Zoologica Scripta

KUNGL. VETENSKAPS-AKADEMIEN
THE ROYAL SWEDISH ACADEMY OF SCIENCES

Genetic divergence and geographical variation in the deep-water *Conus orbignyi* complex (Mollusca: Conoidea)

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Submitted: 9 December 2010
Accepted: 2 April 2011
doi: 10.1111/j.1463-6409.2011.00478.x

Puillandre, N., Meyer, C. P., Bouchet, P. & Olivera, B. M. (2011). Genetic divergence and geographical variation in the deep-water *Conus orbignyi* complex (Mollusca: Conoidea). — *Zoologica Scripta*, *40*, 350–363.

The cone snails (family Conidae) are a hyperdiverse lineage of venomous gastropods. Two standard markers, COI and ITS2, were used to define six genetically divergent groups within a subclade of Conidae that includes *Conus orbignyi*; each of these was then evaluated based on their shell morphology. We conclude that three forms, previously regarded as subspecies of *C. orbignyi* are distinct species, now recognized as *C. orbignyi*, *C. elokismenos* and *C. coriolisi*. In addition, three additional species (*C. pseudorbignyi*, *C. joliveti* and *C. comatosa*) belong to this clade. Some of the proposed species (e.g. *C. elokismenos*) are possibly in turn complexes comprising multiple species. Groups such as Conidae illustrate the challenges generally faced in species delimitation in biodiverse lineages. In the case of *C. orbignyi* complex, they are not only definable, genetically divergent lineages, but also considerable geographical variation within each group. Our study suggests that an intensive analysis of multiple specimens within a single locality helps to minimize the confounding effects of geographical variation and can be a useful starting point for circumscribing different species within such a confusing complex.

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Introduction

The cone snails (family: Conidae) comprise a hyperdiverse group of venomous gastropods. Because the peptides produced by their venom gland have medical potential (Terlau & Olivera 2004; Olivera 2006), there has been intense recent interest in this biodiverse lineage. *Conus orbignyi* Audouin, 1831 (*Bathyconus orbignyi* in the Tucker & Tenorio 2009 classification) is one of the problematic species complexes in the Conidae; traditionally (Röckel *et al.* 1995), it is regarded as a widely distributed species ranging from the southwest Pacific, to southwest Africa and north to Honshu, Japan, where it is one of the few species in Conidae that thrives at such northern latitudes. In the 19th and first half of the 20th century, most specimens were collected by Japanese or Chinese fishing boats. The holotype from China is an example of the Northern Pacific form of *C. orbignyi*.

Specimens assigned to *C. orbignyi* from South Africa, Mozambique and Madagascar are generally regarded as a geographical subspecies, *C. orbignyi elokismenos* Kilburn, 1975. In the Coral Sea – New Caledonia area, a form known as *C. orbignyi coriolisi* Moolenbeek & Richard (1995) has been described. There are two species that also are somewhat nodulose that appear to be closely related to *C. orbignyi*: one is *C. pseudorbignyi* Röckel & Lan (1981), described from Taiwan, and the other is *C. joliveti* Moolenbeek *et al.* 2008, from Fiji. Because cones are a collectable group of seashells, many of the recently named species, especially those based on a small number of specimens, are sometimes viewed with suspicion by other biologists, and admittedly the taxonomy of *C. orbignyi*, as well as its affinities to morphologically similar species has not been thoroughly assessed by non-typological approaches.

The variability of mollusc shells, and in particular Conoidea shells, is known to be difficult to interpret (e.g. Duda *et al.* 2008; Puillandre *et al.* 2010), due to extended polychromatism and rampant homoplasy. The variability of molluscan shells, especially in the case of small samples, is difficult to attribute *a priori* to individual variability, within species geographical variation or differences between species (see e.g. Appleton & Palmer 1988; Meyer *et al.* 2005). Molecularly, well-defined species may have very subtle conchological differences, but in the meantime morphologically distinct clusters of specimens could correspond merely to geographical variants, with different environmental conditions acting differentially on the shape and ornamentation of the shell. Consequently, investigating whether these forms correspond to different species or not requires analysis of characters not determined by the environment. DNA sequences meet this requirement, and are now commonly used to delimit species (e.g. Wiens 2007; Wheeler 2009). In the genus *Conus*, Duda *et al.* (2008) recently published a DNA-based analysis of the species diversity in the *C. sponsalis* complex, another complex in the Conidae, where morphological characters alone were unable to accurately define species limits. Several other studies have also been carried out on *Conus*-related groups belonging to the superfamily Conoidea (e.g. Puillandre *et al.* 2009, 2010).

We present a similar approach to the *orbignyi* complex, analysing first DNA variation to delimit putative species, then morphological variation to link these putative species to the different forms described in literature. Although our analysis includes specimens collected in other geographical regions, we focus on forms related to *C. orbignyi* from the Philippines, as the starting point of this research was an expedition carried out at Aurora, Luzon Island in 2007 ('Aurora 2007') that recovered over 100 specimens that could be assigned to the *C. orbignyi* complex.

We applied several species delimitation criteria in the analysis of our *C. orbignyi* dataset (see e.g. De Queiroz 1998, 2007; Samadi & Barberousse 2006). First, specimens that belong to the same species are supposed to be more similar to each other than to any other species; we used the *COI* gene to delimit groups of specimens. Then, phylogenetic analyses were carried out not only using the *COI* gene, but also the 12S and 16S genes to establish the monophyly of each group. However, discrepancies between gene and species tree can occur, due, for example, to mtDNA introgression or incomplete lineage sorting (Funk & Omland 2003; Maddison & Knowles 2006; Linnen & Farrell 2008; Petit & Excoffier 2009). To overcome this difficulty, an independent nuclear marker ITS2 was assessed: if the phylogenetic relationships inferred with each gene are congruent at the interspecific level (i.e. between putative species), then interspecific gene trees can be equated to the species tree (Gaines *et al.* 2005; Knowles & Carstens 2007; Edwards 2009). Finally, an attempt was made to define morphological differences between the genetic groups, taking into account geographical variation.

Materials and methods

Alcohol-preserved specimens

Specimens morphologically identified as belonging or closely related to the *C. orbignyi* complex were collected in Taiwan, Philippines, Solomon and Chesterfield Islands, Vanuatu and Madagascar during several expeditions conducted between 2003 and 2009 (Table 1). The known geographical distribution of different species, together with the sampling sites, is shown in Fig. 1. A piece of foot was cut from the living animal and preserved in 95% alcohol for molecular analyses, and the corresponding shell was kept for further reference. For the phylogenetic analyses, three species were used as outgroups: *C. mahogani* (*Ximenoconus mahogani* in the Tucker & Tenorio 2009 classification), *C. vimineus* (*Viminiconus vimineus* in the Tucker & Tenorio 2009 classification) and *C. ichinoseanus* (*Yeddoconus ichinoseanus* in the Tucker & Tenorio 2009 classification). Within *Conus*, all are included in the small major clade (*sensu* Duda & Kohn 2005). *C. vimineus* and *C. ichinoseanus* are placed together with the *C. orbignyi* complex in Conolithinae by Tucker & Tenorio (2009). With the exception of a Taiwan specimen housed at FLMNH, all specimens are vouchered in MNHN and registered in the Barcode of Life Database (Table 1).

