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Short Communication

When everything converges: Integrative taxonomy with shell, DNA and venomic data reveals *Conus conco*, a new species of cone snails (Gastropoda: Conoidea)

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ABSTRACT

Cone snails have long been studied both by taxonomists for the diversity of their shells and by biochemists for the potential therapeutic applications of their toxins. Phylogenetic approaches have revealed that different lineages of *Conus* evolved divergent venoms, a property that is exploited to enhance the discovery of new conotoxins, but is rarely used in taxonomy. Specimens belonging to the Indo-West Pacific *Conus lividus* species complex were analyzed using phenetic and phylogenetic methods based on shell morphology, COI and 28S rRNA gene sequences and venom mRNA expression and protein composition. All methods converged to reveal a new species, *C. conco* n. sp. (described in Supplementary data), restricted to the Marquesas Islands, where it diverged recently (~3 mya) from *C. lividus*. The geographical distribution of *C. conco* and *C. lividus* and their phylogenetic relationships suggest that the two species diverged in allopatry. Furthermore, the diversity of the transcript sequences and toxin molecular masses suggest that *C. conco* evolved unique toxins, presumably in response to new selective pressure, such as the availability of new preys and ecological niches. Furthermore, this new species evolved new transcripts giving rise to original toxin structures, probably each carrying specific biological activity.

1. Introduction

Far ahead of snakes and together with scorpions and spiders, cone snails are among the three most promising groups of venomous animals for pharmaceutical studies given the diversity of their toxins (King et al., 2008). Recent proteomic approaches reveal that each cone snail species may be able to produce at least 200 unique conotoxins or other proteins (Violette et al., 2012) and possibly even thousands if all variants and fragments are explored (Dutertre et al., 2013). Furthermore, as many as 761 species are now considered valid (www.marinespecies.org) and this number is expanding. This would lead to an estimate of at least 150,000 toxins, given that thousands of marine molluscs of the Conoidea superfamily remain to be investigated.

The diversity of the conotoxins, driven by either mechanisms such as recombination or gene duplication and positive selection (Duda, 2008), has been invoked for to explain the higher rates of diversification of cone snails compared to other predatory – but non venomous – neogastropods (Olivera, 2006). It has been shown that (i) even closely related species of cone snails may exhibit different feeding specializations (Kohn, 2001) and (ii) each cone species possesses its own arsenal of toxins, weakly overlapping with the arsenal of other species (Kaas et al., 2010; Olivera, 2006). Duda (2008) thus suggested that prey shifts after speciation induces a strong positive selection on venom, and the emergence of new toxins more adapted to the new prey, in line with the hypotheses proposed for snakes (Barlow et al., 2009) and scorpions (Kozminsky-Atias et al., 2008).

We present here an integrative approach, including morphological analysis, DNA sequencing and venom characterization that led

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to the recognition of a new species of cone snails in the *Conus lividus* complex, which we name *Conus conco* new species (description is provided in Supplementary Data 1). *Conus conco* is restricted to the Marquesas Archipelago, where it had earlier been mistaken for *C. sanguinolentus* or *C. lividus* (Moolenbeek et al., 2008). We used phenetic approaches for morphological characters and phylogenetic methods for DNA sequences (COI and 28S genes) to distinguish this new species from other species that belong to the same complex (*C. diadema*, *C. sanguinolentus* and *C. lividus*). A new approach is also proposed: the presence/absence of the different toxins detected by MALDI-TOF-MS analysis of the venom of multiple specimens from *C. sanguinolentus*, *C. lividus* and *C. conco* n. sp. can be coded in a matrix, similar to traditional analyses of morphological characters. Chemical signals, including toxins, have previously been used as characters to confirm an existing phylogeny, using classic phylogenetic approach or clustering methods (Nascimentoa et al., 2006), but rarely to help recognizing a new species. These data, together with venom gland transcriptome investigation, revealed that *C. conco* evolved specific conotoxins not found in its sister-species. The patterns of phylogenetic relationships, divergence estimation, geographical distribution and venom diversity, within and between species, are discussed to suggest a speciation scenario that led to the emergence of this new species of cone snail.

