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

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3 **An integrative approach to species delimitation in *Benthomangelia***
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6 **(Mollusca: Conoidea)**
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49 Integrative taxonomy in *Benthomangelia*
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Abstract

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 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 to both the results obtained with the independent nuclear 28S gene and to 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.

Keywords

28S rRNA - COI gene – *Benthomangelia* – 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 it 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 done 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 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 (e.g. Röckel, Rolan & Monteiro (1980) vs 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

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3 terminal taxa were clarified, advocating for the use of an integrative framework. De Queiroz
4 (1998), followed by Samadi & Barberousse (2006), first suggested that a unified view of
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6 “what is a species” is possible if species are considered as definitively diverging lineages and
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8 most of the other so-called “species concepts” as criteria for delimiting species (Sites &
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10 Marshall, 2003). In this context, molecular characters are classically used to propose primary
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12 hypotheses of species delimitation based on genetic distances (Floyd *et al.*, 2002; Vogler &
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14 Monaghan, 2007). Contrary to the morphological characters used in most mollusc species
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16 description, molecular characters are strictly heritable and reproducible. Within an integrative
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18 framework (De Queiroz, 2007) these primary molecular hypotheses can then be tested against
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20 several criteria, namely: (i) monophyly of primary species hypotheses (Wheeler & Meier,
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22 2000; Meyer & Paulay, 2005), (ii) independent genetic markers, such as a nuclear gene, as
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24 gene trees do not necessarily reflect the species tree (Nichols, 2001; Funk & Omland, 2003),
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26 and (iii) morphological analysis to discuss species delimitations based on genetic data
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28 (Bichain *et al.*, 2007; Pfenninger *et al.*, 2006).
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36 In this integrative context, we aim to combine several criteria to propose new hypotheses of
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38 species delimitations in the genus *Benthomangelia* (Conoidea, Conidae). This is a widely
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40 distributed genus of bathyal and abyssal marine molluscs (Bouchet & Warén, 1980). It was
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42 described by Thiele (1925), grouping together species previously placed in other genera.
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44 Since 1925, new species have been described, based on morphological characters such as
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46 ornamentations and shape of the shell (Bouchet & Warén, 1980; Sysoev & Ivanov, 1985;
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48 Sysoev, 1988). Ten species are now considered as valid (Tucker 2004), of which six are
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50 present in the Pacific. Several potential new forms have been recently collected during cruises
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52 organized by the Museum National d’Histoire Naturelle (MNHN) and the Institut de
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54 Recherche pour le Développement (IRD), but the great and sometimes continuous
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3 morphological variability complicate the delimitation of species based solely on these
4 characters (Bouchet & Sysoev, 2001).
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8 We demonstrate the delimitation of genetic groups within the genus *Benthomangelia*, using
9 molecular characters and a tree-based method. Two independent genes are used, one
10 mitochondrial (COI) and one nuclear (28S) gene. An Elliptic Fourier Analysis (EFA; Rohlf,
11 1996) of the shape of the last whorl of the shell is also performed, allowing detailed analysis
12 of complex structures as a whole (Monti, Baylac & Lalanne-Cassou, 2001). Contrary to shell
13 characters used in most molluscan species descriptions, EFA has the advantage to be
14 formalized and thus reproducible. EFA analysis complements the DNA-based species
15 delimitation, as morphological characters are indispensable for the delimitation of species in
16 an integrative framework. Indeed, the genetically defined groups can be described
17 morphologically, on the basis of shell characters linked to interspecific genetic variation, as
18 opposed to the morphological variability linked to geographical or ecological factors.
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36 **Material & Methods**

37 Sampling

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39 Specimens of *Benthomangelia* were collected between 2004 and 2007 during several deep-sea
40 cruises conducted by the MNHN and the IRD in Philippines, Vanuatu and Solomon Islands
41 (Table 1, Figure 1). Living specimens were anesthetized in MgCl₂ and fixed in 95 % ethanol.
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43 Shells were kept intact, so the same individuals were used for both molecular and
44 morphological analyses.
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52 Sequencing

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54 DNA was extracted from a piece of foot, using 6100 Nucleic Acid Prepstation system
55 (Applied Biosystem). Two gene fragments were amplified: (i) a fragment of 658 bp of
56 Cytochrome Oxidase I (COI) mitochondrial gene using universal primers LCO1490 and
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3 HCO2198 (Folmer *et al.*, 1994) and (ii) a fragment of 900 bp of the rDNA 28S gene, using
4 the primers C1 and D3 (Jovelin & Justine, 2001). All PCR reactions were performed in 25 μ l,
5 containing 3 ng of DNA, 1X reaction buffer, 2.5 mM MgCl₂, 0.26 mM dNTP, 0.3 μ M of each
6 primer, 5% DMSO and 1.5 units of Q-Bio Taq (MPBiomedicals) for all genes. COI gene
7 amplifications were performed according to Hebert *et al.* (2003); for 28S gene, they consisted
8 in an initial denaturation step at 94°C for 4', followed by 30 cycles of denaturation at 94°C for
9 30'', annealing at 52°C and extension at 72°C for 1'. The final extension was at 72°C for 10'.
10 PCR products were purified and sequenced by the Genoscope (genbank accession numbers
11 EU015528, EU015628, EU015644, EU015743 and EU428956-EU429039). In all cases, both
12 directions were sequenced to confirm accuracy of each haplotype sequence.

