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Mitogenomic phylogeny of cone snails endemic to Senegal

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ABSTRACT

Cone snails attain in Senegal one of their highest peaks of species diversity throughout the continental coast of Western Africa. A total of 15 endemic species have been described, all placed in the genus *Lautoconus*. While there is ample data regarding the morphology of the shell and the radular tooth of these species, virtually nothing is known regarding the genetic diversity and phylogenetic relationships of one of the most endangered groups of cones. In this work, we determined the complete or near-complete (only lacking the control region) mitochondrial (mt) genomes of 17 specimens representing 11 endemic species (*Lautoconus belairensis*, *Lautoconus bruguieresi*, *Lautoconus cacao*, *Lautoconus cloveri*, *Lautoconus* cf. *echinophilus*, *Lautoconus guinaicus*, *Lautoconus hybridus*, *Lautoconus senegalensis*, *Lautoconus mercator*, *Lautoconus taslei*, and *Lautoconus unifasciatus*). We also sequenced the complete mt genome of *Lautoconus guanche* from the Canary Islands, which has been related to the cones endemic to Senegal. All mt genomes share the same gene arrangement, which conforms to the consensus reported for Conidae, Neogastropoda and Caenogastropoda. Phylogenetic analyses using probabilistic methods recovered three major lineages, whose divergence coincided in time with sea level and ocean current changes as well as temperature fluctuations during the Messinian salinity crisis and the Plio-Pleistocene transition. Furthermore, the three lineages corresponded to distinct types of radular tooth (robust, small, and elongated), suggesting that dietary specialization could be an additional evolutionary driver in the diversification of the cones endemic to Senegal. The reconstructed phylogeny showed several cases of phenotypic convergence (cryptic species) and questions the validity of some species (ecotypes or phenotypic plasticity), both results having important taxonomic and conservation consequences.

1. Introduction

Cone snails (Conidae, Caenogastropoda) represent the paradigm of a species rich clade of marine animals (Röckel et al., 1995; Duda and Kohn, 2005; Tucker and Tenorio, 2013; Kohn, 2014; Puillandre et al., 2014), and therefore are an excellent group for studying the evolutionary processes underlying biological diversification. The more than 800 described species of cone snails (WoRMS, accessed October 2016; Bouchet and Gofas, 2010) are widely found in all tropical and subtropical seas from intertidal zones to deep waters associated to rocky shores, coral reefs, and sandy bottoms, preying on marine worms, snails, and fishes (Tucker and Tenorio, 2013). Cone snails are best known for their harpoon-like radular teeth and for having one of the most sophisticated venom strategies of the animal kingdom (Olivera et al., 2012): within a specialized venom gland, cones produce a cocktail composed of small peptides named conotoxins with both predatory and defensive functions (Dutertre et al., 2014).

The species diversity of cones is highest in the Indo-West Pacific region (Röckel et al., 1995; Duda and Kohn, 2005; Puillandre et al., 2014), and consequently for many years, studies on ecology, natural history, and conotoxin diversity of cone snails focused on species from this area (e.g., Duda et al., 2001) to the detriment of others such as Western Africa (e.g., Monteiro et al., 2004; Cunha et al., 2005; Duda and Rolan, 2005) or the Western Atlantic (Kohn, 2014). Cone species in the Indo-West Pacific region attain maximum diversity in the tropics and tend to show relatively widespread distributions (Cunha et al., 2014). In contrast, studies focused on Western African cones have revealed high levels of endemicity and peaks of species diversity concentrated in subtropical areas around Senegal and Angola in the continent, and most prominently in the Cabo Verde archipelago, which may harbor about 10% of cone species diversity worldwide (Cunha et al., 2014). These remarkable differences in species richness distribution indicate that distinct diversification processes may be acting

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in both regions, provided that the observed patterns rely on a consistent estimation of the number of cone species in both areas. Thus far, however, species delimitation in cones has been mostly based on the shape, color, and banding patterns of the shell, which may show in some instances important levels of homoplasy (Duda et al., 2008). In many cases, it is unclear whether different shell morphotypes represent distinct species or different forms of the same species (Duda and Palumbi, 1999). Therefore, determination of genetic variation and inference of phylogenetic relationships are timely in this hyperdiverse group in order to discern in which instances conchology can be used as basis for the recognition of species (Duda et al., 2008).

In this study, we focus on cone snails endemic to Senegal (Monteiro et al., 2004). This country, and in particular the Cape Verde peninsula (now entirely occupied by the urban growth of Dakar) has one of the highest peaks of diversity of cone snails in the Western African coast (Cunha et al., 2014). Although the cones of Senegal were already known in the times of Linnaeus, it was not until recently that a comprehensive monograph was produced upon exhaustive sampling and detailed morphological comparisons (Pin and Leung Tack, 1995). A total of 11 different endemic species were identified (see Table 1), all belonging to the subgenus *Lautoconus* (Puillandre et al., 2015), which some authors have elevated to the generic status (Tucker and Tenorio, 2009); we follow herein the latter taxonomic proposal. Moreover, *Lautoconus taslei* from the Petite-Côte region of Senegal was not considered in the revision. Afterwards, *Lautoconus trencarti* (Nolf and Verstraeten, 2008), *Lautoconus tacomae* (Boyer and Pelorce, 2009) and *Lautoconus dorotheae* (Monnier and Limpalaër, 2010) that live in deeper waters were added to the list. Recently, *Lautoconus senegalensis* (Gulden et al., 2017) was described. It corresponds to specimens previously known as *Lautoconus* cf. *mediterraneus* from Senegal (Pin and Leung Tack, 1995). We will use the new name henceforth. Importantly, seven species of *Lautoconus* from Senegal are considered endangered and another three vulnerable according to the IUCN Red List (Peters et al., 2013). In addition, several non-endemic species are found in Senegal including *Genuanoconus genuanus*, *Kalloconus pulcher*, *Kalloconus byssinus*, *Monteiroconus tabidus*, *Monteiroconus ambiguus*, and the amphi-Atlantic *Chelyconus ermineus* (Pin and Leung Tack, 1995).

Thus far, several molecular phylogenies have been reported either for the family Conidae (Puillandre et al., 2014; Uribe et al., 2017) or focused in particular geographic regions including the Indo-West Pacific (Duda and Palumbi, 1999; Duda and Kohn, 2005), Cabo Verde archipelago (Cunha et al., 2005; Duda and Rolan, 2005; Cunha et al., 2008), Canary Islands (Cunha et al., 2014), Mozambique (Pereira et al., 2010) and Saint Helena Island (Tenorio et al., 2016), but none has studied in a comprehensive fashion the cone snails endemic to Senegal.