2. Material and methods

2.1. Sampling

Most specimens were collected during three expeditions in 2007 to Tahiti and the Marquesas and Chesterfield Islands. Specimens were photographed, the shell was broken and the animal was then directly dissected on site. A piece of foot tissue was preserved in ethanol, radular sacs were stored dry at −80 °C. Extracts of the venom duct were dissolved in 10% acetic acid in water, dried under vacuum and stored at −80 °C. The remaining venom gland was preserved in RNALater (Ambion) for further transcriptomic studies. Whenever possible, shells were reconstructed for use as vouchers. Additional comparative material originates from expeditions in the Philippines (Panglao 2004), Vanuatu (Santo 2006) and Madagascar (Atimo Vatae 2011). A piece of foot tissue was cut from the body and placed in 95% ethanol. All specimens were collected in shallow water (between 0 and 10 m), and were identified in the field as *C. lividus* or *C. sanguinolentus* (following the taxonomy of Röckel et al., 1995, and referred to as *Lividoconus* in Tucker and Tenorio, 2013).

Outgroups for phylogenetic analyses were selected according to the last available classification of cone snails (Tucker and Tenorio, 2013) and on the available phylogenetic analyses for the group. *Conus muriculatus* was used as closely related outgroup. Other species of *Conus* (*C. virgo*, *C. consors* and *C. marmoreus*) were also used as outgroups. *Conasprella pagoda* (Conidae) and *Bathytoma carnicolor* (Conoidea, Borsoniidae) were used as distant outgroups. Finally, in the COI gene dataset, *Conus quercinus* was also added to date the species divergences (see Section 2.2).

2.2. DNA sequencing and phylogenetic analyses

DNA was extracted using the Epmotion 5075 robot (Eppendorf). Fragments of the COI and rDNA28S genes of 658 and 750 bp, respectively, were amplified using the protocol described in Kantor et al. (2013). PCR products were purified and sequenced by Eurofins in both directions. Available COI and 28S sequences for *C. lividus*, *C. diadema* and *C. sanguinolentus* in GenBank were downloaded and added to the dataset. GenBank accession numbers are provided in Supplementary Data 2.

Sequences were manually (COI gene) or automatically (28S gene) aligned using ClustalW multiple alignment (BioEdit 7.0.5.3, Hall, 1999). The best model of evolution was selected for each gene using Modelgenerator V.85 (Keane et al., 2006), following the Hierarchical Likelihood Ratio Tests (with four discrete gamma categories). The best substitution models are TrN + G for both COI and 28S genes. Bayesian Analyses (BA) were performed running two parallel analyses in MrBayes (Huelsenbeck et al., 2001), consisting each of eight Markov chains of 10,000,000 generations. A GTR + G model was applied to each gene. Tracer 1.4.1 (Rambaut and Drummond, 2007) was used to check that ESS values were all greater than to 200. A consensus tree was then calculated after omitting the first 25% trees as burn-in.

The divergences of the genus *Conus* from the other conoideans, and of *C. quercinus* from *C. lividus*, respectively estimated at 55 mya and 11 mya based on fossil records (Duda and Kohn, 2005), were used as calibration points to date the divergences between the different species included in the *C. lividus* complex. BEAST 1.8.0 (Drummond and Rambaut, 2007) was used to reconstruct a phylogenetic tree based on a concatenation of the COI and 28S sequences including one specimen per species. The priors of the t_{MRCA} of the cone snails and of the clade including *C. quercinus*, *C. lividus*, *C. sanguinolentus* and *C. conco* were set to follow a lognormal distribution. An uncorrelated lognormal relaxed clock with a "birth–death incomplete sampling" speciation model was applied *and* independent models of substitutions (TN93 + G) were defined for each gene. Two MCMC chains were run for 100,000,000 generations. Convergence of the runs was tested as described previously. Tree annotator 1.8.0 (http://beast.bio.ed.ac.uk) was used to summarize the tree data generated by BEAST.

