Published in final edited form as:

Copeia. 2016 ; 104(1): 83–93. doi:10.1643/CH-14-210.

Phylogeny of Mental Glands, Revisited

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Abstract

Mental glands and their associated delivery behaviors during courtship are unique to the plethodontid salamanders. Because previous interpretations of the evolution of these features were conducted using older phylogenetic hypotheses, we reanalyzed these traits with newer courtship descriptions and contemporary phylogenetic methods. Using Bayesian ancestral state reconstruction methods that have been developed since the first phylogenetic analyses were conducted in the mid-1990s, we reconstructed mental gland and courtship behavior evolution on a Bayesian phylogeny of the nuclear gene *Rag1*. The most probable ancestral condition for plethodontids was resolved as presence of a mental gland. Loss of a mental gland occurred in each subfamily and was recovered as the most probable ancestral condition for the Spelerpinae. In contrast, parsimony reconstruction recovered the presence of a mental gland in the ancestor to Spelerpinae with multiple secondary losses. We hypothesize that that absence of a mental gland is possibly ancestral in some clades (i.e., Spelerpinae) and secondary in others (e.g., paedomorphic *Eurycea*). The most probable ancestral form of the mental gland is likely to be the large pad-type distributed extensively in Plethodontinae and Bolitoglossinae. Desmognathans have the most unique mental glands, occurring in an anterior protrusion or bifurcated form (in *Desmognathus wrighti*). Fan-shaped mental glands evolved independently in *Eurycea* and *Oedipina*. Small pads arose independently in Bolitoglossinae, Plethodontinae, and Spelerpinae. Head-rubbing behavior for mental gland delivery mode was recovered as the most probable and parsimonious ancestral state for the Plethodontidae, with independent losses of this behavior in Plethodontinae and Spelerpinae. Because head-rubbing was observed in outgroups, we hypothesize that head-rubbing behavior predated mental gland evolution. Pulling, snapping, slapping, and biting behaviors evolved independently in the Plethodontinae and Spelerpinae and are not homologous with head-rubbing. All hypotheses of mental gland and courtship evolution invoke homoplasy.

In salamanders of the family Plethodontidae, males of many species use courtship pheromones that are secreted by a gland called the mental gland. The mental gland occurs in the dermis of the lower jaw and is a feature only found in plethodontids (Noble, 1927; Trufelli, 1954; Lanza, 1959; Sever, 1976). The pheromones are delivered to the females by

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DATA ACCESSIBILITY

Additional supporting information available from the Dryad Digital Repository: <http://dx.doi.org/10.5061/dryad.hm202>. List of species *Rag1* sequences (Sever et al., 2016: Table S1), topologies generated with Bayesian and parsimony analyses (Sever et al., 2016: Figs. S1–2), and parsimony ancestral state reconstructions (Sever et al., 2016: Figs. S3–9).

several different behaviors and increase female receptivity both before and (in some species) during the tail straddle walk (Houck et al., 2007, 2008). In the tail straddle walk, which is stereotypic for plethodontids, the female follows the male with her chin on his dorsal tail base (Noble, 1929). The male deposits a spermatophore and leads the female over it until the spermatophore comes in contact with her cloacal orifice.

In the late 1980s and early 1990s, the notion of mapping traits (mostly behavioral traits at that time) on phylogenies reconstructed from independent characters was a new idea (Wanntorp et al., 1990; Block et al., 1993; Miles and Dunham, 1993). Houck and Sever (1994) used the concept of trait-mapping to develop evolutionary hypotheses about aspects of mental gland occurrence, anatomy, and delivery using the then-timely hypotheses of plethodontid relationships of Wake (1966), Larson (1984), and Lombard and Wake (1986). They found support for the hypothesis that the ancestral plethodontid possessed a mental gland, although the anatomy of this gland and delivery mode were equivocal. Absence of a mental gland in some taxa represented a secondary loss of the mental gland and associated delivery behavior.

