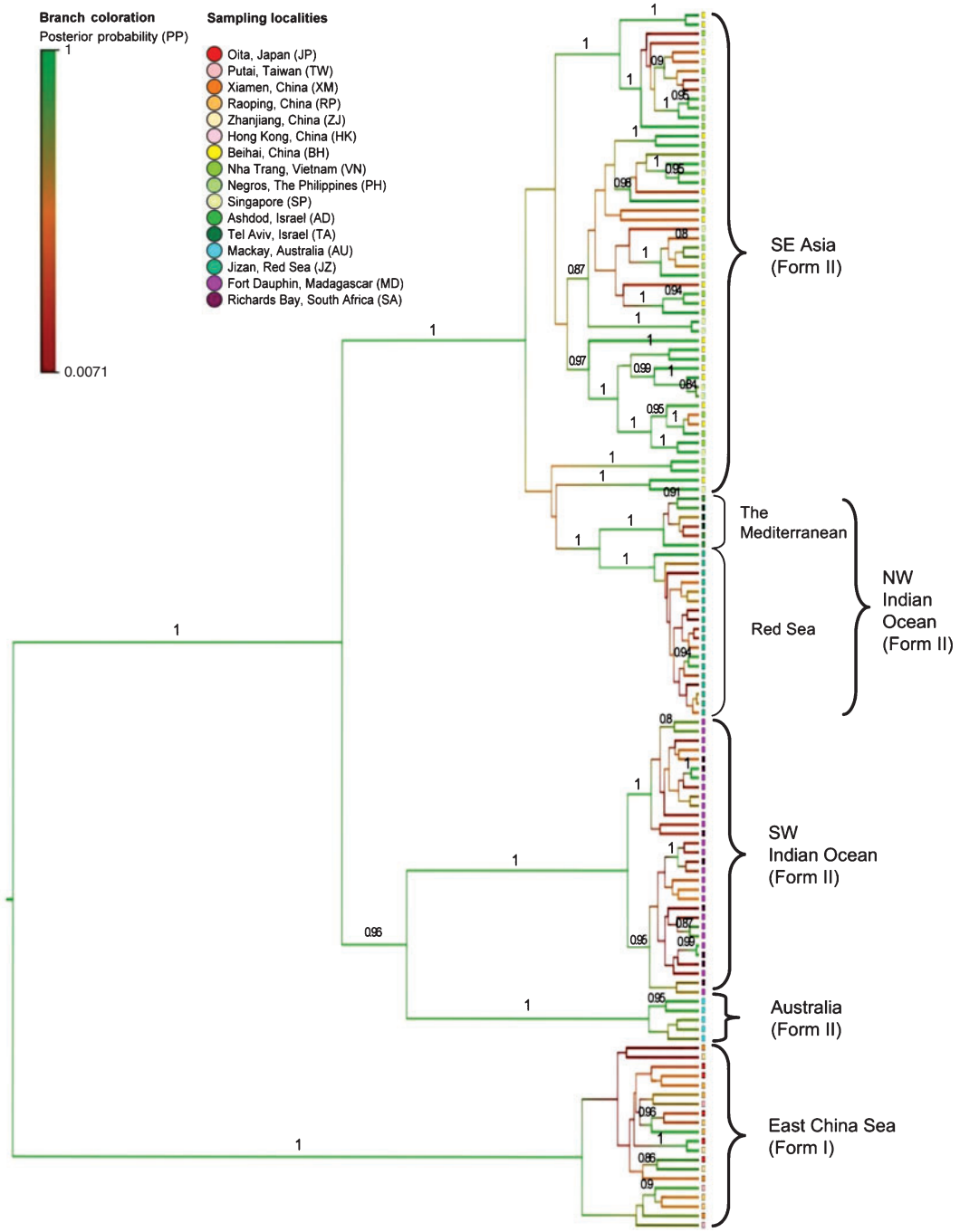

Fig. 4. Bayesian Inference phylogenetic tree of the control region dataset. Numbers above branches indicate posterior probability (PP) values (only PP values ≥0. 8 are shown).

from the others (F_{ST} = 0.707–0.923, P < 0.05). Other genetically distinct clades exhibited relatively lower F_{ST} values (F_{ST} = 0.488–0.881, P < 0.001). The phylogenetic results revealed a close genetic relationship between the populations of the Red Sea and the Mediterranean in the NW Indian Ocean clade but indicated no recent gene flow between the two regions (F_{ST} = 0.784, P < 0.001). The SW Indian Ocean clade was genetically homogeneous with an insignificant F_{ST} value (P > 0.05). No genetic drift or bottleneck was detected in any of the populations as supported by insignificant values of Tajima's D, and Fu & Li's D (Table 5). Yet a significant and negative value in the SW Indian Ocean population revealed the occurrence of population expansion (Fu & Li's D = –2.616, P < 0.05).