2.3. Venom MALDI-TOF-MS fingerprinting

MALDI-TOF-MS analyses were carried out on an Ultraflex TOF–TOF mass spectrometer operated in positive reflector mode (*m/z* 480–5000) under the control of the FlexControl 2.2 software (Bruker, Bremen, Germany). Samples (listed in Supplementary Data 2) were deposited on a 384 AnchorChip 600 plate using an affinity method based on manufacturer's guide to sample preparation. External calibration was carried out and checked in the 700–3500 Da mass range with an error 50 ppm.

The FlexAnalysis 2.2 software (Bruker) was used for data processing and analysis. Only the best representative mass spectrum of each venom was selected for data interpretation, i.e. the one with the highest number of signals and the best signal-to-noise ratio.

Each ionized molecular species obtained in the 41 mass spectra was considered as a different character with two different states, present (1) or absent (0). A total of 444 characters were coded, and the matrix obtained was analyzed using MrBayes (Huelsenbeck et al., 2001). Two parallel analyses were performed, each consisting of eight Markov chains of 10,000,000 generations. Convergence of the runs was tested as described previously. A consensus tree was calculated after omitting the first 25% trees as burn-in. The absence of outgroup in this analysis is due to the unavailability of venom for closely related species, and to the fact that venom available for more distant species had almost no mass in common with the three species of the *lividus* complex.

2.4. Venom gland mRNA transcriptomic sequencing

Six venom glands (two per species) were used for transcriptomic analyses (specimens listed in Supplementary Data 2). Total RNA was extracted from the glands using a TissueLyzer LT (Qiagen) and

the method developed by Chomczynski (1993). Messenger RNA was purified using the Dynabeads mRNA purification kit (Invitrogen) and the quality of total and mRNA was verified using a 2100 Bioanalyzer (Agilent technologies). The construction of the whole transcriptome libraries, the sample preparation for RNA-Seq sequencing and the sequencing were performed according to the Ion Torrent protocols using the Ion Torrent Personal Genome Machine (PGM).

Reads obtained from the Torrent Suite Software (version 3.6) were converted to FastQ format for subsequent data analysis. Sequencing quality was assessed by aligning the reads to housekeeping genes of Gastropods (CEGMA database and NCBI BLAST version 2.2.26). Data belonging to rRNA and identical sequences were removed by matching reads against public rRNA sequences (Silva database and NCBI BLAST version 2.2.26) followed by clustering together reads of 100% identity (CD-HIT-454 version 4.5.4). *De novo* assembly was performed using MIRA (version 3.9.15) with default parameters for Ion Torrent data. Contigs represented by less than 10 reads were discarded. Sequences coding for toxins were detected using GeneWise (EMBL-EBI version 2.4.1 – (Birney et al., 2004)) and HMMER2 models based on public sequences of conotoxins (ConoServer and UniProt). Signal sequences in toxins were identified using an in-house (Koua et al., 2013).

3. Results

3.1. Morphological analyses

The specimens collected in the Marquesas Islands, and preliminarily identified as *C. sanguinolentus*, formed two morphologically separable clusters (Fig. 1). One morphotype, with an average total shell length of 48 mm, is characterized by a lavender to purple background color; the other morphotype, with an average total shell length of 35 mm, is olive or yellowish brown and lacks the lavender to purple background color (Supplementary Data 1). The larger morphotype was only found in the Marquesas Islands, where it seemed to replace *C. lividus*, which is found throughout the Indo-Pacific except in the Marquesas. This purple morphotype likely represent a new species (and this hypothesis is supported by other evidence – see below), and it is described as *Conus conco* new species (Supplementary Data 1).

3.2. Phylogenetic analyses

Analyses of the COI and 28S genes resulted in phylogenetic trees with identical topology (Fig. 2). Specimens identified as *C. lividus* all belonged to a single clade (Posterior Probability PP = 0.97 for the COI, 0.98 for the 28S). Similarly, the two COI sequences of *C. diadema* from GenBank clustered in a highly supported clade (PP = 1). However, specimens identified as *C. sanguinolentus* clustered in two different clades (each with PP = 1). One of them included only specimens now identified as *C. conco* n. sp., and was the sister-group of *C. lividus*. These four clades were all grouped in a single clade (PP = 0.99 for COI, 1 for 28S), sister-group of *C. quercinus*.