DNA extraction and sequencing

Genomic DNA was extracted from a piece of foot or hepatopancreas tissue, using the 6100 Nucleic Acid PrepStation system (Applied Biosystem, Carlsbad, CA, USA), or the Gentra PUREGENE DNA Isolation Kit (Gentra Systems, Minneapolis, MN). Four genes were amplified: the 'barcoding' fragment of cytochrome oxidase I (COI) mitochondrial gene, using universal primers LCO1490 and HCO2198 (Folmer *et al.* 1994), the *ITS2* gene, using primers mITS-3D and mITS-4R (Nam *et al.* 2009), a fragment of the 12S gene, using the primers 12S1 and 12S3 (Simon *et al.* 1991) and a fragment of the 16S gene, using the primers 16Sar and 16Sbr (Palumbi 1996). All PCR reactions were performed in 25 μL, containing 3 ng of DNA, 10× reaction buffer, 2.5 mM $MgCl_2$, 0.26 mM dNTP, 0.3 μM of each primer, 5% DMSO and one unit of Advantage 2 Polymerase Mix (Clontech Laboratories, Mountain View, CAn USA). Amplification consisted of an initial denaturation step at 95 °C for 5 min, followed by 35 cycles of denaturation at 95 °C for 1 min, annealing at 50 °C for the *COI* gene, 61 °C for the *ITS2* gene, 54 °C for 12S gene and 52 °C for 16S for 30 s,

Table 1 List of specimens analysed molecularly, with collection data, species identification and GenBank and BOLD (Barcode of Life Database) numbers

						GenBank numbers				
ID	Geographic region	Expedition	Coordinates and depth (m)	Group	Species	COI	ITS2	16S	12S	BOLD ID
MNHN IM200730681	Chesterfield	EBISCO	21°05′S, 160°45′E – 297-378	1	*comatosa*	GU131299	GU131322	GU131286	GU131274	FRANZ906-09
MNHN IM200730660	Vanuatu	Santo 2006	15°39′S, 167°01′E – 266-281	2	*coriolisi*	GU131297	GU131320	GU131285	GU131273	FRANZ907-09
MNHN IM200730666	Vanuatu	Santo 2006	15°39′S, 167°01′E – 266-281	2	*coriolisi*	GU131298	GU131321	–	–	FRANZ908-09
MNHN IM200730823	Vanuatu	Santo 2006	15°40′S, 167°02′E – 272-286	2	*coriolisi*	GU131306	GU131328	–	–	FRANZ909-09
MNHN IM200730836	Chesterfield	EBISCO	21°06′S, 158°36′E – 356-438	2	*coriolisi*	GU131307	GU131329	–	–	FRANZ910-09
MNHN IM200730842	Chesterfield	EBISCO	21°06′S, 158°36′E – 356-438	2	*coriolisi*	GU131308	GU131330	–	–	FRANZ911-09
MNHN IM200730899	Philippines	Aurora 2007	15°54′N, 121°42′E – 189-189	3	*pseudorbignyi*	GU131312	GU131333	GU131289	GU131277	FRANZ912-09
MNHN IM200730942	Vanuatu	BOA 1	15°07′S, 166°54′E – 131-308	3	*pseudorbignyi*	GU131316	GU131337	GU131292	GU131280	FRANZ913-09
MNHN IM200730925	Solomon Is.	Salomon 2	8°38′S, 157°22′E – 195-197	4	*joliveti*	GU131313	GU131334	GU131290	GU131278	FRANZ914-09
UF 327736	Taiwan	–	–	5	*orbignyi*	GU131317	–	GU131293	GU131281	–
MNHN IM200717921	Philippines	Panglao 2005	9°29′N, 123°44′E – 271-318	5	*orbignyi*	EU015721	–	–	–	CONO296-08
MNHN IM200730729	Philippines	Panglao 2005	9°39′N, 123°48′E – 255-268	5	*orbignyi*	GU131301	GU131324	GU131288	GU131276	FRANZ915-09
MNHN IM200730773	Philippines	Panglao 2005	8°43′N, 123°19′E – 259-280	5	*orbignyi*	GU131302	GU131325	–	–	FRANZ916-09
MNHN IM200730785	Philippines	Aurora 2007	16°03′N, 121°53′E – 189-189	5	*orbignyi*	GU131303	GU131326	–	–	FRANZ917-09
MNHN IM200730813	Philippines	Aurora 2007	16°03′N, 121°53′E – 189-189	5	*orbignyi*	GU131304	GU131327	–	–	FRANZ918-09
MNHN IM200730815	Philippines	Aurora 2007	16°03′N, 121°53′E – 189-189	5	*orbignyi*	GU131305	–	–	–	FRANZ919-09
MNHN IM200730714	Vanuatu	BOA 1	15°05′S, 166°54′E – 400-350	6	*elokismenos*	GU131300	GU131323	GU131287	GU131275	FRANZ920-09
MNHN IM200730846	Vanuatu	Santo 2006	15°07′S, 166°53′E – 328-354	6	*elokismenos*	GU131309	GU131331	–	–	FRANZ921-09
MNHN IM200730848	Vanuatu	Santo 2006	15°07′S, 166°53′E – 328-354	6	*elokismenos*	GU131310	GU131332	–	–	FRANZ922-09
MNHN IM200730893	Vanuatu	BOA 1	15°05′S, 166°54′E – 400-350	6	*elokismenos*	GU131311	–	–	–	FRANZ923-09
MNHN IM200730938	Vanuatu	BOA 1	15°05′S, 166°54′E – 400-350	6	*elokismenos*	GU131314	GU131335	–	–	FRANZ924-09
MNHN IM200730939	Solomon Is.	Salomon 2	8°41′S, 157°24′E – 248-253	6	*elokismenos*	GU131315	GU131336	GU131291	GU131279	FRANZ925-09
MNHN IM20097513	Madagascar	Miriki 2009	12°41′S, 48°17′E – 231-237	6	*elokismenos*	GU131318	GU131338	GU131294	GU131282	FRANZ926-09
OUTGROUPS					*ichinoseanus*	GU131319	–	GU131296	GU131284	–
					mahogani	FJ868119	–	FJ868057	FJ868049	–
					vimineus	GU134378	–	EU682306	EU682297	–

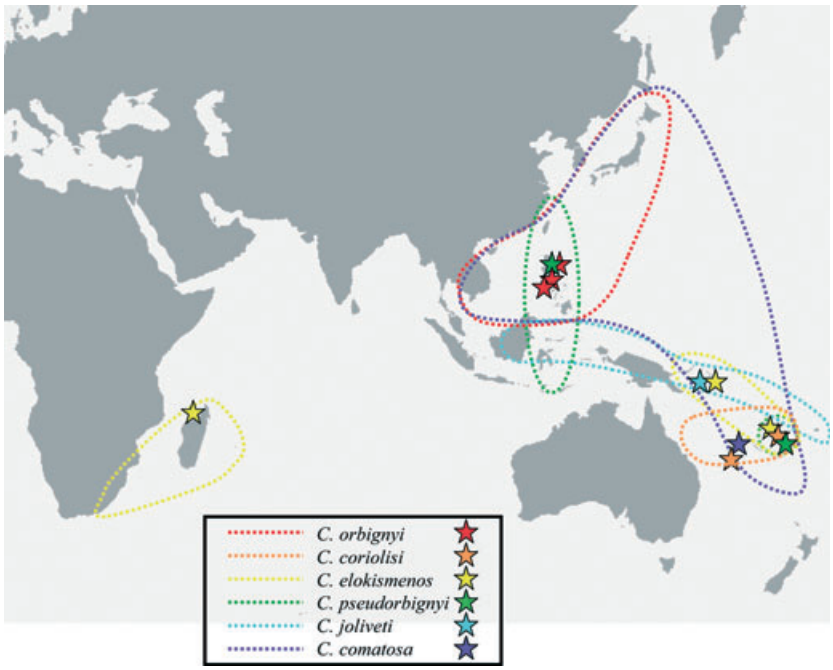

Fig. 1 Known geographic distribution and sampling sites of the six delimited species.

followed by extension at 68 °C for 30 s. The final extension was at 68 °C for 5 min. PCR products were purified using the Qiagen gel extraction kit (Qiagen, CA, USA) or the High Pure PCR Product Purification Kit and sequenced by the Health Sciences Center Core Sequencing Facility, University of Utah. In most cases, both directions were sequenced to confirm accuracy of each haplotype sequence. All sequences were submitted to GenBank (Table 1).

