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Conidae phylogenomics and evolution

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Abstract

Understanding the relative role of different evolutionary processes leading to the extraordinary morphological, ecological and species diversity of cone snails requires a robust phylogeny, which thus far has been elusive. Here, we constructed a mitochondrial (mt) genome data set, which included four newly sequenced mt genomes, 25 publicly available mt genomes and 24 data sets with all mt protein-coding and rRNA genes assembled from venom gland transcriptomes. In total, we analysed 42 different species representing 27 genera of cone snails, that is, about one third of the generic diversity of the group. In addition, we used the RNA-Seq reads to assemble 21 nuclear genes, which were concatenated in a nuclear data set. Finally, a combined data set including mt and nuclear genes was also constructed. The three data matrices were analysed with probabilistic methods, site-homogeneous and site-heterogeneous models, and with protein-coding genes both at the amino acid and nucleotide levels. Diet specialization, radular morphology and the type of protoconch (paucispiral or multispiral indicating lecithotrophic or planktonic larvae, respectively) as well as conotoxin diversity were mapped onto the reconstructed mt phylogeny, and a chronogram dating mayor cladogenetic events within the group was also reconstructed.

KEYWORDS

Conidae, conotoxins, mitogenome, phylogenomics, transcriptomics

1 | INTRODUCTION

In few decades of existence, the field of molecular phylogenetics has come of age and undoubtedly represents a quantum leap for inferring the evolutionary history of organisms. For many years, one of the main limitations of molecular phylogenetics derived from the use of single or few genes, which could often render unresolved trees (Bleidorn et al., 2009; Rokas, Williams, King, & Carroll, 2003). This problem has been particularly severe in the case of evolutionary radiations (i.e., major diversification events occurring in a relatively short period of time) since phylogenetic relationships among taxa are difficult to disentangle due to the limited associated phylogenetic signal, which is visualized as short internal nodes in the tree (Philippe et al., 2011). Upon the advent of the next-generation sequencing (NGS) techniques, the capacity for generating genomewide markers for virtually any taxon was thought to overcome this limitation and open the possibility of reconstructing fully resolved trees (Rokas et al., 2003). Although NGS results are encouraging, it has become apparent that just adding many genes is not enough, as the taxon sampling (including outgroup selection), fit of the evolutionary models and marker congruence, among others, also have capital effects on the results (Hedtke, Townsend, & Hillis, 2006; Jeffroy, Brinkmann, Delsuc, & Philippe, 2006; Philippe et al., 2011). A new field, phylogenomics, has emerged to uncover drawbacks associated with large sequence data sets and develop new analytical methods, which are effectively helping in the resolution of most recalcitrant nodes in the "Trees of Life" (Vargas & Zardoya, 2014) of diverse groups such as molluscs (Smith et al., 2011), annelids (Weigert et al., 2014), vertebrates (Irisarri et al., 2017), arthropods (Espeland et

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al., 2018; Sharma et al.., 2018) or land plants (Wickett et al., 2014), among many others rapidly accumulating.

Cone snails constitute a paradigm of a natural group, which has diversified and evolved through radiation events. Their rich fossil record shows that successive radiations along cone evolutionary history have contributed to their current great species diversity (Kohn, 1990), including most recent diversifications in the Cabo Verde archipelago (Cunha, Castilho, Rüber, & Zardoya, 2005; Duda & Rolán, 2005), the Senegalese coast (Pin & Leung Tack, 1995), Madagascar (Monnier, Tenorio, Bouchet, & Puillandre, 2018) or the Caribbean Sea (Kohn, 2014). Altogether, old and recent radiations have led to astonishing species diversity with a worldwide distribution. Currently, there are more than 900 species of cone snails described and this number grows steadily every year (MolluscaBase, 2018), being present in a broad range of depths in all tropical and subtropical seas (Tucker & Tenorio, 2013).

Cones are a key ecological component in intertidal and subtidal habitats, where most species feed mainly on worms, but also some on molluscs and others on fishes (Kohn, 1959). Cone predatory capacity relies on a sophisticated venom system, formed by hollow harpoon-like radular teeth, which inject a cocktail of hundreds of different peptides named conotoxins (Li et al., 2017; Norton & Olivera, 2006; Peng et al., 2016). The great specificity and biological potential of conotoxins have attracted the interest of pharmacological research (Miljanich, 2004; Yang et al., 2017), and venom gland transcriptomics are currently the main tool for cataloguing the cocktail composition in the different species (Abalde, Tenorio, Afonso, & Zardoya, 2018; Barghi, Concepcion, Olivera, & Lluisma, 2015; Dutertre et al., 2014; Hu, Bandyopadhyay, Olivera, & Yandell, 2012; Li et al., 2017; Peng et al., 2016). In this regard, understanding the evolutionary processes involved in conotoxin diversification and adaptation to different preys requires a robust phylogeny of cones.

The extraordinary species diversity of cones has made their systematics particularly challenging. For many years, the consensus was to classify cone snails into the single genus *Conus*, but recent morphological (Tucker & Tenorio, 2009) and molecular (Duda & Kohn, 2005; Puillandre et al., 2014; Uribe, Puillandre, & Zardoya, 2017) phylogenetic studies discovered enough divergence among main lineages inside the group to ultimately propose the splitting of genus *Conus* into several genera. A first molecular phylogenetic study (Puillandre et al., 2014) recognized four main genera: *Profundiconus*, *Californiconus*, *Conasprella* and *Conus*; the latter holding most of the species diversity with up to 60 monophyletic groups classified as subgenera. A subsequent molecular phylogenetic study (Uribe, Puillandre, et al., 2017) added two more genera: *Lilliconus* and *Pseudolilliconus* (later redefined *Pygmaeconus*; Puillandre & Tenorio, 2017). Alternatively, an exhaustive review of morphological characters classified cones into 84 extant genera (Tucker & Tenorio, 2009), 63 of them included within the family Conidae (equivalent to the genus *Conus* in Puillandre et al., 2014). These numbers were raised in Tucker and Tenorio (2013) to 114 extant genera, and 90 within the family Conidae. Therefore, both the morphological and the molecular proposals agreed on the need of going beyond the single genus classification, were generally congruent in the definition of the groups and only differed in the taxonomic rank that should be used. Furthermore, these groups are monophyletic, share shell and radula synapomorphies and often correspond to biogeographical assemblies, suggesting that the use of the corresponding generic names may be more suitable and convenient. Therefore, we will follow here the classification of Tucker and Tenorio (2009) with the updates of Tucker and Tenorio (2013) and focus on the phylogenetic relationships among genera within Conidae sensu these authors.

Therefore, the challenge is going beyond phylogenies of cones based on few genes (Aman et al., 2015; Puillandre et al., 2014) and reconstruct robust phylogenetic relationships among cone genera sensu Tucker and Tenorio (2009) based on multilocus sequences data sets such as the recent one based on concatenating hundreds to thousands of exon sequences (Phuong & Mahardika, 2018). An additional source of molecular markers for phylogenetic inference is mitochondrial (mt) genomes, which have been widely used in gastropods and proven to be particularly useful for resolving phylogenies at the family level (Osca, Templado, & Zardoya, 2015; Uribe, Williams, Templado, Abalde, & Zardoya, 2017) as well as for disentangling recent radiation events (Abalde, Tenorio, Afonso, & Zardoya, 2017; Abalde, Tenorio, Afonso, Uribe, et al., 2017). Although there are currently about 150 mt genomes of the family Conidae available in GenBank at NCBI (https://www.ncbi.nlm.nih.gov/), the total diversity of the group is clearly underrepresented as these mitogenomes belong to only 11 out of the currently 89 described genera (the genus *Trovaoconus* of Tucker and Tenorio (2009) was recently synonymized with *Kalloconus* by Abalde, Tenorio, Afonso, Uribe, et al., 2017). Alternatively, the many recent Illumina RNA-Seq studies of cone venom glands have deposited millions of raw reads on the Sequence Read Archive (SRA) at NCBI, which belong to 19 different genera, and could be used for cone phylogenomics. Here, we used all these publicly available sequence data and four newly sequenced mt genomes aiming to (a) reconstruct a robust phylogeny for the family Conidae, based on mitochondrial and nuclear data; (b) infer the evolution of diet specialization, radular morphology and conotoxin diversity; and (c) date major cladogenetic events within the family.

