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An integrative approach to species delimitation in *Benthomangelia* (Mollusca: Conoidea)

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DNA sequences are currently used to propose primary hypotheses of species delimitation, especially when morphological variability is difficult to assess. In an integrative taxonomy framework, these hypotheses are then compared with other characters, such as morphology or geography, to produce robust species delimitations. For this purpose, the cytochrome oxidase subunit *I* (COI) gene has been sequenced for almost 50 specimens of the genus *Benthomangelia*, a deep-sea marine gastropod genus, collected in the South-West Pacific. Five genetic groups, displaying low and high genetic distances respectively within and between groups, were defined. COI hypotheses were compared with both the results obtained with the independent nuclear 28S gene and with an elliptic Fourier analysis of the shape of the last whorl of the shell. 28S gene analysis confirmed the same well-supported groups as COI, and elliptic Fourier analysis identified several morphological characters that vary similarly to genetic variability.

ADDITIONAL KEYWORDS: 28S rRNA – COI gene – DNA taxonomy – elliptic fourier analysis – integrative taxonomy – molluscs.

INTRODUCTION

Given the sizeable number of species that remains to be described, especially in the context of a rapidly increasing rate of species extinctions, we urgently need to rethink the work of alpha-taxonomy to improve the rate of species description. In marine environments, the amount of unknown diversity is important, especially for molluscs (Bouchet *et al.*, 2002). Among marine molluscs, the Conoidea are one of the most diverse taxa (Bouchet, 1990; Taylor, Kantor & Sysoev, 1993) and includes a significant portion of undescribed species. Moreover, most of the described taxa from shallow water and deeper ecosystems remain largely underexplored. The difficulty of sampling in such environment and the lack of specialists for most of these groups explain this taxonomic impediment (Boyle *et al.*, 2004).

Until recently, most species descriptions of shelled molluscs were based exclusively on shell characters, leading to brief diagnoses that, in some cases, apply to more than one taxon. Furthermore, shell variability is difficult to characterize with discrete characters (Pfenninger, Cordellier & Streit, 2006) and, consequently, the analysis of such characters, as performed traditionally, is difficult to reproduce. Finally, the broad plasticity of shell characters for some well known species was demonstrated (Hollander *et al.*, 2006; Brookes & Rochette, 2007) but this problem is rarely integrated in mollusc taxonomy. For all these reasons, taxonomists may differ in how they interpret variability of shell characters: what one specialist

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interprets as geographical or bathymetrical variation can be interpreted by another as specific differences. As a consequence, quite contradictory opinions about species delimitations in literature are not rare [Röckel, Rolan & Monteiro (1980) versus Monteiro, Tenorio & Poppe (2004) (Cape Verde *Conus*)].

The difficulty of confronting different opinions is also increased by the lack of a solid theoretical and methodological framework, *de facto* rendering many taxonomical opinions untestable hypotheses. Recently, concepts and methods underlying the delimitation of terminal taxa were clarified, advocating for the use of an integrative framework. De Queiroz (1998), followed by Samadi & Barberousse (2006), first suggested that a unified view of 'what is a species' is possible if species are considered as definitively diverging lineages and most of the other so-called 'species concepts' as criteria for delimiting species (Sites & Marshall, 2003). In this context, molecular characters are classically used to propose primary hypotheses of species delimitation based on genetic distances (Floyd *et al.*, 2002; Vogler & Monaghan, 2007). By contrast to the morphological characters used in most mollusc species description, molecular characters are strictly heritable and reproducible. Within an integrative framework (De Queiroz, 2007), these primary molecular hypotheses can then be tested against several criteria: (1) monophyly of primary species hypotheses (Wheeler & Meier, 2000; Meyer & Paulay, 2005); (2) independent genetic markers, such as a nuclear gene, because gene trees do not necessarily reflect the species tree (Nichols, 2001; Funk & Omland, 2003); and (3) morphological analysis to discuss species delimitations based on genetic data (Pfenninger *et al.*, 2006; Bichain *et al.*, 2007).

In this integrative context, we aimed to combine several criteria to propose new hypotheses of species delimitations in the genus *Benthomangelia* (Conoidea, Conidae). This is a widely distributed genus of bathyal and abyssal marine molluscs (Bouchet & Warén, 1980). It was described by Thiele (1925), grouping together species previously placed in other genera. Since 1925, new species have been described, based on morphological characters such as ornamentations and the shape of the shell (Bouchet & Warén, 1980; Sysoev & Ivanov, 1985; Sysoev, 1988). Ten species are now considered as valid (Tucker, 2004), of which six are present in the Pacific. Several potential new forms have been recently collected during cruises organized by the Museum National d'Histoire Naturelle (MNHN) and the Institut de Recherche pour le Développement (IRD) but the great and sometimes continuous morphological variability complicates the delimitation of species based solely on these characters (Bouchet & Sysoev, 2001).

We demonstrate the delimitation of genetic groups within the genus *Benthomangelia*, using molecular characters and a tree-based method. Two independent genes are used, one mitochondrial (cytochrome oxidase subunit *I*; COI) and one nuclear (28S) gene. An elliptic Fourier analysis (EFA; Rohlf, 1996) of the shape of the last whorl of the shell is also performed, allowing detailed analysis of complex structures as a whole (Monti, Baylac & Lalanne-Cassou, 2001). By contrast to shell characters used in most molluscan species descriptions, EFA has the advantage to be formalized and thus reproducible. EFA analysis complements the DNA-based species delimitation because morphological characters are indispensable for the delimitation of species in an integrative framework. Indeed, the genetically defined groups can be described morphologically, on the basis of shell characters linked to interspecific genetic variation, as opposed to the morphological variability linked to geographical or ecological factors.

MATERIAL AND METHODS

Sampling

Specimens of *Benthomangelia* were collected between 2004 and 2007 during several deep-sea cruises conducted by the MNHN and the IRD in Philippines, Vanuatu, and Solomon Islands (Fig. 1, Table 1). Living specimens were anesthetized in $MgCl_2$ and fixed in 95% ethanol. Shells were kept intact and the same individuals were used for both molecular and morphological analyses.

Sequencing

DNA was extracted from a piece of foot, using 6100 Nucleic Acid Prepstation system (Applied Biosystems). Two gene fragments were amplified: (1) a fragment of 658 bp of the COI mitochondrial gene using universal primers LCO1490 and HCO2198 (Folmer *et al.*, 1994) and (2) a fragment of 900 bp of the rDNA 28S gene, using the primers C1 and D3 (Jovelin & Justine, 2001). All polymerase chain reactions (PCR) were performed in 25 μL, containing 3 ng of DNA, 1 × reaction buffer, 2.5 mM $MgCl_2$, 0.26 mM dNTP, 0.3 μM of each primer, 5% DMSO and 1.5 U of Q-Bio Taq (MPBiomedicals) for all genes. COI gene amplifications were performed according to Hebert, Cywinska & Ball (2003); for the 28S gene, they consisted of an initial denaturation step at 94 °C for 4 min, followed by 30 cycles of denaturation at 94 °C for 30 s, annealing at 52 °C and extension at 72 °C for 1 min. The final extension was at 72 °C for 10 min. PCR products were purified and sequenced by the Genoscope (genbank accession numbers EU015528, EU015628, EU015644, EU015743, and EU428956–