Species delimitation using the COI gene

Specimens belonging to the same species are supposed to be phenetically similar. We used this property to delimit putative species within the *orbignyi* complex by sequencing a standard portion of the *COI* gene (the fragment used for most animals in DNA bar-coding projects) for all alcohol-preserved specimens. Sequences were aligned manually, and the genetic p-distances were calculated between all sequences (excluding outgroups), using MEGA 3.1 (Kumar *et al.* 2004). Their distribution was visualized on a pairwise distances histogram, and groups of genetically similar specimens were proposed. A phylogenetic tree was then reconstructed using the *COI* gene dataset. The best model of evolution identified using the HLRT test implemented in Model generator (Keane *et al.* 2006) is the TVM+I+G (with $I = 0.37$ and $\alpha = 0.23$). Maximum likelihood analyses were performed using RAXML 7.0.4 (Stamatakis 2006), with 20 independent runs, and the GTRGAMMAI model. Robustness of the nodes was assessed with 200 bootstrap replicates (with five searches for each of them). Bayesian analyses were performed using MRBAYES (Huelsenbeck *et al.* 2001) and consisted of two independent analyses (eight Markov chains and five swaps at each sampling). A six substitution categories model associated to a gamma-distributed rate variation across sites and a proportion of invariable sites was used. Convergence of each analysis was evaluated using TRACER 1.4 (Rambaut and Drummond 2007), and analyses were terminated when ESS values were all superior to 200. A consensus tree was then calculated after omitting the first 25% of the trees as burn-in.

ITS2 gene

To avoid problems linked to the use of a single gene in species delimitation, the *ITS2* gene of several specimens for each putative species delimited with the *COI* gene was also sequenced. When specimens from different geographical region were included in a single putative species, the *ITS2* gene of at least one specimen from each region was sequenced. Sequences were automatically aligned using BIOEDIT 7.0.5.3 (Hall 1999). The best model of evolution for the *ITS2* gene is TrN+G ($\alpha = 0.23$). Phylogenetic relationships using the *ITS2* gene were inferred following the methodology described for the *COI* gene.

Phylogenetic relationships between species

To infer the relationships between species, we also sequenced for several specimens representative of the COI

variability of each putative species, two fragments of mitochondrial genes: the 12S and 16S. The two genes are less variable than the *COI* gene, and generally more informative to resolve deeper relationships. As for the *ITS2* gene, sequences were aligned using BIOEDIT. The best models of evolution for the 12S and 16S genes are respectively HKY+I+G (with $I = 0.47$ and $\alpha = 0.21$) and HKY+I+G (with $I = 0.59$ and $\alpha = 0.20$). As the independent trees were congruent, we concatenated these two genes plus the *COI* gene in a single dataset. Five partitions were defined: three for each position of the *COI* gene, one for the 12S gene and another for 16S gene. All partitions were unlinked, each following the GTRGAMMAI model (RAxML) or a model with six categories of substitution (MRBAYES). Once again, phylogenetic relationships using these genes were then inferred following the methodology described for the *COI* gene.

Morphological analysis of living and dead specimens

Shells were *a posteriori* examined to identify differences between the different genetic groups. In addition to the living specimens analysed both molecularly and morphologically, several dead shells collected in the Philippines (and in particular during the Aurora 2007 expedition – Table 2) were integrated in the analysis. As all species and subspecies descriptions are based on shell characters, linking genetic groups to discrete morphological entities was the only way to attribute available taxon names to the different putative species of the *orbignyi* complex. The major character states used for differentiation were the ground colour of the body whorl (white or brown), the banding pattern on the body whorl (number of darker brown bands, how continuous the bands are), how nodulose the spire sutural ridges are (very nodulose or almost obsolete) and the presence or absence of dark brown blotches between the spire nodules.

Results

Molecular analyses

The 26 COI sequences alignment (23 in groups and three outgroups) is 658 bp long. The pairwise p-distances ranges from 0 to 11.5% (excluding outgroups), and four modes can be recognized in the pairwise distribution (Fig. 2). This pattern is somewhat different from the classic bimodal distribution of pairwise genetic distance, with low distances corresponding to intraspecific distances and high genetic distances to interspecific distances (e.g. Hebert *et al.* 2004; Ward *et al.* 2005; Puillandre *et al.* 2010). The different thresholds between four modes could be used to delimit groups of specimens in the COI tree (Fig. 3), but identifying which threshold corresponds to the species level is not straightforward.

Table 2 List of the Aurora 2007 expedition stations and number of dead shells for each Philippines species (CP, beam trawl; DW, dredge)

Station	Coordinates	Depth (m)	*C. o. orbignyi*	*C. o. coriolisi*	*C. pseudorbignyi*
CP2655	16°03′N 121°53′E	189-189	6	3	–
CP2662	15°47′N 121°44′E	253-253	1	–	–
CP2666	15°57′N 121°45′E	198-199	14	7	–
CP2672	14°57′N 121°44′E	346-276	2	–	–
CP2709	15°11′N 121°35′E	296-244	6	–	–
CP2710	15°15′N 121°33′E	207-216	1	–	–
CP2711	15°20′N 121°32′E	200-184	3	–	–
CP2712	15°21′N 121°30′E	140-139	–	–	1
CP2715	14°32′N 121°42′E	233-249	10	–	–
CP2716	14°31′N 121°61′E	335-356	1	–	–
CP2717	14°29′N 121°42′E	361-311	17	–	–
CP2719	14°27′N 121°48′E	160-155	6	–	–
CP2721	14°24′N 121°47′E	367-360	4	–	–
CP2723	14°25′N 121°49′E	156-147	1	–	–
CP2724	15°12′N 121°35′E	280-229	2	–	–
CP2741	16°03′N 121°55′E	194-203	3	–	–
CP2742	16°03′N 121°53′E	182-205	3	2	1
CP2748	15°56′N 121°45′E	249-247	5	–	–
CP2760	15°55′N 121°41′E	100-100	–	–	1
CP2762	15°52′N 121°37′E	66-66	–	–	1
DW2670	14°52′N 121°49′E	180-187	3	–	–
DW2726	15°04′N 121°41′E	323-313	5	–	–
DW2758	15°55′N 121°50′E	173-151	–	–	1
TOTAL	–	–	93	12	5

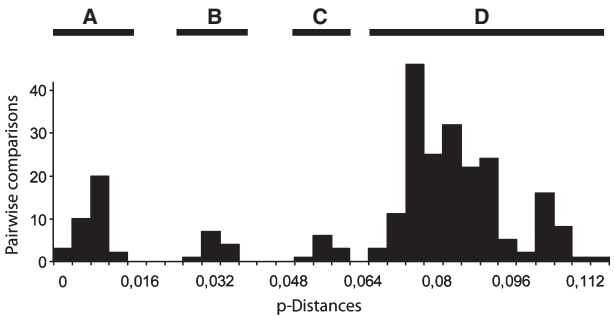

Fig. 2 Pairwise COI genetic p-distances between all the specimens. Four modes are indicated. (A) Distances between specimens within group 2, 5 (Philippines specimens only) and 6 (Vanuatu specimens only), (B) distances within group 3, 5 (Taiwan vs. Philippines) and 6 (Solomon vs. Vanuatu), (C) distances within group 6 (Madagascar vs. others – see text for Discussion), and (D) distances between groups.