TABLE 1 Mitochondrial genomes analysed in this study

Species	Diet	Data source	Transcriptome		mt genome	
			SRA No.	Reference	GenBank Acc. No.	Reference
Africonus borgesi (Trovão, 1979)	Vermivorous	GenBank	—	—	NC_013243	Cunha, Grande, and Zardoya (2009)
Africonus infinitus (Rolán, 1990)	Vermivorous	GenBank	—	—	KY864967	Abalde, Tenorio, Afonso, Uribe, et al. (2017)
Africonus miruchae (Röckel, Rolán & Monteiro, 1980)	Vermivorous	GenBank	—	—	KY864971	Abalde, Tenorio, Afonso, Uribe, et al. (2017)
Calamiconus quercinus (Lightfoot, 1791)	Vermivorous	GenBank	—	—	KY609509	Gao, Peng, Chen, Zhang, and Shi (2018)
Calamiconus quercinus (Lightfoot, 1791)	Vermivorous	SRA	SRR2609537	Phuong, Mahardika, and Alfaro (2016)	—	This study
Chelyconus ermineus (Born, 1778)	Piscivorous	GenBank	—	—	KY864977	Abalde, Tenorio, Afonso, Uribe, et al. (2017)
Chelyconus ermineus (Born, 1778)	Piscivorous	SRA	SRR6983166, 68, 69	Abalde et al. (2018)	—	This study
Conus marmoreus Linnaeus, 1758	Molluscivorous	SRA	SRR2609532	Phuong et al. (2016)	—	This study
Cylinder gloriamaris (Chemnitz, 1777)	Molluscivorous	GenBank	—	—	NC_030213	Chen, Hsiao, Huang, et al. (2016) unpublished
Cylinder gloriamaris (Chemnitz, 1777)	Molluscivorous	SRA	SRR5499408	Robinson et al. (2017)	—	This study
Cylinder textile (Linnaeus, 1758)	Molluscivorous	GenBank	—	—	NC_008797	Bandyopadhyay et al. (2008)
Cylinder victoriae (Reeve, 1843)	Molluscivorous	SRA	SRR833564	Robinson *et al*. (2014)	—	This study
Darioconus episcopatus (da Motta, 1982)	Molluscivorous	SRA	DRR034332	Lavergne *et al*. (2015)	—	This study
Dendroconus betulinus (Linnaeus, 1758)	Vermivorous	SRA	SRR2124881	Peng et al. (2016)	—	This study
Eugeniconus nobilis (Linnaeus, 1758)	Molluscivorous	GenBank	—	—	KX263253	Uribe, Puillandre, et al. (2017)
Fulgiconus goudeyi (Monnier & Limpalaër, 2012)	Vermivorous	PCR	—	—	KY864975	This study
Gastridium geographus (Linnaeus, 1758)	Piscivorous	SRA	SRR503413-16	Hu et al. (2012)	—	This study
Gastridium tulipa (Linnaeus, 1758)	Piscivorous	GenBank	—	—	NC_027518	Chen, Hsiao, Huang, et al. (2016)
Genuanoconus genuanus (Linnaeus, 1758)	Vermivorous (Amphinomidae)	PCR	—	—	KY864974	This study

(Continues)

TABLE 1 (Continued)

Species	Diet	Data source	Transcriptome		mt genome	
			SRA No.	Reference	GenBank Acc. No.	Reference
Harmoniconus sponsalis (Hwass in Bruguière, 1792)	Vermivorous	SRA	SRR2609541	Phuong *et al*. (2016)	—	This study
Kalloconus ateralbus (Kiener, 1850)	Vermivorous	GenBank	—	—	KY864970	Abalde, Tenorio, Afonso, Uribe, et al. (2017)
Kalloconus pulcher ([Lightfood]), 1786)	Vermivorous	GenBank	—	—	KY864972	Abalde, Tenorio, Afonso, Uribe, et al. (2017)
Kalloconus pulcher ([Lightfood]), 1786)	Vermivorous	GenBank	—	—	KY864973	Abalde, Tenorio, Afonso, Uribe, et al. (2017)
Kalloconus trochulus (Reeve, 1844)	Vermivorous	GenBank	—	—	KY864969	Abalde, Tenorio, Afonso, Uribe, et al. (2017)
Kalloconus venulatus (Hwass in Bruguière, 1792)	Vermivorous	GenBank	—	—	KX263250	Uribe, Puillandre, et al. (2017)
Kioconus lenavati (da Motta & Röckel, 1982)	Vermivorous	SRA	SRR1803942	Barghi et al. (2015)	—	This study
Kioconus tribblei (Walls, 1977)	Vermivorous	GenBank	—	—	NC_027957	Barghi, Concepcion, Olivera, and Lluisma (2016)
Kioconus tribblei (Walls, 1977)	Vermivorous	SRA	SRR1802610	Barghi et al. (2015)	—	This study
Kioconus tribblei (Walls, 1977)	Vermivorous	SRA	SRR1803938	Barghi et al. (2015)	—	This study
Lautoconus guanche (Lauer, 1993)	Vermivorous	GenBank	—	—	KY801847	Abalde, Tenorio, Afonso, and Zardoya (2017)
Lautoconus hybridus (Kiener, 1847)	Vermivorous	GenBank	—	—	KX263252	Uribe, Puillandre, et al. (2017)
Lautoconus ventricosus (Gmelin, 1791)	Vermivorous	GenBank	—	—	KX263251	Uribe, Puillandre, et al. (2017)
Lindaconus spurius (Gmelin, 1791)	Vermivorous	PCR	—	—	KY864976	This study
Lividoconus lividus (Hwass in Bruguière, 1792)	Vermivorous	SRA	SRR2609539	Phuong et al. (2016)	—	This study
Miliariconus coronatus (Gmelin, 1791)	Vermivorous	SRA	SRR2609545	Phuong et al. (2016)	—	This study
Miliariconus miliaris (Hwass in Bruguière, 1792)	Vermivorous	SRA	SRR1548185	Weese and Duda (2015)	—	This study
Monteirococonus tabidus (Reeve, 1844)	Vermivorous	PCR	—	—	KY864968	This study
Pionoconus consors (G. B. Sowerby I, 1833)	Piscivorous	GenBank	—	—	NC_023460	Brauer et al. (2012)
Pionoconus consors (G. B. Sowerby I, 1833)	Piscivorous	SRA	SRR1955039	Leonardi et al. (2012)	—	This study
Pionoconus striatus (Linnaeus, 1758)	Vermivorous	GenBank	—	—	NC_030536	Chen et al. (2016b)

(Continues)

TABLE 1 (Continued)

Species	Diet	Data source	Transcriptome		mt genome	
			SRA No.	Reference	GenBank Acc. No.	Reference
Puncticulis arenatus (Hwass in Bruguière, 1792)	Vermivorous	SRA	SRR2609544	Phuong et al. (2016)	—	This study
Rhizoconus capitaneus (Linnaeus, 1758)	Vermivorous	GenBank	—	—	NC_030354	Chen et al. (2016a)
Rhizoconus vexillum (Gmelin, 1791)	Vermivorous	SRA	SRR2890189	Prashanth et al. (2016)	—	This study
Rhombiconus imperialis (Linnaeus, 1758)	Vermivorous	SRA	SRR2609542	Phuong et al. (2016)	—	This study
Rolaniconus varius (Linnaeus, 1758)	Vermivorous	SRA	SRR2609543	Phuong et al. (2016)	—	This study
Splinoconus biliosus (Röding, 1798)	Vermivorous	SRA	SRR1956759	Unpublished	—	This study
Virgiconus virgo (Linnaeus, 1758)	Vermivorous	SRA	SRR2608262	Phuong et al. (2016)	—	This study
Virroconus ebraeus (Linnaeus, 1758)	Vermivorous	SRA	SRR2609538	Phuong et al. (2016)	—	This study
Conasprella wakayamaensis (Kuroda, 1956)	Vermivorous	GenBank	—	—	KX263254	Uribe, Puillandre, et al. (2017)
Californiconus californicus (Reeve, 1844)	All	GenBank	—	—	KX263249	Uribe, Puillandre, et al. (2017)
Californiconus californicus (Reeve, 1844)	All	SRA	SRR2609536	Phuong et al. (2016)	—	This study
Profundiconus teramachii (Kuroda, 1956)	Vermivorous	GenBank	—	—	KX263256	Uribe, Puillandre, et al. (2017)
Tomopleura sp.	—	GenBank	—	—	KX263259	Uribe, Puillandre, et al. (2017)

Note. Genbank: published mtDNA; PCR: amplified mtDNA; SRA: RNA sequences.

TABLE 2 Nuclear genes analysed in this study

Gene	Length (bp)	Length (aa)
Translocon-associated protein subunit alpha	921	307
Cathepsin Z	942	314
Eukaryotic translation elongation factor 1 beta 2-like	660	220
Transmembrane protein 59-like	1,023	341
RWD domain-containing protein 1-like	750	250
Eukaryotic translation initiation factor 3 subunit G-like	825	275
Alcohol dehydrogenase class-3-like protein	1,143	381
Eukaryotic translation initiation factor 3 subunit F-like	879	293
Sodium-/potassium-transporting ATPase subunit beta-like	894	298
Ferritin	522	174
Syntenin-1	909	303
Bax inhibitor-1	600	200
CD63 antigen	720	240
Translocation protein SEC62-like	1,185	395
Cyclin-I	990	330
Nucleoside diphosphate kinase B	507	169
Mitochondrial import receptor subunit TOM20	459	153
Ragulator complex protein LAMTOR3-A	378	126
Coatomer subunit epsilon-like	903	301
Nascent polypeptide-associated complex subunit alpha-like protein	630	210
Elongation factor 1-gamma	1,284	428
Total	17,124	5,708

2 | MATERIALS AND METHODS

2.1 | Taxon sampling

We studied 48 specimens belonging to 41 different species and 27 genera within the family Conidae (Table 1). As outgroup, we selected five specimens of another four highly divergent genera (*Californiconus*, *Conasprella* and *Profundiconus* of cone snails and *Tomopleura* of the family Borsoniidae). During November 2017, a total of 25 mt genome and 24 RNA-Seq entries were downloaded from GenBank and SRA databases at NCBI, respectively (Table 1). We also sequenced the nearly complete (only missing the control region and flanking regions) mt genomes of four species: *Fulgiconus goudeyi* (voucher MNCN/ADN 95093; from New Caledonia), *Genuanoconus genuanus* (voucher MNCN/ADN 95096; from Cabo Verde), *Lindaconus spurius* (voucher MNCN/ADN 95097; from Aruba) and *Monteiroconus tabidus* (voucher MNCN/ADN 95098; from Cabo Verde).