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

Genetic analysis

COI and 28S sequences were manually aligned, as no ambiguous indels were found. Standard
molecular diversity indices were calculated using Arlequin 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 the program Modeltest (Posada & Crandall, 2001), in conjunction
with PAUP 4.0b10, following the Akaike information criterion (AIC). These distances are

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3 visualized on a NJ tree calculated using PAUP 4.0b10 to define groups of specimens with low
4 genetic distances within groups and high distances between groups.
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7 *Phylogenetic analyses*

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10 Phylogenetic reconstruction were conducted using Bayesian Analysis (BA), consisting of two
11 Markov chains (2000000 generations each with a sampling frequency of one tree each
12 hundred generations) run in four parallel analyses using Mr. Bayes (Huelsenbeck, Ronquist &
13 Hall, 2001). When the log-likelihood scores were found to stabilize, a consensus tree was
14 calculated after omitting the first 25% trees as burn-in. Only the number of nucleotide
15 substitutions categories was fixed for BA. Phylogenetic analyses were performed on the
16 cluster housed at the MNHN (17 nodes, 2 Go Ram per node, 30 AMDs 64 bits CPU's for the
17 slave nodes and 4 Xeon 32 bits CPU's for the two master nodes)
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28 *Gene flow estimation*

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31 Within each group of specimens defined by genotypic clustering and phylogenetic analyses,
32 population structure was estimated between groups of specimens collected in different
33 geographic region. Arlequin 3.1 was used to perform AMOVA (with a 10000 permutations
34 test) for each pair of populations.
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40 Fourier analysis

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42 Morphometric analyses were performed on the same 46 shells used for the molecular analysis.
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44 The shape of the last whorl has been previously considered informative in the alpha-taxonomy
45 of *Benthomangelia* (Bouchet & Warén, 1980). In order to test the consistency of this
46 hypothesis with the results obtained with the genetic data, the morphometric analysis
47 considered only this part of the shell. Shells were placed horizontally, aperture up, and
48 digitized using a macro stand. To evaluate the potential positioning error, each shell was
49 digitized twice. The same error was also estimated by digitizing three shells (chosen as three
50 very similar ones) five times each (Position Test Dataset, PTD). As the labrum of some shells
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3 was broken, the lateral orientation of the shell could variate from one shell to another. To
4 estimate potential error due to this problem of parallax, three shells were digitized five times,
5 turning the shell around the columellar axis between each picture but keeping the columellar
6 axis parallel to the support (Orientation Test Dataset, OTD).
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11 Six homologous landmarks (Bookstein 1991) were defined in the last whorl of the shell,
12 corresponding to the junction with the previous whorl (landmarks 2 and 3) and the aperture
13 (landmark 5) and to the apex of the peripheral chord (landmarks 1, 4 and 6; Figure 2). As
14 these six points covered only the upper part of the whorl, we also defined a seventh point
15 located at the apex of the siphonal canal. The position of this point could be problematic for
16 some shells, as the extremity of the canal can be broken, but the aim was to cover the whole
17 outline. The seven landmarks and the outlines were digitized using TpsDig (Rohlf, 1996). We
18 always used the first landmark as starting point. As the labrum of some shells was broken,
19 two outlines were defined: the first corresponds to the whole last whorl (outline 1) and the
20 second corresponds to the whole last whorl except the labrum by joining the extremity of the
21 siphonal canal to the upper part of the aperture following the columellar lip (outline 2, Figure
22 2). These two outlines were analysed for the 46 shells photographed twice (complete dataset =
23 92 pictures) and for the PTD and OTD. All pictures and outlines were taken by the same
24 person (NP).
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45 Outlines were used as input for an EFA (Baylac & Friess, 2005). The seven landmarks were
46 used as control points to rotate the outlines into the same orientation. For the analysis of the
47 outline 2, the sixth landmark, placed on the labrum of the shell, was removed (Figure 2B).
48 The images were then centred and normalised for size (using square roots of the surface). A
49 visualization of Fourier reconstructions using different numbers of harmonics, compared to
50 the original outline, shows that 40 harmonics are sufficient to reconstruct the outlines with
51 high accuracy (Figure 2).
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3 The variates resulting from the EFA and used in the subsequent analyses correspond to the
4 different Fourier coefficients, as described in Rohlf 1996. PTD and OTD were studied using
5 Principal Components Analysis (PCA) for both outlines 1 and 2. In order to eliminate
6 potential error due to the positioning of the shell, the mean of each pair of Fourier outlines
7 corresponding to the two replicates of each specimen was used for the complete dataset,
8 resulting in the study of 46 outlines. As few specimens were used, an exploratory analysis was
9 first performed for the complete dataset using PCA in order to evaluate the level of variability
10 within and among the groups then tested with Canonical Variate Analysis (CVA). For CVA
11 analysis, variability was maximised following two different grouping variables: the genetic
12 groups as defined by the molecular analysis and the cruise of collection (Table 1).
13 Visualizations of the outline deformations along the canonical axes were made using the
14 procedure described in Monti *et al.* (2001). Multivariate regression parameters of Fourier
15 coefficients were calculated using the depth of collection of each specimen as the independent
16 grouping variable (the depth of collection is calculated as the mean between the depth at the
17 beginning of the trawl and the depth at the end of the trawl, see table 1). Effect of the size of
18 the last whorl on shape was also estimated, plotting the shape of the shell (measured as the
19 projection on each axis of the CVA of the Fourier coordinates) as a function of the size of the
20 specimens. All analyses were performed using specially devised MATLABv5.2 functions
21 implemented by MB.
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50 **Results**