Using two calibration points, the ages of the clade including *C. sanguinolentus*, *C. lividus* and *C. conco* (*C. diadema* was not included in this analysis, because no 28S sequence was available for it) and the clade including *C. lividus* and *C. conco* were estimated at 7.84 mya (5.12–12.46) and 2.738 mya (1.11–4.73) respectively (Fig. 3).

3.3. Toxin MS profile and venom gland RNA-Seq transcriptomes

A total of 444 monoisotopic signals (consisting of a single isotope) could be detected from the 41 venoms of *C. lividus*, *C. sanguinolentus* and *C. conco* in the mass range *m/z* 480–5000 corresponding to the molecular masses of most known conopeptides (Conoserver; Kaas et al., 2010). Each signal was generally found in only a few specimens (mean = 3.27) reflecting a high level of intra-specific venom variation, and only 30 were found in more than 10 specimens. Each specimen of *C. lividus*, *C. sanguinolentus* and *C. conco* included a mean of 35.6, 31.5 and 37.19 different conotoxin masses, respectively. Eleven signals (given in Da) found in at least 50% of the *C. conco* specimens, but never in *C. lividus* or *C. sanguinolentus* were identified: 1053 (found in 17 specimens of *C. conco*), 1077 (12), 1585 (17), 1615 (18), 1754 (17), 1772 (13), 1812 (12), 1932.8 (14), 1970 (14), 2299 (16), 2321 (12). The phylogenetic analysis of the 41 mass spectra resulted in a tree with three main groups, each including only specimens from the same species (Fig. 4).

The sequencing of these *Conus* venom glands libraries yielded around 2 million reads that were then assembled into 100,000 contigs on average. Transcriptome analyses confirmed the high diversity of toxins found in the three species. In total, 88 different toxin precursors were found in the six specimens analyzed, attributable to 12 different superfamilies using the signal sequences (following the classification in Puillandre et al., 2012), plus one set of 8 sequences (found in the three species) characterized by a signal sequence different from the known superfamily of conotoxins (Supplementary Data 3). Most precursor amino-acid sequences are unique to a single species, but three are shared by the three species, four by *C. lividus* and *C. sanguinolentus* and nine by *C. conco* and *C. lividus*, in agreement with the fact that *C. conco* and *C. lividus* are closely related and diverged from each other more recently than from *C. sanguinolentus*. Correlating the MALDI-TOF-MS signals and the transcriptome data to infer potential mature conotoxin sequences was judged to be too hypothetical due to the elevated number of matches resulting from unknown precursor cleavages sites, undefined post-translational modifications and the lack of any previously characterized toxins from these species.

4. Discussion

Congruence of the phylogenetic trees resulting from the two unlinked markers (COI, mitochondrial, and 28S, nuclear) is a strong, although indirect, evidence for reproductive isolation among *C. sanguinolentus*, *C. lividus* and *C. conco*. Moreover, they are characterized by highly divergent sets of toxins and different shell characters. All these evidences strongly support that *C. lividus* and *C. conco* are isolated species – and not divergent populations within a single species that would be still able to exchange genes – and this justifies the description of *C. conco* as a new species.

To understand how *C. conco* diverged from *C. lividus*, several patterns must be taken into account. First, the geographical distributions of the two species are clearly allopatric. *Conus lividus* is present in all the Indo-West Pacific except in the Marquesas Islands, and our analyses show that specimens from the two extremes of the range (Madagascar and Tahiti) are genetically indistinguishable (Fig. 2). Conversely, *C. conco* is known only from the Marquesas Islands. Second, phylogenetic analyses show that *C. conco* and *C. lividus* are sister groups. Third, *C. conco* and *C. lividus* diverged recently (~2.738 mya), i.e. between 2 and 6 my after the elevation of the first island of the Marquesas above sea level (Eiao Island is estimated to have emerged between 4.99 and 8.72 mya – Clouard and Bonneville, 2005). Fourth, *C. conco* expressed specific precursor sequences and toxin molecular masses not found in *C. lividus* and *C. sanguinolentus* (Fig. 4). In particular, 11 toxin masses are present in most *C. conco* specimens and not in the other two species, suggesting that this is not just a sampling bias. Finally, the protoconchs of all *C. conco* specimens were eroded, but that