2.2 | Mitochondrial DNA extraction, PCR amplification, sequencing and annotation

Total genomic DNA was isolated from up to 1–3 mg of foot tissue of one individual of *F. goudeyi*, *G. genuanus, L. spurius* and *M. tabidus* following a standard phenol-chloroform extraction. The mtDNA was PCR-amplified in two or three overlapping fragments following Uribe, Puillandre, et al. (2017). Long PCR products from the same mitogenome were pooled together in equimolar concentrations, and an indexed library was constructed using the NEXTERA XT DNA library prep kit (Illumina, San Diego, CA, USA). The four libraries were sequenced together with others from different projects in a single run of an Illumina MiSeq platform (2 × 150 paired-end reads) at Sistemas Genómicos (Valencia, Spain).

Raw reads were uploaded to the TRUFA webserver (Kornobis et al., 2015), where the 150 bp reads were trimmed and filtered out if the PHRED quality was below 20 using PRINSEQ v.0.20.3 (Schmieder & Edwards, 2011) and de novo assembled using Trinity r2012-06-08 (Grabherr et al., 2011) with default parameters. The resulting contigs with a minimum length of 5 kb were selected and used as reference to map all remaining reads using Geneious® 8.1.8. The mapping requirements were a minimum overlap of 60 bp with a 100% of identity and one mismatch allowed.

Newly sequenced mitogenomes were annotated using already published mtDNAs of other cone snails as a reference with Geneious. The open reading frames (ORFs) of the 13 mt protein-coding genes were manually checked for potential misannotations in the start and stop codons. All mt transfer RNA (tRNA) genes were identified using tRNAscan-SE 1.21 (Lowe & Chan, 2016), which infers cloverleaf secondary structures (with a few exceptions that were determined manually). The mt ribosomal RNA (rRNA) genes were identified by comparison with orthologous genes in other Conidae mt genomes and assumed to extend to the boundaries of adjacent genes (Boore, Macey, & Medina, 2005).

2.3 | Transcriptome assembly and annotation

The raw reads from the different cone venom gland transcriptome projects were downloaded from the SRA repository at NCBI and trimmed, filtered and de novo assembled in the TRUFA webserver, using default parameters in PRINSEQ v.0.20.3 (Schmieder & Edwards, 2011) and Trinity r2012-06-08 (Grabherr et al., 2011). All contigs with more than 200 bp were kept for annotation.

The different mt genes were identified by BLASTn searches against a custom database, formed by the already published mt genomes of cone snails. The sequences of the assembled mt genes are presented in Supporting Information Table S1. In addition, nuclear ORFs were identified using TransDecoder (Haas, 2016). Those genes which were common to all studied species were selected using the AGALMA pipeline (Dunn, Howison, & Zapata, 2013). Briefly, all homologous sequences were identified through an all-by-all blast approach. The resulting gene clusters were aligned and cleaned, and a phylogenetic tree for each gene cluster was built using RAxML v.8.1.16 (Stamatakis, 2014). Orthologs were identified by looking for repeated species trees in the gene tree (indicative of gene duplications). Only those orthologs present in at least 21 of the 24 analysed individuals were kept. For each of the selected orthologs, sequences were aligned using TranslatorX (Abascal, Zardoya, & Telford, 2010) and manually curated and filtered, as suggested by Philippe et al. (2011) in order to detect translational frameshifts and local sequencing errors as well as unusually divergent sequences indicating potential contaminations. Finally, the 21 most complete and divergent nuclear gene sequences were chosen (Table 2) in order to obtain similar amounts of nuclear and mt sequence data in the final combined data set.

As a proof of concept, in those cases for which transcriptomic and mitogenomic sequence data were available independently for the same species (*Calamiconus quercinus*, *Californiconus californicus*, *Chelyconus ermineus*, *Cylinder gloriamaris*, *Kioconus tribblei* and *Pionoconus consors*), the number of different positions and percentages of similarity were examined.

2.4 | Sequencing alignment and phylogenetic analyses

Three data sets were compiled: (a) mt genome, which included 13 mt protein-coding and two rRNA genes; (b) nuclear, which included 21 nuclear genes; and (c) combined, which included mt and nuclear genes. Protein-coding genes were analysed both at the nucleotide and the amino acid levels. They were individually aligned using TranslatorX (Abascal et al., 2010), whereas the nucleotide sequences of the rRNA genes were aligned using MAFFT v7 (Katoh & Standley, 2013). All ambiguously aligned positions were removed using GBlocks v.0.9.1b (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. Sequences were format converted for further analyses using the ALTER webserver (Glez-Pena, Gomez-Blanco, Reboiro-Jato, Fdez-Riverola, & Posada, 2010). Finally, the different single alignments were concatenated using Geneious. Alignments can be accessed at TreeBase (http://purl.org/phylo/treebase/phylows/study/TB2:S22475).

The best-fit partition schemes and models of substitution for each data set were identified using PartitionFinder (Lanfear, Calcott, Ho, & Guindon, 2012) with the Bayesian information criterion (BIC; Schwarz, 1978). The following partitions were tested: all genes together, all mt genes together versus all nuclear genes together, all genes arranged in subunits (*atp*, *cob*, *cox*, *nad, rrn* and *nuclear*) and all genes separated (except *atp6-atp8* and *nad4-nad4L)*. In addition, for those data sets in which protein-coding genes were analysed at the nucleotide level, we also tested separately the three codon positions.

Phylogenetic relationships were inferred using maximum likelihood (ML; Felsenstein, 1981) and Bayesian inference (BI; Huelsenbeck & Ronquist, 2001). For ML, we used RAxML v.8.1.16 (Stamatakis, 2014) with the rapid hill-climbing algorithm and 10,000 bootstrap pseudoreplicates. BI analyses were conducted using (a) MrBayes v3.1.2 (Ronquist & Huelsenbeck, 2003), performing two independent runs (to increase the chance of adequate mixing of the Markov chains and of convergence) with four simultaneous Markov chains for 10 million of generations, sampling every 1,000 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; and (b) PhyloBayes MPI v1.5 (Lartillot, Rodrigue, Stubbs, & Richer, 2013), running two independent chains under a site-heterogeneous CAT-GTR model and based only on

FIGURE 1 Phylogeny of the family Conidae. The reconstructed BI tree using best-fit partitions and site-homogeneous models based on the mt genome data set (concatenated 13 protein-coding genes analysed as amino acid sequences plus two rRNA genes at nucleotide level) is shown. Bayesian posterior probabilities (BPP) supporting nodes are shown as black (BPP = 1) and white (BPP = 0.95–0.99) dots. Bold names indicate those species whose genes were assembled from transcriptomic data. Branch colours and silhouettes (downloaded from PhyloPic) represent ancestral character state reconstruction under unordered parsimony of diet specializations: blue, vermivory; brown, vermivory on amphinomids; green, molluscivory; red, piscivory. In the inset, radular teeth from (Tucker & Tenorio, 2009) corresponding to the different feeding modes are illustrated. Shell pictures are from the authors or from Alexander Medvedev (www.coneshells-am.ruwileyonlinelibrary.com]). Scale bar indicates substitutions/site. [Colour figure can be viewed at wileyonlinelibrary.com]

Radular type
Piscivorous
Molluscivorous
Vermivorous (Amphinomidae)
Generalist
Vermivorous

Cylinder gloriamaris
Cylinder gloriamaris
Cylinder victoriae
Cylinder textile
Darioconus episcopatus
Conus marmoreus
Eugeniconus nobilis
Dendroconus betulinus
Chelyconus ermineus
Chelyconus ermineus
Puncticulis arenatus
Harmoniconus sponsalis
Fulgiconus goudeyi
Gastridium tulipa
Gastridium geographus
Pionoconus striatus
Pionoconus consors
Pionoconus consors
Africonus borgesi
Africonus infinitus
Africonus miruchae
Lautoconus guanche
Lautoconus hybridus
Lautoconus ventricosus
Kalloconus trochulus
Kalloconus venulatus
Kalloconus ateralbus
Kalloconus pulcher
Kalloconus pulcher
Miliariconus coronatus
Virroconus ebraeus
Miliariconus miliaris
Lindaconus spurius
Calamiconus quercinus
Calamiconus quercinus
Lividoconus lividus
Virgiconus virgo
Genuanoconus genuanus
Rhombiconus imperialis
Rhizoconus capitaneus
Rhizoconus vexillum
Kioconus tribblei (454)
Kioconus tribblei **(Illumina)**
Kioconus tribblei
Kioconus lenavati
Splinoconus biliosus
Monteiroconus tabidus
Rolaniconus varius
Californiconus californicus
Californiconus californicus
Conasprella wakayamaensis
Profundiconus teramachii
Tomopleura sp. (Borsoniidae)

0.09

the protein-coding genes at the amino acid or nucleotide levels. Convergence between chains was assessed a posteriori using the *bpcomp* and *tracecomp* tools implemented in PhyloBayes.

The outgroups used with the mt genome data set were *Conasprella wakayamaensis*, two specimens of *C. californicus*, *Profundiconus teramachii* and *Tomopleura sp*. (Family Borsoniidae). The outgroup used with the nuclear and combined data sets was one specimen of *C. californicus*.

Ancestral character state reconstructions of diet specialization, radular morphology and the type of protoconch (paucispiral or multispiral indicating lecitotrophic or planktonic larvae, respectively) as described in Tucker and Tenorio (2009) were performed using unordered maximum parsimony with Mesquite (Maddison & Maddison, 2018) and mapped onto our best working hypothesis for the phylogeny of Conidae (see Results), that is, the one recovered by the BI analysis based on the mt genome data set with protein-coding genes analysed at the amino acid level.