51 Molecular analysis

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53 Forty-four specimens were sequenced for COI, resulting in a 658 bp fragment. No indels were
54 found. Forty-four specimens were sequenced for 28S (42 in common with COI, table 1),
55 resulting in a 908 bp fragments after the alignment. The Kimura-3-parameter model
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3 (K81uf+I+G, with I = 0.4654 and G = 0.5381) model for the COI gene and the Tamura-Nei
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5 model (TrN+I+G, with I = 0.6746 and G = 0.6616) model for the 28S gene were defined as
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7 the best fitting models.
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10 *Genetic clustering*

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12 For the COI gene, 25 different haplotypes were found among the 44 specimens, displaying
13
14 186 polymorphic loci, and a high haplotypic diversity (0.948). The distribution of pairwise
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16 genetic distances for the COI gene is clearly bimodal (Figure 3). Indeed, the genetic distance
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18 between two specimens is either lower than 2.5% or higher than 7.5%. These two categories
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20 of distances are visualized in the NJ tree (not shown) as five long branches at the end of
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22 which from 1 to 28 genetically similar specimens are clustered. For the clarity of the
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24 discussion, we will refer to these branches as “groups” numbered from 1 to 5 (Table 1),
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26 although one includes only one specimen (17981). Specimens are from Panglao 05, BOA 1
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28 and Salomon 2 for group 1, Aurora 07 and BOA 1 for group 2, Salomon 2 for group 3,
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30 Panglao 05 and Santo 06 for group 4 and Salomon 2 for group 5.
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36 Results obtained with the 28S are congruent with those obtained with COI gene. Overall
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38 variability for the 28S gene is less than for the COI gene, with only 5 different haplotypes
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40 among the 44 specimens, 36 polymorphic loci and a haplotypic diversity of 0.584. This low
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42 level of variability does not allow the recognition of a gap between short and large genetic
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44 distances, but specimens displaying short distances with the COI gene possess exactly the
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46 same 28S sequence. This complete congruency between both genes is also visualized in the
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48 figure S1 (supplementary material). Indeed, the same five groups are recognized in the 28S
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50 and COI trees.
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53 *Phylogenetic analysis*

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

10 11 12 13 14 15 16 17 18 19 20 21 *Gene flow estimation*

22 It was possible to estimate gene flows only for group 1 because it was the only one that
23 sampled several specimens from each of the different geographic regions. Population
24 structure was evaluated only with the COI gene since the 28S gene did not vary within
25 groups. Three populations were defined: the first including five specimens from BOA 1
26 (Vanuatu), the second 13 specimens from Panglao 05 (Philippines) and the last 10 specimens
27 from Salomon 2 (Solomon Islands). The pairwise populations comparisons (F_{st}) among the
28 three populations are 0.066 (p-value = 0.106) between BOA 1 and Salomon 2, 0.321 (p-value
29 = 0.014) between BOA 1 and Panglao 05 and 0.239 (p-value = 0.003) between Salomon 2 and
30 Panglao 05.
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43 Fourier analysis

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46 Analyses of potential error linked to positioning (PTD) or orientation (OTD) both show that
47 variability between the replicates of the same shell is always less than the variability between
48 different shells, as revealed by the PCA, except for the PTD with outline 1 (results not
49 shown).
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55 For both outlines 1 and 2, the PCA revealed high variability within and between genetic
56 groups, allowing separation of two groups (genetic groups 1 and 3 vs genetic groups 2, 4 and
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5; supplementary material, Figure S2A). The groups of specimens that correspond to different cruises of collection are not separated (Figure S2B).

The variable “genetic groups” is significantly discriminant using CVA (F test associated to the Wilk’s lambda = 2.65, $df = 80$, $p < 10^{-4}$ and $F = 2.90$, $df = 80$, $p < 10^{-4}$ for outline 1 and 2 respectively). Axes 1 and 2 represent together 91.13% of the variance for outline 1 (Figure 5A) and 86.19% for outline 2 (Figure 5C). They allow the separation of the five genetic groups, except the groups 4 and 5 for outline 2. The variable “cruise of collection” is also significantly discriminant ($F = 3.27$, $df = 80$, $p < 10^{-4}$ and $F = 2.27$, $df = 80$, $p < 10^{-4}$ for outline 1 and 2 respectively). Axes 1 and 2 represent together 83.2% of the variance for outline 1 (Figure 5B) and 80.84% for outline 2 (Figure 5D). However, contrary 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 can be due to the strong relationships between these two variables (Chi² test: $p < 10^{-4}$). On the second axis, genetic groups are separated by the length of the siphonal canal (Figure 5A and C), and specimens from different cruises of collection are separated by the shape of the curvature at the beginning of the siphonal canal (Figure 5B and D), more marked for some specimens collected during Panglao 05.

The following axes do not allow a better separation of the specimens from different cruises. The same patterns are conserved if the unique specimen of the 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).