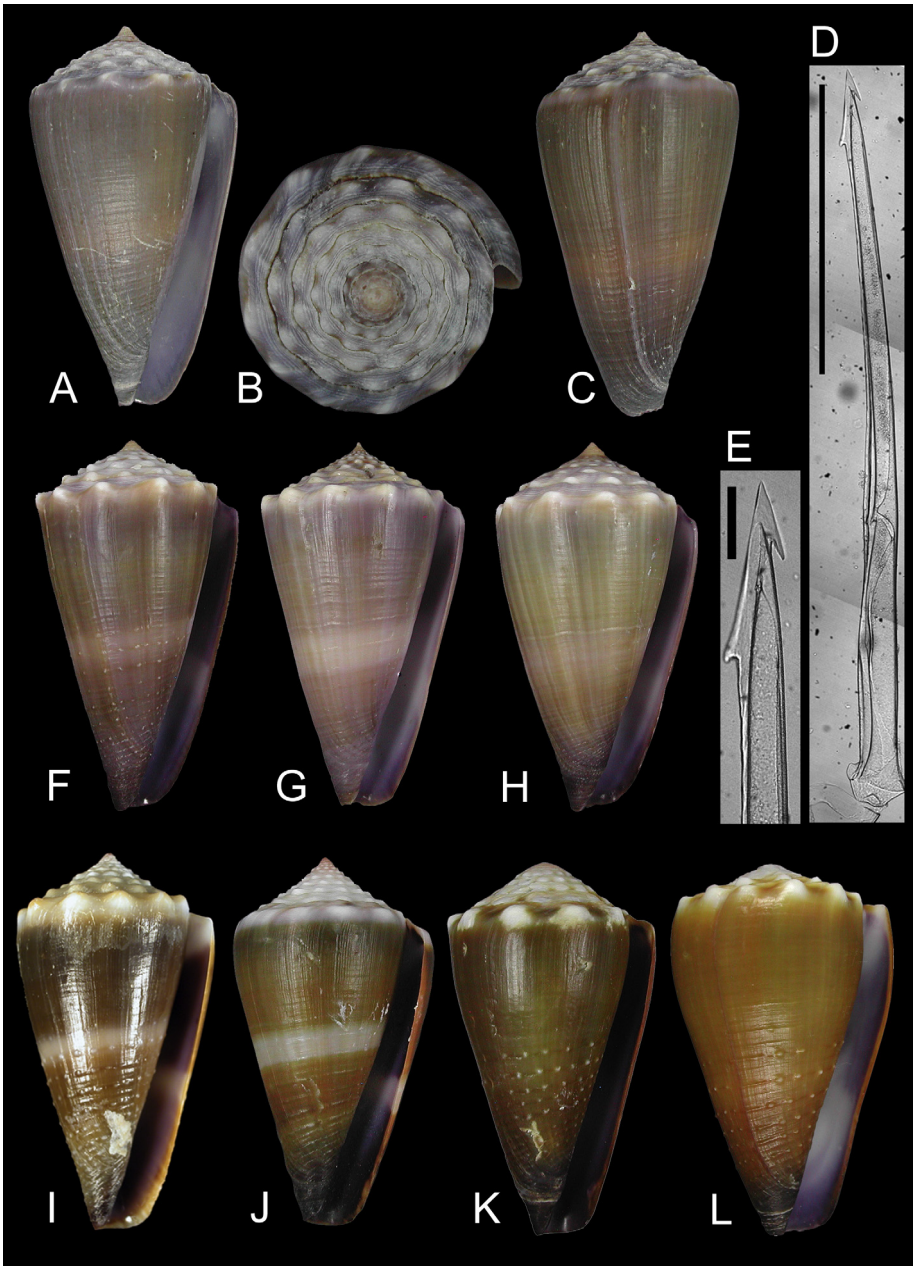

Fig. 1. Illustrations of shell and radula morphology. (A–C): *Conus conco* n. sp., holotype MNHN IM-2000-23355 (= MA071111AF); (D and E): *Conus conco* n. sp. radula (scale = 1 mm and 100 μm respectively). (F): *Conus conco* n. sp., paratype 7. (G): *Conus conco* n. sp., paratype 8. (H): *Conus conco* n. sp., paratype 9. (I): *Conus lividus*, MNHN IM-2007-30891, Santo, Vanuatu. (J) *Conus lividus*, 39.8 mm, Ile Ste Marie, Madagascar, coll. Monnier. (K): *Conus sanguinolentus*, 42.2 mm, Ile Ste Marie, Madagascar, coll. Monnier. (L): *Conus sanguinolentus*, 52.8 mm, Taiohae, Nuku Hiva, coll. Monnier.

of its sister species *C. lividus* is multispiral (four whorls – (Röckel et al., 1995)), suggesting a high dispersal potential (Jablonski and Lutz, 1980).

Given the geographic distribution of *C. conco* and *C. lividus* and the fact that the speciation event occurred after the emergence of the Marquesas archipelago, it is more likely that the speciation process occurred in the Marquesas Islands. If the speciation process that led to the emergence of *C. conco* and *C. lividus* has occurred in sympatry, e.g. as a result of a prey shift, then the two species diverged after the colonization of the Marquesas Islands by *C. lividus*. This hypothesis is supported by the differentiation of the toxin repertoire in *C. conco* (thus linked to the prey shift). *Conus lividus* is known to prey on polychaetes (direct observations, Kohn, 1959), a taxonomically and ecologically very diversified group, and it is probable that the guild of polychaete species found in the Marquesas differs significantly from that occurring elsewhere in French Polynesia or the Indo-West Pacific. However, the sympatric hypothesis does not explain the current geographical distribution. If *C. conco* and *C. lividus* are now adapted to different habitats (i.e. different preys), they are no longer competitors (Quenouille et al., 2011) and there is no simple explanation why *C. lividus* is absent from the Marquesas Islands. Alternatively, the divergence may also be the result of a peripatric speciation event, where a small peripheral population of *C. lividus* colonized the Marquesas Islands, in agreement with the geographic distribution of the two species. However, and following the peripatric hypothesis, the differentiation of the toxin repertoire in *C. conco* would be more difficult to explain. Duda and Lee (2009) suggested that ecological release,