2.5 | Estimation of divergence times

Divergence times were estimated following a Bayesian approach using the software BEAST v.1.7.5 (Drummond & Rambaut, 2007). An uncorrelated relaxed molecular clock was used to infer branch lengths and nodal ages. The tree topology was fixed using our best working hypothesis for the phylogeny of Conidae but with only one tip per species and fixing only those nodes with high statistical support (BPP = 0.95–1) . 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. Concatenated mt protein-coding genes (amino acids) plus rRNA (nucleotides) genes were analysed. We used the mtREV (+I+G) substitution model for amino acids (the closest model available in the programme to the one selected by PartitionFinder) and the GTR (+I+G) substitution model for the rRNA genes (see Results). The final Markov chain was run twice for 26 million generations,

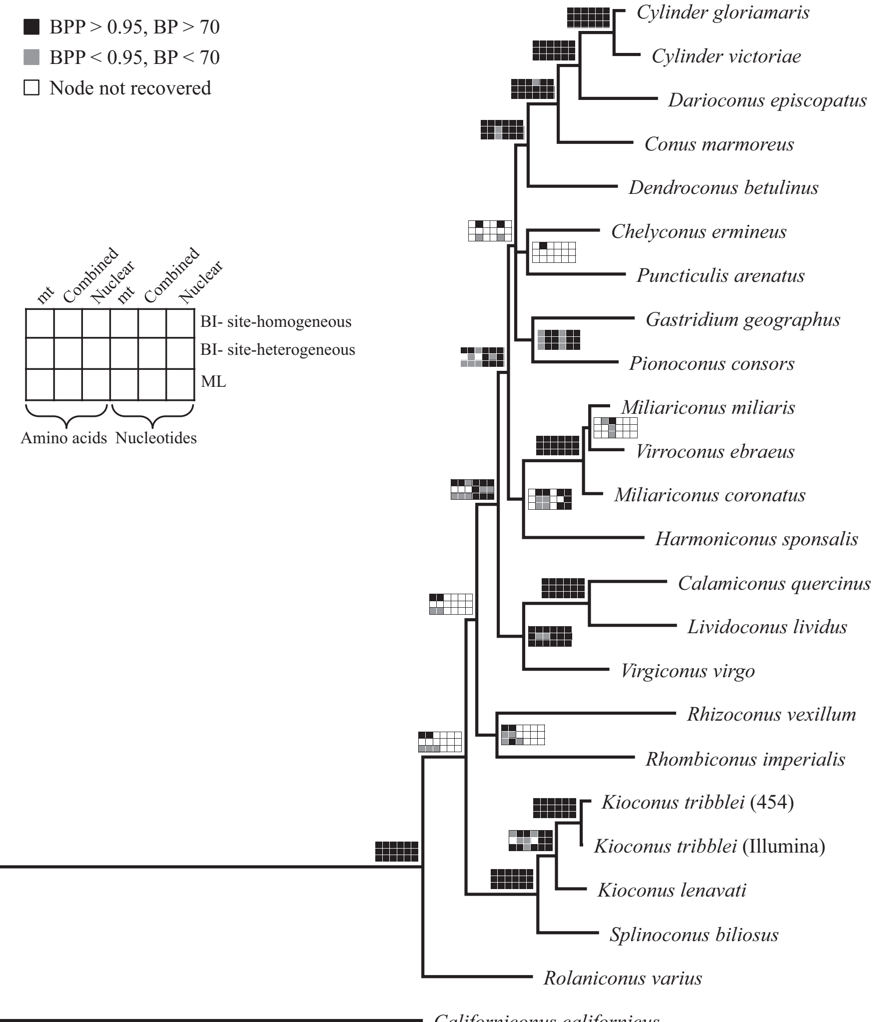

FIGURE 2 The reconstructed BI tree using best-fit partitions and site-homogeneous models based on the combined (mt + nuclear) data set with protein-coding genes analysed at the amino acid level. For each node, statistical support based on the 18 different phylogenetic analyses is shown: A black square represents BPP > 0.95 or BP > 70% in BI and ML, respectively; a gray square represents BPP between 0.90 and 0.95 or BP between 50% and 70%; a white square indicates no recovery of the node. Scale bar indicates substitutions/site. The genus *Californiconus* was used as outgroup

sampling every 10,000 generations, and the first 1,000 trees were discarded as part of the burn-in process, according to the convergence of chains checked with Tracer v.1.6. (Rambaut & Drummond, 2007). The ESS of all parameters was above 200 except for seven of 42 tmrca (time to most recent common ancestor) statistics, which were above 150.

Although there are many reported fossils of cone snails, their identification is not always straightforward, since shell morphology is prone to homoplasy (Abalde, Tenorio, Afonso, Uribe, et al., 2017; Duda, Bolin, Meyer, & Kohn, 2008). Hence, the use of cone fossils for calibration has to be done with caution. We tried two different approaches to calibrate the molecular clock: (a) The first known fossil of a cone snail was used to date the divergence between *Tomopleura sp*. (Borsoniidae) and Conidae (57 million years ago –mya–; Tracey, Craig, Belliard, & Gain, 2017) and the age of formation of Sal, the oldest island of Cabo Verde (28 mya; Holm et al., 2008), was used to date the divergence between *Africonus* and (paraphyletic) *Lautoconus*, as the colonization and diversification of *Africonus* species endemic to the different Cabo Verde islands was reported to occur shortly after island emergence (Abalde, Tenorio, Afonso, Uribe, et al., 2017); (b) the two previous references and three well-recognized fossils, which belong to *Conasprella* (Squire, 1987), *Kioconus* (Beu & Maxwell, 1990) and *Cylinder* (Shuto, 1969) lineages that would allow us testing their reliability as calibration points.

The calibration points were included in the analysis as follows. For the date of origin of Conidae (at least, 57 mya), we used a log-normal distribution, enforcing the mean to 58 ($SD = 0.05$, offset = 0.0001). The origin of Sal island (28 mya) was defined by a log-normal distribution, with mean in 24.5 ($SD = 0.05$, offset = 0.7). The three fossils were calibrated using normal distributions, whose means were 44.25 ($SD = 5.4$) for *Conasprella*, 19.5 ($SD = 1.8$) for *Kioconus* and 9.5 ($SD = 3.3$) for *Cylinder*.

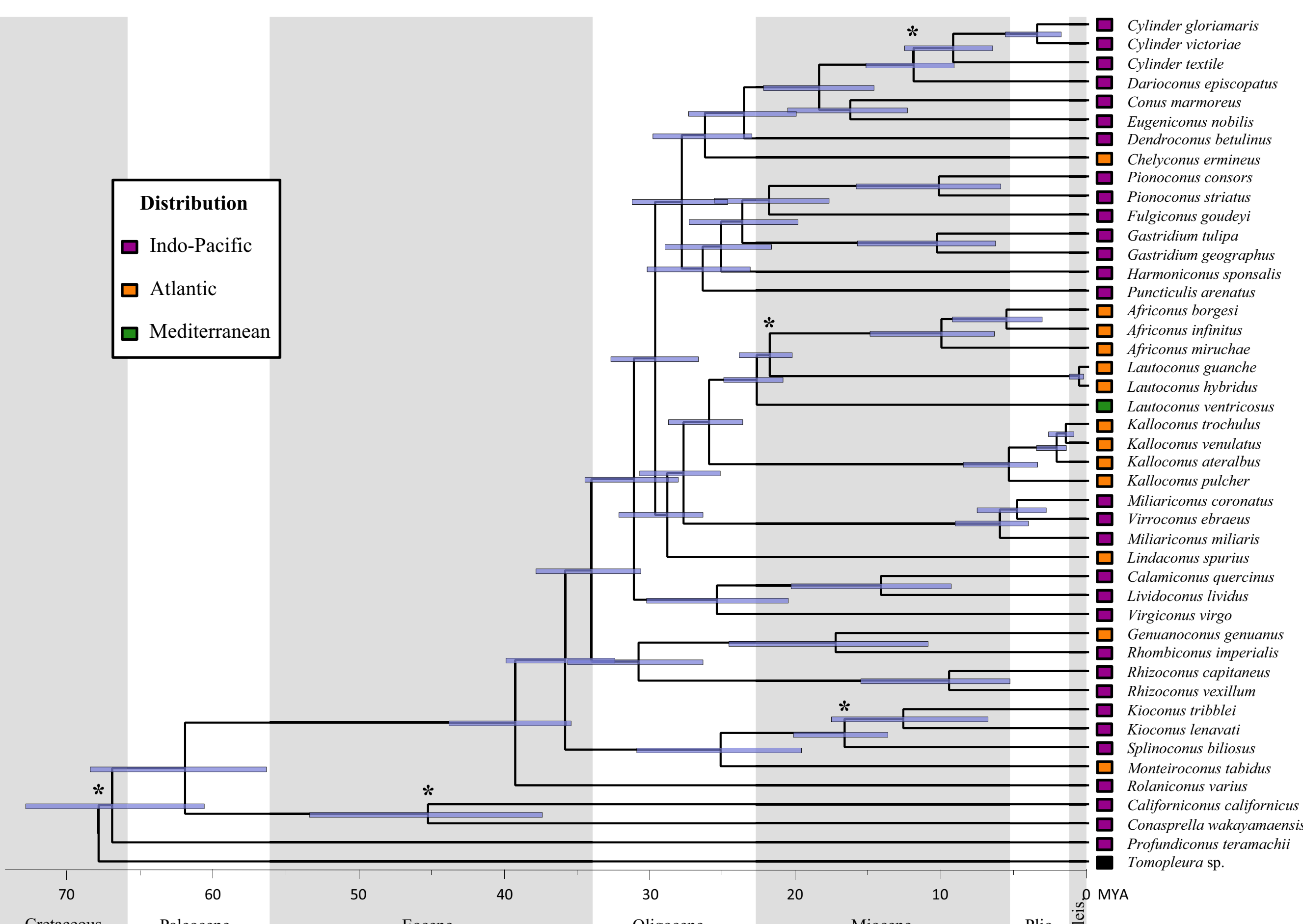

FIGURE 3 Chronogram based on the mitogenome data set (concatenated 13 protein-coding genes analysed as amino acid sequences plus two rRNA genes at nucleotide level) using the topology shown in Figure 1 (only nodes with high statistical support [BPP = 0.95–1] were fixed). A Bayesian uncorrelated relaxed lognormal clock with geographic- and fossil-based calibration priors (denoted by asterisks) was used in BEAST. Horizontal bars represent 95% credible intervals for time estimates; dates are in millions of years. Geological ages are highlighted as gray-white intervals. Square colours indicate cone distributions: purple, Indo-Pacific Ocean; orange, Atlantic Ocean; and green, Mediterranean Sea [Colour figure can be viewed at wileyonlinelibrary.com]