The *ITS2* gene was sequenced for 19 specimens, resulting in a 574 bp fragment after alignment. The ITS2 phylogenetic tree presented in Fig. 3 is highly congruent with the COI tree. All specimens placed in a single cluster using the third threshold defined by the four modes of the pairwise COI genetic distances distribution (<6.4%), are characterized by highly similar ITS2 haplotypes (<1% genetic divergence) and, conversely, specimens separated by more than 6.4% of divergence with the *COI* gene have different ITS2 haplotypes. Each of these groups (numbered from 1 to 6) correspond to highly supported monophyletic groups in both *COI* and *ITS2* gene trees, except the distinction between groups 5 and 6 in the ITS2 marker. Based on ITS2, group 6 is a nested monophyletic clade within an undifferentiated group 5.

Moreover, the identification of the threshold between intra- and interspecific COI distances in comparison with the *ITS2* gene tree allows an interpretation of the different modes found in the pairwise distribution shown in Fig. 2. The first (A) would correspond to intraspecific distances, mostly found between specimens collected in the same geographical region. The second mode (B) corresponds to distances between specimens collected in different geo-

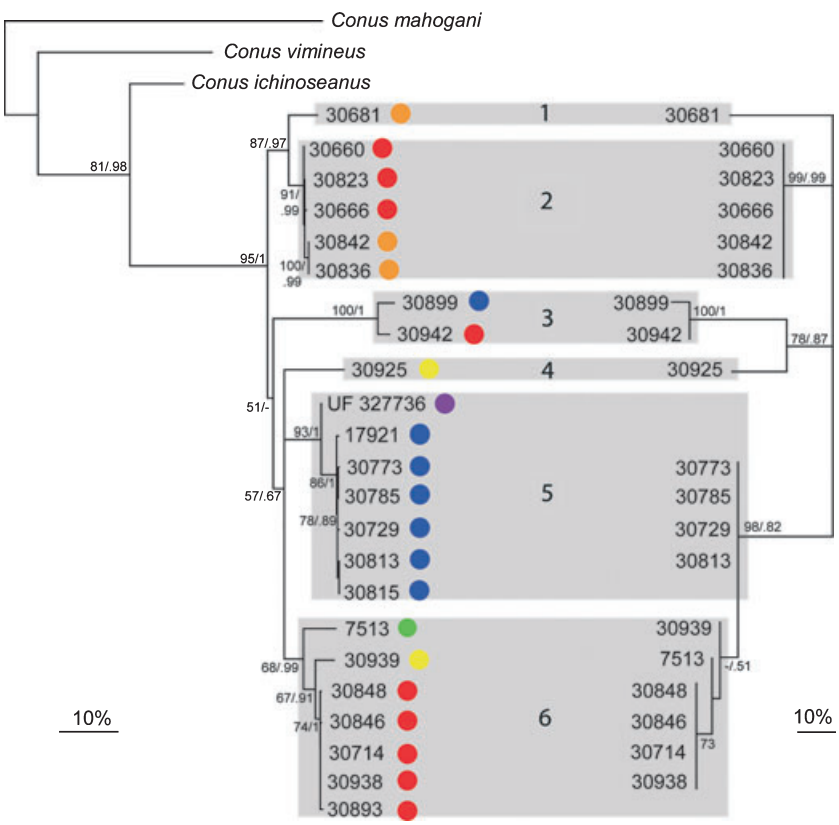

Fig. 3 Left: COI phylogenetic tree (RAxML, best tree over 20 replicates), with bootstraps values (200 replicates) and posterior probabilities indicated for each node (when superior to 50 and 0.5 respectively). At the tip of each branch the geographic location is indicated (colours are used as in the Fig. 1). Right: ITS2 phylogenetic tree (RAxML, best tree over 20 replicates), with bootstrap values (200 replicates) and posterior probabilities indicated for each node (when superior to 50 and 0.5 respectively). The congruency between both trees is highlighted with grey boxes, each of them representing a putative different species (groups 1–6).

graphical regions, but included in the same group (3, 5, 6). The third mode (C) corresponds to distances between the specimen from Madagascar and other specimens of group 6 and the final mode (D) to distances between different groups. Specimen MNHN IM20097513 from Madagascar is separated from the other specimens of group 6 by 5 to 6% divergence in the *COI* gene.

The 12S and 16S genes were used to infer the relationships between all the defined groups. The resulting fragments are 546 and 484 bp long respectively after alignment. In the resulting tree of the concatenated dataset (Fig. 4), the inclusion of one specimen from each geographical location within the groups 3, 5 and 6 confirms their respective monophyly. As found with the *COI* and *ITS2* genes, the *C. orbignyi* complex corresponds to a highly supported monophyletic group. However, most of the relationships between each group within this complex are not well-supported.

Species assignments for the six genetic groups

Shell characters traditionally used in *Conus* taxonomy were investigated in all the specimens analysed molecularly. The goal was to identify diagnostic characters in each of the six genetic groups that would allow an attribution of each of these groups to a species or subspecies name available in literature. To do so, we mainly refer to the name-bearing types of the relevant forms shown in Fig. 5, and the description of shell variation and map ranges provided by Röckel *et al.* (1995). Protoconchs in all species of the *C. orbignyi* complex are non-diagnostic; all are multispiral and indicate planktotrophic larval development. In contrast, teleoconchs differ by subtle but apparently stable differences:

Group 1 – *Conus comatosa*. As shown in Fig. 6A, the identification of the Chesterfield specimen as *C. comatosa* seems unambiguous, based on its mostly smooth, rather than tuberculate, spiral shoulder and four spiral colour bands, rather than three. All other members of the *orbignyi* complex included in this study have nodulose spiral shoulders, making *C. comatosa* conchologically distinctive. The dark brown colour of the anterior end of the body whorl is another distinctive character absent in the other forms.

