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Phylogenetic and morphometric differentiation reveal geographic radiation and pseudo-cryptic speciation in a mangrove crab from the Indo-West Pacific

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ABSTRACT

The presence of boundaries to dispersal has been recently documented for many Indo-West Pacific (IWP) species with planktonic propagules and a widespread distribution. We studied the phylogeography of the mangrove crab *Neosarmatium meinerti* (Brachyura: Sesarmidae) and the phylogenetic relationship to its presumed sister species *N. fourmanoiri* in the IWP in order to compare intraspecific with interspecific diversity. Portions of the mitochondrial genes 16S and CoxI were sequenced for 23 specimens of *N. meinerti* and 5 *N. fourmanoiri*, while a fragment of the 28S was obtained for a subset of specimens. Genetic data are supplemented by morphometric and based on 37 adult males of *N. meinerti* and 9 males of *N. fourmanoiri*.

The conserved nuclear 28S reveals the existence of a genetic break between the Indian and Pacific oceans. Otherwise, mitochondrial genes as well as morphometry clearly support the presence of a species complex within *N. meinerti* composed by four well structured and geographically defined lineages: East African coast; western Indian Ocean islands; South East Asia; and Australia.

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1. Introduction

In the Indo-West Pacific (IWP), a widespread distribution has been historically documented for many marine species belonging to different taxa, supporting the idea that speciation is a rare event in the marine realm (Becker et al., 2007). Conversely, in recent years, many marine boundaries for organisms with pelagic larvae have been described (Knowlton, 2000) and the number of cryptic species descriptions has dramatically increased (Bickford et al., 2007; Pfenninger and Schwenk, 2007). Notwithstanding the apparent lack of geographic barriers, several genetic studies conducted on marine fauna and flora dispersed by planktonic propagules (Fairweather, 1991) agree in recording a high genetic divergence between populations of the western Indian Ocean (WIO) and the eastern Indian/western Pacific Ocean (EIO/WPO) populations, as for example in the starfish *Linckia laevigata* (see Williams and Benzie, 1998), the tiger prawn *Penaeus monodon* (see Benzie et al., 2002; Duda and Palumbi, 1999), the limpet *Patelloidea profunda* (see Kirkendale and Meyer, 2004), the mangrove and estuarine fish *Mugil cephalus* (see Rossi et al., 1998), the tuna fish *Thunnus obesus* (see Alvarado Bremer et al., 1998; Appleyard et al., 2002) the reef fish *Chlorurus sordidus* (see Bay et al., 2004), mangrove crabs of the genus *Scylla* (see Gopurenko et al., 1999), the buckler crabs of the genus *Cryptopodia* (see Chiong and Ng, 1998), the box crab of the genus *Calappa* (see Lai et al., 2006; Lai and Ng, 2006; Ng et al., 2002), and the red algae *Spyridia filamentosa* (see Zuccarello et al., 2002). Another crab species showing an interesting subdivision in this area is the swimming crab *Portunus pelagicus* with four genetically and geographically defined clusters supported by high bootstrap values (Lai et al., accepted for publication).

Moreover, in some studies, an additional genetic break has been reported between the EIO and the WPO, identifying two biogeographic regions, Southeast Asia (the triangular area including New Guinea, Philippines and the Malay Peninsula) and Australia plus the Pacific Islands (Benzie, 1999; Duke et al., 1998; Rossi et al., 1998; Williams and Benzie, 1997; Wray et al., 1995).

Our study focuses on the sesarmid crab *Neosarmatium meinerti* (De Man, 1887) (Brachyura: Thoracotremata: Sesarmidae). Like most mangrove crabs, *N. meinerti* releases its larvae in synchrony with spring tides, with a preference for dry season, at least in East Africa (Skov et al., 2005). The larval phase consists of five zoeal stages, with a supposed duration of about one month, according to laboratory rearing (Pereyra Lago, 1989). During this period, larvae may be transported by oceanic currents and thus have the potential of being dispersed over long distances. This semi-terrestrial crab is widespread in the rearward belt of mangrove forests throughout the IWP, with a relative continuous geographic range including the East African coast and its offshore islands, India, Sri Lanka, Andamans, Southeast Asia, Taiwan and northwestern Australia (Davie, 1994; Ng et al., 1996, 2008; Schubart and Ng, 2002). A recent

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population genetic study conducted on *N. meinerti* from the western Indian Ocean recorded a sharp genetic break between populations of the East African coast and the population of Mahé Island, Seychelles (Ragionieri et al., accepted for publication). Such finding suggests that biogeographic boundaries may be present within the *N. meinerti* geographic range, as evident in other marine species dispersed by planktonic propagules in the IWP. Within this framework, an extensive phylogeographic study was therefore performed including specimens covering the entire geographic range of this species, by means of two mitochondrial markers (the subunit 1 of the cytochrome oxidase gene, Cox1, and the large ribosomal RNA subunit gene, 16S). In addition, portions of the nuclear large ribosomal subunit 28S, commonly employed for phylogenetic studies (Porter et al., 2005) was sequenced as further evidence for potential taxonomic implications (Avise, 2000). The putative sister species of *N. meinerti*, *N. fourmanoiri* Serène, 1973, and the type species of the genus, *N. smithi* (H. Milne Edwards, 1853), were also included in this study to compare the genetic diversity within *N. meinerti* to the one between sister species and other congeners.

2. Materials and methods

2.1. Specimen collection and DNA extraction

A total of twenty-three specimens from the IWP identified as *Neosarmatium meinerti* were sequenced for two selected mitochondrial genes (Fig. 1 and Table 1). Most of the samples from the East African coast and northern Australia were specifically collected for this study by the authors. For each sample, a walking leg was detached and conserved in 96% ethanol, while the rest of the animals was archived and catalogued at the Zoological Museum of the University of Florence (MZUF) (Table 1) as morphological vouchers. The other samples have been recovered from museum collections, some dating back up to 40 years (Table 1). The specimens of *N. meinerti* collected from northern Australia (Darwin) belong to two colour morphs, red and yellow chelae, as reported by Davie (1994). In the phylogenetic analysis we also included five individuals of *N. fourmanoiri* and, as outgroup, two individuals of *N. smithi* (Table 1). DNA was isolated from muscle tissues using the Puregene Kit (Gentra System), resuspended in TE buffer or water and then preserved at −20 °C.