Table 2. Mean values and range (in parentheses) of K2P distance matrices of control region between populations

Populations		East China Sea	SE Asia	Red Sea	Mediterranean	SW Indian Ocean	Australia
East China Sea							
SE Asia		0.198 (0.164–0.230)					
NW Indian Ocean	Red Sea	0.220 (0.190–0.242)	0.072 (0.047–0.095)				
	Mediterranean	0.205 (0.185–0.220)	0.086 (0.061–0.104)	0.057 (0.047–0.067)			
SW Indian Ocean		0.221 (0.193–0.239)	0.114 (0.083–0.147)	0.132 (0.114–0.149)	0.145 (0.134–0.162)		
Australia		0.235 (0.221–0.244)	0.147 (0.141–0.170)	0.157 (0.141–0.170)	0.156 (0.144–0.171)	0.140 (0.119–0.164)	

Table 3. Pairwise comparison of genetic differentiation (presented in F_{ST}) between five populations based on the control region data

*$P < 0.05$, **$P < 0.00001$

Populations		East China Sea	SE Asia	Red Sea	Mediterranean	SW Indian Ocean	Australia
East China Sea							
SE Asia		0.793**					
NW Indian Ocean	Red Sea	0.906**	0.488**				
	Mediterranean	0.865**	0.511**	0.784**			
SW Indian Ocean		0.895**	0.669**	0.881**	0.874**		
Australia		0.876**	0.707**	0.923**	0.884*	0.858**	

Table 4. Hierarchical analysis of molecular variance (AMOVA) of Control Regions with grouping between (*A*) Forms I and II, and (*B*) among five populations groups: East China Sea, SE Asia, NW Indian Ocean, SW Indian Ocean and Australia

*$P < 0.05$, **$P < 0.00001$

Source of variation	d.f.	Sum of square	Variance component		Percentage variation
A					
Among groups	1	1777.920	49.728	Va	65.910
Among populations within groups	14	1944.878	16.001	Vb	21.210
Within population	115	1118.183	9.723	Vc	12.890
Total	130	4840.980	75.452		
Overall Φ_{CT}	0.659*				
B					
Among groups	4	1829.292	23.721	Va	70.680
Among populations within groups	5	60.781	0.239	Vb	0.710
Within population	101	969.872	9.603	Vc	28.610
Total	110	2859.945	33.563		
Overall Φ_{CT}	0.707**				

Minimum spanning networks

CR haplotypes were nested according to the forms to which they belonged and the geographical localities. Haplotypes 01–20 and 21–128 were categorized into Forms I and II, respectively (Fig. 5). These two major assemblages were discriminated by 74 mutational events. Form II was further separated into four clades – the Australian, NW and SW Indian Ocean clades appeared to have originated from the South-east Asian clade. The Australian clade was the most distantly related to the other Form II clades, as separated by more than 53 mutational steps. The NW Indian Ocean clade was separated from the South-east Asian clade by 22 mutational steps. Within the NW Indian Ocean clade, the Mediterranean subclade was split from the Red Sea subclade by 22 steps as well. No haplotype was shared among the Mediterranean and Red Sea subclades.

Table 5. Neutrality test of Tajima's *D*, and Fu & Li's *D*
*$P < 0.05$ (Tajima 1989; Fu and Li 1993)

Parameter	Populations						Total
	East China Sea	SE Asia	NW Indian Ocean		SW Indian Ocean	Australia	
			Red Sea	Mediterranean			
Number of sequences	20	52	18	6	30	5	131
Number of haplotypes	20	51	17	6	29	5	128
Total number of mutations	107	169	27	22	72	29	316
Number of polymorphic sites	95	146	27	22	70	29	231
Haplotype diversity (h)	1.000 ± 0.016	0.999 ± 0.004	0.993 ± 0.021	1.000 ± 0.096	0.998 ± 0.009	1.000 ± 0.126	1.000 ± 0.001
Nucleotide diversity (π)	0.038 ± 0.003	0.050 ± 0.001	0.011 ± 0.001	0.017 ± 0.002	0.021 ± 0.001	0.026 ± 0.005	0.104 ± 0.004
Average number of nucleotide difference	19.1	25.3	5.6	8.5	10.4	13.2	51.7
GC ratio	0.183	0.179	0.183	0.196	0.192	0.203	0.183
Neutrality Tests: Tajima's *D*	–1.507	–1.160	–1.120	–0.717	–1.615	–0.386	–0.359
Neutrality Tests: Fu & Li's *D*	–1.866	–1.030	–0.916	–0.801	–2.616*	–0.386	–0.315