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3 Multivariate regressions using the depth of collection as the independent grouping variable
4 are not significant, whatever the number of axes used: $\alpha = 0.86, 0.65, 0.50$ and 0.79 for
5 outline 1 and $\alpha = 0.77, 0.85, 0.63$ and 0.62 for outline 2, for 3, 5, 10 and 20 components
6 respectively.
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11 To avoid problems due to the correlation of the different variables, and to estimate the level of
12 morphological variability between different cruises of collection within a genetic group, CVA
13 was performed within the one genetic cluster with multiple samples from multiple localities
14 (group 1: 6 specimens from BOA 1, 13 from Panglao 05 and 10 from Salomon 2) to test for
15 geographic signal within group. The three different cruises are clearly separated on the two
16 first axes, for both outline 1 and 2 (results shown only for outline 1, Figure 6). The first axis
17 represents more than 87% of the variability and distinguishes Panglao 05 specimens from
18 Salomon 2 and BOA 1 specimens, based again on the shape of the curvature at the beginning
19 of the siphonal canal, as found with CVA including the complete dataset.
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34 Multivariate regression using the depth of collection was also performed for the genetic
35 groups 1 and 2: no significant correlation is found (group 1, outline 2: $F = 0.505, p = 0.770$;
36 group 2, outline 1: $F = 1.281, p = 0.396$; group 2, outline 2: $F = 0.898, p = 0.0546$), except
37 for the group 1 with the outline 1 ($F = 6.53, p < 10^{-4}$). However, when removing one of the
38 specimen (17987), whose labrum is broken, test is not significant anymore ($F = 1.018, p =$
39 0.1879).
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48 Finally, a potential effect of size on the shape of the last whorl between genetic groups or
49 cruises of collection was tested: no significant correlation were found, as the entire range of
50 size is found in each numerous groups (genetic groups 1 and 2 and cruise of collection
51 Panglao 05, BOA 1, Aurora 07 and Salomon 2). Results are shown only for the genetic
52 groups, using the first axis of the CVA (supplementary material, Figure S3).
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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. Since 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 3 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 *B. 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 that the moderate genetic structure within group 1, especially between the Philippines (Panglao 05 cruise) and the two others archipelagos

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3 (Solomon Islands and Vanuatu), is due to the presence of several species. This structure can
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5 also be interpreted as intraspecific genetic structure linked to geographic differentiation, as
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8 Philippines and Solomon islands are separated by more than 4000 km, and Philippines and
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10 Vanuatu by more than 5000 km. Similarly, weak but non-negligible genetic structure is found
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12 between Solomon Islands and Vanuatu, separated by only 1200 km.

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15 The only group present in three different geographic regions is also the group with the most
16
17 specimens. Consequently, the apparently limited geographic distribution of some species,
18
19 illustrated by the strong correlation between the two variables “genetic groups” and “cruise of
20
21 collection”, can only be an artifact due to undersampling of some species. Furthermore, it is
22
23 important to note that our data set does not cover the whole genus since the whole Pacific is
24
25 not sampled and putative distinct species of *Benthomangelia* from the Atlantic are not
26
27 represented (Bouchet & Warén, 1980).

28 29 30 Comparison with morphological results

31
32 While the heritability of morphologic characters is difficult to document, the use of objective
33
34 and repeatable morphometric analysis allows retrieving the five genetic groups. A large part
35
36 of the morphological variability of the last whorl of the shell gives valuable characters to
37
38 delimit species. Specimens from genetic group 1 are characterized by a more elongated shell
39
40 and a long canal, those from group 2 also by a long canal, but a relatively short last whorl, and
41
42 those from group 4 by a short shell and a short canal. Outline 2 does not distinguish genetic
43
44 group 4 and 5, suggesting that the labrum could also be a valuable character to delimit species
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46 (Kohn, pers. com.).

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48 However, the morphometric analysis also revealed that a part of the variability of the shape of
49
50 the shell imparts the separation of specimens collected during different cruises within group 1.
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52 Thus, specimens of group 1 collected during Panglao 05 are characterized by a more vaulted
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54 siphonal canal, in opposition to the specimens collected during Salomon 2 and BOA 1. The
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3 morphologic differentiations between geographic groups within group 1 can be either
4 associated to the genetic structure found between the same groups or to a morphologic
5 plasticity of the shell in response to different environment conditions encountered in the
6 different archipelagos. The potential combination of genetic and environmental effects on the
7 shape of the shells constitutes one of the reasons why it is difficult to use solely the
8 morphological characters in such a problematic groups.
9

17 On the use of shell characters to delimit species

18
19 Shell characters are known to be highly plastic, and several studies clearly identify characters
20 that vary according to environmental factors: shells can be thicker (Brookes & Rochette,
21 2007) and shape can be modified in response to the presence of predators (Palmer, 1990), to
22 flow velocity (Baker *et al.*, 2004) or biochemical conditions (Chiu *et al.*, 2002), and
23 ornamentation of the shell can vary along with the environment (Yeap, Black & Johnson,
24 2001). The analysis of several character sets, such as DNA and morphology, is needed to
25 avoid the use of characters that reflects environmental differences rather than taxonomic
26 differences (Kantor *et al.*, unpublished data; Samadi *et al.*, 2000; Bichain *et al.*, 2007). For
27 example, in the pulmonate genus *Radix*, shell characters are correlated with environmental
28 factors, and do not differ constantly among the species defined with DNA (Pfenninger *et al.*,
29 2006). These authors suggest that erroneous species delimitations based on unsuitable
30 morphological characters are likely to be widespread in molluscs with species description
31 based solely on shell characters. Without a test of morphological characters using an
32 integrative approach, taxonomists risk defining species based on non-genetically determined
33 characters (Godfray, 2007). Mating trials are useful to test for species boundaries (Ribi &
34 Porter, 1995; Pickles & Grahame 1999), but such tests are difficult with deep-sea groups such
35 as *Benthomangelia*. In these cases, combining genetic and morphological analyses appears to
36 be a robust method to propose hypotheses of species delimitations.
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Conclusion

In *Benthomangelia*, we demonstrated that the morphological characters analysed are congruent with the groups recognized by genetic analyses. They can thus be used in systematic 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 more replicates but also other species that are not represented here. 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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Figure caption

Figure 1: South-west Indo-Pacific map, with emphasis on Philippines (1), Solomon Islands (2) and Vanuatu (3). Sampling sites for each cruise are represented, with the same symbols used for CVA analyses (Figures 5, 6 and S2).