In this study, we used complete mitochondrial (mt) genomes, which have proven to be very useful in reconstructing relatively highly resolved phylogenies of different gastropod groups including Neogastropoda (Cunha et al., 2009) and in particular, Conidae (Uribe et al., 2017). At present, the complete or near-complete mt genomes of 13 species belonging to the family Conidae are publicly available. Here, we sequenced the complete or nearly complete mt genomes of 17 individuals representing different populations and species of *Lautoconus* endemic to Senegal. In addition, we sequenced the complete mt genome of *Lautoconus guanche* from the Canary Islands (also occurring from Northern Mauritania to Morocco), which is included in the same genus (Tucker and Tenorio, 2009). We aimed to: (1) reconstruct a robust phylogeny of cones endemic to Senegal; (2) study radular tooth evolution in the group; (3) provide a first genetic hypothesis of species delimitation within the group; and (4) date major events in the diversification of Senegal endemic cones.

2. Materials and methods

2.1. Samples and DNA extraction

The complete list of specimens analyzed in this study corresponding to different populations and species of *Lautoconus* from Senegal and the Canary Islands (Spain) is shown in Table 1, along with details on the respective sampling localities and museum vouchers. Specimens were collected by snorkel at 1–3 m depth, or picked by hand at low tide. All samples were stored in 100% ethanol, and total DNA was isolated from 5–10 mg of foot tissue following a standard phenol-chloroform extraction (Sambrook et al., 1989).

Table 1
New mitochondrial (mt) genomes analyzed in this study.

ID	Species	Location	Coverage		Length (bp)	GenBanc Acc. No	Voucher (MNCN/ADN)	Voucher (shell) (MNCN)
			No. reads	Mean depth				
1258	*Lautoconus mercator* (Linnaeus, 1758)	Les Almadies, Dakar, Senegal	193,127	1902	15,332	KY801864	91278	15.05/78419
1266	*Lautoconus hybridus* (Kiener, 1845)[a]	NGor, Dakar, Senegal	123,000	1208	15,507	KY801863	91279	15.05/78427
1278	*Lautoconus mercator* (Linnaeus, 1758)	NGor, Dakar, Senegal	180,207	1774	15,329	KY801862	91280	15.05/78439
1282	*Lautoconus guinaicus* (Hwass, 1792)	Ndayane, Senegal	163,070	1608	15,316	KY801861	91281	15.05/78443
1290	*Lautoconus unifasciatus* (Kiener, 1845)[a]	Ndayane, Senegal	144,388	1424	15,506	KY801860	91282	15.05/78451
1296	*Lautoconus cloveri* (Walls, 1978)	Ndayane, Senegal	67,122	659	15,323	KY801859	91283	15.05/78457
1301	*Lautoconus cacao* (Ferrario, 1983)	Ndayane, Senegal	179,361	1767	15,318	KY801858	91284	15.05/78462
1302	*Lautoconus cacao* (Ferrario, 1983)	Ndayane, Senegal	19,742	192	15,327	KY801857	91285	15.05/78463
1312	*Lautoconus senegalensis* (Gulden et al., 2017)	Ndayane, Senegal	200,965	1806	15,317	KY801856	91286	15.05/78473
1315	*Lautoconus taslei* (Kiener, 1845)	Joal-Fadiouth, Senegal	116,067	1202	15,314	KY801855	91287	15.05/78476
1321	*Lautoconus mercator* (Linnaeus, 1758)	Île de Gorée, Dakar, Senegal	107,938	1063	15,328	KY801854	91288	15.05/78482
1335	*Lautoconus guinaicus* (Hwass, 1792)[a]	Île de Gorée, Dakar, Senegal	124,435	1227	15,506	KY801853	91289	15.05/78496
1336	*Lautoconus bruguieresi* (Kiener, 1845)	Île de Gorée, Dakar, Senegal	86,860	910	15,318	KY801852	91290	15.05/78497
1338	*Lautoconus bruguieresi* (Kiener, 1845)	Île de Gorée, Dakar, Senegal	144,761	1424	15,340	KY801851	91291	15.05/78499
1341	*Lautoconus* cf. *echinophilus* (Petuch, 1975)	Île de Gorée, Dakar, Senegal	219,541	2164	15,319	KY801850	91292	15.05/78502
1343	*Lautoconus belairensis* (Pin and Leung Tack, 1989)	Terrou-Bi, Dakar, Senegal	74,962	738	15,321	KY801849	91293	15.05/78504
1350	*Lautoconus guinaicus* (Hwass, 1792)	Terrou-Bi, Dakar, Senegal	148,308	1456	15,323	KY801848	91294	15.05/78511
CG13	*Lautoconus guanche* (Lauer, 1993)[a]	Lanzarote, Canary Islands, Spain	318,448	2915	15,506	KY801847	91295	–

[a] Complete.

2.2. Radular tooth preparation

The radular sac was dissected from the cone snail and soft parts were digested in concentrated aqueous potassium hydroxide for 24 hours. The resulting mixture was then placed in a petri dish and examined with a binocular microscope. The entire radula was removed with fine tweezers and rinsed with distilled water, then mounted on a slide using Aquatex (Merck, Germany) mounting medium, and examined under a compound microscope. Photographs were obtained with a charge-coupled device (CCD) camera attached to the microscope. Terminology for radular morphology follows Tucker and Tenorio (2009), with abbreviations following Kohn et al. (1999).

2.3. PCR amplification and sequencing

Complete or near-complete (without the control region; see results) mt genomes were amplified through a combination of standard and long PCRs using the primers and following the protocols of Uribe et al. (2017). Standard-PCR products were sequenced using Sanger technology. Long-PCR products were subjected to massive parallel sequencing. Briefly, products were purified by ethanol precipitation and amplified fragments from the same mt genome were pooled together in equimolar concentrations. For each mt genome a separate indexed library was constructed using the NEXTERA XT DNA library prep kit (Illumina, San Diego, CA, USA) and run in an Illumina MiSeq platform (v.2 chemistry; 2×150 paired-end) at Sistemas Genómicos (Valencia, Spain).