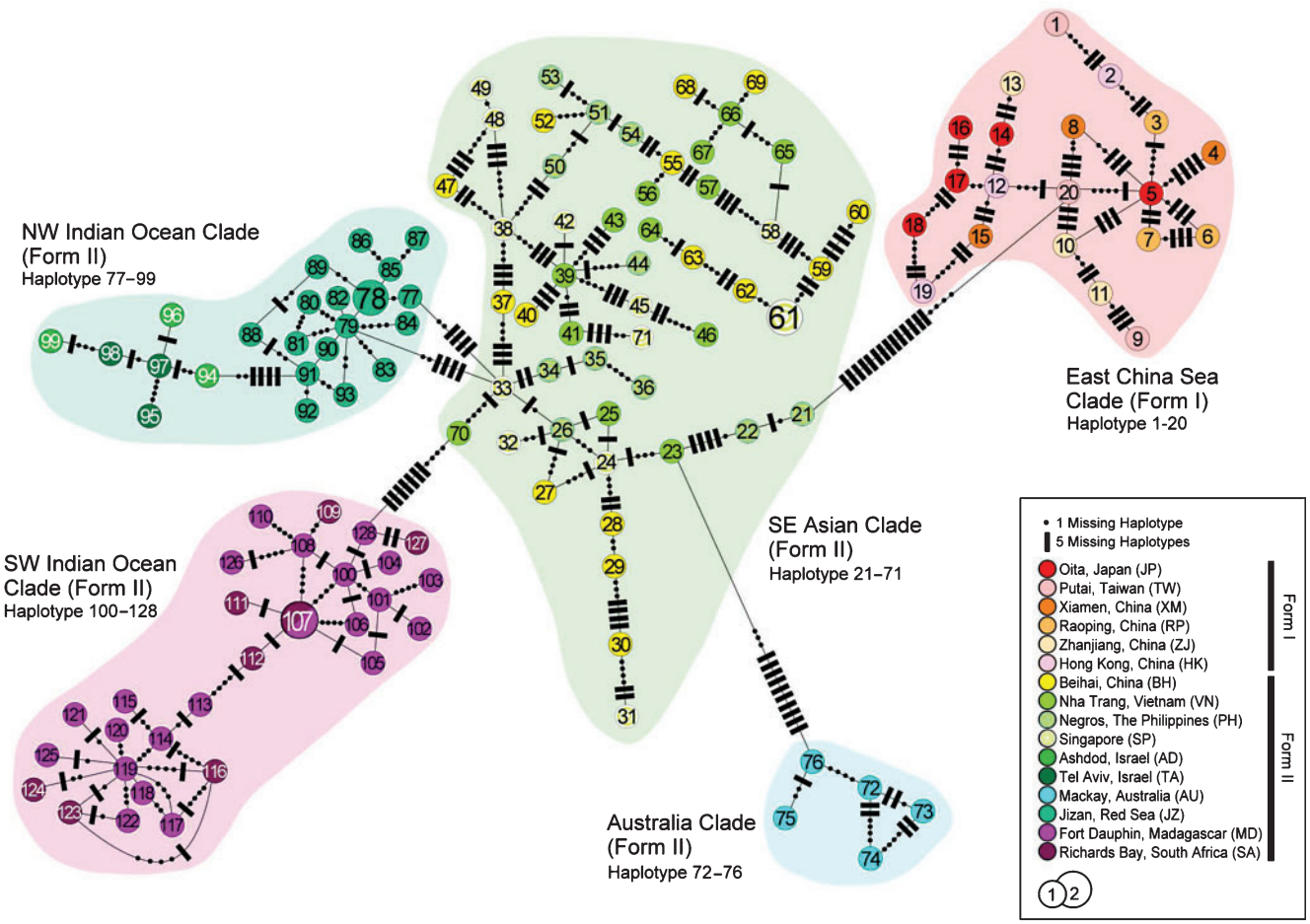

Fig. 5. Minimum spanning network of control region haplotypes. The frequency of the haplotype occurrence is indicated by the circle area.

Discussion

Cryptic species differentiation

Cryptic diversity

Our previous studies have identified two genetically distinct colour forms of *P. japonicus* and revealed their different geographical distributions (Tsoi *et al*. 2005, 2007). The present study extends the analysis of the CR dataset to new populations, further supporting the occurrence of cryptic species. Significant F_{ST} and the high genetic divergence (74 mutational events) support that these two genetically distinctive lineages are phylogenetic species. In addition, Tsoi *et al*. (2005, 2007) did not perceive any shared composite haplotypes between the two forms in all mtDNA markers used. Using AFLP, hybridization is also not detected across the forms collected from the sympatric zone in AFLP diploid nuclear loci, supporting our contention that the two reproductively isolated forms also likely represent biological species.

Studies from other research groups also support the occurrence of two genetically distinct taxa in the South China Sea (Shih *et al*. 2011; Tan *et al*. 2009). However, Tan *et al*. (2009) reported a new clade from Sanya of China. This new clade was claimed to be genetically diverged from Forms I and II at the level of 8% in the 16S rRNA marker. Our previous studies show that genetic divergences of the two forms are only 1–1.5% for 16S rRNA and 6–7% for a more variable marker COI (Tsoi *et al*. 2005, 2007). Such a high level (8%) of divergence in the conserved 16S marker is rarely observed in cryptic species delineation of penaeid shrimps. The highest value of the sequence divergence in 16S rRNA data among various *Penaeus* spp. in *Melicertus* Rafinesque-Schmaltz, 1814 is 7.3% (Lavery *et al*. 2004). The 16S rRNA divergence between *P. vannamei* Boone, 1931 and *P. subtilis* (Pérez Farfante, 1967) attains 8. 2% yet these species belong to two different subgenera *Farfantepenaeus* Burukovsky, 1972 and *Litopenaeus* Pérez Farfante, 1969, respectively (De Francisco and Galetti 2005). Our BLAST search of the shrimp sequences (EU056320–22 and EU056324) of the 'new clade' reported by Tan *et al*. (2009) shows that it probably represents *P. semisulcatus* De Haan, 1844 as its nucleotide alignment achieved 99% of the sequence identity to *P. semisulcatus*, whereas the highest value only reaches 93% in comparing with sequences of *P. japonicus* obtained from other studies. Therefore the presence of one more cryptic species (other than Forms I and II) of *P. japonicus* proposed by Tan *et al*. (2009) is most likely due to misidentification and cannot be substantiated.