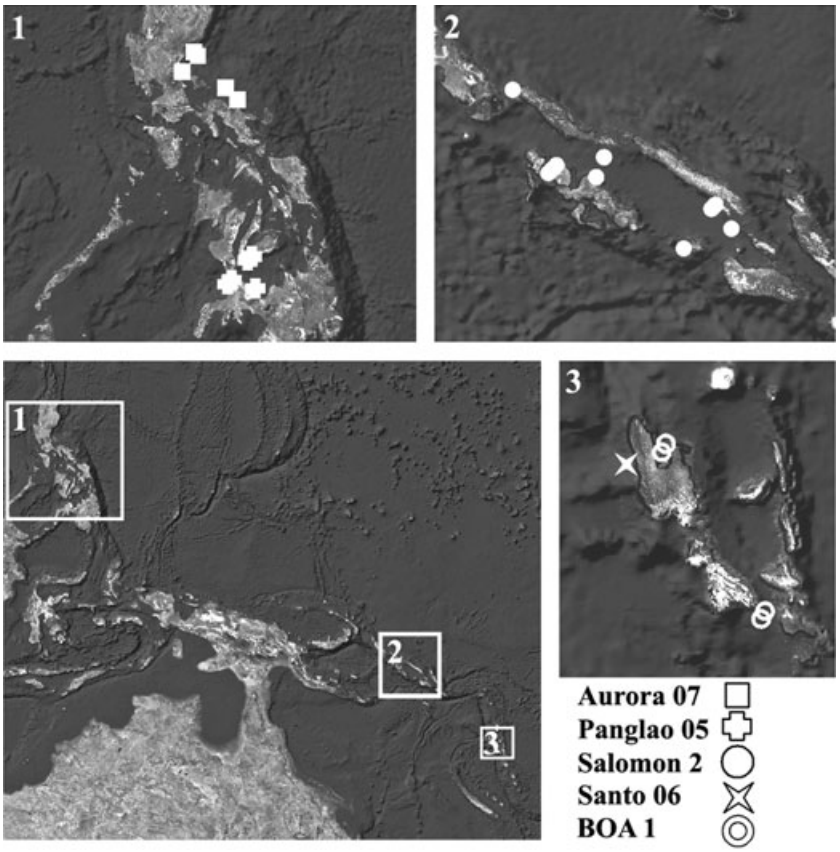

Figure 1. South-west Indo-Pacific map, with emphazis on Philippines (1), Solomon Islands (2) and Vanuatu (3). Sampling sites for each cruise are represented, with the same symbols used for canonical variate analysis (Figs 5, 6; see also Supporting information, Fig. S2).

EU429039). In all cases, both directions were sequenced to confirm accuracy of each haplotype sequence.

A specimen of the sister group of *Benthomangelia* (Puillandre *et al.*, 2008), the genus *Toxicochlespira* (17925, genbank accession number: EU015738 and EU015623 for the COI and 28S genes, respectively) and two other Conoidea (specimens 17866, *Mangelia*, EU015688 and EU015573 and 17754, *Turris*, EU015677 and EU015562) are used as the outgroup. Outgroups were chosen to form a non-monophyletic group, as recommended by Darlu & Tassy (1993).

Genetic analysis

The COI and 28S sequences were manually aligned because no ambiguous indels were found. Standard molecular diversity indices were calculated using ARLEQUIN, version 3.1 (Excoffier, Laval & Schneider, 2005).

Genotypic clustering

Genetic pairwise distances (excluding outgroups) for each gene separately were calculated with PAUP 4.0b10 (Swofford, 2002), using the best fitting model of nucleotide substitution for each gene as defined by MODELTEST (Posada & Crandall, 2001), in conjunction with PAUP 4.0b10, following the Akaike information criterion. These distances are visualized on a Neighbour-joining tree (NJ) tree calculated using PAUP 4.0b10 to define groups of specimens with low genetic distances within groups and high distances between groups.

Phylogenetic analyses

Phylogenetic reconstruction were conducted using Bayesian analysis (BA), consisting of two Markov chains (2 000 000 generations each with a sampling frequency of one tree each hundred generations) run in four parallel analyses using MrBayes (Huelsenbeck, Ronquist & Hall, 2001). When the log-likelihood scores were found to stabilize, a consensus tree was calculated after omitting the first 25% trees as burn-in. Only the number of nucleotide substitutions categories was fixed for BA. Phylogenetic analyses were performed on the cluster housed at the MNHN (17 nodes, two Go Ram per node, 30 AMDs 64-bit CPUs

Table 1. Identification number, cruise, station, depth, coordinates, and species number, as determined by the molecular analysis, are given for each specimen

ID	Cruise	Station	Depth (minimum to maximum)	Latitude	Longitude	DNA group	COI	28S
17950	Aurora 07	CP2678	507–540 m	14°46.54N	123°09.69E	2		x
17952	Aurora 07	CP2729	593–600 m	15°19.04N	121°37.02E	2	x	x
17953	Aurora 07	CP2729	593–600 m	15°19.04N	121°37.02E	2	x	x
17954	Aurora 07	CP2734	453–460 m	15°56.41N	121°48.71E	2	x	x
17955	Aurora 07	CP2734	453–460 m	15°56.41N	121°48.71E	2	x	x
17956	Aurora 07	CP2734	453–460 m	15°56.41N	121°48.71E	2	x	x
17957	Aurora 07	CP2749	473 m	15°56.38N	121°49.46E	2	x	x
17958	Aurora 07	CP2749	473 m	15°56.38N	121°49.46E	2	x	x
17959	Aurora 07	CP2749	473 m	15°56.38N	121°49.46E	2	x	x
17960	BOA 1	CP2427	745–827 m	14°56.2′S	166°54.8′E	1	x	x
17961	BOA 1	CP2422	667–750 m	14°55.1′S	166°55.4′E	1	x	x
17835	BOA 1	CP2462	618–641 m	16°37.5′S	167°57.4′E	1	x	x
17962	BOA 1	CP2462	618–641 m	16°37.5′S	167°57.4′E	1	x	x
17963	BOA 1	CP2462	618–641 m	16°37.5′S	167°57.4′E	2	x	x
17964	BOA 1	CP2461	582–614 m	16°35.5′S	167°57.9′E	1		x
17965	BOA 1	CP2461	582–614 m	16°35.5′S	167°57.9′E	1	x	x
17966	Panglao 05	CP2333	584–596 m	9°38.2′N	123°43.5′E	1	x	x
17967	Panglao 05	CP2334	606–631 m	9°37.5′N	123°40.2′E	1	x	x
17968	Panglao 05	CP2358	569–583 m	8°52.1′N	123°37.1′E	1	x	x
17969	Panglao 05	CP2388	762–786 m	9°26.9′N	123°34.5′E	1	x	x
17970	Panglao 05	CP2388	762–786 m	9°26.9′N	123°34.5′E	1	x	x
17971	Panglao 05	CP2388	762–786 m	9°26.9′N	123°34.5′E	1	x	x
17972	Panglao 05	CP2381	259–280 m	8°43.3′N	123°19.0′E	4	x	x
17973	Panglao 05	CP2384	624–647 m	8°46.2′N	123°16.1′E	1	x	x
17974	Panglao 05	CP2386	2120–2149 m	8°49.3′N	123°01.9′E	1	x	x
17975	Panglao 05	CP2389	784–786 m	9°27.9′N	123°38.4′E	1	x	x
17976	Panglao 05	CP2388	762–786 m	9°26.9′N	123°34.5′E	1	x	x
17977	Panglao 05	CP2389	784–786 m	9°27.9′N	123°38.4′E	1	x	x
17978	Panglao 05	CP2389	784–786 m	9°27.9′N	123°38.4′E	1	x	x
17979	Panglao 05	CP2392	242–400 m	9°29.0′N	123°41.1′E	4	x	x
17980	Panglao 05	CP2396	609–673 m	9°36.3′N	123°42.0′E	1	x	x
17981	Salomon 2	CP2268	632–640 m	7°48.7′S	156°53.3′E	5	x	x
17982	Salomon 2	CP2175	579–585 m	9°05.8′S	158°59.9′E	1	x	x
17983	Salomon 2	CP2219	650–836 m	7°58.3′S	157°34.4′E	3	x	x
17984	Salomon 2	CP2219	650–836 m	7°58.3′S	157°34.4′E	3	x	x
17985	Salomon 2	CP2182	762–1060 m	8°47′0S	159°37.9′E	1	x	
17930	Salomon 2	CP2269	768–890 m	7°45.1′S	156°56.3′E	3	x	x
17986	Salomon 2	CP2196	724–765 m	8°25.6′S	159°25.9′E	1	x	x
17987	Salomon 2	CP2196	724–765 m	8°25.6′S	159°25.9′E	1	x	x
17988	Salomon 2	CP2196	724–765 m	8°25.6′S	159°25.9′E	1	x	x
17989	Salomon 2	CP2264	515–520 m	7°52.4′S	156°51.0′E	1	x	x
17990	Salomon 2	CP2195	543–593 m	8°25.5′S	159°26.4′E	1	x	x
17991	Salomon 2	CP2194	440–521 m	8°24.8′S	159°26.7′E	1	x	
17992	Salomon 2	CP2213	495–650 m	7°38.7′S	157°42.9′E	1	x	x
17993	Salomon 2	CP2213	495–650 m	7°38.7′S	157°42.9′E	1	x	x
17994	Santo 06	AT105	408–444 m	15°03.0′S	166°34.5′E	4	x	x
17925	Salomon 2	CP2227	508–522 m	6°37.2′S	156°12.7′E	*Toxicochlespira*	x	x
17866	Panglao 04	S19	3–4 m	9°42.1′N	123°51.4′E	*Mangelia*	x	x
17754	Panglao 04	R42	8–22 m	9°37.1′N	123°52.6′E	*Turris*	x	x