2.2. Gene amplification

Polymerase chain reaction (PCR) was used for amplification of the three DNA-fragments analysed in the present study. From fresh material we obtained a 658 base pair (bp) fragment of the CoxI and a 597 bp fragment of 16S (excluding the primer regions) by a standard PCR protocol (40 cycles: 45 s 94°/1 min 48–50°/1 min 72° denaturing/annealing/extension temperatures). To obtain the same fragment from older specimens it was necessary to amplify and combine shorter fragments with a shorter PCR program (40 cycles; 30 s 94°/45 s 46°/45 s 72° denaturing/annealing/extension temperatures) and using internal primers. For the CoxI we used the following primer combinations: HCOI2198 and COL6b for the long fragments and combined with the new internal primers COH7Nm and COL19Nm for the short fragment (Table 2). For the 16S region we used the primers 16L29 and 16H10 and alternatively combinations with 16L12 and 16H7 for slightly shorter fragments (Table 2). For the amplification of 28S, the primers 28L4 and 28H4 were used (40 cycles: 75 s 97°/1 min 54°/1 min 72° denaturing/annealing/extension temperatures). This combination yields a 632 bp fragment, including the D2 and D3 loops of this nuclear ribosomal gene. The 28S rRNA gene has a low variability in comparison to the two mitochondrial genes; therefore a reduced number of specimens ($N = 9$) representing the entire distribution area of *N. meinerti* was considered adequately informative. Two specimens of *N. fourmanoiri* were also sequenced for this gene.

PCR products were purified with Sure Clean (Bioline), resuspended in water, and subsequently sequenced with the ABI Big Dye terminator mix (Big Dye Terminator® V 1.1 Cycle Sequencing kit; Applied Biosystems) in an ABI Prism automated sequencer (ABI Prism™ 310 Genetic Analyzer; Applied Biosystems). The resulting sequence files were corrected manually with Flinch TV 1.4.0 (Geospiza®) and aligned with BioEdit (Hall, 1999).

2.3. Statistical analysis

MODELTEST version 3.6 (Posada and Crandall, 1998) was used for determining the best fitting model of sequence evolution by the hLRT criterion. The best model and the likelihood parameters were calculated first for the two mitochondrial genes separately and subsequently for the combined alignment. Phylogenetic con-

Fig. 1. Map of the Indo–West Pacific Ocean. The black and white dots indicate the sample sites of *N. meinerti* and *N. fourmanoiri*, respectively.

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Table 1
Specimens of the genus *Neosarmatium* used for the phylogenetic reconstruction.

Species	Museum collection number	Nation	Morpho type	CoxI	16S	28S
N. meinerti	MNHN B-31905	Nosy Bé, Madagascar	Red	FN392143	FN392173	
N. meinerti	MNHN B-31906	Nosy Bé, Madagascar	Red	FN392144	FN392174	
*N. meinerti**	MNHN B-30342	Tuléar, Madagascar.	Red			
*N. meinerti**	MNHN B-30342	Tuléar, Madagascar.	Red			
*N. meinerti**	MNHN B-30342	Tuléar, Madagascar.	Red			
*N. meinerti**	MNHN B-30342	Tuléar, Madagascar.	Red			
*N. meinerti**	MZUF_2931	Mahe Islands, Seychelles	Red			
*N. meinerti**	MZUF_2940	Mahé Islands, Seychelles	Red			
N. meinerti	MZUF_3664	Mahé Islands, Seychelles	Red	FN392149	FN392179	FN392201
*N. meinerti**	MZUF_3663	Aldabra, Seychelles	Red	FN392148	FN392178	
*N. meinerti**	MZUF_3661	Rodriguez, Republic of Mauritius	Red	FN392146	FN392176	
*N. meinerti**	MZUF_3662	Rodriguez, Republic of Mauritius	Red	FN392147	FN392177	FN392202
*N. meinerti**	MZUF_3660	Mauritius, Repubblic of Mauritius	Red	FN392145	FN392175	
*N. meinerti**	MZUF_3665	Mauritius, Repubblic of Mauritius	Red			
*N. meinerti**	MZUF_3666	Kenya	Red			
N. meinerti	MZUF_3669	Lamu, Kenya	Red	FN392139	FN392169	FN392198
N. meinerti	MZUF_3767	Mida Creek, Kenya	Red	FN392140	FN392170	FN392199
N. meinerti	MZUF_3768	Inhaca Island, Mozambique	Red	FN392141	FN392171	FN392200
*N. meinerti**	MZUF_2731	Mida Creek, Kenya	Red			
*N. meinerti**	MZUF_1025	Gazi Bay, Kenya	Red			
*N. meinerti**	MZUF_1025	Gazi bay, Kenya	Red			
*N. meinerti**	MZUF_2967	Mida Creek, Kenya	Red			
*N. meinerti**	MZUF-pending	Mida Creek, Kenya	Red			
*N. meinerti**	MZUF-pending	Mida Creek, Kenya	Red			
*N. meinerti**	MZUF_677	Jumbo River, Somalia	Red			
*N. meinerti**	MZUF-pending	Jumbo River, Somalia	Red			
*N. meinerti**	MZUF_678	Jumbo River, Somalia	Red	FN392138	FN392168	
*N. meinerti**	MNHN-B31275	Natal, South Africa	Red			
N. meinerti	MZUF 2511	South Africa, Natal	Red	FN392142	FN392172	
*N. meinerti**	MZUF_2968	Darwin, East Point, Australia	Yellow	FN392158	FN392188	FN392204
*N. meinerti**	MZUF_3658	Darwin, Univ of Darwin, Australia	Red	FN392156	FN392186	
*N. meinerti**	MZUF_2971	Darwin, CDNP, Australia	Red			
*N. meinerti**	MZUF_2971	Darwin, CDNP, Australia	Yellow			
*N. meinerti**	MZUF_2971	Darwin, CDNP, Australia	Red			
*N. meinerti**	MZUF_2971	Darwin, CDNP, Australia	Red			
*N. meinerti**	MZUF_2969	Darwin, Univ. of Darwin, Australia	Red			
N. meinerti	QMW21260	Kimberly Coast, Australia	Red	FN392160	FN392190	
N. meinerti	QMW25080	Kakadu NP, Australia	Yellow	FN392159	FN392189	
*N. meinerti**	MZUF_2969	Darwin, Univ. of Darwin, Australia	Red			
*N. meinerti**	MZUF_2969	Darwin, Univ. of Darwin, Australia	Red			
*N. meinerti**	MZUF_3659	Darwin, Univ. of Darwin, Australia	Red	FN392157	FN392187	FN392203
N. meinerti	MZUF_3769	Galle, Sri Lanka	Red	FN392155	FN392185	FN392206
*N. meinerti**	NCHU_13077	Dajhong Temple, Taiwan	Yellow	FN392152	FN392182	
*N. meinerti**	NCHU_13076	Dajhong Temple, Taiwan	Red			
*N. meinerti**	MZUF_2516	Paoli stream, Taiwan	Yellow			
N. meinerti	ZRC_2001.1898	Phuket, Thailand	Red	FN392154	FN392184	FN392205
*N. meinerti**	ZRC_2001.1083	Phuket, Thailand	Red	FN392153	FN392183	
*N. meinerti**	MNHN-B31277	Indonesia (Aquarium trade)	Red	FN392150	FN392180	
N. meinerti	MNHN-B31276	Sulawesi (Aquarium trade)	Red	FN392151	FN392181	
N. fourmanoiri	NCHU_13078	Dajhong Temple, Taiwan	Yellow	FN392163	FN392193	FN392208
N. fourmanoiri	SMF 25170	Viti Levu Island, Fiji	Red	FN392161	FN392191	FN392207
*N. fourmanoiri**	UF1602	Viti Levu Island, Fiji	Red			
*N. fourmanoiri**	UF99	Mariana Island, Guam	Yellow			
*N. fourmanoiri**	UF100	Mariana Island, Guam	Red			
*N. fourmanoiri**	QMW_24964	Dako Nating, Solomon Islands	Red	FN392164	FN392194	
*N. fourmanoiri**	QMW_24964	Dako Nating, Solomon Islands	Yellow			
*N. fourmanoiri**	QMW_12901	Lideman Island	Red	FN392165	FN392195	
*N. fourmanoiri**	ZRC_2001.0715	Merizio, Guam	Yellow			
N. fourmanoiri	ZRC_2002-0179	Apra Harbor, Guam	Red	FN392162	FN392192	
*N. fourmanoiri**	MZUF_2517	Apra Harbor, Guam	Red			
*N. fourmanoiri**	QMW_19558	Menou, New Caledonia	Red			
N. smithi	MZUF 2504	Mida Creek, Kenya		FN392167	FN392197	
N. smithi	MZUF 2510	South Africa (leg. Emerson)		FN392166	FN392196	