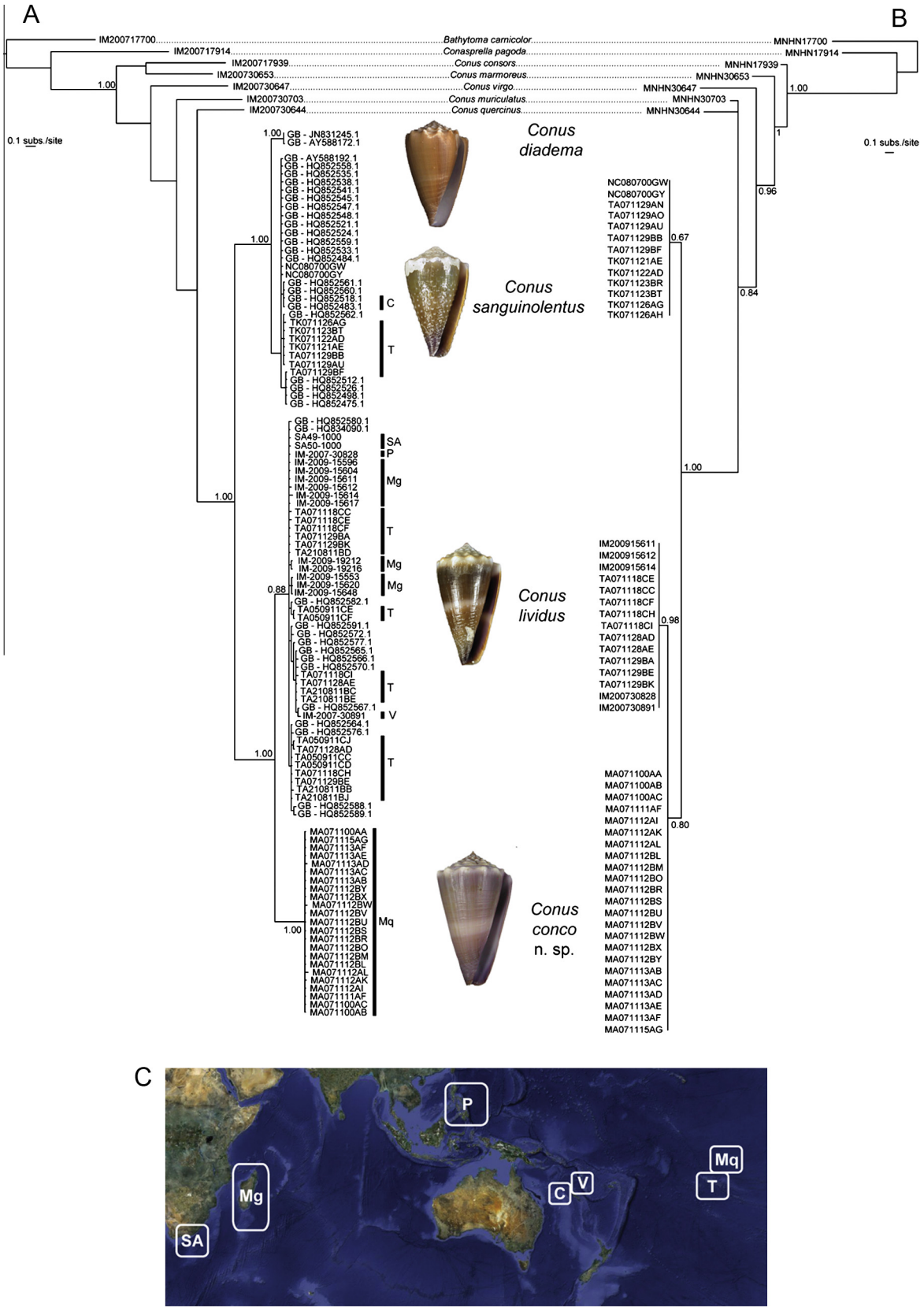

Fig. 2. Bayesian phylogenetic trees (MrBayes) based on (A) the COI gene and (B) the 28S gene. Labels of the specimens correspond to the sample_ID in Supplementary Data 2 (GB = GenBank). PP (>0.80) are indicated for each node. Geographic distribution (when known) is indicated as vertical bars: C = Chesterfield Islands; Mg = Madagascar; Mq = Marquesas Islands; P = Philippines; SA = South Africa, T = Tahiti; V = Vanuatu. (C) map showing the different sampling localities in the Indo-West Pacific.

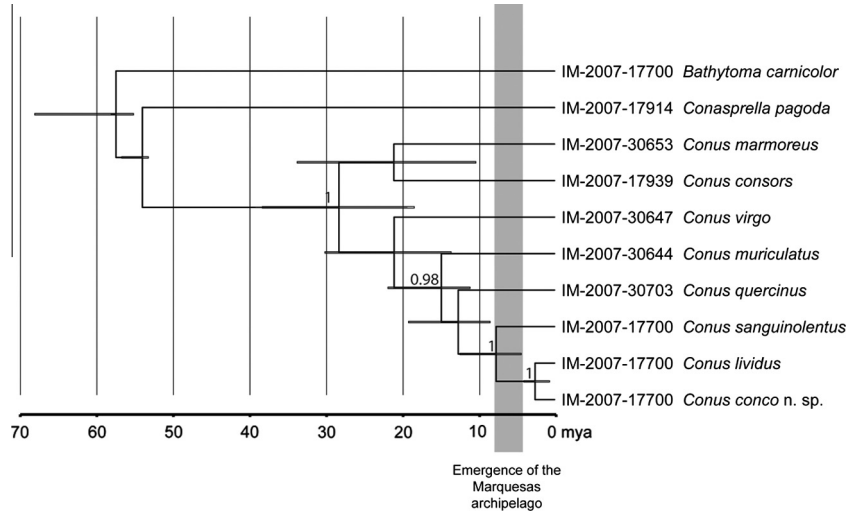

Fig. 3. Bayesian phylogenetic tree (BEAST) based on a concatenation of the COI and 28S genes with estimate of the age of each node (with confidence interval). PP (>0.95) are indicated for each node.