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.

Figure 3: Histogram of genetic distances for COI gene.

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

Figure 5: 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.

Figure 6: 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.

Table 1: Identification number, cruise, station, depth, coordinates and species number as determined by the molecular analysis are given for each specimen. In the column COI and 28S, a cross indicates that the specimen has been successfully sequenced for the gene.

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

Figure S1: Comparison of 28S and COI genetic distances for each specimen.

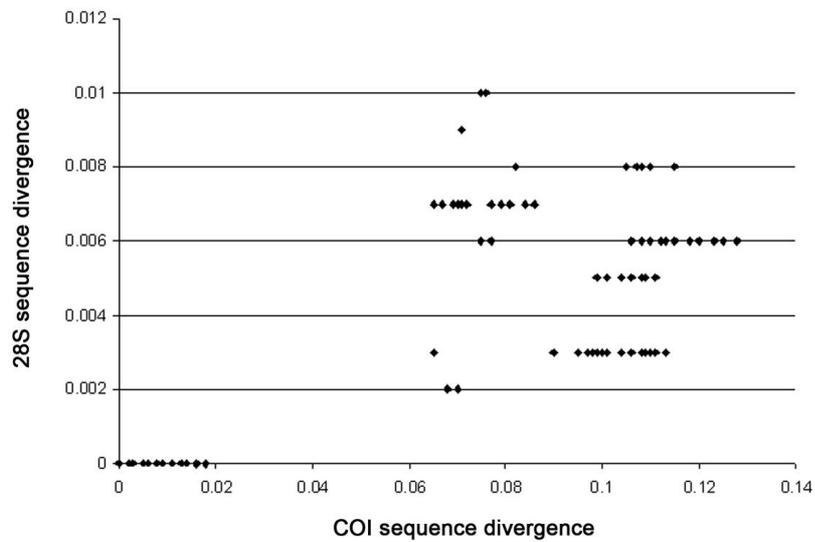


Figure S2: PCA for the complete dataset, with a visualisation of the genetic groups (A) and the cruise of collection (B). Only results for the first outline are are shown. Superimposed outlines for minimum (dotted line) and maximum (full line) projections onto the two principal axes are represented.

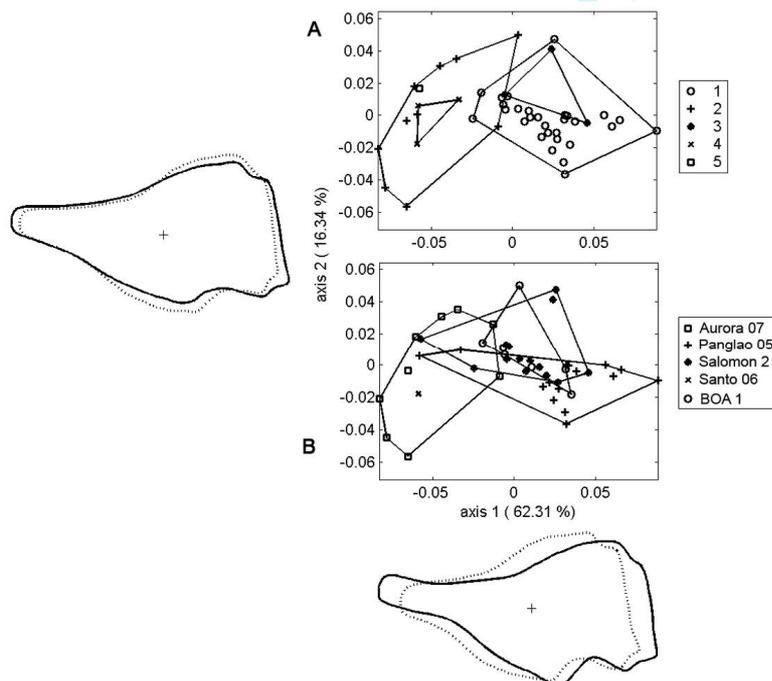
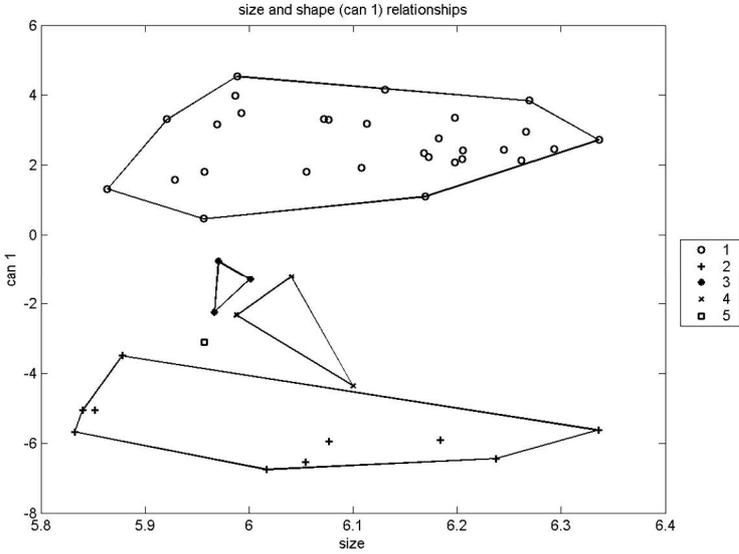


Figure S3: Plots of the shape of the last whorl of the shell as a function of the size. The five genetic groups are represented. Size of the shells was measured as the square root of the area.