In the column COI and 28S, a cross indicates that the specimen has been successfully sequenced for the gene.

for the slave nodes and 4 Xeon 32-bit CPU for the two master nodes).

Gene flow estimation

Within each group of specimens defined by genotypic clustering and phylogenetic analyses, the population structure was estimated between groups of specimens collected in different geographic region. ARLEQUIN, version 3.1 was used to perform analysis of molecular variance (with a 10 000 permutations test) for each pair of populations.

Fourier analysis

Morphometric analyses were performed on the same 46 shells used for the molecular analysis.

The shape of the last whorl has been previously considered informative in the alpha-taxonomy of *Benthomangelia* (Bouchet & Warén, 1980). To test the consistency of this hypothesis with the results obtained with the genetic data, the morphometric analysis considered only this part of the shell. Shells were placed horizontally, aperture up, and digitized using a macro stand. To evaluate the potential positioning error, each shell was digitized twice. The same error was also estimated by digitizing three shells (chosen as three very similar ones) five times each (position test dataset; PTD). As the labrum of some shells was broken, the lateral orientation of the shell could variate from one shell to another. To estimate potential error due to this problem of parallax, three shells were digitized five times, turning the shell around the columellar axis between each picture but keeping the columellar axis parallel to the support (orientation test dataset; OTD).

Six homologous landmarks (Bookstein, 1991) were defined in the last whorl of the shell, corresponding to the junction with the previous whorl (landmarks 2 and 3) and the aperture (landmark 5) and to the apex of the peripheral chord (landmarks 1, 4, and 6; Fig. 2). Because these six points covered only the upper part of the whorl, we also defined a seventh point located at the apex of the siphonal canal. The position of this point could be problematic for some shells because the extremity of the canal can be broken, but the aim was to cover the whole outline. The seven landmarks and the outlines were digitized using TpsDig (Rohlf, 1996). We always used the first landmark as starting point. Because the labrum of some shells was broken, two outlines were defined: the first corresponds to the whole last whorl (outline 1) and the second corresponds to the whole last whorl except the labrum by joining the extremity of the siphonal canal to the upper part of the aperture following the columellar lip (Fig. 2, outline 2). These two outlines were analysed for the 46 shells photographed twice (complete dataset = 92 pictures) and for the PTD and OTD. All pictures and outlines were taken by the same person (N.P.).

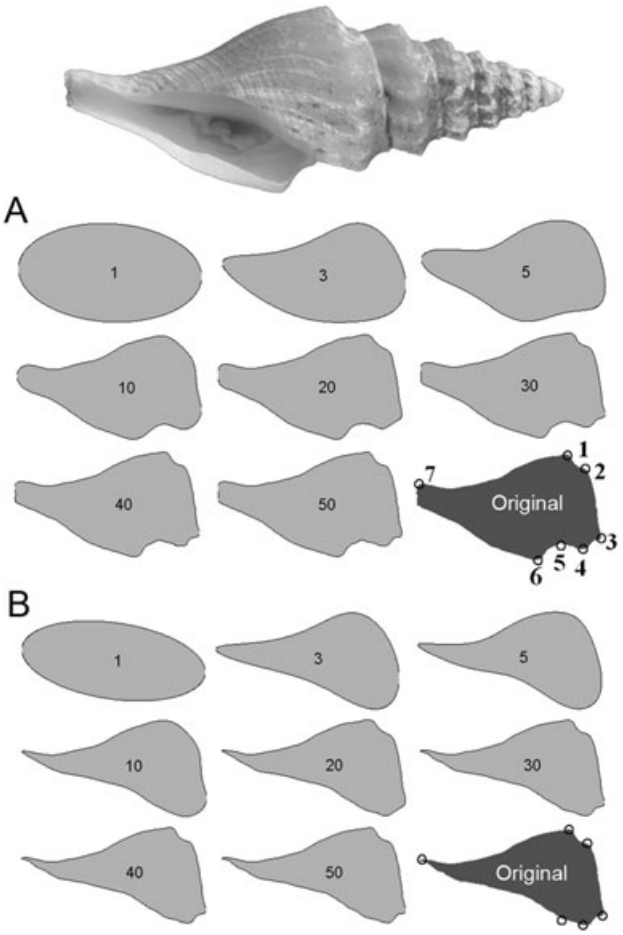

Figure 2. Outlines reconstructions with increasing number of harmonics indicated within outlines, for outline 1 (A) and outline 2 (B). The seven landmarks are represented on the original outline 1; only six landmarks were used for the second outline 2.

Outlines were used as input for an EFA (Baylac & Friess, 2005). The seven landmarks were used as control points to rotate the outlines into the same orientation. For the analysis of the outline 2, the sixth landmark, placed on the labrum of the shell, was removed (Fig. 2B). The images were then centred and normalized for size (using square roots of the surface). A visualization of Fourier reconstructions using different numbers of harmonics, compared to the original outline, shows that 40 harmonics are sufficient to reconstruct the outlines with high accuracy (Fig. 2).