Group 2 – *Conus coriolisi*. The molecular data obtained were from specimens collected in Vanuatu and Coral Sea; despite the close genetic distance using the various molecular markers in the two populations (<1%), they are unexpectedly morphologically divergent (see Figs 6E–I and 7). The Chesterfield/Coral Sea material is similar to the holotype of *C. orbignyi coriolisi* (type locality: Capel Bank, Coral Sea) with weak to almost absent spiral ribbons and white ground colour. The Chesterfield specimens, while somewhat broader in their shell outline than the type, have the characteristic interrupted brown blotches organized into three bands characterizing the type. The Vanuatu specimens belonging to *C. coriolisi* have stronger spiral sculpture and a pale tan ground colour. The brown bands are both stronger and more continuous than in the Chesterfield specimens including the type. Given the genetic distance (approximately 9%) of group 2 specimens to *C. orbignyi* (group 5) and the fact that *C. comatosa* is its sister species

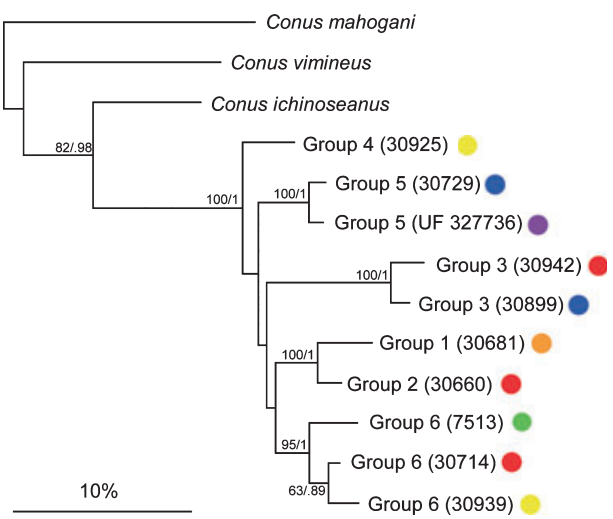

Fig. 4 Phylogenetic tree obtained with a concatenation of three genes (COI, 12S, 16S) with bootstrap values (200 replicates) and posterior probabilities indicated for each node (when superior to 50 and 0.5 respectively).

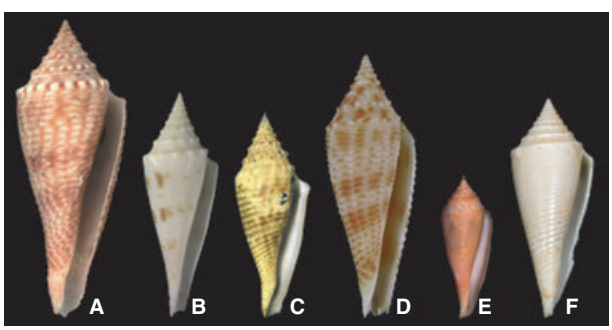

Fig. 5 Illustration of type specimens (scaled). (A) *Conus orbignyi* Audouin, 1831. Holotype MNHN 2532, H 53.2 mm, (B) *C. orbignyi coriolisi* Moolenbeck & Richard, 1995. Holotype MNHN 2570, H 41.5 mm, (C) *C. orbignyi aratus* Kilburn, 1973 (renamed *C. o. elokismenos*). Holotype NM, H 60 mm, (D) *C. jolive*ti Moolenbeek *et al.* 2008;. Holotype MNHN 21036, H 29.1 mm, (E) *C. pseudorbignyi* Röckel & Lan, 1981. Holotype TMT. H 45.5 mm, and (F) *C. comatosa* Pilsbry, 1904. Lectotype (Coomans *et al.* 1985) ANSP 85590, H 43.4 mm. Photos C. Reyens (A, B), A. Robin (D).

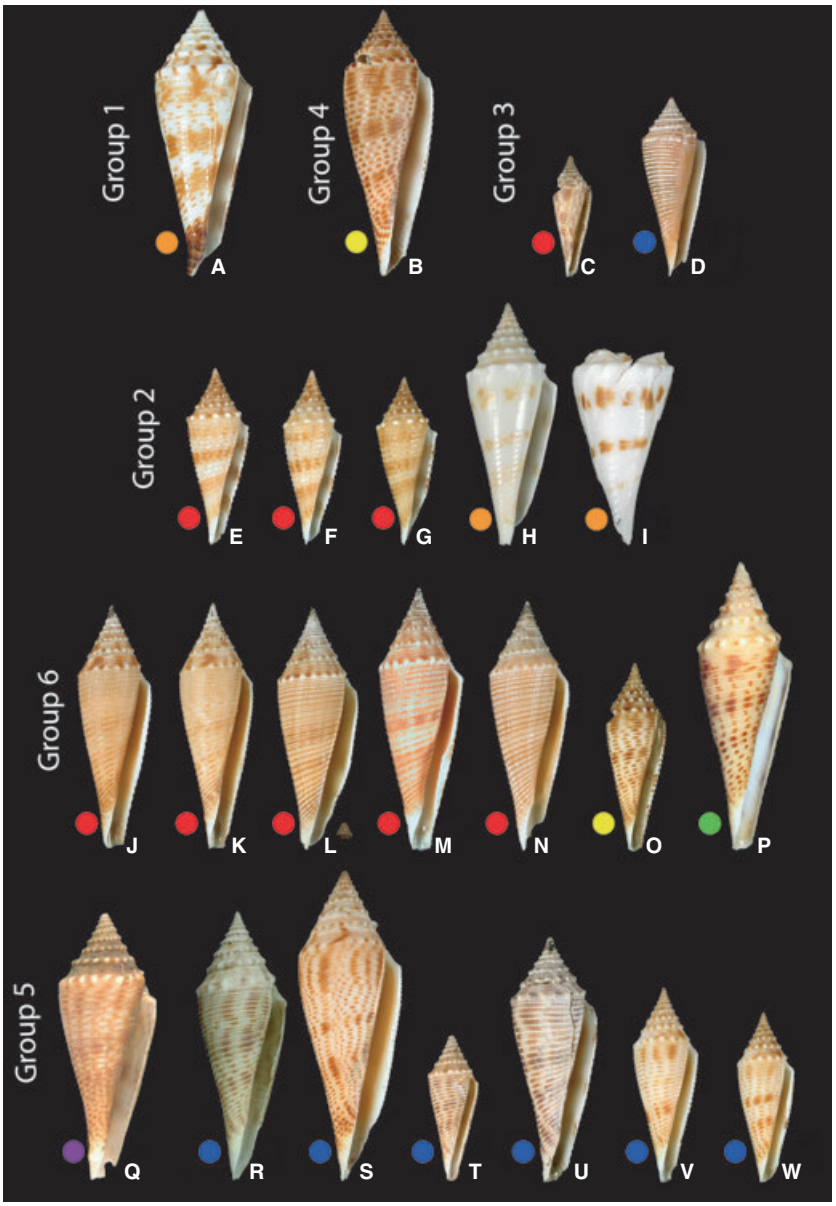

Fig. 6 Specimens used for the molecular analyses (scaled). (A) Group 1 – *C. comatosa*, Chesterfield, MNHN IM200730681 (46.8 mm), (B) Group 4 – *C. joliveti*, Solomons, MNHN IM200730925 (46.9 mm), (C, D) Group 3 – *C. pseudorbignyi*. C: Vanuatu, MNHN IM200730942 (21.2 mm), (D) Philippines, MNHN IM200730899 (31.5 mm), (E–I) Group 2 – *C. coriolisi*. (E–G) Vanuatu. E: MNHN IM200730660 (31 mm), (F) MNHN IM200730823 (30.7 mm). G: MNHN IM200730666 (29.4 mm), (H, I) Chesterfield. H: MNHN IM200730836 (42.5 mm), (I) MNHN IM200730842 (50 mm), (J–P) Group 6 – *C. elokismenos*. J-N: Vanuatu, (J) MNHN IM200730848 (42.5 mm), (K) MNHN IM200730846 (43 mm), (L) MNHN IM200730714 (42.4 mm), (M) MNHN IM200730938 (45.9 mm), (N) MNHN IM200730893 (43.6 mm), (O) Solomons, MNHN IM200730939 (33 mm), (P) Madagascar, MNHN IM20097513 (50.5 mm), (Q–W) Group 5 – *C. orbignyi*, (Q) Taiwan, FLMNH UF 327736 (47 mm), (R–W) Philippines, (R) MNHN IM200717921 (47.3 mm), (S) MNHN IM200730813 (54.4 mm), (T) MNHN IM200730729 (25.5 mm), (U) MNHN IM200730773 (43 mm), (V) MNHN IM200730785 (34.2 mm), and (W) MNHN IM200730815 (29.9 mm).

in both markers (Fig. 3) justify treating *C. coriolisi* as a distinct species and not a subspecies of *C. orbignyi*. It is notable that a very distinctive Philippine morph primarily collected from Aliguay Island and Panglao, but also represented in the Aurora 2007 material as empty shells (see below), is most similar to group 2 specimens from Vanuatu

based on the overall shell shape, structure of the sutural ramp, degree of spiral sculpture, and ground colour. No molecular data on such Philippine material has been obtained, but based on the similar morphology of the Philippine material to the Vanuatu specimens, and based on the molecular results, we tentatively assign the Philippines specimens (Table 2 – see description below) and the group 2 specimens from Vanuatu to *C. coriolisi*, even though they are morphologically distinct from *C. coriolisi* from Coral Sea specimens including the type.