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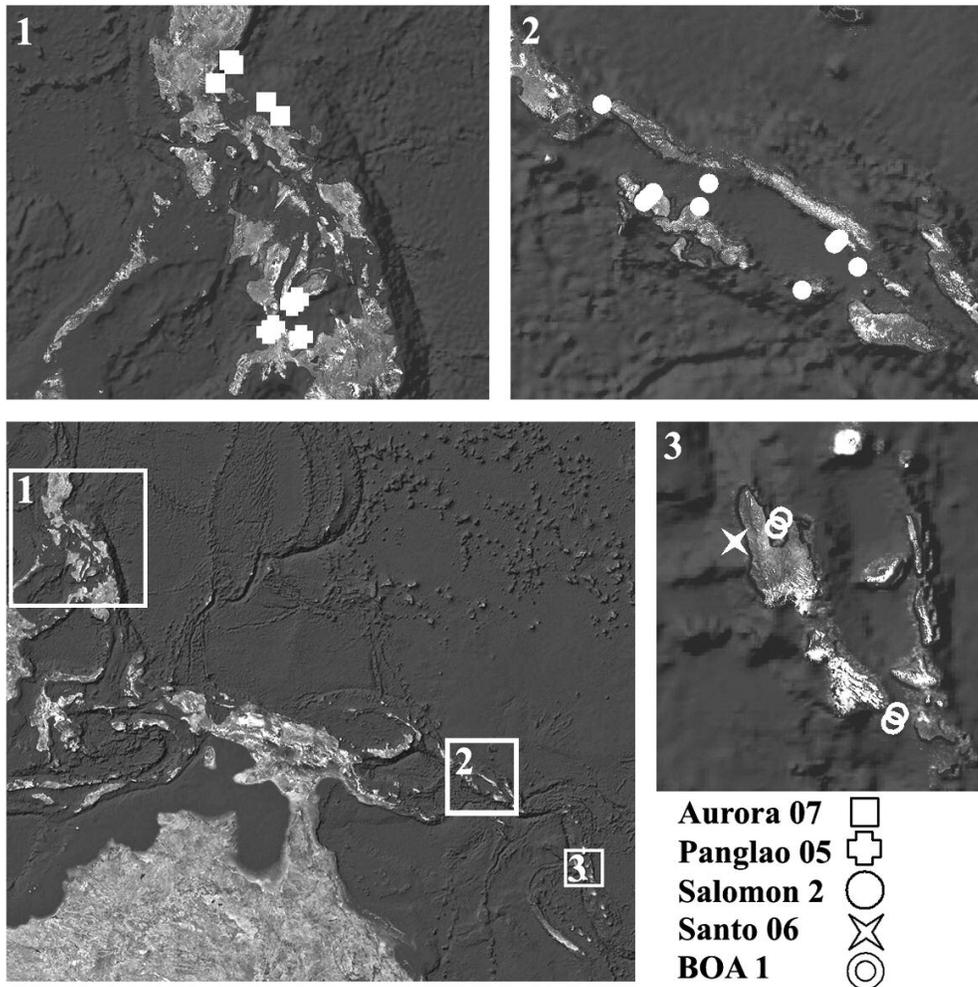


Figure 1: South-west Indo-Pacific map, with emphasis on Philippines (1), Solomon Islands (2) and Vanuatu (3). Sampling sites for each cruise are represented, with the same symbols used for CVA analyses (Figures 5, 6 and S2).

99x102mm (300 x 300 DPI)