Abbreviations: MNHN, Muséum National d'Histoire Naturelle, Paris; MZUF, Museo Zoologico dell'Università di Firenze; NCHU, National Chung Hsing University, Taichung, Taiwan; ZRC, Zoological Reference Collection, Department of Zoology, National University of Singapore; SMF, Senckenberg-Museum, Frankfurt a.M.; QMW, Queensland Museum, Brisbane. Specimens with an asterisk (*) have also been used for morphometric analysis. Still part of the previous version.

gruence among CoxI and 16S data partitions was tested using the incongruence length difference (ILD) test (Farris et al., 1995) implemented in PAUP* as the partition-homogeneity test (Swofford, 1998). For this test, we used random taxon addition, TBR branch swapping, and a heuristic search with 1000 randomisations of the data.

Four methods of phylogenetic inference were applied: neighbor joining (NJ), maximum parsimony (MP), and maximum likelihood (ML) using PAUP* (Swofford, 1998), and Bayesian Inference (BI) as implemented in MrBayes v. 3.0b4 (Huelsenbeck and Ronquist, 2001). For MP, we used a heuristic search with 10 replicates of random sequence addition and tree-bisection–reconnection as branch

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Table 2
Details of the primers used for PCR amplification of the CoxI, 16S and 28S gene fragments.

Name	Sequence	Source
HCOI2198	5′-taaacttcagggtgaccaaaaaatca-3′	Folmer et al. (1994)
COL6b	5′-acaaatcataaagatatygg-3′	Schubart and Huber (2006)
COH7Nm	5′-tgtaatgaaaaaattccta-3′	Present paper
COL19Nm	5′-atagttgaaagaggtgttgg-3′	Present paper
16L29	5′-ygcctgtttatcaaaaacat-3′	Schubart et al. (2009)
16H10	5′-aatcctttcgtactaaa-3′	Schubart (2009)
16L2	5′-tgcctgtttatcaaaaacat-3′	Schubart et al. (2002)
16H7	5′-ccggtctgaactcaaatcatgt-3′	Schubart (2009)
28L4	5′-tatttcccctcgtgatgtaggtc-3′	Present paper
28H4	5′-actccggacagagcaggat-3′	Present paper

swapping option. Gaps were treated as 5th state. Otherwise, default options of PAUP* were used. For the NJ analysis, the parameters of the suggested model of evolution were implemented in PAUP*. The bootstrap method (with 2000 pseudoreplicates) was used for calculating the confidence values for the proposed groups within each inferred tree. For ML, the best fitting models from MODELTEST were implemented in the analysis with PAUP*, and the other settings corresponded to the ones of MP. For these time-consuming calculations the nodal support was estimated after 500 bootstrap pseudoreplicates. BI analyses were calculated with four MCMC chains for 2,000,000 generations, saving trees every 500 generations (with a corresponding output of 4000 trees). The $-\ln L$ converged on a stable value after 8000 generations. The first 8000 generations were thus discarded from the analysis and the posterior probabilities were determined by constructing a 50% majority rule consensus of the remaining trees.

We calculated the mean genetic *p*-distance (±standard error) among inferred groups with MEGA 3.1 (Kumar et al., 2004) with a simple nucleotide model (number of substitutions), a homogeneous pattern among lineages and uniform rate among sites. The constancy of evolutionary rate among two taxa with reference to an outgroup was tested with the Tajima relative rate test (Tajima, 1993) as implemented in MEGA 3.1 (Kumar et al., 2004). If the null hypothesis of homogeneous rates between sister taxa is rejected (i.e. test significant), then the molecular clock hypothesis must be rejected for this set of sequences, whereas a molecular clock can be applied, if the relative rate test is non-significant.

The mean divergence time was estimated by applying the non-Jamaican rate by Schubart et al. (1998), i.e. 1.17% per MYA for the combined 16S-CoxI dataset, with the standard error of divergence time being the standard error of the mean genetic *p*-distance divided by the rate.