Group 3 – *Conus pseudorbignyi*. Two different specimens represent this group, one from Aurora in the Philippines and another from Vanuatu (Fig. 6C, D). The Aurora specimen, as shown in Fig. 8C, though smaller, is generally similar to the holotype of *C. pseudorbignyi*, which was collected in Taiwan. The Vanuatu specimen is quite divergent from both the holotype and the Philippine specimens in shell pattern, having much more distinctive brown blotches. Both the Philippine and Vanuatu material differ in their shell morphology from Taiwanese specimens collected in recent years (see Fig. 8E) and assigned by Röckel *et al.* (1995) to *C. pseudorbignyi*. These specimens have strong alternating brown blotches between the nodules on the prominent sutural ridge and are generally larger than the holotype and other specimens examined in this study.

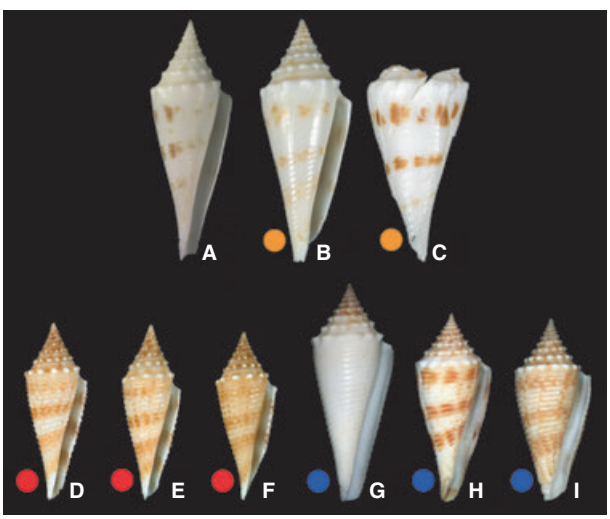

Fig. 7 Specimens assigned to the *C. coriolisi* complex. (A) *Conus orbignyi coriolisi* Moolenbeck & Richard, 1995. Holotype MNHN 2570, H 41.5 mm. (B, C) Chesterfield, (B) MNHN IM200730836 (42.5 mm), (C) MNHN IM200730842 (50 mm). (D, E) Vanuatu, (D) MNHN IM200730660 (31 mm), (E) MNHN IM200730823 (30.7 mm), (F) MNHN IM200730666 (29.4 mm) (G–I) Philippines, (G) Aliguay island (38 mm), (H) Aliguay Island (33 mm), and (I) Aurora 2007, station CP2666 (32 mm). Photos C. Reyens (A).

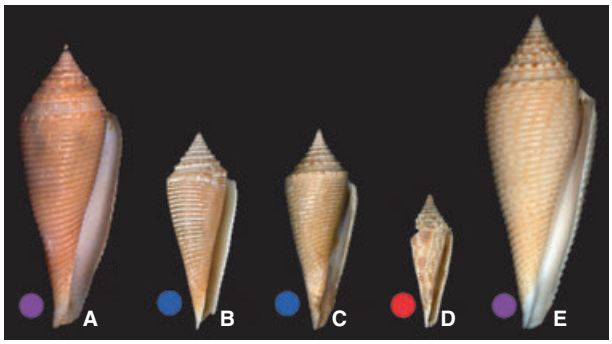

Fig. 8 Specimens assigned to the *C. pseudorbignyi* complex, (A) *Conus pseudorbignyi* Röckel & Lan, 1981. Holotype TMT. H: 45.5 mm, (B, C) Philippines, (B) MNHN IM200730899 (31.5 mm), (C) Aurora 2007, station CP2760 (32 mm), (D) Vanuatu, MNHN IM200730942 (21.2 mm), and (E) Taiwan, Olivera collection, *C. pseudorbignyi* (50 mm).

No molecular data are available for these Taiwanese specimens of *C. pseudorbignyi* to assess their genetic relationship to the Philippine and Vanuatu forms. We provisionally assign all of the specimens as shown in Fig. 8 to *C. pseudorbignyi*, despite the divergence in shell morphology. A specimen figured by Wilson (1994) from deep water of Western Australia, and identified as *C. orbignyi*, likely belongs to the *C. pseudorbignyi* complex. This assignment would extend the distribution of *C. pseudorbignyi* in the southern hemisphere over a much wider range than has previously reported, from Vanuatu to Port Hedland, Western Australia.

Group 4 – *Conus joliveti*. A single specimen from the Solomon Islands forms a discrete branch in both trees (Figs 3 and 4). The taxonomic identity of this form merits discussion. The specimen in Fig. 6B appears morphologically closest to a specimen illustrated by Moolenbeek *et al.* (2008) from Fiji that these authors assigned to *C. pseudorbignyi* (see Plate 3, fig. 24 of that article). However, the specimen illustrated seems very different from *C. pseudorbignyi*, the holotype of which is from Taiwan, and is morphologically distinct from the specimen here figured from the Solomons. In the same article, the authors describe a new species, *C. joliveti*. This form appears more closely related to the specimen labelled *C. pseudorbignyi* than does *C. pseudorbignyi* from Taiwan. It is clear that because only one specimen is available, more material – notably topotypical material from Fiji – needs to be analysed before the identity of this species can be definitively established. We tentatively designate this branch as *C. joliveti*.

Group 5 – *Conus orbignyi*. The morphology and geographical location of specimens from both Taiwan and the

Philippines are consistent in their nodulose sutural ridges with alternating brown blotches on the spire, their slender body whorl outline and the brown banding pattern with assigning these to *C. orbignyi* (Fig. 6Q–W). Within the Philippines, live specimens were analysed from both Panglao Island, in the Central Philippines, and the Aurora 2007 expedition (Table 1).

Group 6 – *Conus elokismenos*. Three morphologically and geographically distinct classes of specimens are included in this group: a single specimen from Madagascar, similar to the name-bearing type of the subspecies *C. o. elokismenos*, a single specimen from the Solomons, and a majority of specimens from Vanuatu (see Fig. 6J–P). The specimens from Vanuatu are morphologically distinct from the Solomon Island and Madagascar specimens, being less nodulose, and with a more restrained pattern of brown markings in the body whorl, without the distinctive bold dashes on the ribbons characteristic of both the Solomon Island and Madagascar specimens. Furthermore, *C. orbignyi* is not reported from Western Australia (Röckel *et al.* 1995), and the discontinuous distribution of *C. elokismenos* is thus probably real. Together with the high geographical distances, particularly between Madagascar and the South Pacific, this would justify the separation of the various forms into subspecies, or even species. Tentatively, we designate the diverse morphological forms in this group as the '*C. elokismenos* complex'.