After an analysis of courtship behavior of *Gyrinophilus porphyriticus*, Beachy (1997) proposed a different hypothesis regarding the phylogeny of mental glands. Males of *G. porphyriticus* lack mental glands, and the taxon is considered to have a morphology and life history similar to ancestral plethodontids (Wake, 1966). This species and its sister taxon that also lacks a mental gland, *Pseudotriton ruber*, exhibit a behavior called “head-sliding” (Arnold, 1976), during which the male rubs his head over the snout and other body parts of the female (Organ and Organ, 1968; Beachy, 1997). Beachy (1997) proposed that the ancestral condition in the Plethodontidae was absence of a mental gland and possession of head-sliding. The term “head-sliding” has been superseded by “head rubbing” (Dyal, 2006).

Recently, molecular hypotheses of plethodontid evolutionary history have resulted in a massive rearrangement of clades and their phylogenetic relationships (e.g., Chippindale et al., 2004; Mueller et al., 2004; Macey, 2005). Herein, we use Bayesian analyses of the nuclear gene *Rag1* to revisit the phylogeny of mental gland occurrence, anatomy, and delivery mode.

MATERIALS AND METHODS

Representative taxa with known mental gland morphology were used to reconstruct a phylogenetic tree using Bayesian analyses in MrBayes. The *Rag1* gene sequence was used for reconstruction of the phylogeny. A surrogate was used for *Nototriton* (*N. abscondens*), as *Rag1* sequences from a species within this genus with a known mental gland morphology (*N. picadoi*) were not available on GenBank. *Oedipina* was also excluded from the analysis, as the only *Rag1* sequence available was much shorter than the other taxa. All other taxa available that met our requirements for use in the reconstruction (*Rag1* gene sequences, gland morphologies, and behavioral secretion delivery modes available) were used as terminals for ancestral state reconstruction for mental glands, mental gland morphology, and mental gland secretion delivery modes.

Forty-nine representative taxa with known mental gland morphologies were analyzed. For each taxon, a nuclear recombination-activating gene 1 (*Rag1*) sequence was obtained from GenBank (Sever et al., 2016: Table S1). *Rhyacotriton* and *Amphiuma* were included as outgroup taxa for all analyses. Overall, the analysis included 75% of the extant genera of Plethodontidae, with approximately 28% of those genera represented by multiple terminals.

Sequences were aligned using an internet implementation of MUSCLE (<http://www.ebi.ac.uk/Tools/msa/muscle/>). The aligned sequences were trimmed to 1020 base pairs (bp) to reduce the amount of missing sequence data. After alignment, only three taxa had sequences shorter than 1020 bp: *N. abscondens* (715 bp), *Bolitoglossa lincolni* (732 bp), and *Thorius macdougalli* (745 bp). The alignment was unambiguous and contained no indels. Phylogenetic relationships were inferred with MrBayes 3.2.4 (Ronquist et al., 2011). A general time reversible model with a proportion of invariant sites and gamma-distributed rate heterogeneity was chosen using MrModelTest 2.25 (Nylander, 2004).

Bayesian analyses were performed by Markov Chain Monte Carlo (MCMC) sampling for 30 million generations. Two independent runs of four Metropolis-coupled chains were run simultaneously using uniform prior probabilities and appropriate model parameters estimated on a randomly generated starting phylogeny. Trees were sampled from the posterior-probability distribution once every one hundred generations. Stationarity was reached within the first three million generations, but the first 25% of samples (75,000 trees) from each run was conservatively discarded as burn-in. The remaining 225,000 trees from each run were combined to determine final posterior probabilities.

Mental gland states were coded as '0' for absent and '1' for present. Mental gland morphology was coded as '0' for absent, '1' for small pad, '2' for large pad, '3' for anterior protrusion, '4' for anterior protrusion with bifurcation, and '5' for fan-shaped. States for the latter character were treated as unordered to eliminate any bias on the trajectory of the evolution of mental gland morphology (Figs. 1, 2). Five distinct courtship behaviors involving the mental glands were independently coded as absent '0' or present '1': head-rubbing, pulling, snapping, slapping, biting/restraint. For a definition of each mental gland morphology and for each behavior associated with mental gland delivery, see Houck and Sever (1994).

