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## **Renewed taxonomy : phylogeny and species delimitation in an integrative framework**

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### **Abstract**

*The vast diversity of species included in the genus Conus and in the superfamily Conoidea make them one of the most promising taxa for the discovery of new toxin-derived drugs. To rationalize the search for new peptides, integrative taxonomy can be used to quickly identify new lineages that potentially evolved new toxins. In this context, molecular characters are now a major tool, whether for specimen identification ("barcoding"), species delimitation (alpha-taxonomy) or phylogeny. At the species level, different criteria have been successfully applied to analyze DNA sequences and propose hypotheses of species delimitation in the genus Benthomangelia that are supported by other characters such as morphology. At the phylogenetic level, molecular characters were also used to define highly divergent lineages within Conoidea and to infer their relationships. The methodology used, based on clearly stated concepts and integrating different criteria and characters allow the definition of robust, reproducible and testable hypotheses. Furthermore, discriminating these lineages (families, subfamilies, genera or species) is of great value in toxin research, as illustrated with the family Terebridae where three different lineages have been identified each of them susceptible to have evolved their own set of toxins.*

### **Le renouveau de la taxonomie : phylogénie et délimitation d'espèces dans un contexte intégratif**

*La grande diversité spécifique incluse dans le genre Conus et dans la superfamille des Conoidea font de ces groupes certains des plus prometteurs pour la découverte de nouvelles thérapies dérivées de toxines. Pour rationaliser la recherche de nouveaux peptides, une approche de taxonomie intégrative peut permettre d'identifier rapidement des lignées évolutives qui auraient potentiellement développé de nouvelles toxines. Dans ce contexte, les caractères moléculaires sont devenus un outil majeur, que ce soit pour l'identification de spécimens (« barcoding »), la délimitation d'espèces (alpha-taxonomie) et la phylogénie. Au niveau spécifique, différents critères ont été appliqués avec succès pour analyser des séquences ADN et proposer des hypothèses de délimitation d'espèces au sein du genre Benthomangelia, hypothèses soutenues par d'autres caractères (morphologiques). Au niveau phylogénétique, les caractères moléculaires ont été également utilisés pour définir différentes lignées au sein des Conoidea. La méthodologie utilisée, basée sur des concepts clairement établis et intégrant différents critères et caractères, permet de proposer des hypothèses robustes, reproductibles et testables. De plus, discriminer ces lignées (familles, sous-familles, genres et espèces) est important pour la recherche de toxines, comme le montre l'analyse de la famille des Terebridae, où trois différentes lignées ont été identifiées, chacune susceptible d'avoir développé des toxines uniques.*

**Keywords** : Conoideans, conotoxin, DNA barcoding, integrative taxonomy, molecular phylogeny.

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## **Introduction**

Cone snails are certainly one of the most diverse genus of marine invertebrates, with more than 600 described species (Kohn, 1990 ; Duda and Kohn, 2005). Their ornamentation, their accessibility and the "deadly" fascination for these venomous animals make this group one of the most famous in the naturalist collectors community. Furthermore, cone snails are also of great scientific value particularly because of the therapeutic applications of their toxins. Several different *Conus* peptides are now being clinically tested and one is currently commercialized under the name Prialt (Olivera, 2006). These toxins are also characterized by their huge diversity, as each species displays its own set of 100-200 specific toxins, which lead to the estimated number of 60,000-120,000 different toxins in this single genus.

Several species have already been investigated and several hundreds of toxins are now described. Known as conotoxins, they all share a common structure (Olivera, 2008) and are classified in ~ 20 superfamilies. Some of these toxins are functionally characterized, and a huge diversity of molecular targets has been found (Terlau and Olivera, 2004), echoing the vast potential therapeutic applications of these conotoxins.

However, despite the considerable amount of data accumulated, only a few *Conus* species have been analyzed proportionately. Furthermore, recent phylogenetic analyses have shown that most of the analyzed species are concentrated in a few clades within *Conus* and are thus not representative of the diversity of the genus (Olivera, 2008 ; unpublished results). To balance this bias, Olivera proposed a “concerted discovery” strategy, where modern taxonomic tools, such as molecular phylogeny and integrative taxonomy, could be used to identify independent lineages within *Conus*, where different toxins may have evolved, and thus accelerate the discovery of new toxins (Olivera, 2006).