3 | RESULTS

3.1 | Sequencing, assembly and annotation

The nearly complete mt genomes of *F. goudeyi* (length = 15,261 bp; mean coverage = 5,321×; number of reads = 538,094), *G. genuanus* (15,328 bp; 7,301×; 741,250 reads), *L. spurius* (15,329 bp; 2,003×; 203,397 reads) and *M. tabidus* (15,368; 2,821×; 287,156 reads) were sequenced only lacking a fragment including the *trnF*, the control region and the beginning of the *cox3* gene, which was not PCR-amplified. All these mt genomes encode for 13 protein-coding, two rRNA and 22 tRNA genes (but note that the presence of the *trnF* gene could not be determined) and share the same gene order. All genes were encoded by the major strand, except those forming the cluster MYCWQGE (*trnM*, *trnY*, *trnC*, *trnW*, *trnQ*, *trnG*, *trnE*) and the *trnT* gene. The complete annotations of the newly determined mt genomes including start and end of each gene, start and stop codons of protein-coding genes, and the position and length of intergenic sequences are provided in Supporting Information Table S2. The start codon of all protein-coding genes is ATG, except in the case of the *nad4* gene of *F. goudeyi*, *G. genuanus* and *L. spurius*, which is GTG. The stop codon showed more variation among genes (TAG, TAA and TA−).

The transcriptomes downloaded from the SRA database were sequenced using various platforms, which rendered different read depths (details in Supporting Information Table S3). We could identify the 13 protein-coding and two rRNA genes in most cases, but one gene was missing for *C. californicus* and *K. tribblei* (Illumina), two for *Gastridium geographus* and *Rolaniconus varius*, three for *Rhizoconus vexillum*, four for *K. tribblei* (454) and six genes for *Splinoconus biliosus* (Supporting Information Figure S1 and Table S3). Regarding the nuclear data set, the matrix completeness (in terms of presence/absence of genes) was 94%. The species that presented more missing data were *K. tribblei* (454; nine missing genes), *Darioconus episcopatus* (five missing genes) and *C. californicus* and *Splinoconus biliosus* (four missing genes; Supporting Information Figure S1 and Table S3).

For six species, it was possible to compare the sequences of the mt genes assembled from the transcriptomes to the corresponding ones from the mt genomes available in GenBank. The percentage of sequence similarity was above 98% for all genes (slight sequence differences may reflect either distinct geographic origins, individual variability or sequencing errors; see Supporting Information Table S4), which confirms the reliability of our pipeline and the data obtained.

3.2 | Phylogenetic analyses

The phylogenetic relationships within the family Conidae were reconstructed based on three different data sets. The mt genome data set had 13,285 or 5,743 positions depending on whether protein-coding genes were analysed at the nucleotide or amino acid level, respectively; the nuclear data set had 17,124 or 5,708 positions; and the combined data set had 30,266 or 11,456 positions. The best-fit partitions and substitution models according to BIC for each data set can be found in Supporting Information Table S5. A total of 18 phylogenetic trees were reconstructed: Three data sets with protein-coding genes analysed at either the nucleotide or the amino acid level and with either ML or BI (using either site-homogeneous or site-heterogeneous models). The 18 phylogenies are shown in Supporting Information Figure S2, which can be accessed at TreeBase (http://purl.org/phylo/treebase/phylows/study/TB2:S22475), and their log-likelihoods can be consulted in Supporting Information Table S6. Phylogenetic inferences based on BI or ML using site-homogeneous models arrived at very similar or even identical topologies (BI and ML trees of the mt genome data set analysed at the amino acid level; BI and ML trees of the mt genome data set analysed at the nucleotide level; and BI and ML trees of the combined data set analysed at the nucleotide level; Supporting Information Figure S2). Therefore, the main differences among trees were due to the data set or whether the protein-coding genes were analysed at the amino acid or the nucleotide levels. In general, the different trees (Supporting Information Figure S2) agreed on terminal clades, which generally received strong statistical support in all analyses, and differed mainly on those internal nodes, which lacked statistical support (BP < 70; BPP < 0.95) or which directly rendered a polytomy as was the case of trees based on BI using site-heterogeneous models (Supporting Information Figure S2).

The phylogenetic trees with overall better statistical support along the different nodes were reconstructed using BI, site-homogeneous models, and based on protein-coding genes analysed at either the amino acid or the nucleotide levels. Among these trees, the ones including more taxa (53 tips, 27 genera) were those based on mt genomes. Here, we selected arbitrarily the one using protein-coding genes analysed at the amino acid level as our best working hypothesis for the phylogeny of Conidae (Figure 1), as its differences to the corresponding one with protein-coding genes analysed at the nucleotide level are minimum and restricted to statistically unsupported nodes. According to the selected phylogenetic tree, the Conidae are monophyletic and exhibit a long branch, which separate them from outgroup taxa (Figure 1). All the genera with more than one species were recovered as monophyletic (although with low statistical support in the case of *Kioconus*; BPP = 0.63) except *Miliariconus* (due to *Virroconus*, with BPP = 0.54) and *Lautoconus* (due to *Africonus*, with BPP = 0.98). The genus *Rolaniconus* is sister to all remaining Conidae (BPP = 0.99). Within the latter, a well supported clade (BPP = 1) including *Monteiroconus* sister to *Splinoconus* + *Kioconus*

was the sister group of the remaining taxa (Figure 1). The clade (BPP = 0.99) including *Rhizoconus* sister to *Genuanoconus* + *Rhombiconus* (both preying on worms of the family Amphinomidae) was sister to a clade (BPP = 1) including *Virgiconus* sister to *Lividoconus* + *Calamiconus* and a clade with the remaining analysed Conidae (Figure 1). The latter were arranged into two main clades. One included the Caribbean *Lindaconus* (although with low statistical support; BPP = 0.86) sister to paraphyletic *Miliariconus* + *Virroconus* plus West African cone snails (*Kalloconus* sister to paraphyletic *Lautoconus* + *Africonus*; Figure 1). The paraphyly of *Miliariconus* was due to the close sister group relationship of *Miliariconus coronatus* and *Virroconus ebraeus,* whereas the paraphyly of *Lautoconus* was due to the close sister group relationship of *Lautoconus hybridus* + *Lautoconus guanche* and *Africonus* (Figure 1). The other clade included two lineages: (a) *Harmoniconus* + *Puncticulis* sister to a clade including *Fulgiconus* and piscivorous genera from the Indo-Pacific (*Gastridium* + *Pionoconus*); and (b) the Atlantic and East Pacific piscivorous genus *Chelyconus* sister to *Dendroconus* + the molluscivorous genera (*Eugeniconus* + *Conus* sister to *Darioconus* + *Cylinder*; Figure 1).

The corresponding phylogenetic trees (BI, site-homogeneous models, protein-coding genes at the amino acid level) based on the nuclear and the combined data sets had 24 tips, with all genera but *Cylinder*, *Miliariconus* and *Kioconus* represented by a single species (Figure 2). The combined tree showed more resolution than the nuclear tree and a similar topology to the BI tree based on amino acid mt data, except for the relative positions of *Harmoniconus* (here related to the clade *Virroconus* + paraphyletic *Miliariconus*) and *Puncticulis* (here related to *Chelyconus*). The paraphyly of *Miliariconus* was due to a close sister group relationship between *Miliariconus miliaris* and *V. ebraeus* (Figure 2). There was a general lack of resolution of internal nodes (Figure 2).

The phylogenetic trees based on protein-coding genes using site-homogeneous models and analysed at the nucleotide level showed comparable patterns of resolution and recovered generally similar topologies to the corresponding ones based on amino acid data (Supporting Information Figure S2), although some conflicting nodes were detected: (a) The first offshoot was generally *Rhizoconus*; (b) *Rolaniconus*, the clade *Genuanoconus* + *Rhombiconus* or the clade including *Monteiroconus* sister to *Splinoconus* + *Kioconus* were the second offshoot depending on the analysis; (c) *Harmoniconus* was sister to *Chelyconus* in the BI and ML trees based on the mt genome data set; and (d) the paraphyly of *Lautoconus* was due to the close sister group relationship of *Lautoconus ventricosus* and *Africonus*.