Bayesian and parsimony ancestral state reconstructions were performed on an unconstrained topology (Sever et al., 2016: Fig. S1) and on a topology with *Gyrinophilus porphyriticus*, *Pseudotriton ruber*, and *Stereochilus marginatus* constrained to form a monophyletic group (Sever et al., 2016: Fig. S2). The tree was constrained because all other recent phylogenies recovered *Gyrinophilus*, *Pseudotriton*, and *Stereochilus* as monophyletic with high support (e.g., Pyron and Wiens, 2011; Bonett et al., 2014a). A Shimodaira-Hasegawa test (Shimodaira and Hasegawa, 1999) performed in PAUP* 4.0b10 (Swofford, 2000) found no significant differences between the two topologies (not shown). The constrained topology was used for subsequent analyses presented here because the results of the ancestral state reconstructions did not drastically differ between topologies.

Ancestral state reconstructions were conducted using BayesTraits v2.0 (<http://www.evolution.rdg.ac.uk/BayesTraits.html>) with a reverse jump MCMC model and multistate reconstruction (Pagel et al., 2004). The analyses were performed on 100,000 trees by combining the final 50,000 trees from each of the independent MrBayes runs. Hyper uniform priors from 0 to 100 were used for analysis of each character. Each analysis was run for 50 million generations, with samples taken every 1000 generations. The first 50% of the sampled generations were discarded as burn-in, leaving 25,000 samples for analysis. To broaden our approach, we also optimized each of the above character states as unordered to the consensus Bayesian topology using parsimony ancestral state reconstruction in Mesquite v2.5 (Maddison and Maddison, 2011).

RESULTS

Mental gland absence/presence

Bayesian ancestral state reconstruction resulted in mental glands first appearing on the branch leading to Plethodontidae (Fig. 3). This result coincides with optimization of the character states with parsimony ancestral state reconstruction (Sever et al., 2016: Fig. S3). Mental glands were also recovered as the ancestral state for the Bolitoglossinae, Plethodontinae, and Hemidactylinae using Bayesian ancestral state reconstruction, with three independent losses in the Plethodontinae (*Ensatina eschscholtzii*, *Phaeognathus hubrichti*, and *Plethodon punctatus*) and one loss in the Bolitoglossinae (*Batrachoseps incognitus* + *Batrachoseps gabrieli*; Fig. 3). Parsimony optimization resulted in an identical reconstruction for those subfamilies (Sever et al., 2016: Fig. S3). With Bayesian ancestral state reconstruction, mental glands were lost on the branch leading to Spelerpinae, reappeared on the branch leading to *Eurycea*, lost on the branch leading to *Eurycea spelaea* + *Eurycea tynerensis*, reappeared in *Eurycea spelaea*, and lost in *Eurycea rathbuni* (a total of five evolutionary transitions; Fig. 3). Parsimony ancestral state reconstruction resulted in fewer steps with only three independent losses on the branches leading to *Gyrinophilus porphyriticus* + *Pseudotriton ruber* + *Stereochilus marginatus*, *E. rathbuni*, and *E. tynerensis* (Sever et al., 2016: Fig. S3). The major difference between reconstruction methods for the subfamily Spelerpinae are undoubtedly the result of the short branch leading to the *G. porphyriticus* + *P. ruber* + *S. marginatus* clade pushing the loss of the mental gland from that clade to the relatively longer branch leading to Spelerpinae.