2.4. Genome assembly and annotation

The reads corresponding to the different PCR amplified mt genomes were sorted using the corresponding library indices, and assembly of the different mt genomes was performed in the TRUFA webserver (Kornobis et al., 2015). Briefly, adapters were removed using SeqPrep (StJohn, 2011), quality of the reads was checked using FastQC v.0.10.1 (Andrews, 2010), and raw sequences were trimmed and filtered out according to their quality scores using PRINSEQ v.0.20.3 (Schmieder and Edwards, 2011). Filtered reads were used for *de novo* assembly of each mt genome using default settings (minimum contig length: 200; sequence identity threshold: 0.95) of Trinity r2012-06-08 (Grabherr et al., 2011) in TRUFA, and only retaining contigs with a minimum length of 3 kb. These contigs were finally overlapped in Sequencher 5.0.1 to render the different complete or nearly complete mt genomes included within each index. In order to estimate mean coverage, each assembled mt genome was used as a reference to map the original (raw) reads with a minimum identity of 100% using Geneious® 8.0.3.

The newly determined mt genomes were annotated with the MITOS webserver (Bernt et al., 2013) using the *Africonus borgesi* mt genome (Cunha et al., 2009) as a reference. Annotations of the 13mt protein-coding genes were corroborated manually identifying the corresponding open reading frames using the invertebrate mitochondrial code. The transfer RNA (tRNA) genes were further identified with tRNAscan-SE 1.21 (Schattner et al., 2005), which infer cloverleaf secondary structures (with a few exceptions that were determined manually). The ribosomal RNA (rRNA) genes were identified by sequence comparison with the *A. borgesi* mt genome (Cunha et al., 2009), and assumed to extend to the boundaries of adjacent genes (Boore et al., 2005). GenBank accession numbers of each mt genome are provided in Table 1.

2.5. Sequence alignment and phylogenetic analyses

The newly sequenced complete or nearly complete mt genomes were aligned with the mt genomes of *A. borgesi* (Cunha et al., 2009) from Boa Vista Island, Republic of Cabo Verde, and *Lautoconus ventricosus* (Uribe et al., 2017) from Faro, Portugal, which were used as outgroup taxa based on the phylogeny reported by Puillandre et al. (2014). A sequence data set was constructed concatenating the nucleotide sequences of the 13 mt protein-coding and two rRNA genes. The deduced amino acid sequences of the 13 mt protein-coding genes were aligned separately and used to guide the alignment of the corresponding nucleotide sequences with Translator X (Abascal et al., 2010). Nucleotide sequences of the mt rRNA genes were aligned separately using MAFFT v7 (Katoh and Standley, 2013) with default parameters. Ambiguously aligned positions were removed using Gblocks, v.0.91b (Castresana, 2000) with the following settings: minimum sequence for flanking positions: 85%; maximum contiguous non-conserved positions: 8; minimum block length: 10; gaps in final blocks: No. Finally, the different single alignments were concatenated using Geneious® 8.0.3. Sequences where format converted for further analyses using the ALTER webserver (Glez-Peña et al., 2010).

Phylogenetic relationships were inferred using maximum likelihood (ML, Felsenstein, 1981) and Bayesian inference (BI, Huelsenbeck and Ronquist, 2001). For ML, we used RAxML v8.1.16 (Stamatakis, 2006) with the rapid hill-climbing algorithm and 10,000 bootstrap pseudoreplicates (BP). BI analyses were conducted with MrBayes v3.1.2 (Ronquist and Huelsenbeck, 2003), running four simultaneous Markov chains for 10 million generation, sampling every 1000 generations, and discarding the first 25% generations as burn-in (as judged by plots of ML scores and low SD of split frequencies) to prevent sampling before reaching stationarity. Two independent Bayesian inference runs were performed to increase the chance of adequate mixing of the Markov chains and to increase the chance of detecting failure to converge, as determined using Tracer v1.6 (Rambaut and Drummond, 2007). The effective sample size (ESS) of all parameters was above 200. Node support was assessed based on Bayesian Posterior Probabilities (BPP). A node was considered highly supported with BP and BPP values above 70% and 0.95, respectively.

The best partition schemes and best-fit models of substitution for the data set were identified using Partition Finder (Lanfear et al., 2012) with the Akaike information criterion (AIC, Akaike, 1973). For the protein-coding genes, the partitions tested were: all genes grouped; all genes separated (except *atp6-atp8* and *nad4-nad4L*); and genes grouped by subunits (*atp*, *cob, cox*, and *nad*). In addition, these three partitions schemes were tested taking into account separately the three codon positions. The rRNA genes were tested with two different schemes, genes separated or combined.

2.6. Estimation of divergence times

The program BEAST v.1.7 (Drummond and Rambaut, 2007) was used to perform a Bayesian estimation of divergence times. An uncorrelated relaxed molecular clock was used to infer branch lengths and nodal ages. The tree topology was fixed using the one recovered by the ML and BI analyses. For the clock model, the lognormal relaxed-clock model was selected, which allows rates to vary among branches without any a priori assumption of autocorrelation between adjacent branches. For the tree prior, a Yule process of speciation was employed. Only the protein-coding genes were used. The partitions and models selected by Partition Finder were applied (see results; except for second codon positions that we used the HKY+I model instead of the GTR+I model, as the latter could not converge). The final Markov chain was run twice for 100 million generations, sampling every 10,000 generations, and the first 1000 trees were discarded as part of the burn-in process, according to the convergence of chains

checked with Tracer v.1.5. (Rambaut and Drummond, 2007). The ESS of all parameters was above 200.

Despite the fact that there are many fossils of Conidae, it is difficult in many instances to be certain about species identifications given the important levels of homoplasy in shell shape (Duda et al., 2008). Hence, although there are fossils attributed to *L. ventricosus* (Sacco, 1893) and *L. mercator* (Glibert, 1960), we opted to calibrate the clock using a biogeographical event. The posterior distribution of the estimated divergence times was obtained by specifying one calibration point as prior for the divergence time of the split between *L. ventricosus* and *A. borgesi* in the outgroup. The latter species is endemic to Boa Vista, and we used the age of formation of this island (16.5 Mya; Dyhr and Holm, 2010) as biogeographical calibration point. We applied a log-normal distribution as the prior model for the calibration and enforced the median divergence time to equal 16.5 (s.d. = 0.05, offset = 0.5).