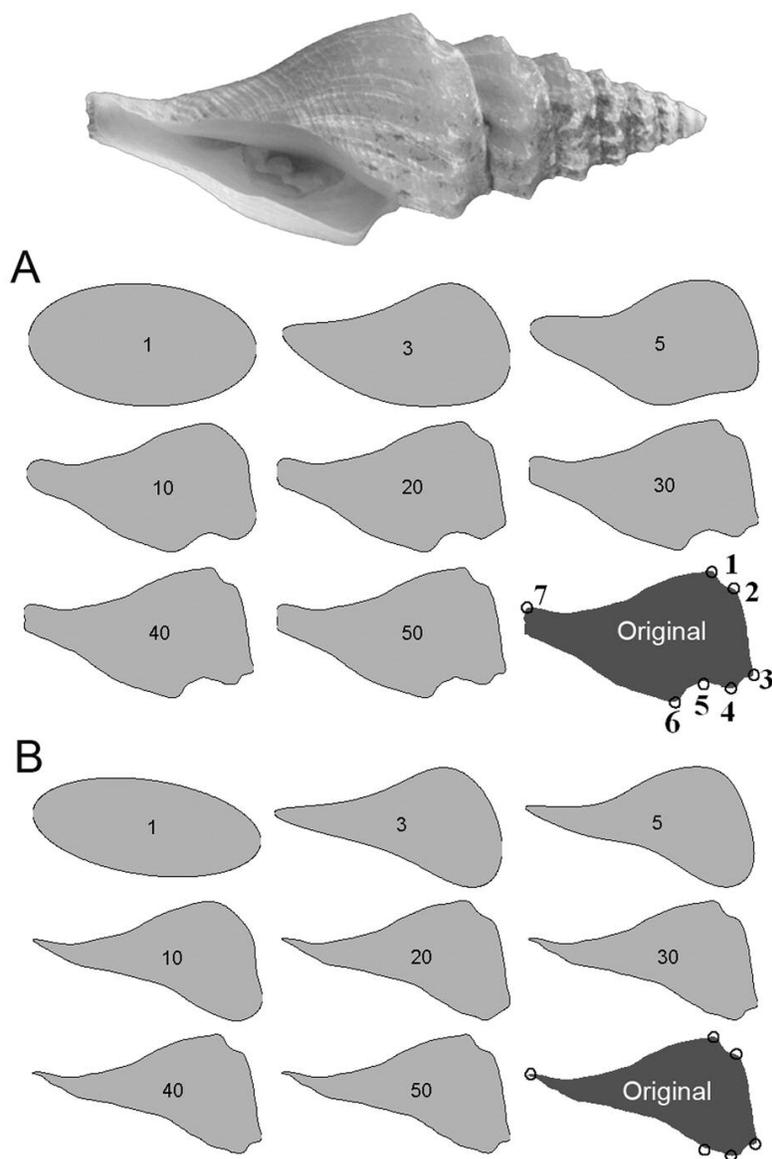


Figure 2: Outlines reconstructions with increasing number of harmonics indicated within outlines, for outline 1 (A) and outline 2 (B). The seven landmarks are represented on the original outline 1; only six landmarks were used for the second outline 2.

80x120mm (300 x 300 DPI)

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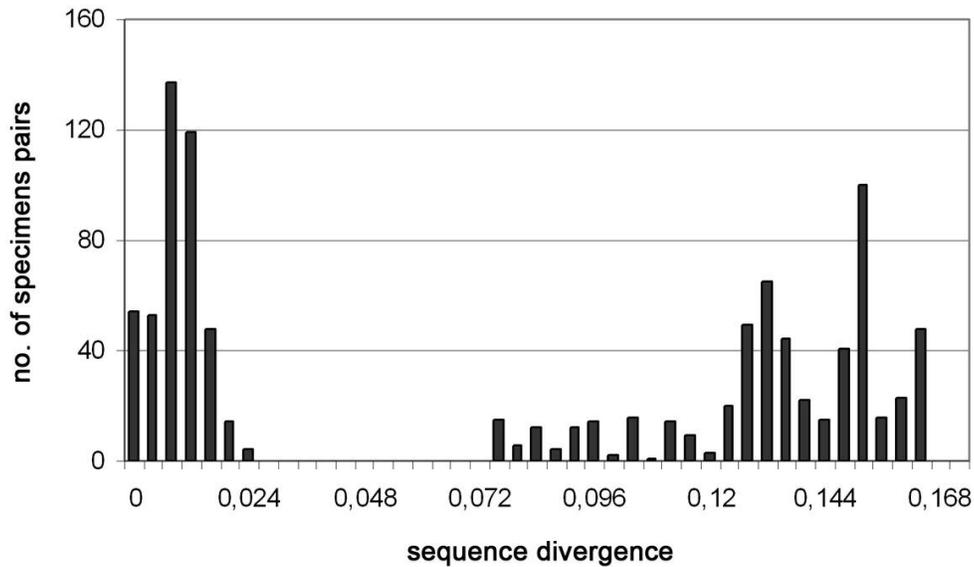


Figure 3: Histogram of genetic distances for COI gene.
99x59mm (300 x 300 DPI)

Review

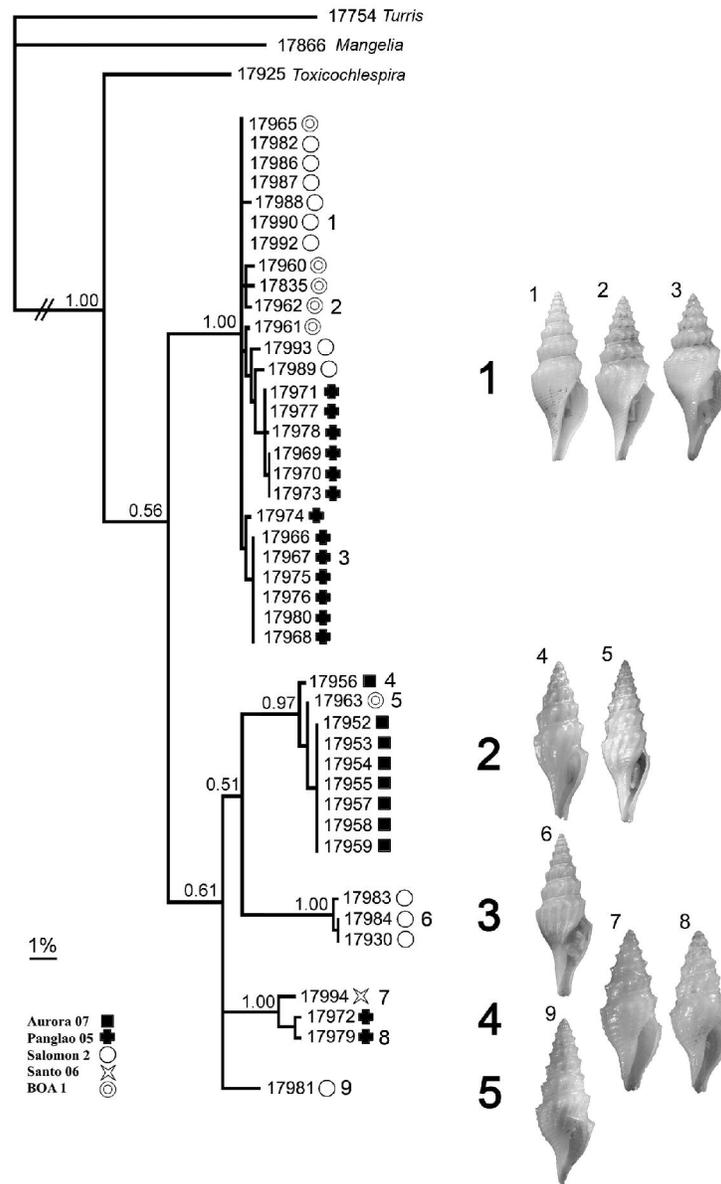


Figure 4: Bayesian tree for combined dataset corresponding to both genes. Posterior Probabilities (superior to 0.5) are given for each node. Groups are numbered top downwards from 1 to 5. For each group and for each cruise within group, one shell is illustrated (numbered from 1 to 9). 127x201mm (300 x 300 DPI)