Gland morphology

Bayesian ancestral state reconstruction resulted in a large pad as the most probable ancestral morphology of the mental gland for Plethodontidae (Fig. 4). Small pads evolved from large pads on the branches leading to *Batrachoseps*, *Nototriton*, *Plethodon cinereus*, and *P. punctatus* + *P. welleri* (Fig. 4). Another transition from large to small either occurred on the branch leading to *Aneides* + *Desmognathus* + *Karsenia* + *Phaeognathus* or *Karsenia*, depending on the state for the former clade, which was recovered as an equal probability between a small and large pad (Fig. 4). Small pads evolved from an ancestor lacking a pad in *Eurycea*, were lost on the branches leading to *E. rathbuni* and *E. spelaea* + *E. tynerensis*, and then reappeared on the branch leading to *E. spelaea* (Fig. 4). The loss of a small pad also occurred on the branches leading to *B. incognitus* + *B. gabrieli* and *P. punctatus* (Fig. 4).

Optimization of the gland morphology states to the tree using parsimony resulted in no unequivocal ancestral states for any subfamily except the monotypic Hemidactylinae (Sever et al., 2016: Fig. S4). In contrast to the Bayesian reconstruction, the states that were equivocal for the branches leading to Bolitoglossinae and Spelerpinae were gland absence and a small pad (Sever et al., 2016: Fig. S4). Thus, parsimony provided a reconstruction that depicted large pads evolving from small pads (or no pads) multiple times versus the Bayesian reconstruction that depicted small pads evolving from large pads multiple times within Plethodontidae.

Bayesian ancestral state reconstruction resulted in an anterior protrusion evolving once on the branch leading to *Desmognathus* with a single emergence of a bifurcated mental gland appearing in *D. wrighti* (Fig. 4). A fan-shaped mental gland evolved on the branch leading to *Eurycea bislineata* + *E. cirrigera* + *E. junaluska* + *E. lucifuga* + *E. quadridigitata* + *E. wilderae* and transitioned to a large pad on the branch leading to *E. lucifuga* (Fig. 4). Note, however, that a fan-shaped gland is also present in Bolitoglossinae (e.g., *Oedipina*; Houck and Sever, 1994), and thus this type of gland also independently arose in Bolitoglossinae. These results were consistent with optimization of the character states with parsimony ancestral state reconstruction. However, the ancestral state for *Desmognathus* was recovered as equivocal in parsimony analysis, with no mental gland, a large pad, a bifurcated mental gland, or anterior protrusion all providing an equally parsimonious reconstruction of the ancestral state (Sever et al., 2016: Fig. S4).

Courtship behavior

Both Bayesian and parsimony ancestral state reconstruction resulted in head-rubbing behavior as the ancestral state for all clades recoverable within Plethodontidae, including *Amphiuma* + Plethodontidae + *Rhyacotriton* and *Amphiuma* + Plethodontidae (Fig. 5; Sever et al., 2016: Fig. S5). The Bayesian analysis was not capable of reconstructing ancestral states in clades with extensive missing behavioral data (e.g., *Batrachoseps*; Fig. 5).

Bayesian ancestral state reconstruction resulted in the evolution of pulling behavior at least five times within Plethodontidae on the branches leading to *Aneides* + *Desmognathus* + *Karsenia* + *Phaeognathus*, *Aneides lugubris*, Bolitoglossinae, *Eurycea bislineata* + *E. cirrigera* + *E. junaluska*, *E. lucifuga* + *E. quadridigitata* + *E. wilderae*, and *Plethodon cinereus* (Fig. 6). Losses of this behavior also occurred five times on the branches leading to *Aneides*, *D. aeneus* + *D. quadramaculatus*, *D. monticola*, *D. wrighti*, and *E. lucifuga* (Fig. 6).

Parsimony ancestral state reconstruction provided a similar picture, with five independent origins of pulling behavior; however, only two losses of the behavior were observed on the branches leading to *Desmognathus aeneus* + *D. quadramaculatus* and *D. monticola* (Sever et al., 2016: Fig. S6). This was due to the emergence of the behavior further up the tree in each clade where the behavior appears; i.e., on the branches leading to *Aneides lugubris*, *Desmognathus*—*D. wrighti*, *Eurycea bislineata* + *E. cirrigera* + *E. junaluska* + *E. wilderae*, and at least in *Pseudoeurycea* of Bolitoglossinae (Sever et al., 2016: Fig. S6).