A minimum spanning network was constructed with TCS version 1.13 (Clement et al., 2000), estimating gene genealogies for the 16S and the 28S gene. The network for the 16S was calculated using 22 sequences (one shorter sequence from Madagascar was excluded) and 5 sequences of *N. fourmanoiri*. The length of the 16S fragment used for the minimum spanning network corresponds to that obtained using the internal primers (i.e. 441 bp excluding the primers), since for this analysis all the sequences need to be of equal length. The 28S network was based on 11 sequences of *N. meinerti* and two of *N. fourmanoiri*. 561 bp long (excluding the first 57 bp and the last 15 bp of the whole fragment amplified by the 28S primer combination). In the networks, every line between two points represents a nucleotide substitution, the connection limit was fixed at 90% and gaps were treated as missing data.

2.4. Morphometrics

For the morphometric comparison, a total of 37 adult males of *N. meinerti* and 9 males of *N. fourmanoiri* were measured (see Table 1). Whenever possible, the genetically analysed specimens were also included in the morphometric analysis (Table 1). The following measurements were taken with a digital caliper (±0.01 mm) as listed in Table 3 and Fig. 2: (1) carapace width (CW); (2) posterior carapace width (PCW); (3) carapace length (CL); (4) body height (BH); (5) maximum frontal width (MFW); (6) minimum orbital width (MOW); (7) telson length (TL); (8) telson width (TW); (9) sixth abdominal distal width (6th ADW); (10) sixth abdominal proximal width (6th APW); (11) sixth abdominal length (6th AL); (12) third pereopod width (3rd PH); (13) third pereopod length (3rd PL); (14) maximum abdominal width (MAW). After assessing normal distribution with the Kolmogorov–Smirnov test (software SPSS 13.0), all the measurements were transformed to ratios (CW/CL; CW/PCW; CW/BH; MFW/MOW; TL/TW; 6th APW/TL; 6th APW/6th AL; 3rd PL/3rd PH; CW/MAW) to reduce the effect of possible allometric growth in specimens of undetermined age. Subsequently, we tested for the existence of morphometric differences in the above defined ratios among *N. meinerti* grouped in four different geographic groups, and *N. fourmanoiri* (applying a one-factor ANOVA and a post hoc Tukey test, as implemented in the software SPSS 13.0), after having assessed the homogeneity of variance by the Levene test. We applied the Bonferroni correction to ANOVA *P* values ($P = 0.01$), as suggested when multiple tests and non-independent data are used. Geographic groups were defined based on our genetic outcome as follows: East African coast including Madagascar (EAC), western Indian Ocean islands excluding Madagascar (WIOIs), Southeast-East Asia (SE-E Asia) and Australia (AUS).

Table 3
List of dimensions and codes used in the morphometric analysis of *N. meinerti* and *N. fourmanoiri*. See also Fig. 2 for a graphic representation.

Carapace (dorsally measured)
CW carapace width: width of the widest section: measured between middle to first epibranchial tooth
PCW posterior carapace width: width of the narrow section e of the carapace where the posterolateral margin begins to converge toward distinct concave posterior carapace margin
CL carapace length: length of the carapace from the frontal margin of the carapace to the posterior margin of the carapace
BH body height: dorso-ventral height of the body, between the antero-median surface with deep Y-shaped groove and abdominal region
MFW maximum frontal width: the greatest width of the of the frontal section
MOW minimum orbital width: the smallest width measured between the orbital hiatus
Abdomen
TL telson length: length of telson, from the mid proximal segment till the tip of the telson
TW telson width: width of the last abdominal segment measured at the joint with the fifth abdominal segment
6th ADW sixth abdominal distal width: width of the sixth abdominal segment measured near the joint with the telson
6th APW sixth abdominal proximal width: width of the sixth abdominal segment measured at the joint with the fifth abdominal segment
6th AL sixth abdominal length: length of the sixth abdominal segment from the mid-anterior margin to the joint with the seventh abdominal segment
Pereiopod
3rd PH third pereopod height: height of the merus of the third pereiopod
3rd PL third pereopod length: ventral length of the merus measured between the triangular tip of the ischium and the distal end of the merus
MAW maximum abdominal width: widest section of the abdomen, corresponding in males at the third abdominal segment

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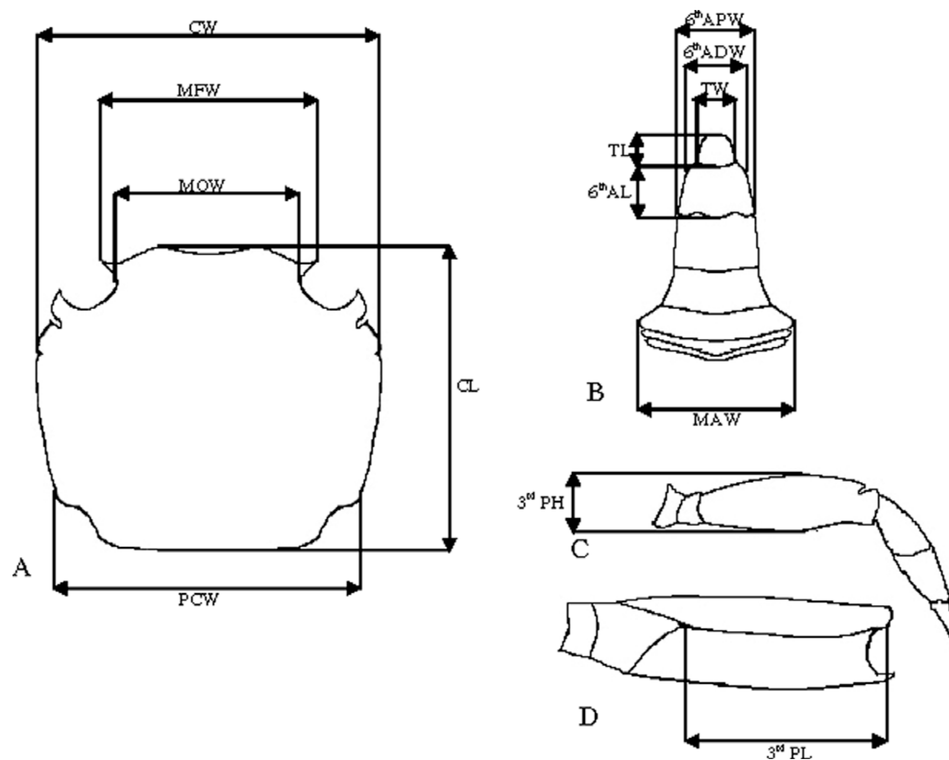

Fig. 2. Diagram showing positions used for morphometric measurements. (A) carapace; (B) abdomen; (C) pereiopod; (D) merus.