3. Results

3.1. Sequencing and assembly

The nucleotide sequences for the mt genomes of *Lautoconus hybridus*, three *Lautoconus guinaicus, Lautoconus unifasciatus, Lautoconus belairensis*, and *L. guanche* were determined to be complete whereas those of three *Lautoconus mercator*, two *Lautoconus bruguieresi*, two *Lautoconus cacao*, *Lautoconus cloveri, Lautoconus senegalensis, Lautoconus* cf. *echinophilus*, and *L. taslei* lacked the *trnF* gene, the control region, and the start of the *cox3* gene because the corresponding fragment could not be PCR amplified (Fig. 1). The number of reads, mean coverage, and length of each mt genome are provided in Table 1. The mt genomes of *L. cacao* from Ndayane and *L. guanche* received the minimum (19,742) and maximum (318,448) number of reads, respectively. The same samples received the minimum (192×) and maximum (2915×) coverage, respectively (Table 1).

3.2. Genome organization and sequence divergence

All sequenced *Lautoconus* mt genomes encode for 13 protein-coding, 2 rRNA and 22 tRNA genes (but note that the *trnF* gene could not be determined in the incomplete mt genomes; see Appendix A). They all share the same genome organization: the major strand encodes all genes, except those forming the cluster MYCWQGE (*trnM*, *trnY*, *trnC*, *trnW*, *trnQ*, *trnG*, *trnE*) and the *trnT* gene (Fig. 1). The genes *nad4*/*nad4L* overlapped in seven nucleotides in all the mt genomes. All protein-coding genes start with ATG (but note that the beginning of *cox3* could not be determined in the incomplete mt genomes; see Appendix A). The stop codons were variable between TAA and TAG, depending on the gene and the species. There were six genes (*cox1*, *cox3*, *nad1*, *nad3*, *nad5* and *nad6*) whose stop codon is TAA. Two genes (*cob* and *nad4L*) ended with TAG. Two genes (*nad2* and *nad4*) had incomplete stop codons (TA-) that become functional after polyadenylation (Chang and Tong, 2012). The remaining three genes (*atp6*, *atp8*, and *cox2*) varied in their stop codon depending on the species (see Appendix A).

Pairwise uncorrected sequence divergences between *L.* cf. *echinophilus*, *L. bruguieresi*, *L. cloveri*, *L. mercator* from Les Almadies and Gorée Island, *L. senegalensis*, and *L. cacao* from Ndayane (specimen #1302) *versus* the remaining analyzed samples averaged 4% (see Appendix A). Pairwise uncorrected sequence divergences between *L. belairensis*, *L. cacao* from Ndayane (specimen #1301) and *L. mercator* from NGor *versus L. unifasciatus*, *L. guanche*, *L. guinaicus*, *L. hybridus*, and *L. taslei* averaged 2.6%. The pairwise uncorrected sequence divergences between *L. guanche versus L. guinaicus*, *L. hybridus*, and *L. taslei* varied 0.4–0.5%. Pairwise uncorrected sequence comparisons (1) between *L.* cf. *echinophilus* and *L. bruguieresi*; (2) among *L. mercator* of Gorée Island, *L. senegalensis*, and *L. cacao* from Ndayane (specimen #1302); (3) among *L. taslei*, and *L. guinaicus* from Terrou-Bi and Ndayane; and (4) between *L. hybridus* and *L. guinaicus* from Gorée Island showed almost no sequence divergence (<0.1%). The mt genomes of *L. bruguieresi* from Gorée Island and Les Almadies had exactly the same sequence (see Appendix A).

3.3. Phylogenetic relationships within Lautoconus

Phylogenetic relationships of cones endemic to Senegal were reconstructed based on the nucleotide sequences of the concatenated 13 mt protein-coding and two rRNA genes using probabilistic methods. The final matrix was 13,582 positions long. According to the AIC, the best partition scheme for the protein-coding genes was the one combining all these genes but analyzing each codon position separately. The best substitution models were GTR+I+G for the first codon position, GTR+I for the second position, and GTR+G for the third codon position. For the rRNA genes, the best scheme had both genes combined under the GTR+I model. Both, ML ($-lnL = 31{,}523.23$) and BI ($-\ln L = 31{,}541.77$ for run 1; $-lnL = 31{,}543.66$ for run 2) arrived at identical topology (Fig. 2), with only slightly differences in branch lengths. All nodes in the reconstructed phylogeny received high statistical support (Fig. 2).

Up to three major lineages could be distinguished within the reconstructed phylogeny (Fig. 2). One lineage included different populations of *L. bruguieresi* and *L.* cf. *echinophilus* sister to a clade that included *L. cloveri* from Ndayane and a group with specimens of *L. mercator* from Gorée Island and Les Almadies, *L. senegalensis* from Ndayane, and one specimen (#1302) of *L. cacao* from Ndayane (Fig. 2). A second lineage included *L. belairensis* from Terrou-Bi sister to *L. mercator* from NGor and another specimen (#1301) of *L. cacao* from Ndayane (Fig. 2). This lineage was sister to another one including the following clades: (1) *L. guinaicus* from Ndayane and Terrou-Bi and *L. taslei* from Joal-Fadiouth sister to (2) *L. hybridus* from NGor and *L. guinaicus* from Gorée Island to the exclusion of (3) *L. unifasciatus* from Ndayane and *L. guanche* from Canary Islands (Fig. 2).

The above-mentioned three major *Lautoconus* lineages corresponded to three distinct types (robust, small, and elongated, respectively) of radular tooth (Fig. 2), all of them of the vermivorous kind. The robust and elongated types were of medium relative size (Shell Length/Tooth Length = 37–42), with a short, pointed barb. They differed in the size of the anterior section of the tooth, which is equal or slightly shorter than the posterior section of the

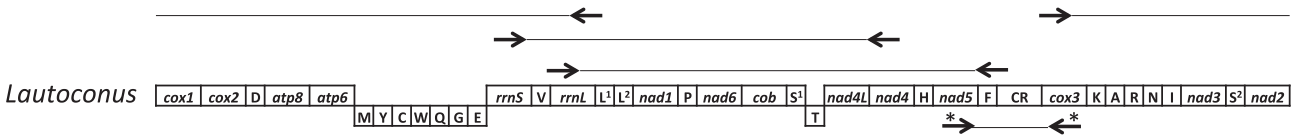

Fig. 1. Mitochondrial gene order of *Lautoconus*. All newly determined mt genomes shared identical genome organization. The genes encoded in the major and minor strands are shown in the top and bottom lines, respectively. The relative position of the primers for long PCR is shown. The two primers labeled with an asterisk were designed to amplify the control region, and failed in several cases rendering incomplete mt genomes.