To do so, a good knowledge of the taxonomy of the group is necessary, which is not currently the case. Traditionally, all cone snails are grouped in a single genus (*Conus*), though several authors have attempted to separate it into several genera (e.g. da Motta, 1991). Recent molecular phylogeny analyses (see Olivera, 2008, *Figure 1*) confirm that several highly divergent lineages are actually included in *Conus*. Investigating toxin diversity in these divergent lineages should lead to the discovery of new toxins. Recent results (e.g. Watkins *et al.*, 2006 ; Imperial *et al.*, 2007) also have shown that *Conus* is only the tip of the iceberg. Actually, *Conus* are included in the superfamily Conoidea, considered as the most diverse groups of marine molluscs (Bouchet *et al.*, 2002). With an estimated species number over 10,000, revealed particularly by recent cruises set up by the Muséum National d'Histoire Naturelle de Paris (Bouchet *et al.*, 2008), conoidean toxins could reach the unlikely number of one or two million...

The potential is evident, but how can we optimize the toxin discovery in such a diverse group? Which species should be investigated first? The “concerted discovery” strategy seems appropriate, but one should not ignore the difficulty encountered by malacologists in their attempt to classify the conoideans. Turrids, one of the three traditionally recognized groups within Conoidea, together with Conidae and Terebridae, is considered “a taxonomic nightmare” (e.g. Tryon, 1884). Whatever the taxonomic level considered (families, subfamilies, genera, species), taxonomists do not agree and contradictions are common : what will be considered as intraspecific variability for one of them will be considered as different species for another. With conoideans, and to some extent with molluscs, taxonomists face two major problems. First, most – if not all – species’ descriptions are based on shell characters. Several studies have shown the plasticity of such characters : they may reflect environmental differences rather than differences between species (Hollander *et al.*, 2006 ; Brookes and Rochette, 2007). Second, taxonomical practices do not allow a clear scientific evaluation of the groups delimited. Species are more opinions than testable hypotheses : specimens are grouped in species using non-formalized characters (a problem also linked to the use of shell characters), generally impossible to reproduce.

Thus, the challenge for taxonomy is to define a new methodology based on proposing hypotheses that are reproducible and testable (Wiens, 2007). Furthermore, as morphological characters are clearly not adapted, new characters will have to be analysed in an integrative framework. I propose here to review some taxonomical practices that are proposed to overcome these difficulties. First will be described the not-so-new character now commonly used in taxonomy : DNA, and its use as an identification tool (*i.e.* the barcoding). Then, methodological and conceptual issues at both the phylogenetic (families and subfamilies) and species levels will be presented, each illustrated by several case studies in conoideans.

