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Diversification in vipers: Phylogenetic relationships, time of divergence and shifts in speciation rates

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ABSTRACT

Snakes of the cosmopolitan family Viperidae comprise around 329 venomous species showing a striking heterogeneity in species richness among lineages. While the subfamily Azemiopinae comprises only two species, 70% of all viper species are arranged in the subfamily Crotalinae or the "pit vipers". The radiation of the pit vipers was marked by the evolution of the heat-sensing pits, which has been suggested to be a key innovation for the successful diversification of the group. Additionally, only crotalines were able to successfully colonize the New World. Here, we present the most complete molecular phylogeny for the family to date that comprises sequences from nuclear and mitochondrial genes representing 79% of all living vipers. We also investigated the time of divergence between lineages, using six fossils to calibrate the tree, and explored the hypothesis that crotalines have undergone an explosive radiation. Our phylogenetic analyses retrieved high support values for the monophyly of the family Viperidae, subfamilies Viperinae and Crotalinae, and 22 out of 27 genera, as well as well-supported intergeneric relationships throughout the family. We were able to recover a strongly supported sister clade to the New World pit vipers that comprises *Gloydius*, *Ovophis*, *Protobothrops* and *Trimeresurus gracilis*. Our results agree in many aspects with other studies focusing on the phylogenetics of vipers, but we recover new relationships as well. Despite the addition of new sequences we were not able to resolve some of the poor supported relationships previously suggested. Time of divergence estimates suggested that vipers started to radiate around the late Paleocene to middle Eocene with subfamilies most likely dating back to the Eocene. The invasion of the New World might have taken place sometime close to the Oligocene/Miocene boundary. Diversification analyses suggested a shift in speciation rates during the radiation of a sub-clade of pit vipers where speciation rates rapidly increased but slowed down toward the present. Thus, the evolution of the loreal pits alone does not seem to explain their explosive speciation rates. We suggest that climatic and geological changes in Asia and the invasion of the New World may have also contributed to the speciation shift found in vipers.

1. Introduction

Vipers form a monophyletic lineage of venomous snakes comprising about 329 species distributed worldwide. Because vipers are considered a medically important group, different aspects of their biology have been widely studied (e.g. Fenwick et al., 2012; Greene, 1997; Martins et al., 2001) but their macroevolutionary dynamics and some aspects of phylogenetic relationships are still poorly understood. Species are currently arranged in 35 genera belonging to three subfamilies: Viperinae, Azemiopinae, and Crotalinae (Uetz and Hosek, 2014). Viperines, or the "true vipers", comprise 98 species whereas Azemiopinae comprises only two and both subfamilies are restricted to the Old World (Phelps, 2010; Uetz and Hosek, 2014). Crotalinae, or the "pit vipers", is the most diverse and widely distributed lineage of vipers, comprising about 229 species (Campbell and Lamar, 2004; Uetz and Hosek, 2014) occurring both in the Old and New World.

In the past years, the access to new Viperidae DNA sequences has greatly improved the limited phylogenetic inferences done solely based on morphological data (see Castoe and Parkinson, 2006) but the few studies (Fenwick et al., 2012; Pyron et al.,

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2013; Wüster et al., 2008) that have used molecular data investigated the phylogenetic relationships of vipers in a broader phylogenetic context. The pioneering study by Wüster et al. (2008) included all except two Viperidae genera in their molecular analysis but was limited to 87 species and therefore explored the phylogenetic relationships among higher taxa. Although recent works by Fenwick et al. (2012) and Pyron et al. (2013) included 220 and 210 Viperidae terminals respectively, the phylogenetic relationships of more inclusive lineages and the tempo of diversification underlying the divergence among those lineages are still unclear and debatable.

The choice of proper fossils and/or biogeographic events (Benton et al., 2009; Ho and Phillips, 2009; Sauquet et al., 2012; see also the supplementary material) is of central importance in dating analyses because it is not possible to estimate absolute ages from molecular data alone (Ho and Phillips, 2009). Wüster et al. (2008) used four fossils and two biogeographic events to calibrate their genus-level tree and Fenwick et al. (2012) two fossils and one biogeographic event. These authors found different diversification times for some lineages, which could be related to differences in sampling effort or choice of calibration points (Parham et al., 2012; Sauquet et al., 2012). A survey of the snake fossil record suggests it is possible to use additional fossils for conducting dating analysis of vipers (see Section 2 and supplementary material) avoiding biogeographic events, which have been suggested to be problematic in dating studies (Sauquet et al., 2012). A calibration setting comprising only fossils and a wider inclusion of current species should therefore greatly improve our understanding of the tempo of viperid diversification and also allow the first proper investigation of the diversification dynamics that gave rise to the current diversity of the family.