Taxonomy

As the type-locality of *P. japonicus* is Japan, this name should be applied to Form I, i. e. the East China Sea Clade. For Form II, the name *P. pulchricaudatus* Stebbing, 1914, with type-locality from the Great Fish Point off the eastern coast of South Africa, is available. However, *P. pulchricaudatus* was known only from the juvenile holotype of ~45 mm body length (Stebbing 1914). As the holotype is a very small juvenile, its sex and exact identity are undetermined. Stebbing (1914) stated that *P. pulchricaudatus* is nearest to *P. japonicus* and indeed it resembles the latter in having only one ventral rostral tooth and with the telson laterally spinose. However, in the SW Indian Ocean, *Penaeus latisulcatus* Kishinouye, 1896 (or *P. latisulcatus hathor* Burkenroad, 1959) also has one ventral rostral tooth and a laterally spinose telson, and juveniles of *P. japonicus* and *P. latisulcatus* cannot be satisfactorily separated without the information of colouration and genetic data (see key provided in Chan 1998). Moreover, the original description and figures provided by Stebbing (1914) indicated that there are eight pairs of lateral spines (four pairs larger) on the telson, a character not known in any other *Penaeus* s. l. species (i. e. having only 0–3 pairs of lateral telson spines). We examined two small juvenile Form II specimens from Madagascar (7.2, 8.4 mm carapace length, corresponding to ~25, 30 mm body length, NTOU M017512) and they are generally similar to Stebbing's *P. pulchricaudatus* but with only three pairs of lateral spines on the telson. Unfortunately, the holotype of *P. pulchricaudatus* (Iziko South African Museum, A 1056) is now lost (L. Hoenson, W. K. Florence, person. comm.) and its exact identity may never be affirmed. In order to fix the name for Form II, a neotype of a South African female specimen used in the present genetic analysis and with coloured photograph (Fig. 2*B*) is now selected for *P. pulchricaudatus*. Therefore, while Form I should be treated as the true *P. japonicus*, Form II now bears the name *P. pulchricaudatus*. Below is a taxonomic account for the neotype of *P. pulchricaudatus*.

Taxonomy

Penaeus pulchricaudatus Stebbing, 1914

(Figs 2*B*, 6)

Material examined

Neotype. South Africa, Richards Bay, 28°72′S, 32°26′E, 30 m, 20 March 2010, female, carapace length 44.2 mm, NTOU M01779 (deposited at the National Taiwan Ocean University, Keelung), COI GenBank accession number KJ765796.

Description

Integument glabrous. Carapace with ridges and grooves well developed but lacking longitudinal and transverse sutures (Fig. 6*A*, *B*). Rostrum, without distinct accessory ridge, extending to distal end of antennular peduncle and armed with 9 dorsal teeth (including 3 teeth on carapace) and 1 ventral tooth. Postrostral carina almost reaching posterior carapace and with deep median groove throughout entire length. Adrostral groove as wide as postrostral carina and extending near to posterior carapace. Orbital angle spiniform and antennal spine very pronounced. Postocular sulcus absent. Gastrofrontal groove distinct and with posterior end divided into two. Gastro-orbital carina long, almost reaching anterior margin of carapace. Orbito-antennal groove well defined. Cervical carina sharp, accompanying groove well marked. Hepatic spine very pronounced. Hepatic carina well marked, curved and with anterior part ventrally inclined. Pterygostomian spine and

