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EVOLUTIONARY REPLACEMENT OF COMPONENTS IN A SALAMANDER PHEROMONE SIGNALING COMPLEX: MORE EVIDENCE FOR PHENOTYPIC-MOLECULAR DECOUPLING

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In this article we explore the evolutionary history of a functional complex at the molecular level in plethodontid salamanders. The complex consists of a proteinaceous courtship pheromone, a pheromone-producing gland on the male's chin, and a set of behaviors for delivering the pheromone to the female. Long-term evolutionary stasis is the defining feature of this complex at both the morphological and behavioral levels. However, our previous assessment of the pheromone gene, plethodontid receptivity factor (PRF), revealed rapid evolution at the molecular level despite stasis at higher levels of organization. Analysis of a second pheromone gene, sodefrin precursor-like factor (SPF), now indicates that evolutionary decoupling in this complex is pervasive. The evolutionary profiles of SPF and PRF are remarkably similar in that: (a) both genes exhibit high levels of sequence diversity both within and across taxa, (b) genetic diversity has been driven by strong positive selection, and (c) the genes have evolved heterogeneously in different salamander lineages. The composition of the pheromone signal as a whole, however, has experienced an extraordinary evolutionary transition. Whereas SPF has been retained throughout the 100 MY radiation of salamanders, PRF has only recently been recruited to a pheromone function (27 million years ago). When SPF and PRF coexist in the same clade, they show contrasting patterns of evolution. When one shows rapid evolution driven by positive selection, the other shows neutral divergence restrained by purifying selection. In one clade, the origin and subsequent rapid evolution of PRF appear to have interfered with the evolution and persistence of SPF, leading to a pattern of evolutionary replacement. Overall, these two pheromone genes provide a revealing window on the dynamics that drive the evolution of multiple traits in a signaling complex.

KEY WORDS: **Courtship signal, pheromone, phospholipase A2 inhibitor, positive selection, rapid evolution, reproductive proteins, sex-related genes.**

In an earlier report we argued that evolution may be decoupled at different levels in a functional complex (Watts et al. 2004). By "functional complex" we mean a set of characters that interact in a coordinated fashion to perform an intricate task, such as pollination, insemination, envenomation, and prey capture. In each of these cases, the functional complex involves interacting characters at multiple levels of organization (molecules, cells, organs). Thus, envenomation in viperid snakes involves a complicated molecular cocktail (the venom) that is produced in a gland and injected into prey through the use of a set of behaviors and morphologies that

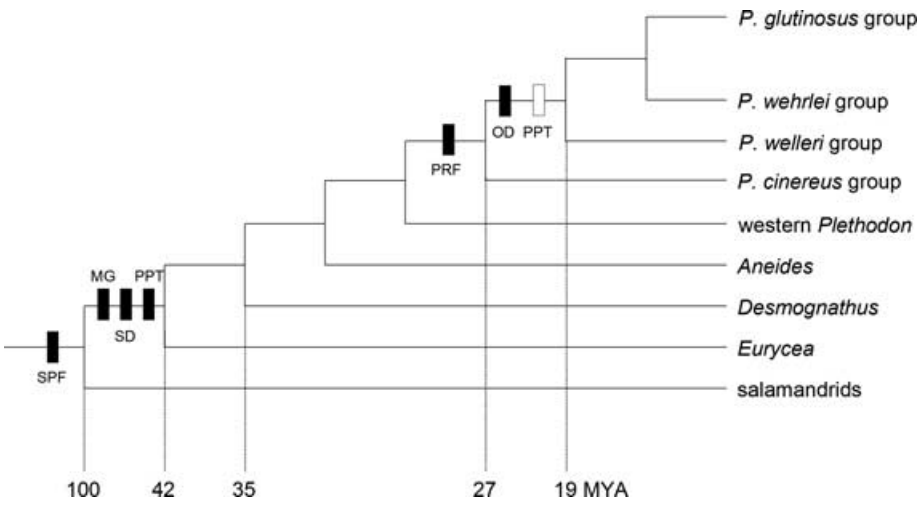

Figure 1. **Cladogram showing the relationships of various clades of plethodontid salamanders and the evolution of characters involved in courtship pheromone delivery. Relationships at the generic level are concordant across studies using both morphological and molecular characters (Chippindale et al. 2004; Macey 2005; Min et al. 2005). Relationships of species groups within the genus *Plethodon* are based primarily on allozyme data (Larson and Highton 1978; Highton 1989; Highton and Peabody 2000, and unpublished). Approximate divergence times (shown at bottom) are based on albumin immunology (Larson et al. 2003). Small rectangular boxes show the point of origin (solid) or loss (open) of various characters: sodefrin-like precursor factor (SPF), mental gland (MG), protruding premaxillary teeth (PPT), scratching delivery of courtship pheromones (SD), plethodontid receptivity factor (PRF), and olfactory delivery of courtship pheromones (OD).**

are dedicated to this task. An expectation for such complexes is that multivariate selection should promote correlations among the characters that interact in the complex (Olson and Miller 1958; Lande 1980; Cheverud 1982; Arnold 2005). One might also expect function-promoting selection to percolate from the morphological to the molecular level, so that traits at different levels of organization in the complex would show similar modes of evolution (Watts et al. 2004). Contrary to this expectation of correlated evolution, our analysis of behavioral, morphological, and molecular evolution in a salamander pheromone delivery system revealed decoupling of evolution at different levels in the functional complex. Although evolutionary stasis prevailed at the morphological and behavioral levels, molecular evolution of a pheromone gene was rapid and incessant.