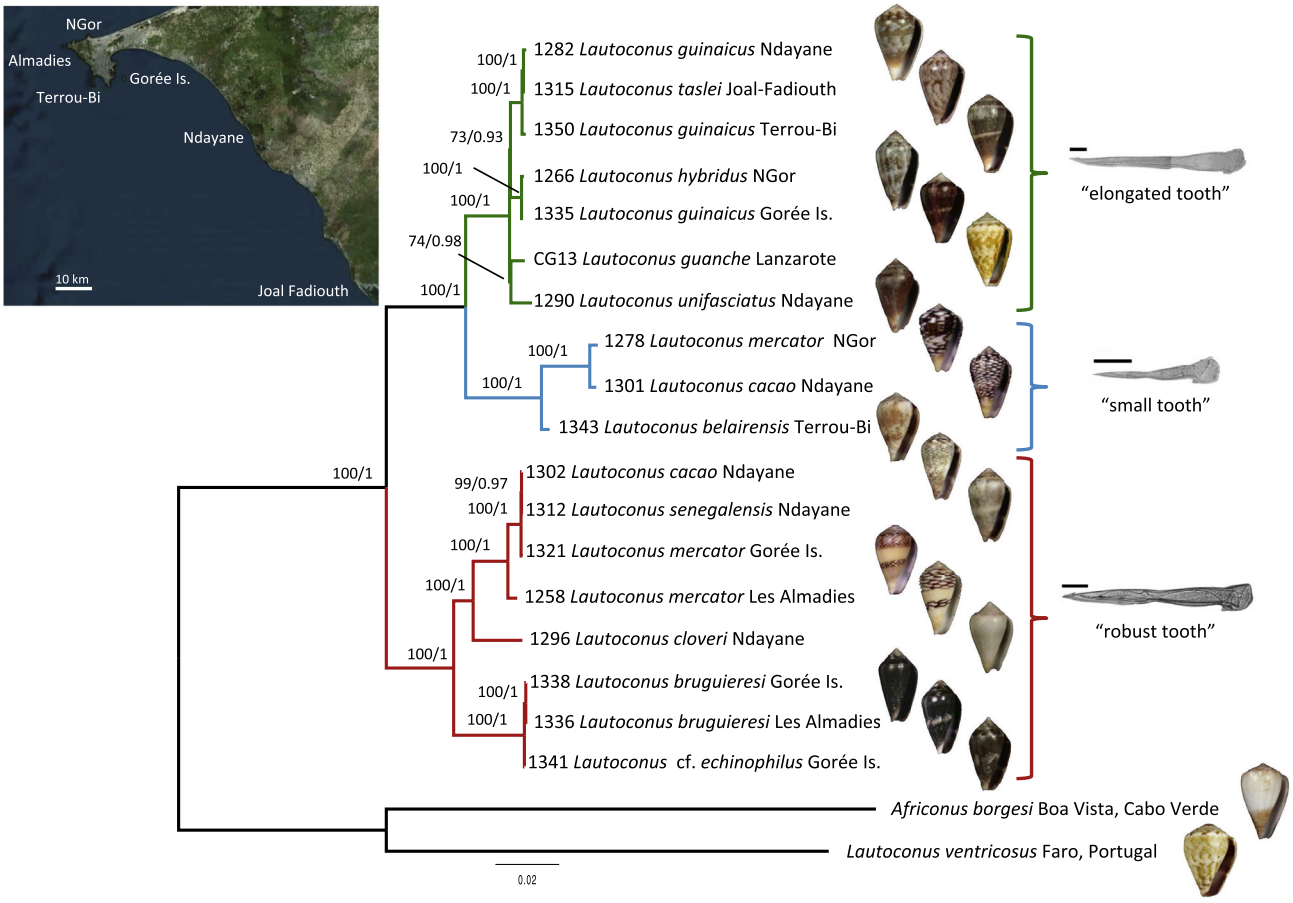

Fig. 2. Phylogenetic relationships of *Lautoconus* based on complete mt genomes (concatenated protein coding plus rRNA genes analyzed at the nucleotide level). The reconstructed ML phylogram using *A. borgesi* from Boa Vista, Cabo Verde archipelago, and *L. ventricosus* from Faro, Portugal as outgroup taxa is shown. Number of specimen, initial species assignment, locality, and a ventral picture of the shell are provided. Three major clades are indicated with different colors (red, blue, and green) that corresponded to three different types of radular tooth: robust, small and elongated, respectively (scale bar equals 0.1 mm; see all radular teeth in Appendix A). A map of Senegal as inset is provided with sampling localities. Numbers at nodes are statistical support values for ML (bootstrap proportions)/BI (posterior probabilities). Scale bar indicates substitutions/site. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

tooth for the robust type (Tooth Length/Anterior Portion Length = 2.0–2.1), but much longer for the elongated type (Tooth Length/Anterior Portion Length = 1.6–1.7). They also differed in the extension of the anterior portion covered by the blade (80–85% in the robust type *versus* 40–46% in the elongated type) and the number of denticles present in the serration (19–30 in the robust type, but more than 40 in the elongated type). The radular tooth of the small type had, as the name indicates, a much smaller relative size (Shell Length/Tooth Length = 85–96). In this case, the anterior section of the tooth was significantly shorter than the posterior section (Tooth Length/Anterior Portion Length = 2.3–2.5). The blade covered 64–70% of the anterior portion, and there were 15–20 small denticles in the serration arranged on a single row. The base of the tooth was large and broad (see Appendix A).

3.4. Divergence times

Major cladogenetic events within *Lautoconus* were dated using an uncorrelated relaxed molecular clock model, which was calibrated using the age of Boa Vista Island at the divergence of *A. borgesi* and *L. ventricosus*. The first event of diversification within the crown group of *Lautoconus* was estimated at a mean of 5.6 (4.5–7.0, credible interval) million years ago (Mya) separating the species with robust radular teeth from the ancestor of the species with elongated and small radular teeth (Fig. 3). The second main split was estimated to occur about 2.8–2.9 (2.1–3.8) Mya separating (1) cones with elongated radular teeth from those with small radular teeth and (2) *L. bruguieresi* and allies from the remaining cones with robust radular teeth (Fig. 3). Finally, it is interesting to note that main speciation events occurred between 0.7–2.0 (0.5–2.7) Mya whereas population divergences were dated about 0.03–0.19 Mya (see discussion regarding species boundaries; Fig. 3).