The variates resulting from the EFA and used in the subsequent analyses correspond to the different Fourier coefficients, as described in Rohlf (1996). PTD and OTD were studied using principal components

analysis (PCA) for both outlines 1 and 2. To eliminate potential error due to the positioning of the shell, the mean of each pair of Fourier outlines corresponding to the two replicates of each specimen was used for the complete dataset, resulting in the study of 46 outlines. As few specimens were used, an exploratory analysis was first performed for the complete dataset using PCA to evaluate the level of variability within and among the groups then tested with canonical variate analysis (CVA). For CVA analysis, variability was maximized following two different grouping variables: the genetic groups as defined by the molecular analysis and the cruise of collection (Table 1). Visualizations of the outline deformations along the canonical axes were made using the procedure described by Monti *et al.* (2001). Multivariate regression parameters of Fourier coefficients were calculated using the depth of collection of each specimen as the independent grouping variable (the depth of collection is calculated as the mean between the depth at the beginning of the trawl and the depth at the end of the trawl; Table 1). The effect of the size of the last whorl on shape was also estimated, plotting the shape of the shell (measured as the projection on each axis of the CVA of the Fourier coordinates) as a function of the size of the specimens. All analyses were performed using specially devised MATLAB, version 5.2 functions implemented by M.B.

RESULTS

Molecular analysis

Forty-four specimens were sequenced for COI, resulting in a 658-bp fragment. No indels were found. Forty-four specimens were sequenced for 28S (42 in common with COI; Table 1), resulting in a 908-bp fragment after the alignment. The Kimura three-parameter model (K81uf+I+G, with I = 0.4654 and G = 0.5381) model for the COI gene and the Tamura–Nei model (TrN+I+G, with I = 0.6746 and G = 0.6616) model for the 28S gene were defined as the best fitting models.

Genetic clustering

For the COI gene, 25 different haplotypes were found among the 44 specimens, displaying 186 polymorphic loci, and a high haplotypic diversity (0.948). The distribution of pairwise genetic distances for the COI gene is clearly bimodal (Fig. 3). Indeed, the genetic distance between two specimens is either lower than 2.5% or higher than 7.5%. These two categories of distances are visualized in the NJ tree (not shown) as five long branches at the end of which from one to 28 genetically similar specimens are clustered. For clarity of discussion, we refer to these branches as 'groups' numbered from 1 to 5 (Table 1), although one includes only one specimen (17981). Specimens are from Panglao 05, BOA 1 and Salomon 2 for group 1; Aurora 07 and BOA 1 for group 2; Salomon 2 for group 3; Panglao 05 and Santo 06 for group 4; and Salomon 2 for group 5.

Results obtained with the 28S are congruent with those obtained with the COI gene. Overall variability for the 28S gene is less than for the COI gene, with only five different haplotypes among the 44 specimens, 36 polymorphic loci, and a haplotypic diversity of 0.584. This low level of variability does not allow the recognition of a gap between short and large genetic distances, but specimens displaying short distances with the COI gene possess exactly the same 28S sequence. This complete congruency between both genes is also provided in the Supporting

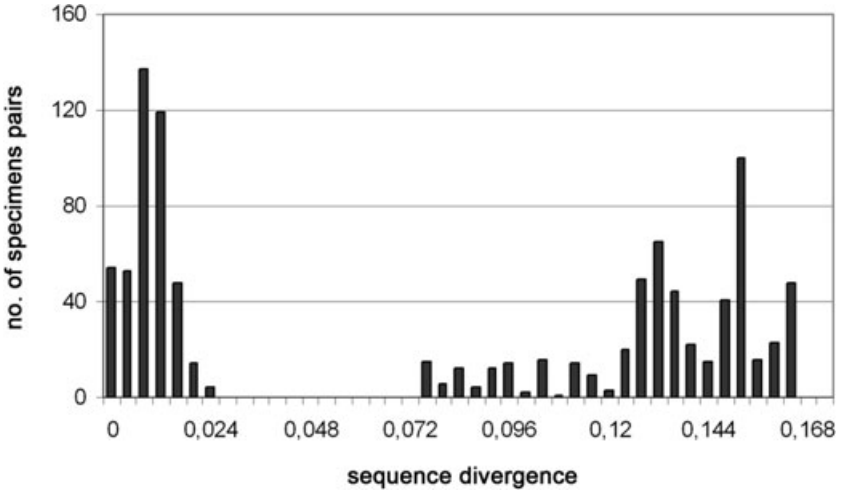

Figure 3. Histogram of genetic distances for the COI gene.

information (Fig. S1). Indeed, the same five groups are recognized in the 28S and COI trees.

Phylogenetic analysis

The four genetic groups containing several specimens are found monophyletic with the BA for the COI gene, although the monophyly of one group is not supported (posterior probabilities > 0.99 for groups 1, 4 and 5, posterior probabilities = 0.91 for group 1; results not shown). Because no incongruence is found between the two genes, a dataset combining the two genes for 42 specimens is created. Here, the data were separated into two different unlinked partitions corresponding to the two analysed genes, each following the best fitting model of substitution estimated for each gene. The four groups are in this case found highly supported, but the relationships among the five groups are not confidently resolved (posterior probabilities < 0.95; Fig. 4).

Gene flow estimation

It was possible to estimate gene flows only for group 1 because it was the only one that sampled several specimens from each of the different geographic regions. Population structure was evaluated only with the COI gene because the 28S gene did not vary within groups. Three populations were defined: the first including five specimens from BOA 1 (Vanuatu), the second 13 specimens from Panglao 05 (Philippines), and the last ten specimens from Salomon 2 (Solomon Islands). The pairwise populations comparisons (F_{ST}) among the three populations are 0.066 (P-value = 0.106) between BOA 1 and Salomon 2; 0.321 (P-value = 0.014) between BOA 1 and Panglao 05; and 0.239 (P-value = 0.003) between Salomon 2 and Panglao 05.

Fourier analysis

Analyses of potential error linked to positioning (PTD) or orientation (OTD) both show that variability between the replicates of the same shell is always less than the variability between different shells, as revealed by the PCA, except for the PTD with outline 1 (results not shown).

For both outlines 1 and 2, the PCA revealed high variability within and between genetic groups, allowing separation of two groups (genetic groups 1 and 3 versus genetic groups 2, 4 and 5; see Supporting information, Fig. S2A). The groups of specimens that correspond to different cruises of collection are not separated (see Supporting information, Fig. S2B).

The variable 'genetic groups' is significantly discriminant using CVA (F-test associated with the Wilk's lambda = 2.65, d.f. = 80, $P < 10^{-4}$ and $F = 2.90$, d.f. = 80, $P < 10^{-4}$ for outliness 1 and 2, respectively). Axes 1 and 2 represent together 91.13% of the variance for outline 1 (Fig. 5A) and 86.19% for outline 2 (Fig. 5C). They allow the separation of the five genetic groups, except groups 4 and 5 for outline 2. The variable 'cruise of collection' is also significantly discriminant ($F = 3.27$, d.f. = 80, $P < 10^{-4}$ and $F = 2.27$, d.f. = 80, $P < 10^{-4}$ for outlines 1 and 2, respectively). Axes 1 and 2 represent together 83.2% of the variance for outline 1 (Fig. 5B) and 80.84% for outline 2 (Fig. 5D). However, by contrast to the results obtained with the genetic groups, specimens from Panglao 05, Salomon 2 and BOA 1 are not completely discriminated.