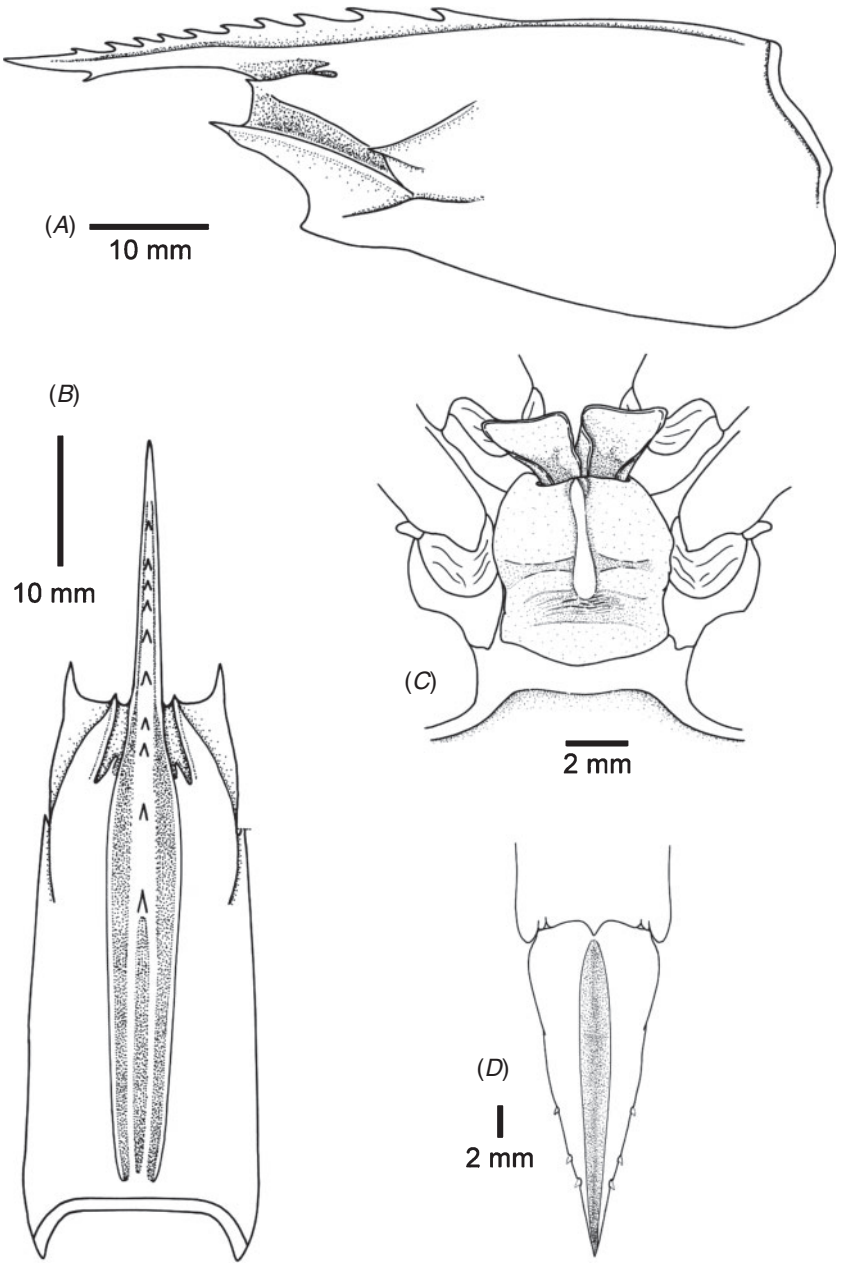

Fig. 6. *Penaeus pulchricaudatus* Stebbing, 1914, neotype female, carapace length 44.2 mm, Richards Bay, South Africa, NTOU M01779. (*A*) Carapace, lateral view. (*B*) Carapace, dorsal view. (*C*) Thelycum with spermatophore attached, ventral view. (*D*) Telson, dorsal view.

branchiocardiac carina absent. First pereiopod with ischial spine indistinct or absent. Abdominal somite VI bearing 3 cicatrices, lacking dorsolateral groove. Thelycum (Fig. 6*C*) pouch-like with double tubes and opened anteriorly. Spermatophore deposited on thelycum as a large subtriangular wing-like process with anteriorly disposed broad base and posteriorly long curved dorsal rod inserted into the tube of thelycum. Telson armed with 3 pairs of movable lateral spines (Fig. 6*D*).

Colouration (Fig. 2*B*)

Body pale yellowish and crossed with dark brown transverse bands, those on carapace extending from top to about mid-carapace, posterior-most band on abdominal somite VI interrupted. Eyes black-brown. Scaphocerite somewhat greenish with white tips, antennal flagella reddish-brown to yellowish-brown. Pereiopods whitish to yellowish. Pleopods yellowish to reddish, with brown and white spots at bases.