4. Discussion

The region around Dakar is considered a hotspot of diversity for cone snails off the Western Africa continent (Cunha et al., 2014). However, rapid expansion of the metropolitan area is seriously compromising the conservation of this extraordinary species richness mainly due to the loss of adequate habitats (e.g., the neighborhood of Bel-Air has a rocky plateau now heavily polluted by the adjacent Port of Dakar. Bel-Air is the type locality of *L. belairensis*, which is not found there anymore). In fact, all 14 cone species included in the highest categories of Critically Endangered and Endangered in the IUCN Red List are endemic to either Senegal or the Cabo Verde archipelago (Peters et al., 2013). Therefore, it is urgent to have a better understanding of the exact number of cone species and their genetic diversity in the region. Moreover, any further comparative analysis aimed at understanding the evo-

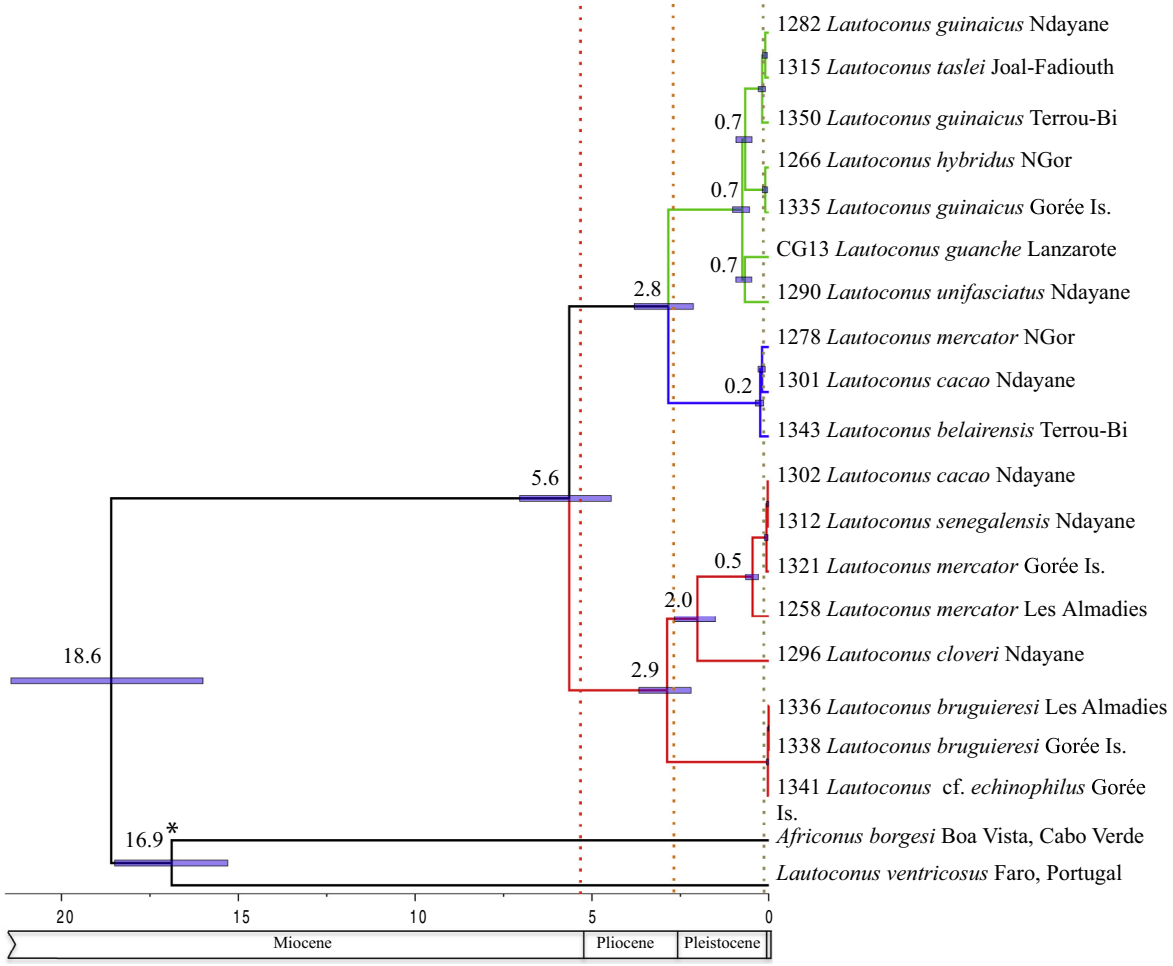

Fig. 3. Chronogram of *Lautoconus* based on complete mt genomes (concatenated protein coding plus rRNA genes analyzed at the nucleotide level) and using the fixed topology of the ML tree shown in Fig. 2. A Bayesian uncorrelated relaxed lognormal clock with a fossil/geographic-based calibration prior (denoted by an asterisk) was used in BEAST. Horizontal bars represent 95% credible intervals for time estimates; dates are in millions of years. The dotted lines represent the age of the Messinian Salinity Crisis (red), the Plio-Pleistocene transition (orange), and the beginning of the Holocene (brown), respectively. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

lutionary process underlying cone diversification in this area needs to be based on a robust phylogeny (Harvey and Pagel, 1991). This primary information was lacking for the cones endemic to Senegal.

Here, we amplified and sequenced complete or near-complete (without the control region) mt genomes of 18 specimens initially assigned to 10 out of the 14 species of genus *Lautoconus* described as endemic to Senegal plus *L. guanche* from Canary Islands. We had no individuals from the four species living in deeper waters (*L. dorotheae*, *L. pineaui*, *L. tacomae* and *L. trencarti*), although *cox1* partial sequences are available for the first two species (Puillandre et al., 2014). The newly determined mt genomes share identical gene arrangement, which conforms to the consensus genome organization reported for Conidae (Uribe et al., 2017), Neogastropoda (Cunha et al., 2009), and Caenogastropoda (Osca et al., 2015).