The monophylies of genera preying on snails and on amphinomid worms, respectively, were recovered in all phylogenetic analyses including the corresponding species. However, in the case of the piscivorous cones, the mitochondrial data set regardless of the phylogenetic analysis recovered Atlantic/East Pacific (*Chelyconus*) and Indo-Pacific piscivorous (*Pionoconus* and *Gastridium*) genera as two independent lineages. The same result was obtained with the BI analysis of the combined data set with protein-coding genes analysed at the amino acid level under the site-homogeneous model and with the BI analysis of the combined data set with protein-coding genes analysed at the nucleotide level under the site-heterogeneous model (Supporting Information Figure S2). The remaining phylogenetic analyses based on the combined data set and all those based on the nuclear data set recovered the monophyly of the piscivorous cones. The evolutionary trends of radular morphology and the type of protoconch (shell of the larvae) were inferred under unordered parsimony and mapped onto the tree recovered under the BI analysis based on the mt genome data set with protein-coding genes analysed at the amino acid level (Supporting Information Figure S3). The basal spur (see this and other radular tooth characters in the drawings of Supporting Information Figure S3 and in more detail in Tucker & Tenorio, 2009) is present in vermivorous cones but absent in molluscivorous and piscivorous cones. The posterior fold is absent in *Pionoconus* and *Chelyconus*. The anterior portion of the radular tooth is very long in piscivorous cones and the molluscivorous *Darioconus* and *Cylinder*. The terminating cusp is absent in *Gastridium* and modified into an accessory process in *Pionoconus* and *Chelyconus*. These two latter genera as well as *Splinoconus*, *Lividoconus*, *Calamiconous* and *Fulgiconus* lack serrations. Among the studied cones, only those endemic to Cabo Verde (genera *Kalloconus* and *Africonus*) show multiple rows of serrations. Finally, cones endemic to Cabo Verde and Senegal, *Lautoconus ventricosus*, plus *Eugeniconus nobilis* show paucispiral protoconch, which is a proxy of lecithotrophic larvae (Supporting Information Figure S3).

3.3 | Estimation of divergence times

The topology of our best and most complete working hypothesis for the phylogeny of Conidae (based on the mt genome data set with protein-coding genes analysed at the amino acid level using BI with site-homogeneous models) was used as a reference for inferring divergence times. Those nodes with low statistical support (BPP < 0.95) were not fixed. Major cladogenetic events in the evolutionary history of Conidae were dated using an uncorrelated relaxed molecular clock model, which was calibrated using two alternative approaches as explained above. Regardless of the calibration method, the same topology and divergence times were obtained (Figure 3). The origin of the family Conidae was estimated around 62 (68–56) mya, and diversification of extant lineages started about 39 (43–35) mya. There was an active period of diversification between 30 and 25 mya, when most

genera diverged, and analysed species within each genus appeared about 10 mya.

4 | DISCUSSION

Understanding the relative role of different evolutionary processes leading to the extraordinary morphological, ecological and species diversity of cone snails requires a robust phylogeny, which thus far has been elusive (Aman et al., 2015; Phuong & Mahardika, 2018; Puillandre et al., 2014; Uribe, Puillandre, et al., 2017). Several reasons make particularly challenging the reconstruction of the phylogeny of cone snails, including the difficulty of obtaining thorough taxon samplings and the need of gathering large sequence data sets able to accumulate the phylogenetic signal needed to resolve the typical short nodes associated to evolutionary radiations. In recent years, phylogenetic studies of cone snails were based either on (a) medium (>40 species; Aman et al., 2015) or large taxon samplings (>300 species; Puillandre et al., 2014) but few partial mt gene sequences; or (b) in a shorter taxon sampling of most divergent lineages (14 species) but mt genomes (Uribe, Puillandre, et al., 2017), leading to resolution of relatively shallower or deeper nodes of the Tree of Life of cone snails, respectively.

Here, we propose an intermediate approach similar to that of Phuong and Mahardika (2018) based on the analysis of concatenated exons (>4,000; >500,000 bp) from 32 cone species, but complementary and now possible thanks to the ongoing active sequencing of cone venom gland transcriptomes. We gathered up to 41 different species representing 27 genera of Conidae in the mt genome data set and 22 different species belonging to 19 genera of Conidae in the nuclear and combined data sets. Hence, we included 30% and 21% of the genus diversity (Tucker & Tenorio, 2013) in the phylogenetic analyses, respectively. Moreover, we compiled mt genome and nuclear sequence data sets with 15 and 21 complete genes, and 95% and 94% matrix completeness, respectively, adding up to 30,266 or 11,456 positions when combined for phylogenetic analyses based on protein-coding genes analysed at the nucleotide or amino acid levels, respectively.

4.1 | Differences in gene assembly based on the NGS platform

The mt and nuclear genes were assembled from RNA-Seq raw reads, which were generated using five different platforms (Illumina HiSeq 2000, Illumina Genome Analyzer II, Illumina MiSeq, 454 GS FLX and Ion Torrent PGM). These platforms are known to render important differences in terms of read length, depth and quality, which could affect assembly results (Loman et al., 2012). In this regard, the already discontinued 454 GS FLX provided about one order of magnitude less number of reads. This was reflected in that, for instance, the *K. tribblei* assembly generated with this technique presented more missing mt and nuclear genes than any other species (the comparison is particularly illustrative in the case of the assembly of *K. tribblei* based on Illumina raw data, which were generated within the framework of the same study, and only missed the mt *atp8* gene). Similarly, the second assembly missing more of the studied genes was the one of *Splinoconus biliosus*, which was generated upon Ion Torrent PGM raw data. Altogether, Illumina-derived sequence data rendered best assembly results, with many species having the whole set of intended genes for phylogenetic analyses. Among mt genes, *atp8* and *nad5* (the shortest and longest, respectively) were the genes missing in more taxa, whereas the missing nuclear genes appear to be randomly distributed across taxa. In six instances, we could compare the sequences of mt genes assembled from RNA-Seq raw reads to those obtained from more traditional approaches (long PCR amplification and sequencing of complete mt genomes), demonstrating that the assembly pipeline rendered equivalent results (98%–100% sequence similarity).

4.2 | Phylogenetic relationships of cones based on mt and nuclear sequence data

Up to 18 phylogenetic trees were built based on the mt genome, nuclear and combined data sets using ML (site-homogeneous models) and BI (site-homogenous and site-heterogeneous models) with protein-coding genes analysed at the amino acid and nucleotide levels. The method of phylogenetic inference (BI or ML) had little effect on the final reconstructed tree and differences arose from analysing the different data sets or incorporating protein-coding genes as amino acids or nucleotides in the matrices. The general pattern obtained from the different phylogenetic inferences was that most supported nodes were recovered at the tips of the trees and were consistent regardless of the data set and analyses. In contrast, differences were located mostly at most internal nodes, which varied across analyses, and showed, in general, poor statistical support.

Among all reconstructed trees, we consider the two inferred using BI with site-homogeneous models and based on the mt genome data set as the most taxon-rich and with overall higher support. The topologies of these two trees mostly differed on poorly supported internal nodes, and hence, both could be declared our best and most complete working hypotheses for the phylogeny of Conidae. In order to simplify further comparative and evolutionary studies, we chose arbitrarily the one based on protein-coding genes analysed at the amino acid level as reference (also for discussion). This phylogeny recovered the monophyly of the different genera as proposed by Tucker and Tenorio (2009) and based on morphological characters. The only exceptions were

Miliariconus, which was paraphyletic due to *Virroconus* and *Lautoconus*, which was paraphyletic due to *Africonus*. The same relationships were recovered by Puillandre et al. (2014), although these authors consider *Miliariconus* and *Africonus* as synonyms of *Virroconus* and *Lautoconus*, respectively. The recent study by Phuong and Mahardika (2018) based on multiple exons recovered the reciprocal monophylies of *Miliariconus* and *Virroconus*, supporting the validity of both genera. The paraphyly of *Lautoconus*, which is recovered in all our trees, was already reported in a specific phylogenetic study of West African and Mediterranean cones based on mt genomes and a large taxon sampling of endemic species of the region (Abalde, Tenorio, Afonso, Uribe, et al., 2017). In that study, the paraphyly is due to *L. ventricosus* sister to *Africonus* (see also Puillandre et al., 2014), whereas here that relationship is recovered with the analysis of protein-coding genes at the nucleotide level but not with the analysis at the amino acid level, which favours the species from Senegal, *L. guanche* and *L. hybridus* sister to *Africonus*. In this case, given that these phylogenetic relationships are rather shallow, it is likely that the nucleotide data set may have larger phylogenetic signal than the amino acid data set. In our phylogeny, the monophyly of *Kioconus* with respect to *Splinoconus* was recovered in most (with moderate support) but not all analyses (except the mt genome data set analysed with BI and the site-heterogeneous model). However, using a larger number of species representing both genera, Puillandre et al. (2014) recovered a clade with *Kioconus* species intermixed with *Splinoconus* species and considered the former a synonym of the later.

Beyond the monophyly of the different genera, the phylogenetic trees agreed on some sister group relationships among genera: (a) The genus *Darioconus* was consistently recovered sister to *Cylinder* in all phylogenetic analyses. This relationship was also recovered in Puillandre et al. (2014). The genera *Conus*, *Eugeniconus* and *Dendroconus* were placed as closely related to the clade *Darioconus* + *Cylinder*, as in Puillandre et al. (2014), although these authors somewhat unexpectedly recovered *Eugeniconus* within *Cylinder*.