The first axis shows up an opposition between short and large shells on one hand and more elongated and thin shells on the other hand for both CVA analyses (using 'genetic groups' or 'cruise of collection' as variables). This might be due to the strong relationships between these two variables (χ^2: $P < 10^{-4}$). On the second axis, genetic groups are separated by the length of the siphonal canal (Fig. 5A, C), and specimens from different cruises of collection are separated by the shape of the curvature at the beginning of the siphonal canal (Fig. 5B, D), which is more marked for some specimens collected during Panglao 05.

The subsequent axes do not allow a better separation of the specimens from different cruises. The same patterns are conserved if the unique specimen of group 5 is removed (CVA using 'genetic groups' as variables), if the unique specimen of the cruise Santo 06 is removed (CVA using 'cruise of collection' as variables), or if only the groups with the most specimens are considered (genetic groups 1 and 2; CVA using 'genetic groups' as variables).

Multivariate regressions using the depth of collection as the independent grouping variable are not significant, whatever the number of axes used: $\alpha = 0.86$, 0.65, 0.50, and 0.79 for outline 1 and $\alpha = 0.77$, 0.85, 0.63 and 0.62 for outline 2, for 3, 5, 10, and 20 components, respectively.

To avoid problems due to the correlation of the different variables, and to estimate the level of morphological variability between different cruises of collection within a genetic group, CVA was performed within the one genetic cluster with multiple samples from multiple localities (group 1: six specimens from BOA 1, 13 from Panglao 05, and ten from Salomon 2) to test for geographic signal within group. The three different cruises are clearly separated on the two first axes, for both outlines 1 and 2 (results shown only for outline 1; Fig. 6). The first axis represents more than 87% of the variability and distinguishes Panglao 05 specimens from Salomon 2 and BOA 1 specimens, again based on the shape of the curvature at the beginning of the siphonal canal, as found with CVA including the complete dataset.

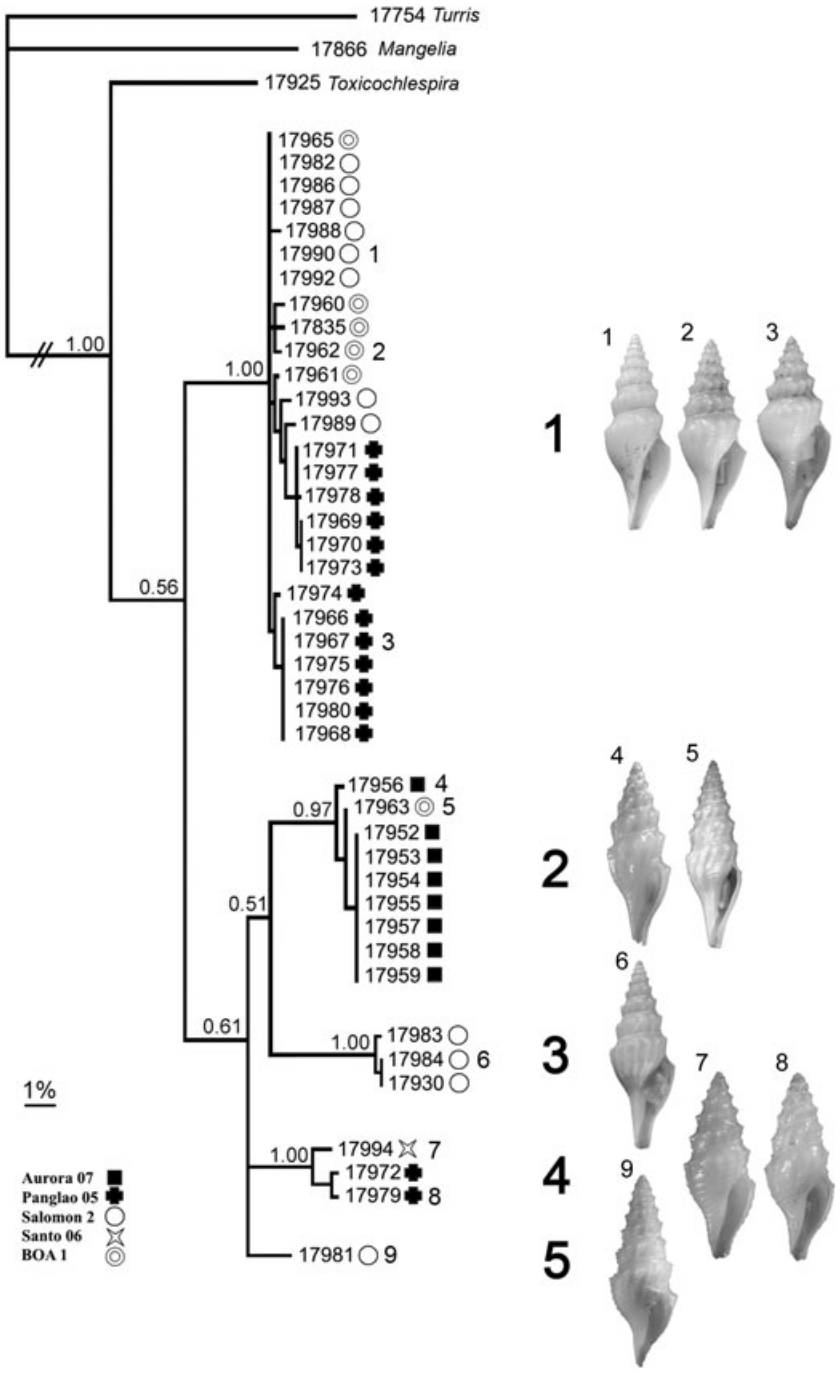

Figure 4. Bayesian tree for combined dataset corresponding to both genes. Posterior probabilities (> 0.5) are given for each node. Groups are numbered top downwards from 1–5. For each group and for each cruise within group, one shell is illustrated (numbered from 1–9).

Multivariate regression using the depth of collection was also performed for the genetic groups 1 and 2: no significant correlation was found (group 1, outline 2: $F = 0.505$, $P = 0.770$; group 2, outline 1: $F = 1.281$, $P = 0.396$; group 2, outline 2: $F = 0.898$, $P = 0.0.546$), except for group 1 with outline 1 ($F = 6.53$, $P < 10^{-4}$). However, when removing one of the specimen (17987), whose labrum is broken, the test was no longer significant ($F = 1.018$, $P = 0.1879$).