Snapping behavior followed an identical evolutionary trajectory to that of pulling behavior with both Bayesian and parsimony ancestral state reconstruction except for the lack of snapping behavior within Bolitoglossinae (Fig. 7; Sever et al., 2016: Fig. S7).

Both Bayesian and parsimony ancestral state reconstruction resulted in one appearance of slapping behavior with Plethodontidae (Fig. 8; Sever et al., 2016: Fig. S8). With the Bayesian analysis, slapping behavior evolved on the branch leading to *Plethodon*—*P. vandykei*, with a loss of the behavior on the branch leading to *P. cinereus* (Fig. 8). Parsimony ancestral state reconstruction resulted in one less transition with the trait only appearing on the branch leading to *Plethodon* with the exclusion of *P. cinereus* and *P. vandykei* (Sever et al., 2016: Fig. S8).

Both Bayesian and parsimony ancestral state reconstruction resulted in two appearances of biting/restraint behavior on the branches leading to *Desmognathus aeneus* and *D. wrighti* (Fig. 9; Sever et al., 2016: Fig. S9).

DISCUSSION

The classical phylogenies of Wake (1966), Larson (1984), and Lombard and Wake (1986) had four major groups: the Desmognathinae and the three plethodontine taxa (the Hemidactyliini, Plethodontini, and Bolitoglossini). The modern topologies (e.g., Chippindale et al., 2004; Mueller et al., 2004; Macey, 2005; Vieites et al., 2011) made major modifications by (1) placing the desmognathans within the paraphyletic *Plethodon*, (2) moving *Hydromantes* from one sister-group position (i.e., to the *Batrachoseps/Bolitoglossa* clade) to a new sister group position to the *Plethodon/Aneides/Ensatina* clade, and (3) movement of *Hemidactylium scutatum* from the spelerpines (*Eurycea*, *Gyrinophilus*, *Pseudotriton*, and *Stereochilus*) to the branch that also contains the *Batrachoseps/Bolitoglossa* clade (where it is the sister group to this clade).

Whereas the classical phylogeny recognizes four major groups, the newer hypotheses are simpler and include three major branches: the plethodontines (which includes *Karsenia*, *Hydromantes*, *Plethodon*, *Ensatina*, *Aneides*, and the desmognathans), the spelerpines, and the *Batrachoseps/bolitoglossines* (*Hemidactylium scutatum* is a sister taxa to the latter branch). The placement of *H. scutatum* is always important and confounding because it is a metamorphosing form that is now sister group to a direct-developing clade. These changes have caused the reinterpretation of several classical problems, e.g., the evolution of direct development (Chippindale et al., 2004), and these analyses invoke the important role of homoplasy in life history evolution.

Our analyses suggest a reconsideration of the mental gland and courtship behavior in the Plethodontidae. This gland is unique to plethodontids as are the behaviors that are associated with delivery of the pheromones produced by this gland. In our original analyses (Houck and Sever, 1994; Beachy, 1997), we found large amounts of homoplasy in both features. In our reconsideration, homoplasy is still important. Because homoplasy results principally from convergence and reversal, this means that both mental glands and (to a greater extent) delivery modes are subject to repeated sexual selection during plethodontid evolution.

Morphology of the mental gland has the greatest phylogenetic value (i.e., compared to behavior), although the number of changes in gland type is still substantial. This seems sensible given that morphology is considered less plastic than behavior. Because delivery mode exhibits more change, this supports the notion that the mental gland type and behavior for delivering the pheromones are not tightly linked. The same group can have fewer gland types than it has delivery behaviors, e.g., *Plethodon* (Dyal, 2006; Houck et al., 2007).