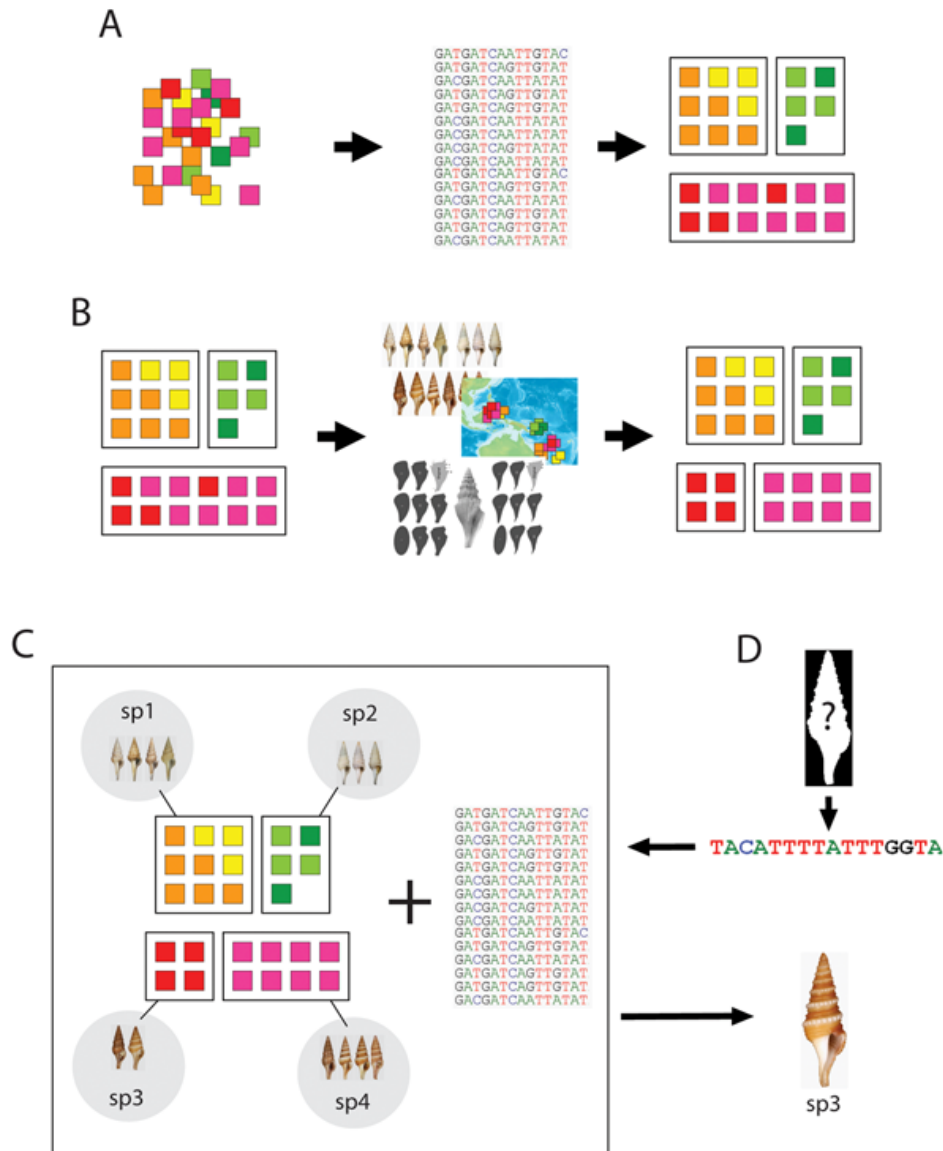
## Molecular characters and barcoding

Although DNA has been applied in taxonomy since the 1980's, its use still remains unconventional. Most species’ descriptions are still primarily based on morphological characters, and DNA is generally restricted to an *a posteriori* confirmation of the species delimitation (e.g. Reid *et al.*, 2006 ; Meyer and Paulay, 2005). However, DNA has several advantages over morphological characters. One of the most important advantages being, by definition, that it is genetically determined : DNA differences are supposed to reflect species boundaries and not environment variability. Furthermore, DNA sequences are now easily accessible and can provide a high number of formalized characters. These advantages have been highlighted recently by Hebert *et al.* (2003) when he proposed to use short DNA sequences as a “molecular barcode” to identify unknown specimens (*Figure 1D*). The principle is not new, but the novelty of the barcoding project resides in the standardization of the technique *i.e.* the same gene fragment, a ~650 bp fragment of the Cytochrome Oxidase I (COI), should be used for all animals.

The barcode system lies on one major principle : the COI sequence of a given specimen is more similar to the COI sequences of conspecific specimens than specimens of other species. The goal of most DNA barcode projects (see [www.barcodinglife.org](http://www.barcodinglife.org)) is to test this assertion by sequencing several specimens of all the species for a given taxa. In most cases, the intraspecific variability is less than the interspecific variability. These sequences can then constitute the database used to identify an unknown specimen; the most similar sequence in the database will provide the species name.

In order to identify marine gastropods’ egg capsules collected in the Philippines in 2007, we used a barcoding approach by comparing mitochondrial sequences (the COI gene, but also fragments of the 12S and 16S genes) to two online databases : BOLD (Barcode Of Life Database) and GenBank (Puillandre *et al.*, 2009b). Two methods were used : the BLAST algorithm based on the similarity between sequences and a phylogenetic analysis where all sequences from GenBank were combined with the unknown ones to construct a phylogenetic tree (bayesian analysis, Huelsenbeck *et al.*, 2001). This methodology has been tested with a known egg capsule (*Erosaria spurca*, Cypraeidae), first identified due to the proximity of the adults (*Figure 2A*) The first hit of the BLAST analysis and the sister-group of the “unknown” sequence were a sequence of *E. spurca*. Several egg capsules were identified this way, and some examples are shown in *Figure 2*. Some identifications were not surprising as the egg capsule morphology is somewhat characteristic of the corresponding group (*Figure 2B*), but others were more unexpected such as the *Granulifusus* (*Figure 2C*) that displays a morphology never found in Fascioliariidae (Knudsen, 1950). However, most egg capsules were not identified at the species level but only at the genus or even familial level. The database’s incompleteness,