Aurora 2007 empty shells: field collection data

A total of 110 adult shells were examined and, on the basis of shell morphology, were tentatively assigned to one of the classes in the *C. orbignyi* complex (Table 2). The allocation of juvenile shells to one or another form was too uncertain, and these are not listed in Table 2. Most of the specimens tentatively assigned to a specific taxon in Table 2 ($N = 93$) correspond to the typical *C. orbignyi* form from the Philippines (Figs 5A and 6R–W). These are strongly nodulose, with the characteristic darker brown bands on a body whorl and a lighter brown ground colour. As explained before, several dead shells (12) presented the same morphology as the specimens collected in Vanuatu and are assigned to *C. coriolisi* (Figs 5B and 6E–I). These have a broader body whorl, a more continuous banding pattern and a distinctly whitish ground colour. Five other specimens were characterized by the typical morphology of *C. pseudorbignyi* from the Philippines (Fig. 5E). These were far less nodulose, with finer spiral sculpture in the body whorl and the brown bands present in the other two forms were absent or obsolete.

All specimens attributed to *C. orbignyi* and *C. coriolisi* were found deeper than 150 m. *C. coriolisi* always co-occurred in the same station as *C. orbignyi*. One specimen of *C. pseudorbignyi* was also collected together with *C. orbignyi* and *C. coriolisi* in a single station, but other specimens of *C. pseudorbignyi* were from stations shallower than 150 m in depth.

Discussion

Success and limits of the molecular approach

The combination of the results obtained with the mitochondrial *COI* gene and the nuclear *ITS2* gene defines at least six distinctive genetic groups within the *C. orbignyi* complex. With both genes, each group is characterized by intra-group distances lower than intergroup distances, and the monophyly of each group is supported when multiple specimens were analysed. It is thus reasonable to consider that each of these six genetic groups corresponds to a different species.

However, we cannot exclude the possibility that some of these groups are themselves species complexes comprising several species, in particular for group 6. The genetic distance between the specimen collected in Madagascar (MNHN IM20097513) and the others is approximately 5–6%, and it could thus be considered as a different species (this exceeds the interspecific thresholds that have been reported for molluscs – Meyer & Paulay 2005; Mikkelsen *et al.* 2007; Duda *et al.* 2008). The alternative hypothesis would be to consider group 6 as an allopatrically structured super-species complex, widely distributed across the southern Indo-Pacific, with a considerable genetic distance between disjunct populations, and adjacent geographical populations still able to exchange genetic material (Knowlton 2000; Klanten *et al.* 2007; Yu & Chu 2006; but see Rhodes *et al.* 2003; Rocha 2004; Meyer *et al.* 2005). The presence or absence of genetically related populations in the intervening geographical areas is a key factor to be evaluated. At the present time, because of the depth range and scarcity of the species involved, the dataset is insufficient to address these hypotheses.

The paucity of the sampling is also certainly responsible for the profile of the histogram of COI distances. In our conservative approach, we considered that the second and third modes of the histogram (Fig. 2) correspond to intraspecific distances. In this case, increasing the sampling by including more specimens from different regions within each species could potentially merge the first three modes into a single one. As emphasized previously, especially in the DNA barcoding literature, the quality of the sampling (both in terms of number of specimens and geographical areas sampled) is a key issue to accurately recover – or discover – species boundaries (Moritz & Cicero 2004; Meyer & Paulay 2005; Eckert *et al.* 2008; Monaghan *et al.* 2009). Although resulting from several years of biodiversity exploration by MNHN (Bouchet *et al.* 2008), our sam-

pling still remains patchy. All the species analysed here live in deep water, and most of them are rare to uncommon. Delimiting species with such uneven sampling is currently one of the main challenges for the molecular taxonomist (Morando *et al.* 2003; Knowles & Carstens 2007; Bouchet *et al.* 2009; Monaghan *et al.* 2009), but pending better methods to discriminate between the two alternative hypotheses 'one broad species with divergent populations' and 'several allopatric species' a conservative approach as applied here seems to be the most prudent course.

Another limitation of our results, not linked to the quality of the sampling but to the taxa analysed, is the lack of resolution using the *ITS2* gene. The monophyly of group 5 is not established using this gene, as group 6 is included in paraphyly, even if several mutations and one indel clearly separate the two groups. Incomplete lineage sorting can explain this pattern (Maddison & Knowles 2006; Elejalde *et al.* 2008), and this could be tested by analysing a more variable nuclear gene. However, the *Conus* genome remains poorly known, and apart from the classic nuclear genes (e.g. 28S, H3, 18S), used to resolve deeper phylogenetic relationships (e.g. Colgan *et al.* 2003, 2007; Puillandre *et al.* 2008) and thus less variable than the *ITS2* gene at the species level, only conotoxins have been used in a similar study (Duda *et al.* 2008). We tested the conotoxin primers used by Duda *et al.* (2008) in the *orbignyi* complex, but because of the high divergence between the complex they studied (*C. sponsalis*, included in the large major clade – Duda & Kohn 2005) and the *orbignyi* complex (included in the small major clade), no amplicons were obtained. Amplifying conotoxin genes from genomic DNA in the small major clade will require developing a new set of primers.

A revised taxonomy for the C. orbignyi complex

By carrying out a combination of a molecular phylogenetic analysis of all available specimens and a morphological survey of material collected at a single site (off Aurora, Luzon Island in the Philippines) and more generally within the Philippines, we provide taxonomic resolution within the *C. orbignyi* complex and gain insights into the biogeography of the putative species. One surprising discovery is that *C. comatosa* is a nested member within the complex. This phylogenetic position implies that the smooth sutural shoulder that differentiates this species from the others is derived from a nodulose plesiomorphic state. Specimens in the *C. orbignyi* clade analysed in this study would have been included in four different species based on shell characters, *C. orbignyi*, *C. pseudorbignyi*, *C. joliveti* and *C. comatosa* (Röckel *et al.* 1995; Moolenbeek *et al.* 2008). *C. orbignyi* was believed to have a discontinuous distribution, with three potential subspecies, *C. o. orbignyi*, *C. o. elokismenos* and *C. o. coriolisi*. Based on the molecular results, we elevate the three subspecies to species rank (*C. orbignyi*, *C. elokismenos* and *C. coriolisi*), two of which coexist in the SW Pacific. Each of these six taxa is discussed in turn.

Two species included in the redefined *orbignyi* complex are represented in the molecular dataset by a single specimen: *C. comatosa* and *C. joliveti*. The recently named *C. joliveti* has been described from material collected in Fiji, and whether or not it is molecularly similar to specimen MNHN IM200730925 identified as *C. joliveti* remains to be assessed. *C. comatosa* is a well-known species, which, unlike other forms in the clade, does not appear to show great geographical variation. The Chesterfield specimen analysed herein is virtually identical to specimens of *C. comatosa* from the Philippines. Unfortunately, no live specimens of *C. comatosa* from the Philippines were available to include in the molecular analysis.