Finally, a potential effect of size on the shape of the last whorl between genetic groups or cruises of collection was tested: no significant correlation was

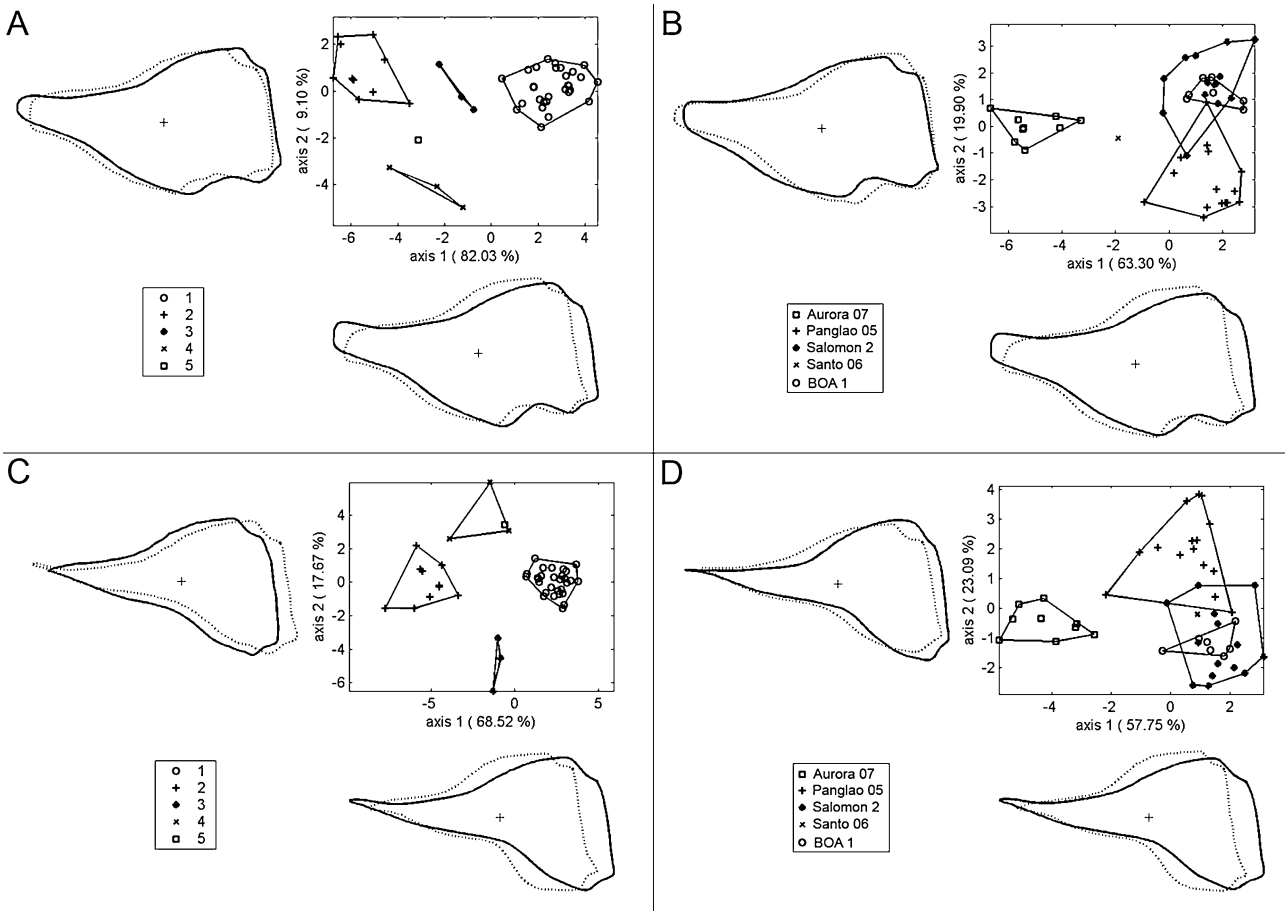

Figure 5. Canonical variate analysis (CVA) for the complete dataset. CVA for the first outline using genetic groups as grouping variable (A); CVA for the first outline using cruise of collection as grouping variable (B); CVA for the second outline using genetic groups as grouping variable (C); CVA for the second outline using cruise of collection as grouping variable (D). Superimposed outlines for minimum (dotted line) and maximum (full line) projections onto the two principal axes are represented.

found because the entire range of size is found in each numerous groups (genetic groups 1 and 2 and cruise of collection Panglao 05, BOA 1, Aurora 07, and Salomon 2). Results are shown only for the genetic groups, using the first axis of the CVA (see Supporting information, Fig. S3).

DISCUSSION

Species delimitation based on genetic data

The number of specimens included in the molecular analysis performed with COI and 28S genes allows for the detection of five genotypic groups, four of which are represented by several specimens. Because there is a strict congruency between the two unlinked genes, our groups are not only clusters of haplotypes but real genotypic clusters (Mallet, 2001; Sites & Marshall, 2004). Phylogenetic analyses also suggest that the groups defined with the genotypic clustering criterion are monophyletic, thus each displaying an independent event of coalescence for two independent genes (COI and 28S), suggesting the absence of genetic exchange between the groups (Ferguson, 2002). Moreover, as three of the five groups include specimens from different geographic regions (e.g. Philippines and Vanuatu), the genetic groups do not reflect a geographic structure among distant populations. The expected dispersal capacity of the larvae of *Benthomangelia* supports the observed genotypic clustering as members of this genus have a planktotrophic protoconch (between 2.5 and three whorls), indicating that the larvae can stay in the water column for a long period and can potentially disperse long distances (Shuto, 1974).

Thus, on the basis of our results, five lineages can be recognized, and we propose these lineages as primary hypotheses of species delimitation in *Benthomangelia*. The first species (group 1) is identified as

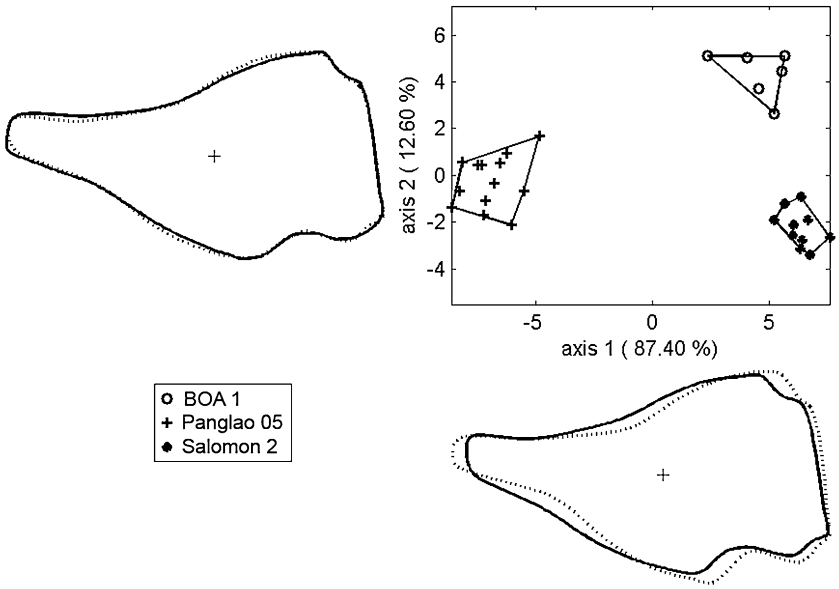

Figure 6. Canonical variate analysis (CVA) on the first outline for the genetic group 1. Superimposed outlines for minimum (dotted line) and maximum (full line) projections onto the two principal axes are represented.

Benthomangelia trophonoidea, and the four other species does not correspond to any of the nine other described species of *Benthomangelia*, and could thus constitute new species (A. Sysoev, pers. com.).

However, recent species are not necessarily characterized by high genetic distances between species or by reciprocal monophyly (Hickerson, Meyer & Moritz, 2006; Knowles & Carstens, 2007). Consequently, we cannot exclude the possibility that the moderate genetic structure within group 1, especially between the Philippines (Panglao 05 cruise) and the two others archipelagos (Solomon Islands and Vanuatu), is due to the presence of several species. This structure can also be interpreted as intraspecific genetic structure linked to geographic differentiation because the Philippines and Solomon islands are separated by more than 4000 km, and the Philippines and Vanuatu by more than 5000 km. Similarly, weak but non-negligible genetic structure is found between Solomon Islands and Vanuatu, separated by only 1200 km.