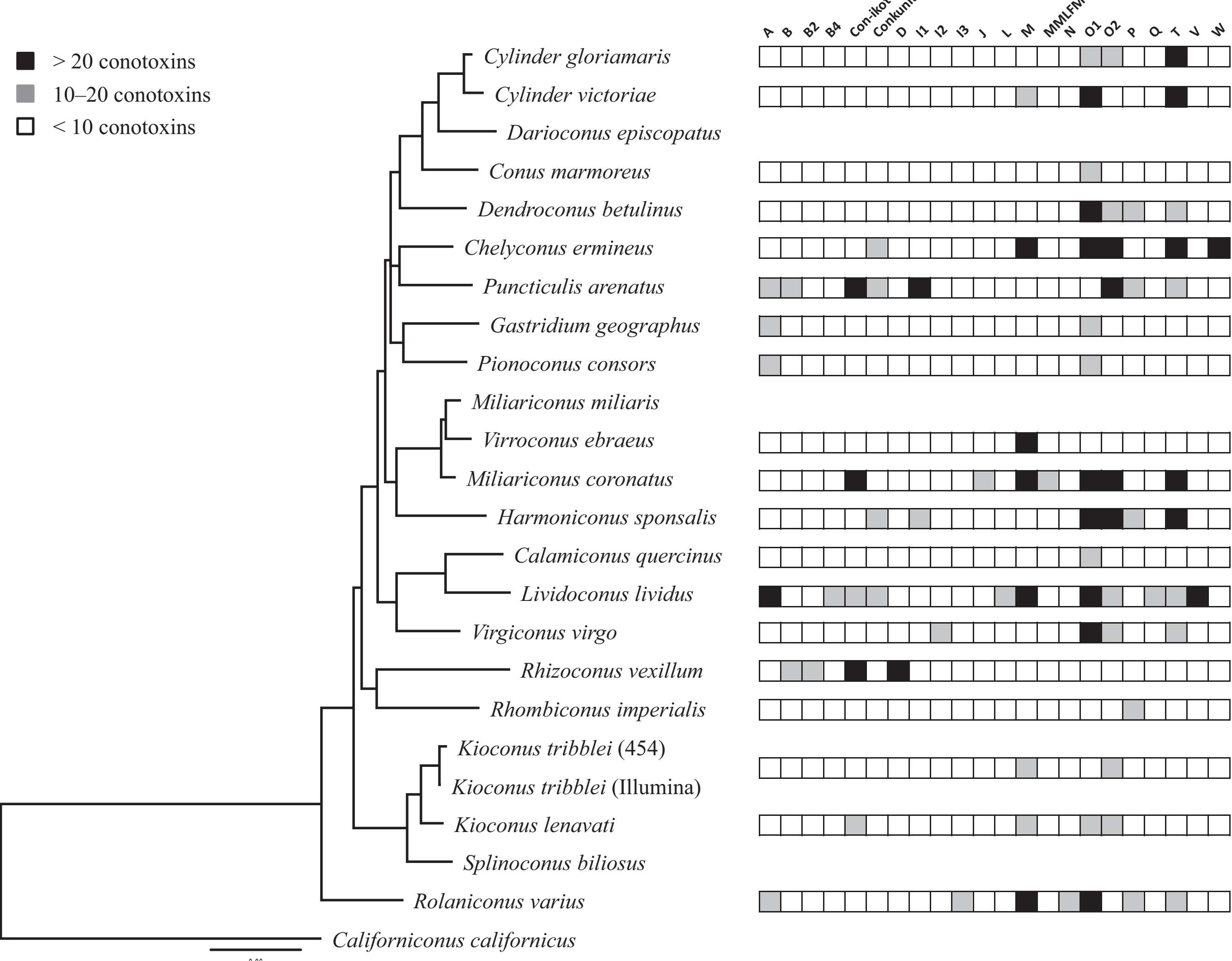

FIGURE 4 Distribution and member diversity of the conotoxin superfamilies across the different studied cone species

Our results are also consistent with the close relationship of *Cylinder* and *Conus* recovered in Aman et al. (2015) and Phuong and Mahardika (2018); (b) the genera *Gastridium* and *Pionoconus* were closely related. This relationship was also recovered in Puillandre et al. (2014), who also included in the clade other genera such as *Phasmoconus* and *Textilia* (see also Aman et al., 2015); (c) the two above-mentioned lineages are related each other and closely related to genera *Fulgiconus*, *Harmoniconus*, *Puncticulis* and *Chelyconus,* although the exact relationship is elusive. The close relationship of all these genera was also found in Puillandre et al. (2014), who synonymized *Fulgiconus* with *Phasmoconus*, and Aman et al. (2015). However, both studies did not include *Fulgiconus goudeyi*, which originally was described as *Phasmoconus goudeyi* (Monnier & Limpalaër, 2012). Puillandre et al. (2014) also included the genera *Lindaconus* and *Virroconus* within this large clade. These two genera were placed as only distantly related in our mt-based phylogenies (although not maximally supported). However, they were included in the large clade in the nuclear-based phylogenies in agreement with Puillandre et al. (2014), but note that the nuclear data set did not include the African/Mediterranean (*Kalloconus*, *Africonus* and *Lautoconus*) cones. The genus *Virroconus* appeared also closely related to *Cylinder* and *Conus* in Aman et al. (2015). A close relationship of *Puncticulis* but not of *Harmoniconus* (see below) to *Pionoconus*, *Cylinder* and *Conus* is recovered in Phuong and Mahardika (2018). In contrast, *Harmoniconus* and *Chelyconus* were sister group in Aman et al. (2015); (d) the close relationship of *Africonus* and *Lautoconus*, and of both to *Kalloconus,* was supported by specific studies on endemic cones of West Africa and the Mediterranean region (Abalde, Tenorio, Afonso, Uribe, et al., 2017) as well as in Puillandre et al. (2014). These three genera were closely related to *Virroconus* and *Miliariconus* in our phylogeny, in contrast to Puillandre et al. (2014). Moreover, *Virroconus* and *Miliariconus* are placed as closely related to *Harmoniconus* in Phuong and Mahardika (2018), as was the case in our phylogenies based on the nuclear and combined data sets, which lacked the West African and Mediterranean cone genera; (e) a strongly supported clade relating *Virgiconus* to *Lividoconus* and *Calamiconus* is recovered in all phylogenetic analyses. This clade is also recovered in the same relative position in the phylogeny of Phuong and Mahardika (2018). In the phylogeny of Puillandre et al. (2014), *Calamiconus quercinus* is considered *Lividoconus quercinus* and recovered as sister to other *Lividoconus*. This genus is sister to *Virgiconus*, and both are placed as closely related to *Kalloconus* and *Lautoconus*, although without support; and (f) *Genuanoconus* and *Rhombiconus* are always recovered as sister taxa. In Puillandre et al. (2014) and Aman et al. (2015), *Rhombiconus imperialis* is considered *Stephanoconus imperialis* and the genus placed as the second offshoot of Conidae after *Fraterconus distans* (and before *Strategoconus*) or sister to *Strategoconus* (and both to *Rhizoconus*), respectively. In Phuong and Mahardika (2018), *R. imperialis* is sister to *Strategoconus* and *Rhizoconus*. With regard to *Genuanoconus genuanus*, Puillandre et al. (2014) consider this species member of *Kalloconus*, as it was recovered deeply nested within this genus. However, a misidentification of the sample in the original work (Cunha et al., 2005) most likely explains this result, and *Genuanoconus* should not be considered a synonym of *Kalloconus* but a distantly related genus.

Most of the differences between inferred trees were concentrated in deepest nodes, affecting the relative position of *Rolaniconus*, *Rhizoconus*, the clade *Rhombiconus* + *Genuanoconus* and the clade including *Monteiroconus* sister to *Splinoconus* + *Kioconus*. In Puillandre et al. (2014) and Phuong and Mahardika (2018), the first diverging lineage of the tree is represented by *F. distans*, a species that we could not incorporate into our analysis. The next diverging lineages in Puillandre et al. (2014) are successively *Stephanoconus* (i.e., *Rhombiconus*), *Strategoconus* (the species *Rolaniconus varius* is considered *Strategoconus varius*) and a clade including, among others, the genus *Turriconus* sister to *Monteiroconus* and *Splinoconus* (including *Kioconus*). Lastly, *Rhizoconus* is recovered in a more derived position as sister to the remaining Conidae. In Phuong and Mahardika (2018), the next diverging lineage includes *Rhombiconus* sister to *Rolaniconus* + *Rhizoconus* (but *Genuanoconus*, *Splinoconus* and *Kioconus* are not included). Finally, in Aman et al. (2015), the first offshoot is *Kioconus* + *Leporiconus*, the second is *Turriconus* and the third is a clade including *Rhizoconus* sister to *Rolaniconus* + *Rhombiconus*. Hence, our phylogenetic analyses and reported trees concur that the above-mentioned species are close to the initial diversification of Conidae but are unable to resolve the exact phylogenetic relationships. Discrepancies among studies could be mainly related to uneven taxon sampling (each study is missing relevant lineages) in the phylogenomic analyses (this work, Phuong & Mahardika, 2018) or lack of enough phylogenetic signal due to relatively small data sets (Aman et al., 2015; Puillandre et al., 2014). The possibility of long branch attraction to the root (Philippe & Laurent, 1998) is less likely as none of these genera shows particularly high evolutionary rates. In any case, it is not possible to shorten the long branch connecting the outgroup and the ingroup as there are no genera more closely related to Conidae than those already included here (Uribe, Puillandre, et al., 2017).