The reconstructed phylogenies using probabilistic methods were fully resolved (all nodes had high statistical support), allowing several further evolutionary inferences. The first striking outcome of our analyses is that cones endemic to Senegal do not form a monophyletic group because *L. guanche* from Canary Islands is recovered as sister to *L. unifasciatus*, nested deep within the reconstructed phylogeny. Its close phylogenetic relationship to cones endemic to Senegal was already hypothesized based on radular tooth comparisons, which prompted the inclusion of the species into the genus *Lautoconus* (Tucker and Tenorio, 2009). This species was also placed along with Angola and Senegal cone endemics in the *cox1* phylogeny of Puillandre et al. (2014), although its exact phylogenetic position could not be fully resolved. Even though *L. guanche* has non-planktonic development (see Appendix A; Cunha et al., 2014), and thus a supposedly limited capacity of dispersal, this species is not endemic to Canary Islands but also found in the Western African coast from Northern Mauritania to Morocco. Phylogenetic studies focused on *L. guanche* showed no differentiation of the species within the Canary archipelago or between the islands and the Western African coast indicating recurrent gene flow (Cunha et al., 2014), which could result from seasonal changes in the direction of the Canary current (Stramma and Siedler, 1988; Navarro-Pérez and Barton, 2001). These findings indicate clearly that the recently introduced taxon *Lautoconus saharicus* (Petuch and Berschauer, 2016) represents the local form of *L. guanche* from Dahkla Bay, Western Sahara. It lacks taxonomical value and must be considered a junior synonym.

The cones endemic to Senegal could be grouped into three main lineages according to our phylogenetic analyses. The members within each lineage share a distinct radular tooth (robust, small, and elongated, respectively). Hence, the type of radular tooth could be used as predictor of the relative position of the missing taxa in our phylogeny. Accordingly, *L. dorotheae*, *L. pineaui*, and *L. trencarti* could be placed within the "elongated tooth" clade, as suggested

by their radular tooth shape (Pin and Leung Tack, 1995; Boyer and Pelorce, 2009; Monnier and Limpalaër, 2010; see Appendix A). Likewise, the radular tooth of *L. tacomae* (Boyer and Pelorce, 2009; see Appendix A) is of the robust type, similar to that of *L. bruguieresi*; we could therefore assume that these two species are closely related.

The inferred chronogram suggests that early diversification in *Lautoconus* was driven by profound changes in the paleoecosystems of continental Western Africa as the two main divergence events in the group were related to drastic shifts in past global climate. The first divergence episode was dated during the Messinian Salinity Crisis (MSC), when the Mediterranean Sea desiccated at the end of the Miocene from 5.96 to 5.33 Mya (Krijgsman et al., 1999), whereas the second one corresponded to the transition between the Pliocene and the Pleistocene about 2.8 Mya. Plate tectonic movements are believed to be responsible of the onset of the MSC (Duggen et al., 2003), but simultaneously there was a glacial period that lasted from 6.26 to 5.50 Mya, and produced an eustatic sea level drop between −10 and −30 m (Hodell et al., 2001). The closure of marine gateways between the Atlantic Ocean and the Mediterranean Sea had likely a great influence on the Canary current and the Atlantic meridional overturning circulation (Ivanovic et al., 2014). Altogether, the glacially driven eustatic sea level changes combined with modifications in surface and deep ocean currents could have seriously affected dispersal patterns in *Lautoconus* given the non-planktonic nature of their larvae (see Appendix A), thus promoting instances of isolation in refugia, restricted gene flow, and the consequent diversification processes. The transition of the Pliocene to the Pleistocene was associated to a shift from a warm to a cold climate, and resulted in oceans completely different in terms of circulation (Filippelli and Flores, 2009). Moreover, this transition marked a pronounced change from a somewhat stable pattern in sea levels to the onset of extreme oscillations concurring with glacial-interglacial periods (Lisiecki and Raymo, 2005). This combination of changes in the sea realm during the Plio-Pleistocene boundary and afterwards could have been determinant for diversification of *Lautoconus* following population expansion-contraction cycles, as has been suggested for other marine organisms (Marko et al., 2010; Shen et al., 2011).

For many years, allopatry was not considered a predominant mode of speciation in the sea due to the high potential for dispersal of many marine organisms (adults and larvae), and the general lack of geographical barriers in the marine realm. However, this view has considerably changed in the recent years due to genetic studies revealing different factors limiting gene flow in the sea, which ultimately provoke population subdivision and genetic differentiation (Palumbi, 1994; Williams and Reid, 2004; Shen et al., 2011). Our results support that allopatry is the main mode of speciation for cone snails having non-planktotrophic larvae, as is the case of *Lautoconus* species (this study) and as it has been suggested for *Africonus* and *Trovaoconus* of the Cabo Verde archipelago, although for these two genera, low sea levels during glacial maxima promoted connection between islands (i.e., gene flow) and posterior sea level rises induced isolation (Cunha et al., 2005; Cunha et al., 2008).

It has been shown that vermivorous, molluscivorous, and piscivorous cone snails have distinct types of radular tooth (Duda et al., 2001). As the type of radular tooth closely correlated with the earliest cladogenetic events in the reconstructed phylogeny, it could be argued that ecological (dietary) adaptation was an additional evolutionary process triggering diversification in the cones endemic to Senegal. It is plausible that ecosystem changes during the MSC and the Plio-Pleistocene transition also enhanced diversification in other marine groups, and polychaetes in particular, triggering trophic specializations in *Lautoconus*. However, within vermivore cone snails, radular tooth specialization has been only clearly documented for those species such as e.g., *Stephanoconus regius* that prey on amphinomids (Duda et al., 2001). Hence, more ecological studies are needed to assess whether a particular tooth shape is directly related to the types of worms that are eaten by a given species or group of species.

Finally, it is important to note that within each of the three main lineages, the different species occupy complementary geographic distributions that cover as a whole all suitable habitats available in the territory (i.e., the different locations in the Cape Verde peninsula, Gorée Island, Ndayane and Joal-Fadiouth). Hence, niche segregation might be a final evolutionary process also contributing to the diversification of the group. Patterns of speciation have been predominantly documented in adaptive radiations, which show how the combination of different evolutionary processes acting successively can promote increasing levels of diversification in relatively short periods of time (Danley and Kocher, 2001; Streelman et al., 2002; Rüber et al., 2003). In addition to the evolutionary processes (vicariance, trophic specialization, niche segregation) that could have been important in generating the diversity of cone snails endemic to Senegal (as here inferred), others have been proposed in the case of adaptive radiations such as ecomorphological and behavioral adaptations, which still need to be investigated in detail for cone snails.