Vipers show a striking heterogeneity in diversity among different lineages, and a number of hypothesis have been proposed to explain the differential species richness of particular clades (e.g. Greene, 2002; Hendry et al., 2014; Lynch, 2009). The early radiation of these snakes is associated with the evolution of a highly derived venom system, which may have allowed the invasion of new niches (Greene, 1997; Pyron and Burbrink, 2012). Furthermore, the evolutionary history of the subfamily Crotalinae is marked by the evolution of a pair of heat-sensing pits on each side of their heads between the eye and the nostril (“loreal pits”) (Goris, 2011; Roelke and Childress, 2007), which have been suggested to represent a key innovation facilitating the radiation of the clade (Rosenzweig et al., 1987; Rosenzweig and McCord, 1991) even though not directly tested. Crotalines also invaded the New World, an event frequently associated with explosive radiation (Burbrink et al., 2012a; Wüster et al., 2002, 2008).

Explosive radiations or “early bursts” have been frequently reported in molecular phylogenies (e.g. Harmon et al., 2003; Morlon et al., 2012) and are usually characterized by very high diversification rates during the early radiation of a lineage followed by a decrease toward the present. The emergence of a key innovation (e.g. Glor, 2010; Losos and Mahler, 2010) and/or the invasion of new areas might allow a lineage to explore previously unavailable niches (Burbrink et al., 2012a,b) and, in theory, could be associated with explosive radiations. Although the predominant explanation regarding diversification slowdowns rely upon speciation mediated by niche differentiation and the subsequent decrease in ecological opportunities (e.g. Burbrink et al., 2012a; Rabosky and Lovette, 2008), recent studies have suggested alternative processes that can also underlie diversification rates slowdowns (see Moen and Morlon, 2014).

In this paper we assembled the most complete time-calibrated molecular dataset of Viperidae to investigate the phylogenetic relationships and diversification dynamics of vipers. We also explored the hypothesis that crotalines, comprising the most diverse subfamily, have undergone an explosive radiation (Rosenzweig et al., 1987; Rosenzweig and McCord, 1991) by investigating if diversification rates significantly increased during the diversification of the Crotalinae.

2. Material and methods

2.1. Taxon sampling and data acquisition

The Reptile Database (Uetz and Hosek, 2014) currently recognizes 329 species in the family Viperidae. From those, we included 260 species as terminal taxa in the ingroup of our phylogenetic tree. Although some researchers considered *B. colombiensis* and *B. isabelae* as synonyms of *B. atrox* (e.g., Campbell and Lamar, 2004; Rivas et al., 2012) others considered both as valid species (e.g. Salomão et al., 1999; Fenwick et al., 2009; Pyron et al., 2013). We chose to include *Bothrops colombiensis* and *Bothrops isabelae* as distinct species in the present study. Castoe et al. (2007) suggest that *C. tortugensis*, an endemic rattlesnake of Tortuga Island, is nested within *C. atrox*, representing a junior synonym of the latter. Murphy et al. (2002) retrieved *C. tortugensis* within *C. atrox* but they considered paraphyly as acceptable in cases of peripheral isolation. In the present study we followed taxonomic arrangement that considers some of the lineages endemic to islands that recently diverged from their sister taxa as valid species, and thus included *C. tortugensis* as distinct species in our analyses (Grazziotin et al., 2006; Grismer, 1999). It is important to highlight to the reader that diversification studies are always prone to be affected by the taxonomic arrangements they follow, but we are confident that only major taxonomic changes in the taxonomy of vipers could affect our results. Thus, our sampling for Viperidae encompasses 263 taxa corresponding to 79% of the diversity presently described for the family. This taxon sampling comprises all the three known subfamilies with the following sampling schemes (sampled species/number of described species): Azemiopinae (1/2), Viperinae (71/98), and Crotalinae (191/232). Additionally, we included as outgroup 97 species from different families: Boidae, Elapidae, Colubridae, Dipsadidae, Homalopsidae, Natricidae, Atractaspididae, Lamprophiidae, Psamophiidae, and Xenodermatidae. The species *Indotyphlops braminus* (Scolecophidia: Typhlopidae) was used to root our phylogenetic tree (Pyron et al., 2013). Details on our sampling strategy and on the curatorial work performed including a list with several issues faced when using sequences from public databases are available in our supplementary material.

Our molecular matrix is composed of sequences from 11 genes, six mitochondrial (12S, 16S, cytb, cox1, nd2, nd4) and five nuclear (bndf, c-mos, jun, nt3, rag1). We used sequences for species of Viperidae available in GenBank up to April 2014 (Table S1). We also included sequences available in the Barcode of Life Database (BOLDSYSTEMS, http://www.boldsystems.org/) to complement information for the Cox1 gene (Table S1). We provided new DNA sequences for 27 viper species (Table S1) for eight genes, including a species not previously included in GenBank (*Causus lichtensteini*), totaling 167 new sequences. All new sequences were obtained following standard PCR and sequencing protocols as described in Grazziotin et al. (2012). Both strands of the PCR products were sequenced, and the trimming and assembling procedures were performed using the default parameters in the program GENEIOUS v.5 (Biomatters, available at http://www.geneious.com).

Our dataset represents a significant improvement in sampling compared to recently published viper phylogenies. We include 43 more species than Fenwick et al. (2012) and 53 more than Pyron et al. (2013). Moreover, our molecular dataset comprises 11 genes and 1186 sequences whereas Fenwick et al. (2012) and Pyron et al. (2013) comprise 784 and 817 sequences for 4 and 11 genes respectively. Therefore, the study presented herein