The only group present in three different geographic regions is also the group with the most specimens. Consequently, the apparently limited geographic distribution of some species, illustrated by the strong correlation between the two variables 'genetic groups' and 'cruise of collection', can only be an artefact due to undersampling of some species. Furthermore, it is important to note that our data set does not cover the whole genus because the whole Pacific is not sampled and putative distinct species of *Benthomangelia* from the Atlantic are not represented (Bouchet & Warén, 1980).

Comparison with morphological results

Although the heritability of morphologic characters is difficult to document, the use of objective and repeatable morphometric analysis allows retrieving the five genetic groups. A large part of the morphological variability of the last whorl of the shell gives valuable characters to delimit species. Specimens from genetic group 1 are characterized by a more elongated shell and a long canal; those from group 2 also by a long canal, but a relatively short last whorl; and those from group 4 by a short shell and a short canal. Outline 2 does not distinguish genetic groups 4 and 5, suggesting that the labrum could also be a valuable character to delimit species (A. J. Kohn, pers. com.).

However, the morphometric analysis also revealed that a part of the variability of the shape of the shell imparts the separation of specimens collected during different cruises within group 1. Thus, specimens of group 1 collected during Panglao 05 are characterized by a more vaulted siphonal canal, by contrast to the specimens collected during Salomon 2 and BOA 1. The morphologic differentiations between geographic groups within group 1 can be either associated with the genetic structure found between the same groups or with the morphologic plasticity of the shell in response to different environment conditions encountered in the different archipelagos. The potential combination of genetic and environmental effects on the shape of the shells constitutes one of the reasons why it is difficult to use solely the morphological characters in such a problematic groups.

On the use of shell characters to delimit species

Shell characters are known to be highly plastic, and several studies clearly identify characters that vary according to environmental factors: shells can be thicker (Brookes & Rochette, 2007) and their shape can be modified in response to the presence of predators (Palmer, 1990), to flow velocity (Baker *et al.*, 2004), or biochemical conditions (Chiu *et al.*, 2002), and ornamentation of the shell can vary along with the environment (Yeap, Black & Johnson, 2001). The analysis of several character sets, such as DNA and morphology, is required to avoid the use of characters that reflects environmental differences rather than taxonomic differences (Samadi *et al.*, 2000; Bichain *et al.*, 2007; Y. I. Kantor *et al.*, in press). For example, in the pulmonate genus *Radix*, shell characters are correlated with environmental factors, and do not differ constantly among the species defined with DNA (Pfenninger *et al.*, 2006). This suggests that erroneous species delimitations based on unsuitable morphological characters are likely to be widespread in molluscs with species description based solely on shell characters. Without a test of morphological characters using an integrative approach, taxonomists risk defining species based on nongenetically determined characters (Godfray, 2007). Mating trials are useful to test for species boundaries (Ribi & Porter, 1995; Pickles & Grahame, 1999) but such tests are difficult to conduct with deep-sea groups such as *Benthomangelia*. In these cases, combining genetic and morphological analyses appears to be a robust method to propose hypotheses of species delimitations.

Conclusion

In *Benthomangelia*, we demonstrate that the morphological characters analysed are congruent with the groups recognized by genetic analyses. Thus, they can be used in systematic studies to delimitate species and identify specimens. Furthermore, the use of reproducible methods, for both genetic and morphological analyses, will allow future tests of these hypotheses of species delimitation, including not only more replicates, but also other species that are not represented in the present study. The identification of taxonomically valuable morphological characters (i.e. genetically determined) is of particular interest for marine gastropods, where the majority of collected specimens are empty shells. In the perspective of a more complete assessment of species diversity of *Benthomangelia*, CVA analyses performed with genetically and morphologically characterized specimens can be used as a guideline to include, in the same analysis, empty shells for which DNA characters are not available.

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REFERENCES

Baker AM, Sheldon F, Somerville J, Walker KF, Hughes JM. 2004. Mitochondrial DNA phylogenetic structuring suggests similarity between two morphologically plastic genera of Australian freshwater mussels (Unionoida: Hyriidae). *Molecular Phylogenetics and Evolution* **32:** 902–912.

Baylac M, Friess M. 2005. Fourier descriptors, Procrustes superimpositions and data dimensionality: An example of cranial shape analysis in modern human populations. In: Slice DE, ed. *Modern morphometrics in physical anthropology*. Chicago, IL: Kluwer, 145–165.

Bichain JM, Boisselier MC, Bouchet P, Samadi S. 2007. Delimiting species in the genus *Bythinella* (Mollusca: Caenogastropoda: Rissooidea): molecular and morphometric approachs. *Malacologia* **49:** 291–311.

Bookstein FL. 1991. *Morphometric tools for landmark data: geometry and biology*. Cambridge: Cambridge University Press.

Bouchet P. 1990. Turrid genera and mode of development: the use and abuse of protoconch morphology. *Malacologia* **32:** 69–77.

Bouchet P, Lozouet P, Maestrati P, Héros V. 2002. Assessing the magnitude of species richness in tropical marine environments: exceptionnally high numbers of molluscs at a new Caledonian site. *Biological Journal of the Linnean Society* **75:** 421–436.

Bouchet P, Sysoev A. 2001. *Typhlosyrinx*-like tropical deep-water turriform gastropods (Mollusca, Gastropoda, Conoidea). *Journal of Natural History* **35:** 1693–1715.

Bouchet P, Warén A. 1980. Revision of the north-east Atlantic bathyal and abyssal Turridae (Mollusca, Gastropoda). *Journal of Molluscan Studies* **SuppI. 8:** 1–19.

Boyle EE, Zardus JD, Chasea MR, Ettera RJ, Rex MA. 2004. Strategies for molecular genetic studies of preserved deep-sea macrofauna. *Deep-Sea Research I* **51:** 1319–1336.

Brookes JI, Rochette R. 2007. Mechanism of a plastic phenotypic response: predator-induced shell thickening in the intertidal gastropod *Littorina obtusata*. *Journal of Evolutionary Biology* **20:** 1015–1027.

Chiu YW, Chen HC, Lee SC, Chen CA. 2002. Morphometric analysis of shell and operculum variations in the viviparid snail, *Cipangopaludina chinensis* (Mollusca: Gastropoda), in Taiwan. *Zoological Studies* **41:** 321–331.

Darlu P, Tassy P. 1993. *La reconstruction phylogénétique. Concepts et méthodes*. Paris: Masson.

De Queiroz K. 1998. The general lineage concept of species, species criteria, and the process of speciation: a conceptual unification and terminological recommendations. In: Howard DJ, Berlocher SH, eds. *Endless forms. Species and speciation*. Oxford: Oxford University Press, 57–75.

De Queiroz K. 2007. Species concepts and species delimitation. *Systems Biology* **56:** 879–886.

Excoffier L, Laval G, Schneider S. 2005. Arlequin ver. 3.0: an integrated software package for population genetics data analysis. *Evolutionary Bioinformatics Online* **1:** 47–50.

Ferguson JWH. 2002. On the use of genetic divergence for identifying species. *Biological Journal of the Linnean Society* **75:** 509–516.