A discriminant analysis, as implemented in the software Statistica 6.0, was carried out using all the above described ratios (Software Statistica 6.0; StatSoft). For each pairwise comparison between defined groups, we calculated the Mahalanobis distances, i.e. the distance of the test point from the mass centre divided by the width of the ellipsoid in the direction of the test point.

3. Results

3.1. Genetic analysis

The ILD test indicated that phylogenetic congruence between the 16S and CoxI gene fragments could not be rejected ($P = 0.97$). Thus, the phylogenetic inference analysis was performed with the combined alignment of the two mitochondrial genes and a total length of 1254 bp after removal of primer sequences. The entire dataset was unambiguously alignable. The best fitting model according to MODELTEST (Posada and Crandall, 1998) for the CoxI is TrN+G (Tamura and Nei, 1993) and for the 16S (Table 4) as well as the combined alignment HKY+I+G (Hasegawa et al., 1985). The TrN model only differs from the HKY in defining two rates of transitional substitution, one between purines and one between pyrimidines and thus the former is a simplification of the latter and HKY+I+G was used throughout. The mtDNA sequences are A–T rich, which is in agreement with the observation of A–T bias of the mitochondrial DNA in arthropods (Simon et al., 1994).

Table 4 summarises the numbers of transversions and transitions together with the number of parsimony-informative sites, the transition/transversion ratio (*R*), the proportion of invariable sites and the shape value of the gamma distribution (as obtained in the model of evolution with MODELTEST) for both mitochondrial genes separately as well as for the combined alignment.

According to the mtDNA sequences, all specimens morphologically identified as *N. meinerti* belong to a monophyletic group, well supported by high confidence values, except for ML with 72 as bootstrap value (Fig. 3). Within the *N. meinerti* monophylum, three main clades are defined. The most basal clade is represented by the populations from southern to eastern Asia (Indonesia, Sri Lanka, Taiwan and Thailand); the second clade includes specimens from Australia (including red and yellow chelar morphs); and the third clade includes the western Indian Ocean populations (from now on summarized as Pan-African populations). This latter clade can be subdivided into two distinct subclades: the EAC group and the WIOIs group. The specimens from northwestern Madagascar (Nosy Bé) group together with the populations from the mainland (Somalia, Kenya, Mozambique and South Africa) and not with the WIOIs group. The samples of *N. fourmanoiri* always occupy a basal position in the mtDNA trees with respect to the *N. meinerti* clades,

Table 4
Information on mtDNA gene fragments amplified for *Neosarmatium* phylogenetic reconstruction.

	T	C	A	G	bp	var	Pi	Ts	Tv	R	Pinvar	α
CoxI+16S	37.4	13.2	33.0	16.3	1254	156	143	36.14	7.91	9.99	0.362	0.0147
CoxI	37.3	16.6	29.2	16.8	657	106	98	26.29	4.51	10.63	0.0	0.0116
16S	37.6	9.3	37.4	15.7	597	50	45	9.84	2.68	5.86	0.506	0.0022
28S	17.5	33.3	13.2	35.9	560	4	3	1.782	0	n/c	0	–

Base frequencies, total number of basepairs (bp), number of variable (var) and parsimony-informative (pi) sites, mean number of pairwise transitions (Ts), transversions (Tv) and transition to transversion ratios (R), proportion of invariant sites (Pinvar) and α value of gamma distribution in the combined CoxI+16S dataset as well as in the two subsets of data.

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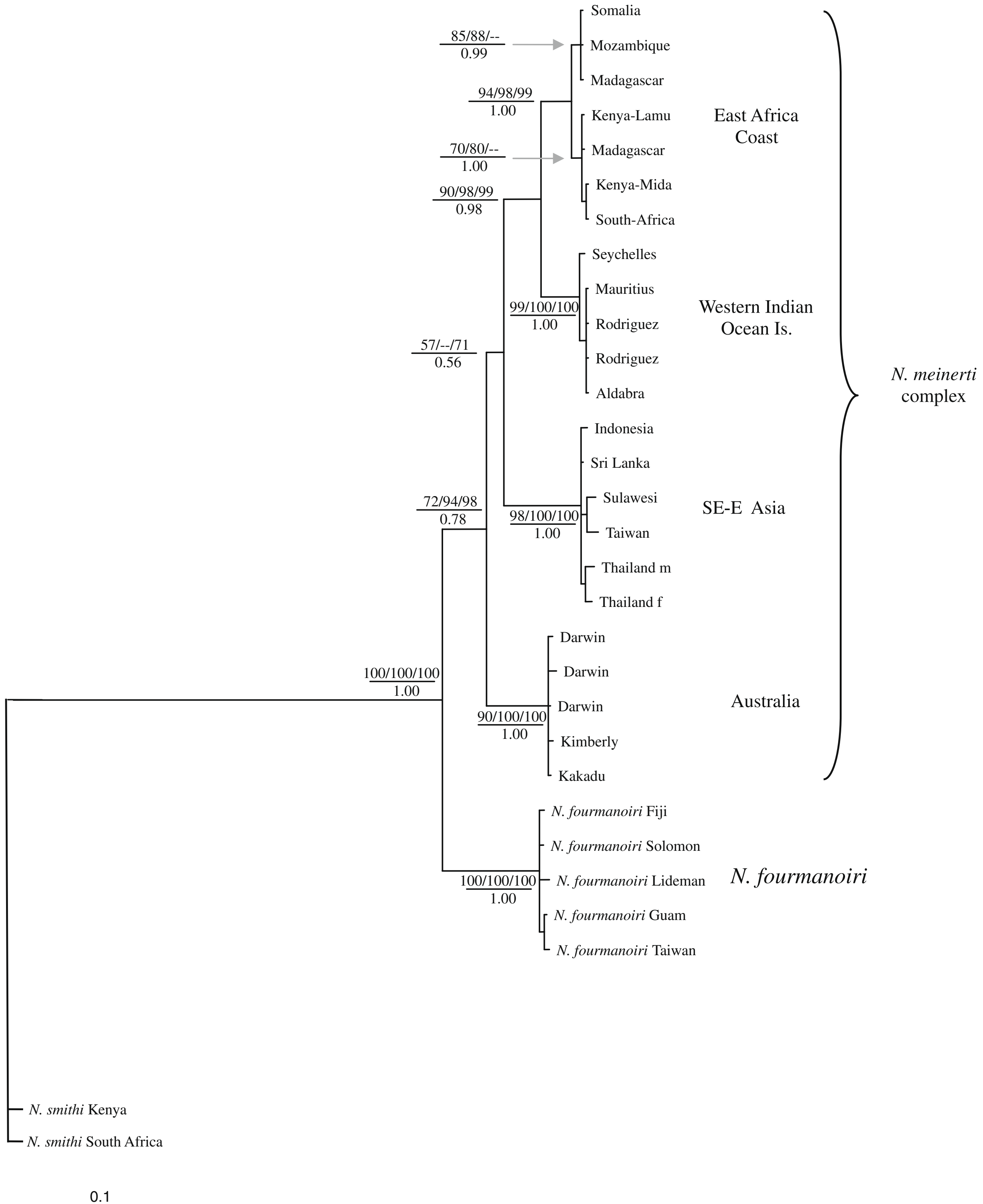