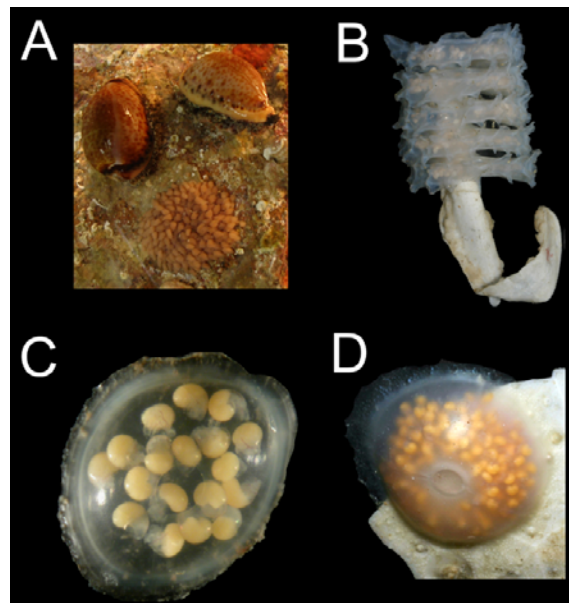
where some molluscan groups are not represented at all, may explain this result.



**Fig. 1.** Integrative taxonomy and Barcoding. **(A)** Several specimens are sequenced and placed in different primary hypotheses of species delimitation. **(B)** These hypotheses are then evaluated with other data (morphology, geography, ecology, etc) and may be modified. **(C)** Each delimited species is then associated in a database to specimens of references ("vouchers") and to their corresponding DNA sequences. **(D)** The DNA sequence from an unknown specimen can then be compared to the sequences included in the database. The most similar sequence will give the species identification.

**Fig. 1.** Taxonomie intégrative et barcoding. **(A)** Plusieurs spécimens sont séquençés et placés dans différents groupes homogènes. **(B)** Ces groupes sont ensuite discutés en analysant d'autres données (morphologie, géographie, écologie...), et peuvent être modifiés. **(C)** Chaque espèce délimitée est ensuite associée dans une base de données à des spécimens de références ("vouchers") et à leurs séquences ADN correspondantes. **(D)** La comparaison d'une séquence ADN d'un spécimen inconnu permet d'identifier à quelle séquence elle ressemble le plus, et donc à quelle espèce le spécimen appartient.

Thus, DNA Barcoding seems to be a powerful tool for species identification. Contrary to morphological characters, it does not need any knowledge about the taxa and can be used whatever the developmental stage (eggs, larvae, adults, etc) or the tissue available (Janzen *et al.*, 2005). However, several limits have been highlighted most of them being linked to the gene chosen : (i) a nuclear copy of the COI can be sequenced instead of the mitochondrial gene (Moritz and Cicero, 2004 ; Lorenz *et al.*, 2005), (ii) the COI gene does not always fulfill the requirement of a molecular barcode as in amphibians where the intraspecific variability is equivalent to the interspecific variability (Smith *et al.*, 2008), or in cnidarians where the intraspecific variability is null (Shearer and Coffroth, 2008), and (iii) the gene tree obtained with the COI (or with another mitochondrial gene) can be different from the species tree (Nichols, 2001 ; Rosenberg and Tao, 2008) because of problems of ancestral polymorphism for example (Fu and Zeng, 2008).



**Fig. 2.** Some egg capsules identified using DNA barcodes. (A) *Erosaria spurca*, Cypraeidae (egg capsule with adults). (B) *Conus* sp., Conidae. (C) *Granulifusus* sp., Fasciolariidae. (D) *Comitas* sp., Turridae.

**Fig. 2.** Quelques capsules d'œufs identifiées par leur Barcode ADN. (A) *Erosaria spurca*, Cypraeidae (œufs et adultes). (B) *Conus* sp., Conidae. (C) *Granulifusus* sp., Fasciolariidae. (D) *Comitas* sp., Turridae.

For all these reasons, many authors claim that DNA barcoding is only an identification tool powerful when species are clearly differentiated with the COI gene (e.g. Ebach and Holdredge, 2005 ; Godfray, 2006). However, DNA barcoding can be used to discover cryptic species. This has been the case, for example, in already well-known groups, such as birds or lepidoptera (Hebert *et al.*, 2004 ; Hajibabaei *et al.*, 2006 ; Vaglia *et al.*, 2008). In these cases, the DNA barcode is used to point out problematic cases, but should not be used solely to describe new species. New standards for taxonomy are now being developed, and if they include the use of molecular data such as DNA barcodes, they also advocate their association with the analysis of other characters and criteria in an integrative framework.