Species delimitation in cone snails has been traditionally based on shell shape and color banding patterns (Tucker and Tenorio, 2013), largely ignoring genetic data. However, shell morphology of snails could be in many cases convergent, reflecting adaptation of genetically distinct populations (ecotypes) or species (sibling or cryptic) to local environments (Knowlton, 1993; Hollander and Butlin, 2010; Dowle et al., 2015). Moreover, phenotypic variation may result not only from genetic differences but also from phenotypic plasticity i.e., the capacity of one genotype to generate different phenotypes in response to distinct environments (Hollander and Butlin, 2010; Dowle et al., 2015). Convergence and phenotypic plasticity may confound taxonomists and could result in under- and overestimations of the number of species in a group, respectively. Hence, the need of detecting such evolutionary processes using robust phylogenies and the comparative method (Harvey and Pagel, 1991), as part of a multidisciplinary approach to species delimitation.

In the case of the cones endemic to Senegal, the history of their taxonomy already reflects controversial decisions regarding the species status of some of the taxa. For instance, some authors considered *L. cacao* a synonym of *L. mercator*, and others proposed that *L. echinophilus* could be a juvenile of *L. bruguieresi* (Pin and Leung Tack, 1995). Moreover, the phenotypic similarity of *L. senegalensis* (Senegal) and *L. ventricosus* (Mediterranean Sea) despite their disjoint geographic distribution is also striking, and calls for a case of potential cryptic species (Bandel and Wils, 1977). According to the reconstructed phylogeny, it is possible to detect two clear cases of phenotypic convergence: (1) the specimen #1335 from Gorée Island initially identified as *L. guinaicus* was recovered as sister to *L. hybridus*, and thus should belong to this latter species. Given the striking shell similarity of specimen #1335 to *L. guinaicus* and in order to discard potential contamination, we sequenced the universal *cox1* gene fragment (Folmer et al., 1994) of extra specimens of *L. guinaicus* from Gorée Island confirming this result (not shown); (2) the different specimens initially identified as *L. mercator* and *L. cacao* were distributed in two distinct clades. The taxonomic implications in this case are more complex (see below).

In contrast, several specimens initially attributed to different species, showed little (<0.1%) or no genetic divergence at all (the time tree dates these divergences less than 100 k years ago). These were the cases of: (1) *L. taslei* and two populations (Terrou-Bi and Ndayane) of *L. guinaicus*; thus, despite its disjoint distribution (southern coast of Senegal), *L. taslei* cannot be considered a valid species (see Appendix A). (2) *L. mercator* from NGor and one spec-

imen (#1301) of *L. cacao* from Ndayane; (3) *L. mercator* from Gorée Island (and possibly from Les Almadies), *L. senegalensis* from Ndayane, and one specimen (#1302) of *L. cacao* from Ndayane; (4) *L.* cf. *echinophilus* and two populations (Les Almadies and Gorée Island) of *L. bruguieresi*. There are several alternative explanations for this pattern including phenotypic plasticity, recent speciation, and mtDNA introgression. Unfortunately, it is not possible to distinguish between them without having the nuclear counterpart. However, for some of these cases there are some independent lines of evidence that could help resolving the conundrum. As mentioned above, our results would be in favor of considering that *L. echinophilus* is a form of *L. bruguieresi* (see Appendix A). However, it is important to note that the specimen that was sequenced in this study was identified as *L.* cf. *echinophilus* as it did not fully match the description of the type specimen of the species. Hence, new specimens ascribed to this species need to be sequenced to resolve the taxonomic status of this species. In the case of *L. cacao* and *L. mercator*, taxonomic problems of synonymy are mixed with evolutionary convergence. The shell morphology of the population of *L. mercator* from Gorée Island matches that of the lectotype of *Conus mercator* L., 1758, whereas specimen #1302 of *L. cacao* from Ndayane compares well with the lectotype of *Conus cacao* Ferrario, 1983 (see Appendix A). According to our results, *L. senegalensis* is a form of *L. cacao*, which in turn becomes a junior synonym of *L. mercator*. On the other hand, specimen #1301 of *L. cacao* from Ndayane was initially considered a juvenile of this species, but the phylogeny indicates that it is actually not related to *L. cacao* (= *mercator*). The shell morphology of this population matches that of the lectotype of *Conus reticulatus* (Born, 1778), which was traditionally considered a junior synonym of *Conus mercator* (see Appendix A). Our data suggest that the taxon *Lautoconus reticulatus* is actually a valid species, distinct from *L. mercator*, and must be reinstated as such. The name *L. reticulatus* (Born, 1778) should be also used for the population of *mercator*-like specimens from NGor. Moreover, *L. belairensis* also stands as a valid species, distinct from *L. mercator* and sister to *L. reticulatus*. Finally, the population of *L. mercator* from Les Almadies is in the limit of sequence divergence that could be associated to speciation events, assuming as threshold for the species status that *L. guanche* from Canary Islands is a valid species given its allopatric geographic distribution, and that it has 0.4–0.5% sequence divergence to closely related endemic species from Senegal in the same clade (more than half My of independent evolution in the chronogram). This threshold lies well within the so-called grey zone of speciation between 0.5–2% (Roux et al., 2016).

5. Conclusions

We reconstructed a robust phylogeny of cone snails endemic to Senegal (genus *Lautoconus*) using complete or near-complete mt genomes. The dating of this phylogeny revealed that major changes in the marine realm during the MSC and the Plio-Pleistocene transition could have produced vicariant events promoting diversification in these cone snails, which have a non-planktonic larval stage. Diversification was further accompanied by radular tooth specializations (which may correlate with dietary adaptations), and followed by speciation in allopatry (an extreme case would be *L. guanche*, whose distribution does not overlap with any cone endemic to Senegal). The reconstructed phylogeny together with sequence divergence (uncorrected *p* distances) data helped recognizing instances of shell convergence and questioned the validity of some species given their little genetic differentiation, although this result needs to be fully validated by sequencing nuclear genes. In any case, our study calls for a thorough revision of species delimitation in the family Conidae using genetic data. Moreover, by providing an evolutionary framework, the results here obtained are particularly important for designing a conservation strategy for Western African cone snails that face serious threats.

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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.2017.04.020.

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