Fig. 3. Bayesian consensus rooted tree of the two mitochondrial genes (16S and CoxI) based on the HKY+I+G evolution model. The Bayesian posterior probabilities are reported below the lines, while the bootstrap values for the Maximum Likelihood, Maximum Parsimony and Neighbour joining are indicated above the lines. Only confidence values higher than 50% are shown in the tree.

but their genetic distance from the outgroup is not higher than distances between eastern *N. meinerti* clades and the outgroup (Table 5).

In the 16S minimum spanning network, the above mentioned clades can be clearly distinguished, even if based on a reduced number of haplotypes. In the network, specimens of the two *N.*

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Table 5
P-distance values ± standard errors in percentage among *Neosarmatium* groups for the mtDNA genes.

	EAC	WIOIs	AUS	SE-E Asia	*N. fourmanoiri*	*N. smithi*
EAC	*	2.8 ± 0.6	4.6 ± 0.8	5.4 ± 0.8	5.0 ± 0.8	8.4 ± 0.1
WIOIs	1.6 ± 0.5	*	4.4 ± 0.8	5.2 ± 0.8	5.8 ± 0.9	8.4 ± 0.1
AUS	2.1 ± 0.6	2.6 ± 0.6	*	5.3 ± 0.8	6.1 ± 0.9	9.6 ± 0.1
SE-E Asia	2.5 ± 0.6	3.2 ± 0.7	1.9 ± 0.5	*	5.8 ± 0.8	8.5 ± 0.1
N. fourmanoiri	2.8 ± 0.7	3.6 ± 0.8	1.8 ± 0.5	2.4 ± 0.6	*	0.74 ± 0.1
N. smithi	4.6 ± 0.9	5.1 ± 0.9	4.2 ± 0.8	4.2 ± 0.7	3.2 ± 0.7	*

Upper diagonal for the CoxI gene and lower diagonal for the 16S gene. EAC: *N. meinerti* East Africa coast; WIOIs: *N. meinerti* West Indian Ocean Islands; Asia: *N. meinerti* southern and eastern Asia; AUS: *N. meinerti* Australia.

meinerti colour morphs, sympatric in northern Australia, share the same haplotype. It is interesting to note that the Australian and Asian clades cluster nearer to *N. fourmanoiri* than to the Pan-African clade. The number of differences increases considerably, if gaps are included or the combined dataset with Cox1 is used. In those cases, it is not possible to build a parsimony network which connects all the major groups.

Resolution of the 28S minimum spanning network is highly reduced, as expected, and only two main clades are identified. The first corresponds to the mitochondrial Pan-African clades and the other one, separated by the former from two fixed positions, corresponds to the Australasian and *N. fourmanoiri* sequences (Fig. 4), whereby *N. fourmanoiri* shares a haplotype with four specimens of *N. meinerti* from the Australasian clade.

The mean genetic *p*-distances show that the three major groups (Pan-Africa, SE-E Asia and AUS) have diverged from each other as much as from *N. fourmanoiri* (Table 5). The Tajima relative rate test with *N. smithi* as outgroup was never significant ($P > 0.05$), suggesting similar mutation rates in all branches. We therefore applied the molecular clock for our combined alignment, using the "non-Jamaican" marine sesarmid rate defined in Schubart et al. (1998). The following divergence times were estimated: between EAC and WIOIs 1.96 ± 0.34 MYA; between WIOIs and AUS 2.99 ± 0.42 MYA; between Pan-Africa and SE-E Asia 3.58 ± 0.42 MYA; between Pan-Africa and AUS 2.99 ± 0.42 MYA; between Pan-Africa and *N. fourmanoiri* 3.7 ± 0.42 MYA; between SE-E Asia and AUS 3.16 ± 0.42 MYA; between SE-E Asia and *N. fourmanoiri* 3.58 ± 0.42 MYA; between *N. fourmanoiri* and AUS 3.50 ± 0.42 MYA.

3.2. Morphometrics

All measurements were normally-distributed according to the Kolmogorov–Smirnov test. The homogeneity of variance was confirmed for all ratios, except for carapace width to posterior carapace width ($P = 0.04$). A one-way ANOVA revealed that there were differences among the groups in five morphometric ratios, and the post hoc Tukey test indicated, which groups are different from each other: carapace width to carapace length (df = 4; $F = 3.461$; $P = 0.015$; EAC *vs* WIOIs); carapace width to posterior carapace width (df = 4; $F = 4.389$; $P = 0.005$; AUS *vs* SE-E Asia; SE-E Asia and *N. fourmanoiri*); carapace width to body height (df = 4; $F = 7.148$, $P < 0.001$; WIOIs *vs* EAC, WIOIs *vs* SE-E Asia; EAC *vs* *N. fourmanoiri*, SE-E Asia *vs* *N. fourmanoiri*); telson length to telson width (df = 4, $F = 5.322$, $P < 0.001$; WIOIs *vs* EAC; EAC *vs* SE-E Asia); sixth abdominal posterior width to telson width (df = 4; $F = 9.593$, $P < 0.001$; EAC *vs* WIOIs; WIOIs *vs* AUS; WIOIs *vs* *N. fourmanoiri*); sixth abdominal posterior width to sixth abdominal length (df 4; $F = 9.021$; $P < 0.001$; EAC *vs* SE-E Asia plus AUS plus *N. fourmanoiri*);