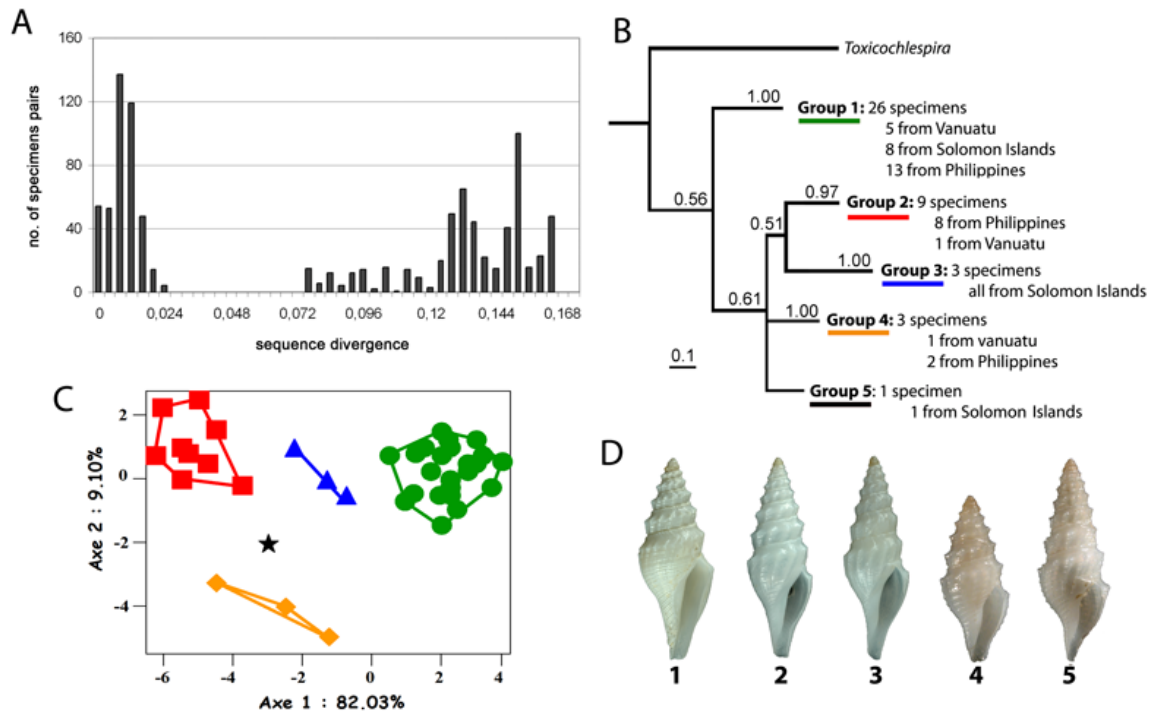
### Integrative alpha-taxonomy

Defining species is certainly one of the most controversial topics in biology. There are dozens of concepts of species available in literature (reviewed in Agapow *et al.*, 2004 or in de Queiroz, 2007), but as de Queiroz (1998) first pointed out, they all correspond to properties shared by some but not all species, and not to a definition that can be applied to all organisms. He thus proposed a unified concept, formalized by Samadi and Barberousse (2006), where species are considered as definitely divergent lineages, known as the Lineage Species Concept (LSC). Because recovering these lineages would necessitate tracing back the network of all relationships between all individuals (who are the parents of who?), which is impossible, the taxonomist will have to indirectly infer this network using different species properties (the so-called "species concepts"). In this framework, species will thus be hypotheses constantly engaged in a process of modification/validation as new characters are analyzed and new criteria applied.

From the LSC, three criteria for defining species can be derived : (i) a species is a reproductive community that share a common gene pool. As a consequence, conspecific specimens will look similar; (ii) specimens from the same species are able to reproduce together, a criterion that can be tested directly (cross tests) or indirectly (no gene exchange between different species); (iii) all the specimens from the same species share a common history and should thus correspond to a monophyletic lineage in a phylogenetic tree. Taxonomists generally agree now that species delimitation should be based on several criteria, as some species may not be found if only one criterion is applied. For example, species that are too recent will share common haplotypes if they did not have enough time to accumulate differences (ancestral polymorphism). A phylogenetic analysis will not successfully recover the species boundaries in this case but another criterion might. Furthermore, the more characters that are analysed, the more supported the hypotheses will be. Even if some characters seem more appropriate (DNA for example), none of them will be infallible, as we saw earlier, and the combination of molecular, morphological, ecological, behavioural, etc, characters is now advocated by most taxonomists (Figures 1A and B). Optimizing the number of criteria and characters to propose testable and reproducible hypotheses of species delimitation constitutes the standards of the integrative taxonomy (Sites and Marshall, 2003 ; Dayrat, 2005 ; Vogler and Monaghan, 2007 ; Wiens, 2007).