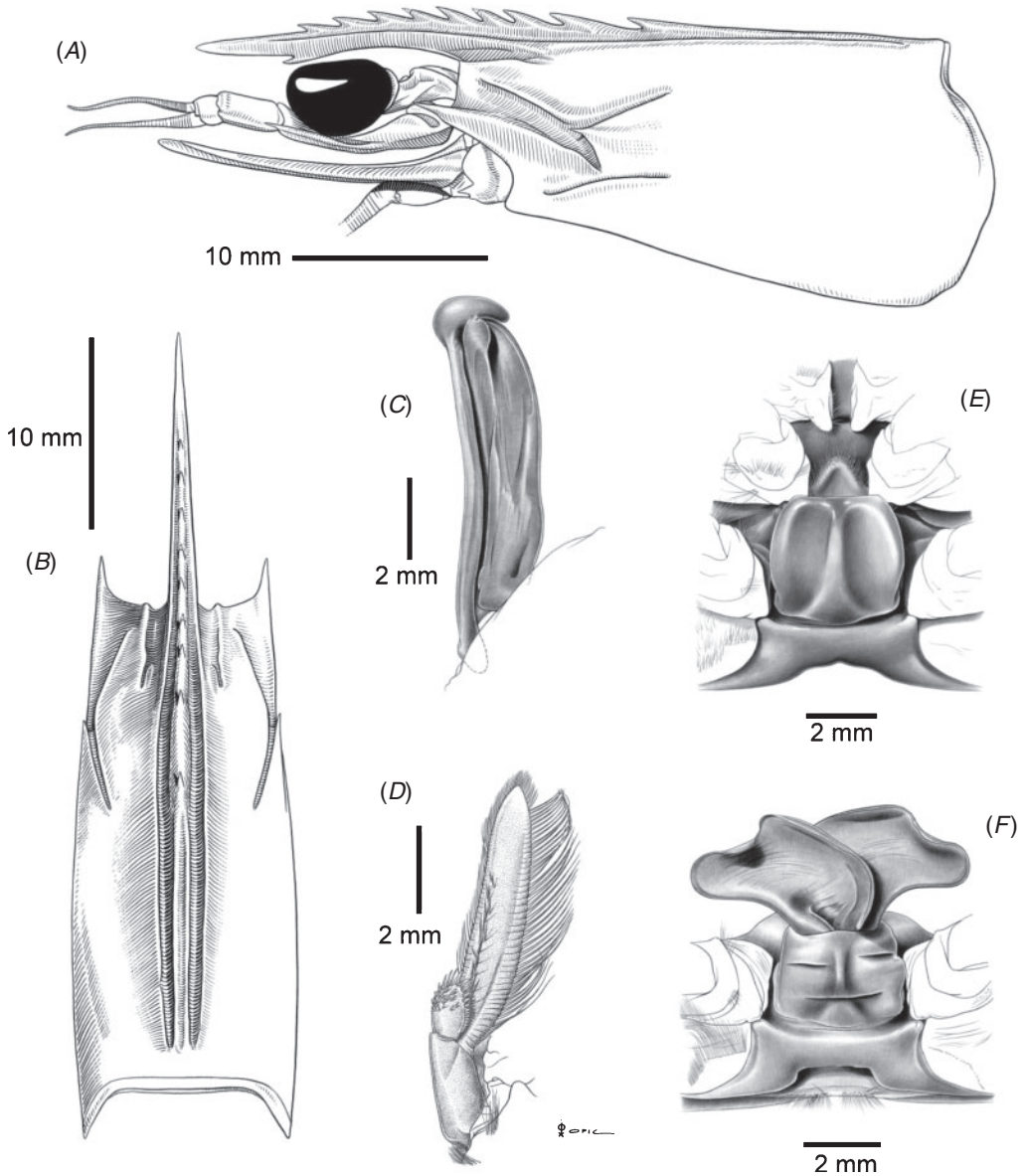

Fig. 7. *Penaeus pulchricaudatus* Stebbing, 1914, Bay of Ambaro, NW Madagascar, MNHN. (*A*, *B*) Male carapace length 25.0 mm. (*C*, *D*) Male carapace length 29.0 mm. (*E*) Female carapace length 30.0 mm. (*F*) Female carapace length 28.5 mm. (*A*) Carapace, lateral view. (*B*) Carapace, dorsal view. (*C*) Right petasma, ventral view. (*D*) Right appendix masculine, dorsal view. (*E*) Thelycum, ventral view. (*F*) Thelycum with spermatophore attached, ventral view.

Distal part of uropods with patch of bright yellow, followed by narrower patch of bright blue and with margins reddish.

Remarks

Since materials from Madagascar and South Africa belong to the same genetic group (Figs 4, 5), additional illustrations for *P. pulchricaudatus* based on Madagascan specimens are provided in Fig. 7 (specimens deposited at the Muséum national d'Histoire naturelle, Paris but could not be located during the present study). Although no male from the topotypic locality eastern coast of South Africa was examined, males from the adjacent locality Madagascar shows that the petasma of this species is symmetrical, semiclosed, and with long distomedian projection overhanging the distal margin of costae (Fig. 7*C*). The appendix masculina is subelliptical, armed with strong marginal spines and a broad patch of short strong spines (Fig. 7*D*). As discussed in the Introduction and Tsoi *et al.* (2005), *P. pulchricaudatus* is almost identical to *P. japonicus* and only different in the colouration of the ventrolateral carapace (Figs 1, 2), which has the dark brown transverse bands on the dorsal carapace terminating at mid-carapace and not extending further downwards.

Phylogenetic analysis and population structure

Geographical distribution

Penaeus japonicus is not widely distributed. The previous description '*P. japonicus* is extensively spread across the Indo-West Pacific' (e. g. Dall *et al*. 1990; Pérez Farfante and Kensley 1997; Chan 1998) is no longer applicable. *Penaeus japonicus* represented by Form I is confined to the East China Sea and the northern South China Sea. The species is genetically homogeneous without any significant population structuring. Genetic analyses of specimens along the coasts of mainland China and Taiwan also support the lack of genetic structure of *P. japonicus*, based on both mtDNA (Shih *et al*. 2011) and nuclear markers (Chu *et al*. 2011). Insignificant population differentiation was also observed in Japanese waters and attributed to high levels of gene flow among the localities (Sugaya *et al*. 2002; Taniguchi and Han 1989).

South-east Asian origin

Penaeus pulchricaudatus is geographically distributed in the South China Sea, Australia and the western Indian Ocean. Phylogenetic analysis shows a strong population structuring. Yet all these clades exhibit a close phylogenetic relationship with the South-east (SE) Asian clade (Fig. 5). Our data reveal a recent population expansion of the species complex. Population clusters of both *P. pulchricaudatus* and *P. japonicus* arrange in a star-like pattern radiating from the centrally located South-east Asian cluster in the CR evolution network. The network reveals that the Australian lineage originated from a haplotype 23 (from Nha Trang, Vietnam in SE Asian clade) which is linked to the haplotype 33 (from Singapore in SE Asian clade). Furthermore the observation that some SE Asian individuals (haplotypes 21, 22, 30 and 31) nested within the NW Indian Ocean clade in the BI cladogram (Fig. 4) indicates that the clade likely had SE Asian origin. It is interesting to note that the haplotype 21 exhibits the closest relationship with *P. japonicus*. Based on the current information, we believe the haplotype 33 and its closely related haplotypes are relatively primitive and associated with the species delineation and population differentiation of this species complex. Our findings on CR evolution network and the highest nucleotide diversity of the SE Asian cluster provide evidence to support that *P. japonicus* and the lineages of *P. pulchricaudatus* were derived from the SE Asian region. The mud crab *Scylla serrata* (Forsskål, 1755) also exhibit the comparable phylogeographic structure and genetically-differentiated geographic clusters in the Indo-West Pacific which were believed to be associated with the seawater level fluctuation and paleo-oceanographic conditions in the Pleistocene (Gopurenko *et al*. 1999; He *et al*. 2011). Geographical barriers emanating from land bridges, historic glacial patterns, and oceanic currents would probably have affected the phylogeographic distribution of *P. pulchricaudatus* and *P. japonicus* (see Tsoi *et al*. 2007). The westward dispersal of the ancestral shrimp might be facilitated by the South Equatorial Current across long distance. Yet the evolutionary origins of these two decapods were found to be different, since ancestral *S. serrata* was originated from the North-west Australia (He *et al*. 2011) whereas the *P. japonicus* complex was likely diversified from SE Asia.