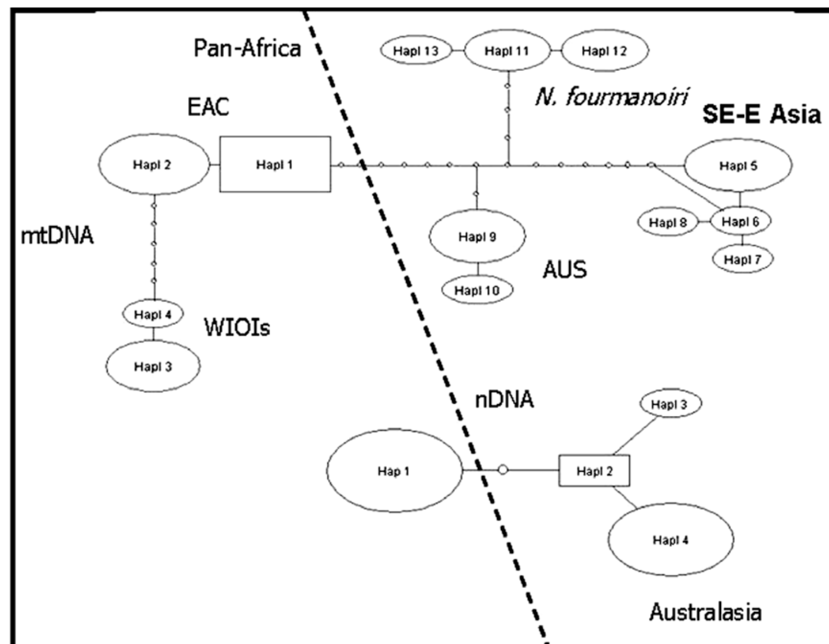

Fig. 4. Minimum parsimony spanning network constructed with TCS (a) of a 441-bp fragment from the 16S gene of *Neosarmatium meinerti* ($N = 22$) and *N. fourmanoiri* ($n = 5$) and (b) of a 560-bp from the nuclear 28S gene *N. meinerti* ($n = 9$) and *N. fourmanoiri* ($n = 2$). The size of the circle is proportional to the frequency of the haplotypes, each line represents one substitution and the spots along a line indicate additional substitutions. The rectangular represents the ancestral haplotype. Australian specimens belong to both of the described colour morphsa (for more details see the text).

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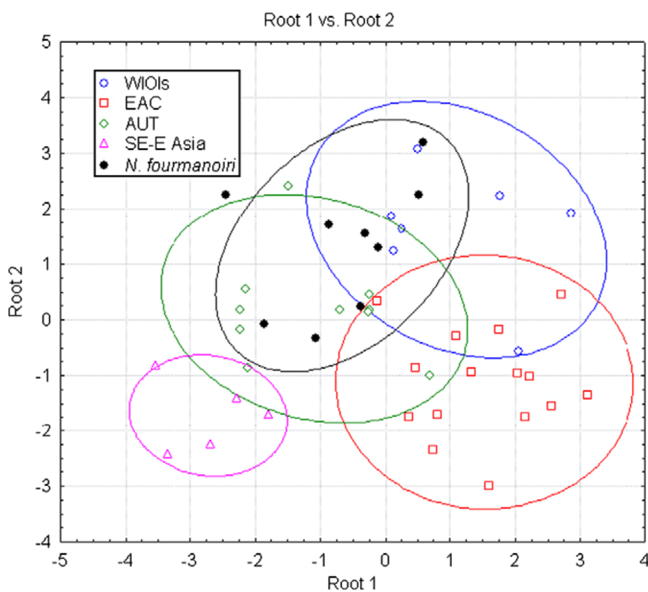

Fig. 5. Canonical analysis depicting discrimination by morphometric measurements of the five groups of *Neosarmatium* spp.: *N. meinerti* western Indian Ocean Islands (WIOIs), *N. meinerti* East African coast plus western Madagascar (EAC), *N. meinerti* Australia (AUS), *N. meinerti* South East Asia (SE-E Asia), *N. fourmanoiri* (*N. fourmanoiri*). Represented is a plot of the first discriminant function (root 1) against the second (root 2).

carapace width to maximum abdominal width (df = 4; $F = 3.785$; $P = 0.010$; EAC *vs N. fourmanoiri*; SE-E Asia *vs N. fourmanoiri*).

The dataset was subjected to canonical analysis (Fig. 5). The discriminant analysis allows to distinguish five groups (Wilk's Lambda: 0.035, $F(36.125) = 5.0293$, $P < 0.0001$). In Table 6 the values of the Mahalanobis distances among the groups are reported. It is interesting to note, that the highest values are not associated to the pairwise comparisons between *N. fourmanoiri* and different groups of *N. meinerti*, but always include the Asian group.

4. Discussion

The present contribution provides new evidence for the apparent prevalence of biogeographic boundaries within the IWP. Notwithstanding a larval phase lasting about one month in the marine plankton (Pereyra Lago, 1989) the mangrove crab *Neosarmatium meinerti* reveals a clear phylogenetic structure within its distributionary range. Mitochondrial and morphological results clearly indicate the existence of four geographically well-defined clades, corresponding to the EAC, the WIOIs, SE-E Asia and AUS. Similar (but not identical) genetic breaks have been reported in other marine decapods species like *Portunus pelagicus* (see Lai et al., accepted for publication) and *Penaeus monodon* (see Benzie et al., 2002; You et al., 2008), and the clam *Pinctada maxima* (see Benzie and Smith-Keune, 2006; Benzie et al., 2003).

Table 6
Pairwise squared Mahalanobis distances among *Neosarmatium* groups.

	EAC	WIOIs	SE-E Asia	AUS	*N. fourmanoiri*
EAC	*				
WIOIs	13.33877	*			
AUS	11.48789	14.63943	*		
SE-E Asia	24.00786	29.75819	14.76693	*	
N. fourmanoiri	13.42082	12.8656	21.50618	6.51371	*

Abbreviations: EAC: *N. meinerti* East Africa coast; WIOIs: *N. meinerti* West Indian Ocean Islands; SE-E Asia: *N. meinerti* southern and eastern Asia; AUS: *N. meinerti Australia.*

All the specimens morphologically identified as *N. meinerti* belong to a monophyletic group according to the mtDNA. However, this monophylum is very closely related to *N. fourmanoiri*, and, in terms of genetic distances, the subgroups of *N. meinerti* are as much differentiated from each other as from the presumed sister species. A confirmation of the genetic similarity between these two species comes from sequence of the nuclear 28S of *N. fourmanoiri*, which is shared with the Australasian clade of *N. meinerti*, but differs in two fixed positions from the Pan-African clade. Consequently, *N. meinerti* appears paraphyletic based on the results of this conserved nuclear gene. However, since the 28S has a mutation rate that is dramatically reduced compared to the rate of the 16S and CoxI, these results must be considered preliminary.