Following these rules, we analyzed the species diversity in the genus *Benthomangelia* (Puillandre *et al.*, 2009a). This genus is included in the subfamily Mangeliinae, closely related to the genus *Conus* (Taylor *et al.*, 1993 ; Puillandre *et al.*, 2008). Several species are already described in *Benthomangelia*, using only morphological characters. Our strategy was to sequence the COI barcode gene of 42 specimens available in the Muséum National d'Histoire Naturelle (MNHN) of Paris, collected recently in South-West Pacific, without any *a priori* hypotheses of species delimitation. As shown in Figure 3A, the analysis of genetic distances

between all specimens reveals a gap between low and high genetic distances. This gap has been used to define five entities, each including specimens separated by low genetic distances. A phylogenetic approach, using both likelihood (PhyML ; Guindon and Gascuel, 2003) and bayesian (Mr. Bayes ; Huelsenbeck *et al.*, 2001) methods found the same five entities (Figure 3B). These entities were also recovered with an independent nuclear gene, a fragment of the 28S, confirming the lack of genetic exchange between them. The three criteria (similarity, common evolutionary history and genetic exchange) are successfully applied to all five entities.



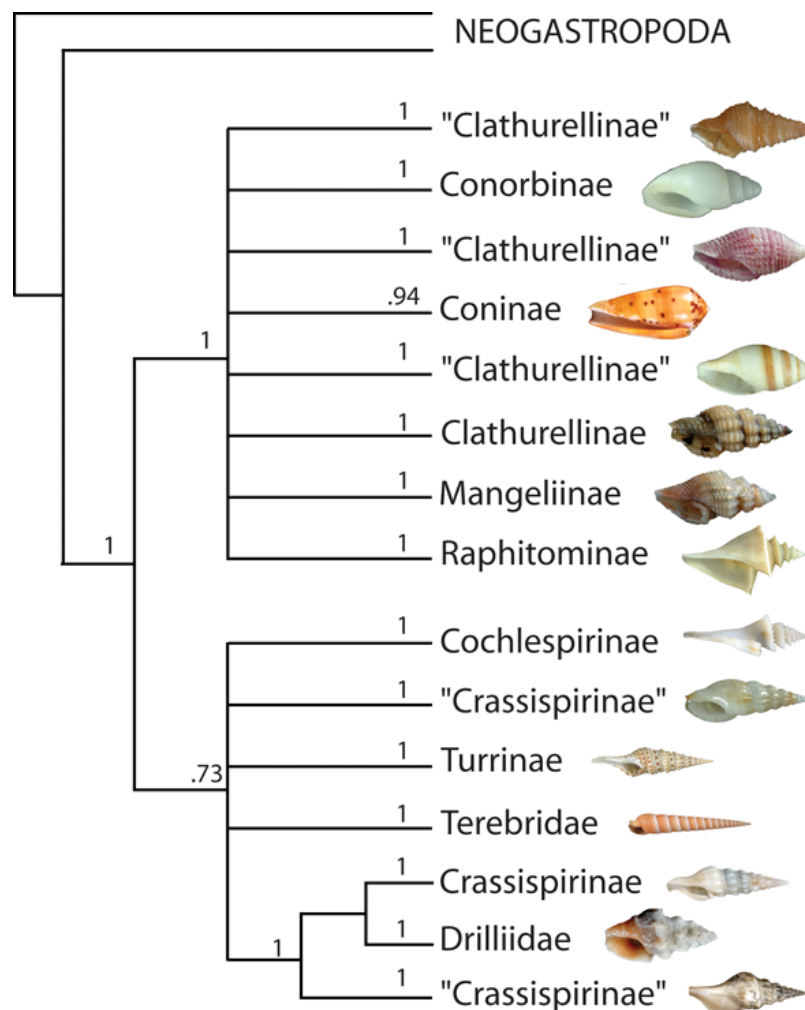
**Fig. 3.** Species delimitation in *Benthomangelia*. (A) Histogram of genetic distances calculated between all sequences. (B) Phylogenetic tree obtained with the COI gene. Five groups are delimited (intra-groups relationships are not shown). (C) Discriminate analysis between genetic groups. (D) One shell is illustrated for each delimited group.