Lessepsian migration

'*Penaeus japonicus*' is considered as an invasive alien species (e.g. Galil and Zenetos 2002; Galil 2006) and regarded as one of the 660 Lessepsian migrants (Galil 2012). Also the shrimp is in the list of the 'top 100 worst invasive alien species' in the Mediterranean because of its serious impacts on biodiversity and socioeconomic aspects (Streftaris and Zenetos 2006). Yet not a single individual of *P. japonicus* was found in either the Red Sea or the Mediterranean populations as all our specimens are *P. pulchricaudatus*. Available photographs and illustrations of '*P. japonicus*' in the Mediterranean actually represent *P. pulchricaudatus* (e. g. Otero *et al*. 2013; p. 81). Thus, the invasive alien species is *P. pulchricaudatus*, not *P. japonicus*.

As all our phylogenetic trees demonstrate the close genetic relatedness between the Red Sea and Mediterranean populations, the Mediterranean stocks are believed to have originated from the Red Sea. The founder stock is thus expected to be genetically homogeneous to its source. But in fact, all haplotypes are found to be private in both populations. Moreover, the significant and high pairwise F_{ST} values reveal no recent gene flow between these two populations, which are separated by at least 22 mutational steps. Such a differentiation level is equivalent to the divergence between the SE Asian and the Red Sea clades. All the evidence reveals that these two stocks are genetically distinct and the vicariance did not appear to be a very recent event. Referring to the calibrated divergence rate at 19%/Myr for the CR marker (McMillen-Jackson and Bert 2003), the Mediterranean and Red Sea stocks of *P. pulchricaudatus* are estimated to be isolated from each other at 150 kya, which is much longer than the time elapsed after the establishment of Mediterranean dispersal via the Suez Canal (<150 years). However, such a phenomenon is not unusual in the region. The mussel *Brachidontes pharaonis* (P. Fischer, 1870) also exhibits a similar lineage divergence in the Mediterranean and Red Sea (Shefer *et al*. 2004). Sirna Terranova *et al*. (2006) further suggested the occurrence of ancient polymorphism of this mussel in the region to explain the high genetic divergence. A question is thus raised on these findings: are the data incompatible with the Lessepsian migration hypothesis for explaining the occurrence of *P. pulchricaudatus* in the Mediterranean?

In fact, private alleles or haplotypes are not uncommon in the Mediterranean pool in various marine fauna e. g. the mussel *B. pharaonis* mentioned above (Shefer *et al*. 2004) and the goatfish *Upeneus pori* Ben-Tuvia and Golani, 1989 (Golani and Ritte 1999). A low level of genetic divergence in the goatfish populations between the Red Sea and the Mediterranean shows good support for the Lessepsian migration hypothesis, yet a rare and single allele undetected in the Red Sea was unexpectedly found in the Mediterranean population (Golani and Ritte 1999). The situation of *P. pulchricaudatus* may be similar in that these private Mediterranean haplotypes may occur in the Red Sea but are absent in our studied samples. Our Red Sea specimens were only collected from the southern part of the Red Sea (Jizan) which is

Table 6. Possible biological differences and reproductive traits of the shrimp from different regions

		Japan/Chinese coast	Mediterranean/Australia
Temperature	Temperature tolerance/ optimal temperature for growth	25–28°C (optimal) (Hudinaga 1942) 25–30°C (optimal) (Liao and Chien 1994) 18–30°C (growth range) (Liao and Chien 1994) 20°C (limiting factor for shrimp distribution) (Zhou 2001) 32°C (critical water temperature as mortality begins) (Liao and Chien 1994)	22–30°C (optimal Italy specimens) (Lumare 1998) 32°C (highest growth rate Australia specimens) (Hewitt and Duncan 2001) 24°C (lowest mean growth); 27°C (highest mean growth); 24°C (highest mean survival) (Coman *et al.* 2002) 32°C (highest survival) (Hewitt and Duncan 2001)
Salinity	Salinity tolerance/ optimal salinity for growth	28–32 ppt (Hudinaga 1942) 21.7–34.3 ppt (Liao and Chien 1994)	30–40 ppt (Dalla Via 1986) 30–35 ppt (Setiarto *et al.* 2004) 20–35 ppt (Lumare 1998)
Reproduction	Spawning patterns	From end of March to the mid-October (Hirata 1975) Sandy bed is required (Taiwan specimens) (Liao and Chien 1990)	From April to November (Mediterranean) (Tom and Lewinsohn 1983) Various spawning season in different localities (Dall *et al.* 1990) Sandy bed is not required (Queensland specimens) (Hansford *et al.* 1993)
Nutrition	Food conversion ratios	1.2–1.5 (Japan specimens) 1.4–2.0 (Taiwan specimens) 2.5–3.0 (China specimens) (Liao and Chien 1994)	2.5 (Australia specimens) (Liao and Chien 1994)
	Inositol requirement	200 mg% (Japan specimens) (Kanazawa *et al.* 1976) 400 mg% (Japan specimens) (Deshimaru and Kuroki 1979)	>400 mg% (France specimens) (Civera and Guillaume 1989)