4.3 | Evolution of diet specialization, radular morphology and conotoxin diversity

The reconstructed phylogeny was used as framework to infer the evolution of different traits relevant to the diversification of the group. One key character in Conidae is the feeding mode (Duda, Kohn, & Palumbi, 2001). Here, the ancestor of Conidae was inferred to prey on marine worms

in agreement with previous studies (Duda et al., 2001; Puillandre et al., 2014). The taxonomic and ecological data on which exact worm species are eaten by the different cone species are rather old, scattered and only the family level is determined (Phuong & Mahardika, 2018), so it is not possible to elaborate further on this subject, although subtle differences in radular tooth morphology may point to the existence of some degree of prey specialization. A striking exception is the case of those cone species hunting on fire worms (family Amphinomidae), which show distinct radular teeth (Nybakken, 1970), and in our phylogeny are recovered together as sister taxa, suggesting a single origin for this specialization (Duda et al., 2001). This is particularly remarkable as genera *Genuanoconus* and *Rhombiconus* are from the Eastern Atlantic and Indo-Pacific oceans, respectively. Other cone species preying on amphinomids belong to the Western Atlantic genera *Stephanoconus* and *Tenorioconus*, which are both placed as closely related to *Rhombiconus* in reconstructed phylogenies (Aman et al., 2015; Puillandre et al., 2014). The shift to feed on snails also occurred once in the evolutionary history of the group according to our phylogeny. This result is consistent across phylogenetic studies (Aman et al., 2015; Duda et al., 2001; Puillandre et al., 2014) and further supported by the characteristic (i.e., synapomorphic) curved and slender radular teeth without waist and spur of all molluscivorous species (Nishi & Kohn, 1999), which are repeatedly shot onto each single prey (Kohn, 2003). Finally, according to our phylogenies, the fish-feeding mode, arguably the most complex hunting behaviour among cones (Olivera, Seger, Horvath, & Fedosov, 2015), may have at least two independent origins in the Indo-Pacific and Atlantic/Eastern Pacific regions, respectively. This result was mainly supported by the mitochondrial data. Instead, the monophyly of piscivorous cones was favoured by all phylogenetic analyses based on the nuclear data set and some based on the combined data set. However, these data sets missed key genera to adequately tackle the question. Thus far, all previous phylogenetic studies have recovered piscivorous cones polyphyletic, although with low support (Aman et al., 2015; Duda et al., 2001; Puillandre et al., 2014). While there is no documented evidence that Indo-Pacific *Gastridium* and *Pionoconus* species feed on other prey than fish, Atlantic/Eastern Pacific *Chelyconus* species may also consume other molluscs (Olivera et al., 2015), which may indicate different evolutionary origins of piscivory in these taxa. Moreover, the comparison of the conotoxin repertoires of Indo-Pacific versus Atlantic/Eastern Pacific cones also supported independent origins of piscivory (Abalde et al., 2018). If true, many of the modifications in the radular teeth that are characteristic of *Pionoconus* and *Chelyconus* would be convergent. The studied radular tooth characters that differed between groups were mostly associated with the overall peculiar radular teeth shape of cone species depending on their diet. Hence, the potential differences and limitations in the ancestral character state reconstructions of these characters depending on the reconstructed phylogeny are the same discussed for the diet specializations. Instead, the protoconch evolutionary trends do not vary when different reconstructed phylogenies are considered, as the involved clades are consistently recovered throughout all analyses.

Another important trait in cone diversification and evolution is related with the diversity of the venom cocktails produced by the different species. Conotoxins are organized into superfamilies according to the signal region of the precursor, which is highly conserved (Puillandre, Koua, Favreau, Olivera, & Stöcklin, 2012). When analysing reported venom gland transcriptomes from various cone species, the emerging general pattern is that several conotoxin superfamilies (e.g., O1, M and T) are widespread among cones and constitute the minimal set required for the effective function of the venom, whereas others are restricted to a few lineages (Duda & Remigio, 2008; Puillandre et al., 2012). The different conotoxin superfamilies show diverse degrees of expansion. Here, we obtained listings of conotoxins of the different species directly from the original literature and mapped the number of described conotoxins per superfamily onto the phylogeny and inferred the evolution of conotoxin superfamily expansions (Figure 4). The genera *Chelyconus*, *Puncticulis*, *Miliariconus*, *Lividoconus* and *Rolaniconus* were the ones showing more superfamilies expanded (Figure 4). In contrast, genera *Conus* and *Calamiconus* had only superfamily O1 expanded, genus *Rhombiconus* only superfamily P and genus *Virroconus* only superfamily M (Figure 4). Superfamily O1 showed more than 20 members in many of the studied genera, although had less than 10 in *Puncticulis*, *Rhizoconus*, *Virroconus* and *Rhombiconus* (Figure 4). Similarly, superfamilies M and T were also highly diverse (>20 members) in several genera, although no specific evolutionary trend was inferred (Figure 4). Superfamily O2 was also expanded in many genera, although in most cases, the number of members varied between 10 and 20. The Indo-Pacific piscivorous genera *Pionoconus* and *Gastridium* showed expansions only in superfamilies A and O1, whereas the Atlantic/Eastern Pacific piscivorous genus *Chelyconus* showed expansions in superfamilies O1, O2, M and T as other genera plus in W and conkunitzin but not in A (Figure 4). The conkunitzins were also expanded in *Puncticulis*, *Harmoniconus* and *Lividoconus*, always with the number of members between 10 and 20. The genus *Rhizoconus* showed very specific expansions in superfamilies B, B2, D and con-ikot-ikot. These general patterns of conotoxin diversity distribution across genera are tentative and should be interpreted with caution as venom gland transcriptomes were obtained

using different methodologies and sequencing platforms as well as were assembled using different reference databases. Evolutionary trends on the expansions of superfamilies should be treated at this point as exploratory since key genera (and species) still need to be added to the phylogeny to reach stronger conclusions.

4.4 | Divergence times of major cladogenetic events

Calibration of the molecular clock using alternative approaches rendered the same chronogram indicating that the ages and lineage ascriptions of the three fossils used in the second approach were consistent with the ages used in the first approach. The inferred chronogram showed that first diversification of extant lineages within family Conidae occurred about 40 mya in the Eocene. We did not include *Fraterconus* in our analyses, a genus that has been recovered as the first offshoot in other phylogenetic studies (Phuong & Mahardika, 2018; Puillandre et al., 2014), and thus, it is likely that the diversification started somewhat earlier. In any case, it is evident that a long gap occurred between the origin of Conidae and the diversification of its extant lineages, indicating important lineage extinction events or eventually low speciation rates during the Late Paleocene–Early Eocene. The main lineages in the phylogeny appeared rapidly in only 5–10 my during the Oligocene, and the origin of many current genera was inferred to be relatively old and could be dated back to the Oligocene–Miocene transition, when there was a major radiation, as stated in the fossil record (Kohn, 1990). At that time, there was a global cooling event (Miller, 2005; Zachos, Flower, & Paul, 1997), accompanied by a sea level drop about 50 m (Beddow, Liebrand, Sluijs, Wade, & Lourens, 2016), which likely produced abrupt changes on the coast morphology and on intertidal habitats triggering diversification events as in other marine species (Davis, Hill, Astrop, & Wills, 2016). Other genera appeared steadily during the Miocene, and it appears that genus diversification was completed by the end of this period. We cannot confidently date when bursts of diversification leading to current species richness within each genus occurred as we had few instances in the phylogeny in which more than one species per genus were included. However, all analysed within-genus diversifications were dated in the Pliocene and Pleistocene concurring with glacial–interglacial events (Lisiecki & Raymo, 2005) in agreement with evolutionary studies analysing the radiation of cones in the Cabo Verde archipelago and the Senegal coast (Abalde, Tenorio, Afonso, & Zardoya, 2017; Abalde, Tenorio, Afonso, Uribe, et al., 2017; Cunha et al., 2005; Duda & Rolán, 2005). The early diversification of Conidae occurred in the Indo-Pacific region, which is consistent with the high species richness of this region. The diverse Atlantic lineages originated independently in different clades but almost all simultaneously about 25 mya during the drastic global climate and sea level changes of the Oligocene–Miocene transition.

5 | CONCLUSIONS

The use of mt genomes and several complete nuclear genes assembled from RNA-Seq raw reads allowed us reconstructing a phylogeny of Conidae including representatives of up to 27 of the 89 currently described genera within this group. This phylogeny had good levels of resolution, although the relative position of the early emerging lineages remains uncertain. The reconstructed phylogeny is comparable in resolution to a very recent one of similar number of genera and based on concatenated exons (Phuong & Mahardika, 2018) and agrees at the tips to the most taxon complete published thus far based on partial mt gene sequences (Puillandre et al., 2014). Hence, our results suggest that until sequencing technologies improve, it may be a sufficient (and more economical) compromise selecting a few tens of (mt and nuclear) genes and having complete data matrices instead of gathering thousands of loci in rather incomplete data sets to achieve similar results in terms of resolved trees, particularly when expanding phylogenetic studies to large taxon samplings. The new phylogeny could be further improved in the future by adding new key taxa representing missing genera and by enlarging the nuclear data set. In any case, this phylogeny provides a robust backbone to further understand the evolutionary processes underlying the great diversification of Conidae, supporting, for example, the single origin of the diet shifts to feeding on amphinomid worms and molluscivory, but the likely independent origins of piscivory in the Indo-Pacific and Atlantic/Eastern Pacific cones, respectively.

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SUPPORTING INFORMATION

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