**Fig. 3.** Délimitation d'espèces au sein du genre *Benthomangelia*. (A) Histogrammes des distances génétiques calculées entre chaque paire de séquences. (B) Arbre phylogénétique obtenu avec le gène COI (les branchements au sein des groupes ne sont pas montrés). (C) Analyse discriminante entre les groupes génétiques. (D) Chaque groupe est illustré par une coquille.

We also performed a morphological analysis on the same 42 specimens. The outline of the last whorl of each shell was reconstructed using an Elliptic Fourier Analysis (EFA) (Rohlf, 1996). This approach is supposed to detect slight differences in shape often not detectable by eye. A discriminate analysis, used to maximize the morphological variability between the five entities defined genetically, revealed strong differences in the shape of the last whorl (Figure 3C). However, morphological differences were also found between geographical region revealing a potential combined effect of genetics and environment on this morphological character. Nonetheless, finding morphological differences between the genetic groups strongly support our hypotheses of species delimitation. We also applied the same methodology to other conoidean groups (*Gemuloborsonia*, *Bathytoma*, Turrinae, etc), each time delimiting cryptic species that were not detected with morphological characters. This is particularly striking in the genus *Bathytoma*, where the species *B. attractoides*, thought to include most of the South-West Pacific variability (Sysoev and Bouchet, 2001), was separated in at least 10 different species (Puillandre *et al.*, submitted).

### Phylogenetic reconstruction

What is true at the species level ("alpha-taxonomy") is also true at higher taxonomic levels ("beta-taxonomy"). The use of molecular characters has changed our understanding of classification by revealing unexpected relationships and by clarifying conoidean evolution. The classification of conoideans (*e.g.* Thiele, 1929 ; Powell, 1942, 1966 ; McLean, 1971) traditionally recognizes three main families : Conidae, with the genus *Conus*, Terebridae ("auger snails"), and Turridae, a heterogeneous group more or less defined as "all conoideans except cone and auger snails". Taylor *et al.* (1993) completely reorganized this classification by including several subfamilies in Conidae previously placed in Turridae. Although most of the previous authors based their conclusions on the analysis of shell and radula characters, Taylor *et al.* mainly relied on anatomical characters. A molecular phylogeny based on several mitochondrial (COI) and nuclear markers (28S, 18S, H3) globally confirmed their results, as the family Turridae (*sensu lato*) was paraphyletic, *i.e.* it included the two other families of the group (Conidae and Terebridae), some turrids being more closely related to *Conus* than to other turrids (Figure 4) (Puillandre *et al.*, 2008).



**Fig. 4.** Molecular phylogeny of Conoidea. Posterior probabilities are given for each node.

**Fig. 4.** Phylogénie moléculaire des Conoidea. Les probabilités postérieures sont précisées pour chaque noeud.

This molecular phylogeny, recently updated by the inclusion of several taxa missing in the tree shown in *Figure 4*, will be used to prepare a new classification for conoideans (Puillandre *et al.*, in preparation). Actually, the genus *Conus* (*i.e.* the Conidae *sensu stricto*) and Terebridae are only two lineages among the fifteen currently recognized. A new classification will have to take into account this result and Turridae *sensu lato* will no longer be a valid family, as it includes lineages as divergent as Conidae and Terebridae could be. This result is not without consequence for the biochemist as each lineage may have evolved different toxins.