Houck and Sever (1994) and Beachy (1997) differed in the interpretation of head-rubbing. Beachy (1997) concluded that the simple suite of behaviors seen in *Gyrinophilus porphyriticus* and *Pseudotriton ruber* (Organ and Organ, 1968), characterized by head-rubbing by the males during the pre-tail straddle walk phase, indicated that head-rubbing could be considered the precursor, i.e., homologous, to the various other elaborate male behaviors that are used to deliver courtship pheromones. Dyal (2006) and Marvin and Hutchison (1996) found pre-tail straddle walk male contact behaviors that were different than slapping or other behaviors. Dyal (2006) showed that these behaviors are likely to be more complex than previously noted by any authors. It is conceivable that these tactile behaviors are found throughout the plethodontids. Coupled with the idea that our data on behavior exhibits more homoplasy, Beachy's (1997) hypothesis that head-rubbing is homologous to pheromone-delivery behaviors is not tenable. This is supported by Dyal's (2006) observations of head-rubbing and other delivery modes during the same courtship.

As noted by Marvin and Hutchison (1996) for *Plethodon*, few descriptions of plethodontid courtship are available. Since 1997, there have been only six descriptions of plethodontid courtship behavior (Verrell, 1999; Kozak, 2003; Dyal, 2006). There are descriptions of courtship behavior for 44 species of plethodontids, i.e., for approximately 9.5% of known species. Our understanding is very good for species of *Desmognathus* (16 species courtship descriptions, approximately 70%), but very poor for the remainder of the family (approximately 88% of plethodontid species are not desmognathans).

Naturally, our understanding of the distribution of mental glands is much better. Understanding how the mental gland is used and how the pheromones are delivered is problematic without courtship descriptions. Additional descriptions for species of *Bolitoglossa* (for both mental glands and delivery modes) would be excellent. In addition, improving the number of descriptions for *Eurycea* would be especially helpful. Mental glands are not strictly a metamorphic feature, as some paedomorphic species of *Eurycea* possess them, whereas others do not (Sever, 1985). The lack of courtship descriptions for the paedomorphic species remains an outstanding omission that contributes to our poor understanding.

This general paucity of descriptions is important. Traits are often conservative enough across taxa that knowing the features of the trait in one species can allow one to assume that the trait is similar (or the same) in related taxa. However, close examination of courtship in plethodontids indicates that each species has idiosyncratic behaviors (Dyal, 2006). While examining and discovering these species-specific courtship patterns is time-consuming, it is a productive research avenue. For example, Dyal's (2006) description of courtship in three species of *Plethodon* provided enough evidence to suggest a novel hypothesis for the

importance and evolution of snapping. It seems imperative that a better understanding of courtship evolution in the Plethodontidae is developed. This family is, by far, the most diverse of the salamanders. While other analyses suggested a decoupling between species diversification and morphological evolution (Adams et al., 2009), it still seems tenable that plethodontid diversity in species is caused in part by the extreme diversity and lability in courtship behavior (Tilley et al., 1990; Bernardo, 1991; Beachy, 1995, 1996; Verrell and Mabry, 2000).

In general, our analysis and reconsideration suggests two important hypotheses. First, the ancestral plethodontid courtship was characterized by presence of the mental gland that secretes courtship pheromones. These pheromones would be delivered principally by a head-rubbing behavior that is seen in nearly all other salamanders for which courtship descriptions are available. In this hypothesis, the mental gland, along with lunglessness and the nabolabial groove, is considered a synapomorphy that is diagnostic for the plethodontid salamanders. Additional pheromone delivery behaviors are not considered homologous to head-rubbing in this scenario, e.g., slapping and snapping, are innovations that can evolve independently in several lineages and can be secondarily lost. This is a novel idea that differs from the hypotheses of Houck and Sever (1994) and Beachy (1997). Secondly, and consequent to this first hypothesis, is that homoplasy is even more extensive than suggested by either Houck and Sever (1994) or Beachy (1997). This is consistent with modern perspectives offered for life history evolution (Bonett et al., 2014a, 2014b) and feeding morphology (Wake et al., 2011) and continues to suggest the perplexing notion that the more we learn about phylogenetic relationships, the more we realize that morphological and behavioral homoplasy makes comparative biology more challenging than ever.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Support during the part of the development of this manuscript was provided by the Major Dyson Endowed Professorship at Southeastern Louisiana University (to DMS) and the National Institute of General Medical Sciences (8 P20 GM103442-12) from the NIH (to CKB).