distant from the Suez Canal. It is thus possible that some Mediterranean haplotypes were missed in a single sampling exercise. In fact, genetic distinctiveness of the populations between the northern and southern populations of the Red Sea has been observed in some organisms, e. g. the Red Sea fishes *Saurida undosquamis* (Richardson, 1848) (Yağlıoğlu and Turan 2012) and *Larabicus quadrilineatus* (Rüppell, 1835) (Froukh and Kochzius 2007). Our data thus only provide the genetic information of a southern Red Sea population but not from the entire region. Thus, the migration hypothesis could not be rejected.

Aquaculture implications

Since the Japanese seed stock (i. e. *P. japonicus*) was introduced to Italy in 1979, the shrimp culture technique has been developed continuously in the Mediterranean (Lumare and Palmegiano 1980; Lumare 1984, 1987, 1998; Türkmen 2007). The kuruma shrimp aquaculture is widespread and even extended to the European coast recently (Türkmen 2007). However most hatcheries still depend upon the breeders collected from nearby wild stocks (Treece 1999; Scordella and Zecca 2002). We believe the farmed stocks might possibly contain *P. pulchricaudatus*. It is noteworthy that an exceptionally high degree of heterozygosity was detected in stocks of '*P. japonicus*' that had been ranched and recollected from the lagoon of Lesina (De Matthaeis *et al*. 1983). Sbordoni *et al*. (1986) also detected peculiar alleles and an unexpectedly high F_{ST} in the introduced hatchery samples. The study mentioned that '*The genetic structure of the new Japanese stock was consistently different from that of the F1 introduced into Italy*' (Sbordoni *et al*. 1986: 243), and thus the introduced shrimps were suspected as originating from different geographic localities. Furthermore, a photograph of shrimps from an Italian shrimp farm (http://www.apulashrimp.it/Sito/English/Penaeus_japonicus_eng.htm) shows that it is *P. pulchricaudatus*. All these scenarios raised uncertainty as to which species, *P. japonicus* or *P. pulchricaudatus* is cultured in Italian or other Mediterranean kuruma shrimp farms. A comprehensive survey on the shrimp cultured in different farms of the region is necessary to resolve this issue.

It appears that previous studies on the biology of the kuruma shrimp were undertaken on different species within the species complex. Incoherent descriptions of the environmental conditions required by the shrimp collected from various regions are noted in relevant literature reviews (Table 6). For instance, different optimal temperatures for growth and survival of the shrimp were recorded: 25–28°C from Japan (Hudinaga 1942), 25–30°C (Liao and Chien 1994) from Taiwan; 22–30°C (Lumare 1998) from Italy and 32°C from Australia (Hewitt and Duncan 2001). According to the geographic distribution of the two species, we presume that the first two studies were on *P. japonicus* or a mixture of the two species, whereas the last two were on *P. pulchricaudatus.* This vague identity of the species studied may inevitably induce confusion and uncertainty in aquaculture practices. For example, Savini *et al*. (2008: 61) described the European alien kuruma shrimp as very resistant as '*cold temperatures are not such a problem for its farming (minimum 5°C)*', yet Zhou (2001) stated that the kuruma shrimp never occurs in regions with sea surface temperature below 20°C in the South China Sea and the water temperature is regarded as the limiting factor affecting its distribution. Furthermore, Quinitio *et al*. (1991) observed the kuruma shrimp in the Philippines exhibits a faster moulting rate than the Japanese shrimp (Shigueno 1975). Also a sandy bed is suggested as an important factor for ovarian maturation

and reproductive success for female shrimp in Taiwan (Liao and Chien 1990), yet the substratum nature is considered not important for females from Australia (Hansford *et al*. 1993). All these findings suggest that *P. japonicus* and *P. pulchricaudatus* may have different environmental requirements and reproductive traits. Further elucidation of the biology of these two species is essential for improving the species-specific culturing techniques in the shrimp farming industry.

Conclusion

The kuruma shrimp is a species complex consisting of two sister species: *Penaeus japonicus* and *P. pulchricaudatus. Penaeus japonicus* is confined to the East China Sea and the northern South China Sea, whereas *P. pulchricaudatus* is widely distributed in the South China Sea, Australia, the western Indian Ocean and the Red Sea. *Penaeus japonicus* is genetically homogeneous, whereas *P. pulchricaudatus* exhibits a strong phylogeographical structure, and probably originated and differentiated from South-east Asia. Our study shows that the invasive Mediterranean population is *P. pulchricaudatus*, which entered the Mediterranean through Lessepsian migration from the Red Sea. Further studies on the biological differences of these two cryptic species are necessary for providing information on their aquaculture.

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