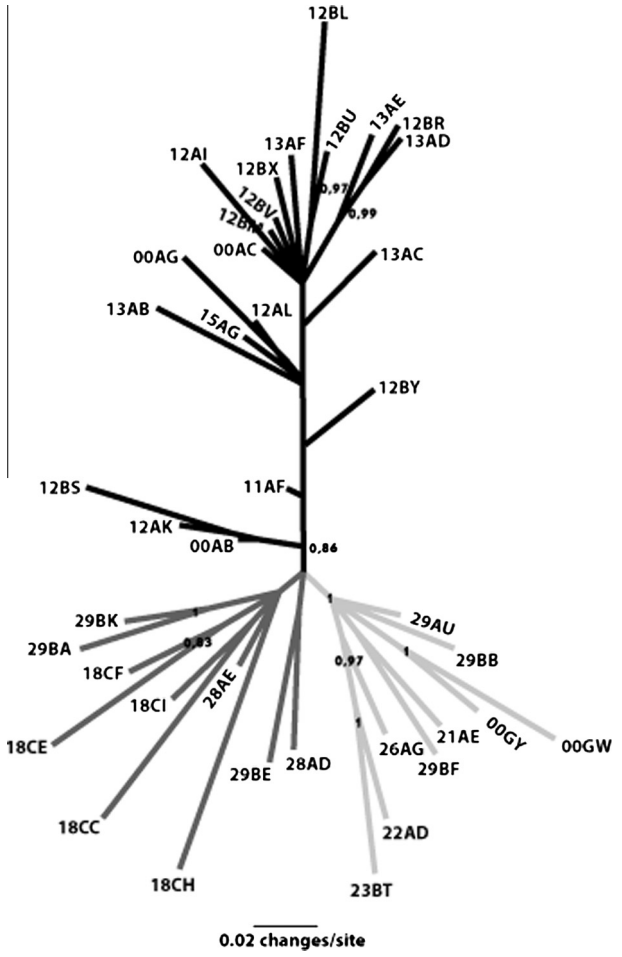

Fig. 4. Phylogenetic tree obtained by coding the presence/absence of each toxin mass for the three species *C. sanguinolentus* (light grey lines), *C. lividus* (dark grey lines) and *C. conco* (black lines).

occurring when an isolated population is under relaxed selective pressure (e.g. predator–prey arm race relaxed), led to the appearance of new toxins, even without prey shift, in *C. miliaris*. This hypothesis may apply to *C. conco*. The absence of *C. lividus* in the Marquesas Islands, although present in close archipelagos, would be explained by competitive exclusion.

Demonstrating ecological speciation and identifying the underlying mechanisms require methods that are difficult to apply to cone snails. Characterizing the prey of *C. conco* and *C. lividus* would be a first step forward, either by direct observation or by analysis of the gut contents. Furthermore, several questions linked to ecological speciation in cone snails remain unanswered. How can two sympatric populations diverge when larvae are highly dispersive? Existing hypotheses relate to the partition in microhabitats that would prevent adults from meeting and maladapted larvae to settle (Krug, 2011). Then, what is the exact role of the toxins in the prey shift? Conticello et al. (2001) suggested that non expressed toxins present in the genomes can serve as a reservoir in prevision of the appearance of a new prey (“lazarotoxins”), but the new toxins may also appear after the prey shift to increase the specificity of the venom. Clearly, a model-based approach is required in order to better characterize the genetic diversity, biology and ecology of the two species.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ympev.2014.06.024.

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