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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