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Fig. 1.

Mental gland types. (A) Character state 1, in *Eurycea spelaea*, from Sever (1976). (B) Character state 2, in *Bolitoglossa*. (1) External appearance in *B. aspera*, from Noble (1927); (2) Horizontal section through the pad of *B. mexicana*, from Truffeli (1954). (C) Character state 3, in *Desmognathus fuscus*. (1) Site of the mental gland ventral and anterior to the mandibular symphysis, from Noble (1927); (2) Mental gland tubules terminating in secretory pores (circles), from Sever (1976). (D) Character state 4, in *Desmognathus wrighti*, from Sever (1976). Glands secrete at the base of anterior mandibular teeth.

A. Fan-shaped *Eurycea***B. Fan-shaped *Oedipina*** **Fig. 2.**

Mental gland shapes continued, character state 5. (A) *Eurycea wilderae* (1) secretory area at apex of mandibles and extent of pad indicated by dotted lines, from Arnold (1976); (2) Pores (circles) and gland tubules, from Sever (1979); (3) Snapping and pulling behavior by which the male delivers pheromones to the female with the aid of sexually dimorphic teeth, from Arnold (1976). (B) *Oedipina*, from Trufelli (1954). (1) *O. uniformis*, ventral view; (2) *O. uniformis*, lateral view; (3) *O. cyclocauda*, sagittal section.

Fig. 3.

Bayesian reconstruction of mental glands within Plethodontidae. Two states were considered: absent (white) and present (black). Pie charts at each node provide the proportional probability of each state. Numerical values indicate the highest probability at each node. The phylogeny was reconstructed using a Bayesian analysis of *Rag1* sequences (see Materials and Methods).

Fig. 4.

Bayesian reconstruction of mental gland morphology within Plethodontidae. Six unordered states were considered: absent (white), small pad (black), large pad (red), anterior protrusion (yellow), bifurcated (green), and fan-shaped (blue). Pie charts at each node provide the proportional probability of each state. Numerical values indicate the highest probability at each node. The phylogeny was reconstructed using a Bayesian analysis of *Rag1* sequences (see Materials and Methods).

Fig. 5. Bayesian reconstruction of head-rubbing behavior within Plethodontidae. Two states were considered: absent (white) and present (black). Pie charts at each node provide the proportional probability of each state. Numerical values indicate the highest probability at each node. The phylogeny was reconstructed using a Bayesian analysis of *Rag1* sequences (see Materials and Methods).

Fig. 6. Bayesian reconstruction of pulling behavior within Plethodontidae. Two states were considered: absent (white) and present (black). Pie charts at each node provide the proportional probability of each state. Numerical values indicate the highest probability at each node. The phylogeny was reconstructed using a Bayesian analysis of *Rag1* sequences (see Materials and Methods).

Fig. 7. Bayesian reconstruction of snapping behavior within Plethodontidae. Two states were considered: absent (white) and present (black). Pie charts at each node provide the proportional probability of each state. Numerical values indicate the highest probability at each node. The phylogeny was reconstructed using a Bayesian analysis of *Rag1* sequences (see Materials and Methods).

Fig. 8. Bayesian reconstruction of slapping behavior within Plethodontidae. Two states were considered: absent (white) and present (black). Pie charts at each node provide the proportional probability of each state. Numerical values indicate the highest probability at each node. The phylogeny was reconstructed using a Bayesian analysis of *Rag1* sequences (see Materials and Methods).

Fig. 9.

Bayesian reconstruction of biting/restraint behavior within Plethodontidae. Two states were considered: absent (white) and present (black). Pie charts at each node provide the proportional probability of each state. Numerical values indicate the highest probability at each node. The phylogeny was reconstructed using a Bayesian analysis of *Rag1* sequences (see Materials and Methods).