Furthermore, neither mitochondrial nor nuclear markers reveal any divergence between the two morph colour (yellow and red), suggesting a phenotypic pattern as already reported for other decapods species (Knowlton, 1986; Reuschel and Schubart, 2007).

The unexpected genetic break between the WIO islands (excluding NW Madagascar) and the African mainland, with no consistent genetic diversity among islands, but marked distances to the continent and NW Madagascar, has so far been unrecorded for other marine invertebrates with larval dispersal, probably due to the limited number of studies in this area. This genetic break is also highlighted in a population genetic study, in which populations of *N. meinerti* from the north of Kenya to South Africa and a population from the Seychelles are compared with higher sample numbers (Ragionieri et al., accepted for publication).

Madagascar deserves particular attention, because the included specimens from Nosy Bé share mtDNA with the continental population. A possible explanation could be the presence of numerous small islands (Comoros) close to the northern tip of Madagascar that may represent stepping stones for dispersal and thus maintain a certain degree of gene flow between populations of the East African coast and the populations of (at least western) Madagascar. Oceanographic studies on the Mozambique currents also highlight the presence of a dipole of upwelling–downwelling system, able to transport ocean waters from the East African coast towards the west side of Madagascar, in approximately 7 days (Hankle and Robertson, 2007).

Overall, population connectivity across the Indian Ocean would only be possible by gene flow between neighbouring populations, because of the huge distances between the geographic extremes. However, even potential genetic mixing between the populations from the relatively close East African coast and the western Indian Ocean islands as well as between southern Asian and northern Australian populations can be ruled out according to this study. This could be due to larval behaviour, driven by active vertical movements in the water column for selecting suitable currents. This type of movement, called ontogenetic or vertical migration, has been reported for species living in estuarine areas, where larvae may "choose" to being retained in near shore waters (with larvae utilising the surface currents generated by onshore winds, for remaining in coastal waters), as in *Callinectes sapidus* and in *Scylla serrata* (see Morgan et al., 1996; Webley and Connolly, 2007), or exported (selecting seaward currents for transport into the open waters and successively the landward currents for returning back), as in *Carcinus maenas* (see Queiroga and Blanton, 2005). Moreover, over the last years, many ethological papers highlighted the importance of physical and chemical cues in larval settlement and recruitment events (Christy, 1989; Diele and Simith, 2007; Gebauer et al., 2002, 1998; Jensen, 1989).

The Southeast Asian biogeographic region is considered a centre of origin for many marine species (Benzie, 1998; Ellison et al., 1999; Palumbi, 1996). Cane and Molnar (2001) argued for a climatic change in the Indian Ocean region and Africa with the reduction of the 'Indonesia through flow' around 3–4 MYA caused by the

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northward movement of the New Guinea-Australian plate and the emergence of much of Halmahera. This event may have played a role for diversification in the *N. meinerti*–*N. fourmanoiri* complex as described above and calculated to have occurred at similar time intervals.

The pairwise *p*-distance values calculated for both the mtDNA genes (CoxI and 16S) are very similar among the three main clades, Pan-Africa, SE-E Asia and Australia, as well as in the pairwise comparisons with *N. fourmanoiri* (Table 5). The lowest *p*-distance value was found between the African mainland and the western Indian Ocean islands and suggests that a second differentiation happened in the western Indian Ocean subsequent to the first radiation. The results of the *p*-distances are illustrated by the 16S minimum spanning network. Overall, three main clades can be identified, Pan-Africa, Australasia and *N. fourmanoiri*, with the latter being more closely related to the Australian group than to any other group.

The mitochondrial phylogenetic inference supports a first split of a common ancestor into *N. fourmanoiri* and a *N. meinerti* species complex, which simultaneously, or shortly later, radiated into four groups. This is reflected in the network and by the *p*-distance values, which could also support a single radiation of a common ancestor into initially three *N. meinerti* groups and *N. fourmanoiri* in the Indo-West Pacific. In contrast, the nuclear DNA phylogenetic inference suggests that the common ancestor of *N. meinerti* and *N. fourmanoiri* differentiated within the Indian Ocean into a western and an eastern form, the former subsequently splitting into three subgroups, of which *N. fourmanoiri* is currently recognized as separate species. Independent of the scenario, both cases give evidence that several evolutionary significant units are currently included within the species *N. meinerti*.

The morphometry of the four clusters of *N. meinerti* that we recognized as distinct groups was also investigated. The discriminant analysis of morphometric ratios support significant separation between all clades, with strongest differentiation of the specimens from Asia, that appear completely separated from the other groups.

Thus our molecular and morphometric results both strongly support the existence of a species complex in what used to be considered a single species, *N. meinerti*. This complex probably comprises four species, three of which are undescribed. Full descriptions of the three so far undescribed species with a summary on their currently recognized distribution will be presented elsewhere (Ragionieri et al., unpublished), because detailed morphological accounts and designation of additional type material would not fit the initial aim of this paper and the scope of this journal.

5. Outlook

Species living in the marine realm with planktonic propagules are believed to have high levels of gene flow across populations, able to maintain panmixis in meta-populations. Nowadays there are examples suggesting that gene flow is not a species-specific trait, but also depends on local environmental conditions and historical events (Sotka et al., 2004). Recently, many sibling and cryptic species have been discovered, even in species with a long-lasting pelagic phase, rejecting the hypothesis of panmixis in population with overlapping habitats (e.g. Patarnello et al., 2007). The resulting idea is that the biogeographic lineage sorting of a species is a complex process depending not only on the presence of barriers to gene flow, but also on its life history and on current stochastic events (Barber et al., 2006, 2002; Patarnello et al., 2007). For these reasons, the phylogeography of marine species appears as a complex process depending on intrinsic characteristics of species. At the same time, a correct classification of taxa is of fundamental importance, especially for ethologists and ecologists, because even small genetic differences could be coupled with ecological and behavioural adaptations as reported, for example, in snapping shrimp belonging to the genus *Alpheus* (see Mathews et al., 2002). There is no defined cut-off point for sequence divergences delimiting species boundaries (e.g. Burton and Davie, 2007). Anyways, the presence of fixed diagnostic differences may indicate the absence of gene flow between putative taxa and the presence of two or more distinct species (Wiens and Servedio, 2000). Moreover, the concordance of several independent genetic markers is a far better criterion for recognizing species boundaries (Avise and Wollenberg, 1997). Will and colleagues (2005) argued that the real cutting-edge future for systematics and biodiversity research is the integrative taxonomy employing a large number of characters, including DNA-barcoding, in delimiting, identifying and describing natural species and taxa.

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