Floyd R, Abebe E, Papert A, Blaxter M. 2002. Molecular barcodes for soil nematode identification. *Molecular Ecology* **11:** 839–850.

Folmer O, Black M, Hoeh W, Lutz R, Vrijenhoek R. 1994. DNA primers for amplification of mitochondrial cytochrome c oxidase subunit I from diverse metazoan invertebrates. *Molecular Marine Biology and Biotechnology* **3:** 294–299.

Funk DJ, Omland KE. 2003. Species-level paraphyly and polyphyly: frequency, causes, and consequences, with insights from animal mitochondrial DNA. *Annual Review of Ecology Evolution and Systematics* **34:** 397–423.

Godfray HCJ. 2007. Linnaeus in the information age. *Nature* **446:** 259–260.

Hebert PDN, Cywinska A, Ball SL, deWaard JR. 2003. Biological identifications through DNA barcodes. *Proceedings of the Royal Society of London Series B, Biological Sciences* **270:** 313–321.

Hickerson JH, Meyer CP, Moritz C. 2006. DNA barcoding will often fail to discover new animal species over broad parameter space. *Systematic Biology* **55:** 729–739.

Hollander J, Collyer ML, Adams DC, Johannesson K. 2006. Phenotypic plasticity in two marine snails: constraints superseding life history. *Journal of Evolutionary Biology* **19:** 1861–1872.

Huelsenbeck JP, Ronquist F, Hall B. 2001. MrBayes: bayesian inference of phylogeny. *Bioinformatics* **17:** 754–755.

Jovelin R, Justine JL. 2001. Phylogenetic relationships within the Polyopisthocotylean monogeneans (Plathyhelminthes) inferred from partial 28S rDNA sequences. *International Journal for Parasitology* **31:** 393–401.

Kantor YI, Puillandre N, Olivera B, Bouchet P. In press. Morphological proxies for taxonomic decision in turrids (Mollusca, Neogastropoda): a test of the value of shell and radula characters using molecular data. *Zoological Science*, in press.

Knowles LL, Carstens BC. 2007. Delimiting species without monophyletic gene trees. *Systematic Biology* **56:** 887–895.

Mallet J. 2001. The speciation revolution. *Journal of Evolutionary Biology* **14:** 887–888.

Meyer PC, Paulay G. 2005. DNA bracoding: error rates based on comprehensive sampling. *PLoS Biology* **3:** 1–10.

Monteiro A, Tenorio M, Poppe G. 2004. *The West African and Mediterranean species of Conus*. Hackenheim: Conch-Books.

Monti L, Baylac M, Lalanne-Cassou B. 2001. Elliptic Fourier analysis of the form of genitalia in two *Spodoptera* species and their hybrids (Lepidoptera: Noctuidae). *Biological Journal of the Linnean Society* **72:** 391–400.

Nichols R. 2001. Gene trees and species trees are not the same. *Trends in Ecology and Evolution* **16:** 358–364.

Palmer AR. 1990. Effect of crab effluent and scent of damaged conspecifics on feeding, growth, and shell morphology of the Atlantic dogwhelk *Nucella lapillus* (L.). *Hydrobiologia* **193:** 155–182.

Pfenninger M, Cordellier M, Streit B. 2006. Comparing the efficacy of morphologic and DNA-based taxonomy in the freshwater gastropod genus *Radix* (Basommatophora, Pulmonata). *BMC Evolutionary Biology* **6:** 100.

Pickles AR, Grahame J. 1999. Mate choice in divergent morphs of the gastropod mollusc *Littorina saxatilis* (Olivi): speciation in action? *Animal Behaviour* **58:** 181–184.

Posada D, Crandall KA. 2001. Selecting models of nucleotide substitution: an application to human immunodeficiency virus 1 (HIV-1). *Molecular Biology and Evolution* **18:** 897–906.

Puillandre N, Samadi S, Boisselier MC, Sysoev AV, Kantor YI, Cruaud C, Couloux A, Bouchet P. 2008. Starting to unravel the toxoglossan knot: Molecular phylogeny of the 'turrids' (Neogastropoda: Conoidea). *Molecular Phylogenetics and Evolution* **47:** 1122–1134.

Ribi G, Porter AH. 1995. Mating between two hybridizing species, *Viviparus ater* and *V. contectus* (Mollusca: Prosobranchia). *Animal Behaviour* **49:** 1389–1398.

Rohlf FJ. 1996. TpsDig. Available at Stony Brook. http://life.bio.sunysb.edu/morph/.

Röckel D, Rolan E, Monteiro A. 1980. *Cone shells from Cape Verde Islands. A difficult puzzle*. Feito: Vigo.

Samadi S, Barberousse A. 2006. The tree, the network, and the species. *Biological Journal of the Linnean Society* **89:** 509–521.

Samadi S, Roumegoux A, Bargues MD, Mas-Coma S, Yong M, Pointier JP. 2000. Morphological studies of Lymnaeid snails from the human Fasciolasis endemic zone of Bolivia. *Journal of Molluscan Studies* **2000:** 31–44.

Shuto T. 1974. Larval ecology of prosobranch gastropods and its bearing on biogeography and paleontology. *Lethaia* **7:** 239–256.

Sites JW, Marshall JC. 2003. Delimiting species: a Renaissance issue in systematic biology. *Trends in Ecology and Evolution* **19:** 462–470.

Sites JW, Marshall JC. 2004. Operational criteria for delimiting species. *Annual Review of Ecology Evolution and Systematics* **35:** 199–227.

Swofford DL. 2002. *PAUP*. Phylogenetic analysis using parsimony* and other methods*. Sunderland, MA: Sinauer Associates.

Sysoev AV. 1988. Ultra-abyssal findings of the family Turridae (Gastropoda, Toxoglossa) in the Pacific Ocean. *Zoologicheskii Zhurnal* **67:** 972.

Sysoev AV, Ivanov DL. 1985. New taxa of the family Turridae (Gastropoda, Toxoglossa) from the Naska Ridge (south east Pacific). *Zoologicheskii Zhurnal* **64:** 197.

Taylor JD, Kantor YI, Sysoev AV. 1993. Foregut anatomy, feedings mechanisms and classification of the Conoidea (= Toxoglossa)(Gastropoda). *Bulletin of the Natural History Museum, London* **59:** 125–170.

Tucker JK. 2004. Catalogue of recent and fossil turrids (Mollusca: Gastropoda). *Zootaxa* **682:** 1–1295.

Vogler AP, Monaghan MT. 2007. Recent advances in DNA taxonomy. *Journal of Zoological Systematics and Evolutionary Research* **45:** 1–10.

Wheeler QD, Meier R. 2000. *Species concepts and phylogenetic theory*. New York, NY: Columbia University Press.

Yeap KL, Black R, Johnson MS. 2001. The complexity of phenotypic plasticity in the intertidal snail *Nodilittorina australis*. *Biological Journal of the Linnean Society* **72:** 63–76.

SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Figure S1. Comparison of 28S and COI genetic distances for each specimen.
Figure S2. Principal components analysis for the complete dataset, with a visualization of the genetic groups (A) and the cruise of collection (B). Only results for the first outline are shown. Superimposed outlines for minimum (dotted line) and maximum (full line) projections onto the two principal axes are represented.
Figure S3. Plots of the shape of the last whorl of the shell as a function of the size. The five genetic groups are represented. Size of the shells was measured as the square root of the area.

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