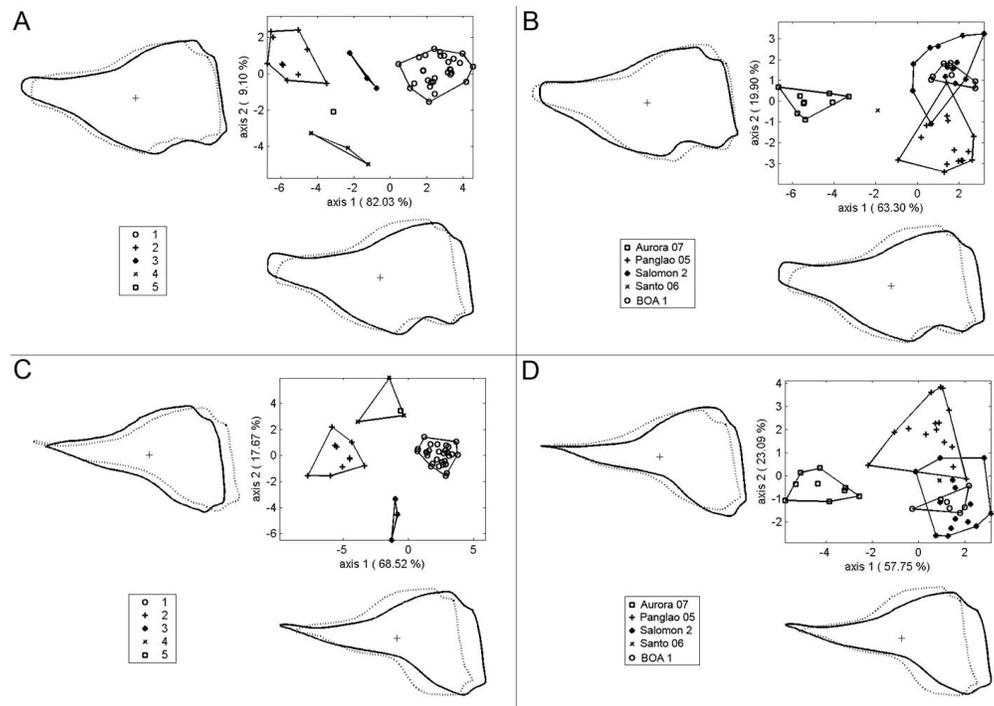


Figure 5: CVA for the complete dataset. CVA for the first outline using genetic groups as grouping variable (A), CVA for the first outline using cruise of collection as grouping variable (B), CVA for the second outline using genetic groups as grouping variable (C), CVA for the second outline using cruise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

119x84mm (300 x 300 DPI)

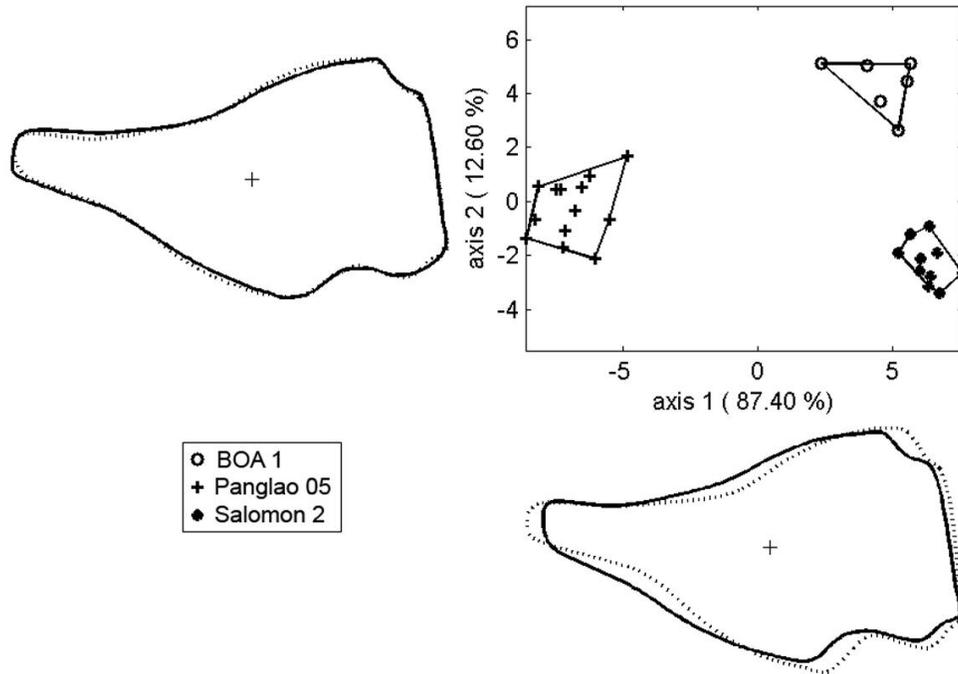


Figure 6: CVA on the first outline for the genetic group 1. Superimposed outlines for minimum (dotted line) and maximum (full line) projections onto the two principal axes are represented.

99x69mm (300 x 300 DPI)