Specimen MNHN IM200730899 (Fig. 6D) matches the illustration of *C. pseudorbignyi* from the Philippines by Röckel *et al.* (1995). However, specimen MNHN IM200730942 from Vanuatu (Fig. 6C), placed in the same genetic group and thus putatively in the same species, seems to have shell characters divergent from *C. pseudorbignyi* from the type locality Taiwan, and a number of distinctive morphological differences from the Philippine specimens as well (see Fig. 5E). Geographical variation was already apparent in the illustration of *C. pseudorbignyi* in Röckel *et al.* (1995) (Plate 56); the Taiwanese specimens are distinctive from both the Philippine specimen as well as from the specimen from Sulawesi. Molecular analysis of *C. pseudorbignyi* from Taiwan (the type locality) is clearly desirable to confirm our assignment of these specimens to *C. pseudorbignyi*. Thus, there appears to be morphological divergence between *C. pseudorbignyi* specimens from Taiwan, Aurora and Vanuatu.

We regard *C. orbignyi* as a rather variable species (Figs 5A and 6Q–W), and the results of the Aurora expedition suggest that at this site, this species is only found in water deeper than 150 m (Table 2). It is likely to be present at a variety of Philippine sites, but is not common in commercially available material; thus the recent book on Philippine molluscs by Poppe (2008) does not illustrate any true *C. orbignyi* as defined by the molecular phylogeny. A typical specimen of *C. orbignyi* from the Philippines has been illustrated earlier by Springsteen & Leobrera (1986) and variation in typical *C. orbignyi* was illustrated by Röckel *et al.* (1995)(Plate 56, specimens 2–4 from Taiwan and Philippines). None of the specimens from southern hemisphere localities was found to belong to this species, suggesting that *C. orbignyi* is a northwestern Pacific species,

found from the Philippines to Japan. During the Aurora 2007 expedition, this was one of the most frequently collected species in the Conidae.

Surprisingly, distant from *C. orbignyi* on the basis of molecular phylogeny is the form previously called *C. orbignyi coriolisi*. We suggest that this is not only a distinct species from *C. orbignyi*, but also one that is morphologically variable as well (Fig. 6E–I). At the present time, specimens assigned to *C. coriolisi* occur in the Western Pacific from Fiji to Queensland, and north to the Central Philippines. Our assignment of Philippine specimens to this species needs to be verified by molecular data. However, these specimens are clearly distinct from the typical *C. orbignyi* and have a much closer morphological similarity to specimens of *C. coriolisi* from Vanuatu (Figs 5A, B and 6E–I). Two of the specimens assigned to *C. orbignyi* by Poppe (2008) (Plate 641, fig. 7a and 7b) as well as one of the specimens illustrated by Springsteen & Leobrera (1986) (Plate 71, fig. 13, rightmost specimen) appear to be *C. coriolisi*. The specimen illustrated in Röckel *et al.* (1995) as *C. coriolisi* is a typical Coral Sea specimen, morphologically similar to the Chesterfield specimens analysed in this study. However, these authors also illustrate a specimen from Queensland assigned to *C. orbignyi* that is similar to *C. coriolisi* from Vanuatu. Thus, *C. coriolisi* appears to be a distinctive species with consistent morphological variation that overlaps with *C. orbignyi* in its geographical range in the Philippines. Based on the specimens examined herein, Chesterfield individuals possess a white ground colour, instead of light brown; and the brown markings are largely restricted to three bands. A distinctive form from Aliguay Island, Philippines, tentatively assigned to *C. coriolisi*, is pure white and highly nodulose; the shell morphology seems most closely related to *C. coriolisi* (see Fig. 5B) than to any other species in the clade, but molecular data may reveal that this is a distinct form.

C. elokismenos is now elevated to species status. Formerly, regarded as a southeast African/Madagascar form, molecular data indicate that specimens in the Solomon Islands and Vanuatu are genetically allied to the Madagascar material. Thus, while *C. orbignyi* is the northern hemisphere species in the complex, the morphologically similar *C. elokismenos* clade exists in the southern hemisphere. As circumscribed herein, there may be a justification for dividing *C. elokismenos* into different subspecies or even different species (as discussed above), turning the *C. elokismenos* clade into a species complex itself. Further sampling would be necessary to justify this step.

Conclusion

The analysis carried out in this study on a clade of cone snails highlight why the taxonomical assignment at the species level based purely on morphology has been extraordinarily challenging for many hyper-diverse lineages. There were four previously recognized species in the complex we have analysed, but we have demonstrated that additional species should be recognized. Some of these species are distributed over a broad geographical area; whereas others appear more geographically restricted. More unexpectedly, the results show that some taxa appear to exhibit a larger geographical morphological variation than others, whereas mode of larval development – and thus inferred dispersal capacity – is the same (planktotrophic) throughout the species complex. However, we refrain from speculating too much on this issue because (a) our specimen sampling is still rather thin, and uneven across the terminal taxa involved (e.g. a single specimen of *C. joliveti* vs. seven specimens of *C. orbignyi*) and (b) some of the taxa here recognized as species (*elokismenos*) may themselves be species complexes.

A useful approach to understanding a complex such as the one analysed in this study is to focus on the morphologically divergent forms found in a single locality, such as was provided by the material from the Aurora 2007 Expedition. At this locality, three clearly separable morphospecies could be unambiguously differentiated (see Fig. 5A, B and E). Two of these were recovered alive, and as expected, they showed a genetic divergence that corresponded to the morphological divergence at this locality. Dead shells from a third distinct morphospecies were recovered, but lacked tissue for genetic testing. All three forms found at Aurora also occur at other localities, and to different degrees, they vary geographically. This intraspecific divergence in morphology as a function of geography in species complexes that are morphologically similar confounds the taxonomy based on morphology alone. To accurately provide a taxonomy for hyper-diverse groups, understanding inter-regional morphological variation in the context of molecular data is highly desirable. Potentially, the situation can be resolved through an intensive analysis of a few geographically separated localities, where the species complex occurs. Thus, expeditions such as Aurora in 2007 and Santo in 2006 provide the type of material that can help elucidate species complexes in hyper-diverse groups such as the Conidae and beyond.

Acknowledgements

This work was supported in part by Program grant GM48677 from the US National Institute of General Medical Sciences (to BMO) and by ICBG grant 1U01TW008163 from the Fogarty Centre, NIH, Margo Haygood, Principal Investigator. Key material originates from the following expeditions. (A) Philippines. The PANGLAO 2005 cruise on board M/V *DA-BFAR* associ-

ated the USC, MNHN (co-PI Philippe Bouchet) and the Philippines Bureau of Fisheries and Aquatic Research (BFAR; co-PI Ludivina Labe). The AURORA 2007 cruise also on board M/V DA-BFAR associated the National Museum of the Philippines (NMP, co-PI Marivene Manuel), MNHN (co-PI Philippe Bouchet) and BFAR, and was made possible through a grant from the Lounsbery Foundation. (B) Vanuatu. Material originates from the MNHN-IRD-PNI Santo 2006 expedition, which was made possible by grants, among others, from the Total Foundation and the Niarchos Foundation. (C) Coral Sea and Solomon Islands. The EBISCO and SALOMON cruises took place on board R/V *Alis* deployed from Nouméa by the Institut de Recherche pour le Développement (IRD). Bertrand Richer de Forges was cruise leader for the Solomons, Coral Sea and Vanuatu expeditions. Ellen Strong and Yuri Kantor are thanked for their role in molecular sampling during these expeditions. The authors thank Virginie Héros for assistance in monitoring Aurora 2007 material and Hans-Jörg Niederhöfer (Staatliches Museum für Naturkunde Stuttgart), Virginie Héros (Muséum National d'Histoire Naturelle, Paris) and Paul Callomon (Academy of Natural Sciences, Philadelphia) for their help in providing pictures of type specimens.

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