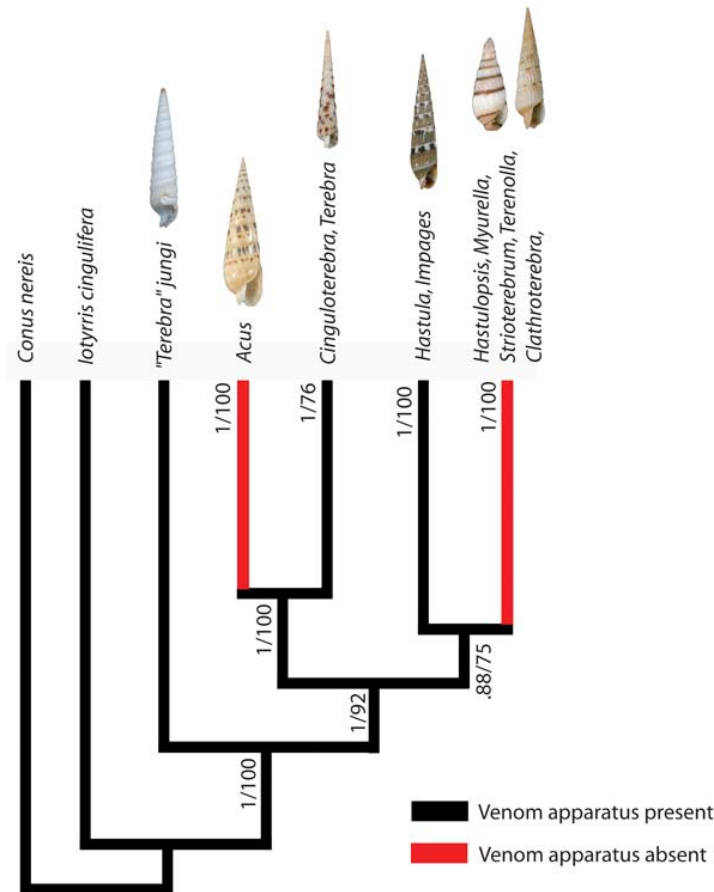
In another analysis, three mitochondrial genes (COI, 12S and 16S) were sequenced and used to infer the phylogenetic relationships between ~50 Terebridae species included in 10 different genera (*Figure 5*) (Holford *et al.*, 2009). Five major lineages were defined: the first included the species "*Terebra*" *jungi*, the second the genus *Acus*, the third the genera *Cinguloterebra* and *Terebra* (both of them being polyphyletic), the fourth the genera *Hastula* and *Impages* (*Impages* being included in *Hastula*), and the last included the genera *Hastulopsis*, *Myurella*, *Strioterebrum*, *Clathroterebra* and *Terenolla*, all of them except *Terenolla* being polyphyletic. We also mapped on the tree the presence and absence of the venom apparatus in most of the species included in the analysis. Two independent lineages do not possess a venom apparatus, suggesting that it has been lost twice independently during the evolution of Terebridae.

This result is of great value to facilitate the discovery of new toxins. The tree constitutes a real "map" to guide the biochemist. Three lineages are susceptible to have evolved different toxins. In particular, the species "*Terebra*" *jungi* - morphologically not suspected to be so different from the other auger snails - is actually the first lineage to diverge in terebrid evolution, and thus constitutes a good target for toxin investigation.

### Conclusion : taxonomy-based toxin discovery

This renewed taxonomy is thus both conceptual, as the taxonomic practices are replaced in a rigorous scientific framework, and methodological, through the use of a combination of several characters and criteria. For marine molluscs, and especially for conoideans, taxonomy and phylogeny integrating molecular characters are now causing a major shift in our perception of the classification and species diversity. At the

species level, morphological approaches generally led to an underestimation of the diversity, and the discovery of new species is promising for the search for new toxins. Analyses within *Conus* also indicate that some species actually include several cryptic species, each of them thought to produce its own sets of conotoxins (Duda *et al.*, 2008 ; unpublished results).



**Fig. 5.** Evolution of the venom apparatus in Terebridae. The molecular phylogeny defined five major lineages, each including several species not represented here. Posterior probabilities and bootstraps are given for each node.

**Fig. 5.** Evolution de l'appareil venimeux chez les Terebridae. La phylogénie moléculaire a permis de définir cinq lignées principales, chacune incluant plusieurs espèces non détaillées ici. Les probabilités postérieures et les « bootstraps » sont précisés pour chaque nœud.

Phylogenetic analyses also proved their utility, as we saw for examples in the family Terebridae. We furthermore performed a phylogenetic analysis of the A-superfamily of conotoxins within the *Pionoconus* clade (Puillandre *et al.*, submitted). Several lineages have been defined, each being the result of duplication events, followed by a rapid evolution by positive selection. Consequently, each defined clade has evolved its own function. This property could be used to accelerate the discovery of new toxins, by focusing the effort on lineages for which functions are still unknown and whose divergence with other lineages might have led to the apparition of unique functions. As illustrated by this direct correlation between the work of the taxonomist and the biochemist, we are entering in a new "gold age" for taxonomy that will not only benefit to a better knowledge, and thus a better conservation of the biodiversity, but also to most other researchers for whom taxa delimitation is a cornerstone.

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