Here we report observations on the evolution of a second pheromone gene in the same functional complex. These observations suggest that evolutionary decoupling is even more dramatic than was previously realized. The two pheromone genes share a number of evolutionary characteristics (e.g., rapid diversification driven by positive selection) that reinforce the picture of decoupled molecular and morphological/behavioral evolution. Additionally, an unexpected evolutionary pattern emerged at the molecular level, namely, replacement of one actively evolving pheromone component by another. To put these new results in perspective, we must first review some details of our salamander pheromone study system.

The behavioral and morphological aspects of courtship pheromone delivery in plethodontid salamanders have remained remarkably stable over a 50–100 million year period (Fig. 1; Houck and Arnold 2003). Plethodontids are a monophyletic group of lungless salamanders that originated in eastern North America. Plethodontids are extraordinary for their sexual conservatism (Fig. 1). All species in the family engage in a tail-straddling walk that aligns sexual partners as they transfer sperm from a spermatophore. Mechanisms of sexual persuasion are also conserved throughout the radiation. Male plethodontids produce pheromones in a gland on their chins (the mental gland) and deliver these pheromones to the female during courtship, prior to sperm transfer.

The ancestral mode of pheromone delivery has been retained in all major clades of plethodontids. In this ancestral mode, the courting male abrades the female's dorsum using specialized premaxillary teeth and then rubs his mental gland over the abraded site. We refer to this ancestral delivery mode as "scratching" (previously referred to as "vaccination" by Watts et al. 2004 and Palmer et al. 2005). All of the morphological and behavioral characters involved in scratching delivery of courtship pheromones have been retained for a 50–100 million year period with only minor evolution in details. These male-limited characters include protruding premaxillary teeth, the mental gland, and a stereotyped series of behaviors (pulling, sliding, snapping) that are used to deliver pheromones to the female (Arnold and Houck 1982; Houck and Arnold 2003).

In particular clades, evolutionary stasis in the functional complex has been interrupted by transitions to new pheromone delivery modes (Houck and Sever 1994). The only major transition, and the one of concern in this article, occurred 19 million years ago within eastern *Plethodon* (Fig. 1). The eastern *Plethodon* clade consists of four distinct species groups (*P. cinereus*, *P. welleri*, *P. wehrlei,* and *P. glutinosus*; Highton and Larson 1979; Highton 1989; Fig. 1). Species in the *P. cinereus* group retain the features of ancestral pheromone delivery, which include small, anteriorly placed mental glands, protruding premaxillary teeth, and scratching delivery behaviors (Dyal 2006). The *P. welleri* and *P. wehrlei* species groups mark the beginning of the transition from scratching to olfactory delivery, exhibiting what we call an intermediate pheromone delivery mode (Palmer et al. 2005; Picard 2005). Males in these two groups lack pre-maxillary teeth and have round, posteriorly positioned mental glands characteristic of olfactory delivery (Highton 1962; Coss 1974), yet olfactory delivery via slapping has rarely been observed (Organ 1960; Arnold 1972; Picard 2005). Instead, pheromone delivery apparently occurs via incidental application of the mental gland to the female's nares and/or passive diffusion through her epidermis as a consequence of male head-rubbing and head-sliding behaviors (Arnold 1972; Picard 2005; Dyal 2006). Males of the most recently derived *P. glutinosus* species group have large, posteriorly positioned mental glands and have lost the enlarged premaxillary teeth and behaviors for scratching delivery (Highton 1962; Arnold 1976). Instead, species in this group deliver pheromones to the female's vomeronasal system by slapping their mental glands directly across the female's nares (Arnold 1976). We will refer to this delivery mode as "olfactory." Apart from this transition from scratching to olfactory pheromone delivery, the major feature that emerges from comparative studies of behavior and morphology is evolutionary stasis within delivery modes.

Viewed against this background of long-term stasis at the behavioral and morphological levels, the evolution of one of the pheromone components produced by the mental gland is especially remarkable. The component, plethodontid receptivity factor (PRF), is a protein that is structurally related to the IL-6-type cytokines (Watts et al. 2004). PRF is expressed exclusively in the male mental gland, and only in eastern *Plethodon* species (Fig. 1, Rollmann et al. 1999; Palmer et al. 2005). Behavioral bioassays using purified PRF show that it reduces time to insemination during courtship (Houck et al. 1998; Rollmann et al. 1999). Surveys of PRF sequences from multiple populations and species of *Plethodon* reveal no molecular signature of the transition from scratching to olfactory delivery that occurs in eastern *Plethodon* (Watts et al. 2004; Palmer et al. 2005). Instead, these surveys indicate that PRF is prone to incessant change driven by positive selection at numerous sites and has undergone repeated gene duplication in nearly all *Plethodon* species groups. An important exception to this picture of incessant evolution occurs in species having an intermediate type of pheromone delivery, such as species in the *P. welleri* group (Fig. 1). In these species, PRF evolves in a manner typical of most proteins (neutral divergence restrained by purifying selection) (Palmer et al. 2005). PRF is not, however, the only pheromone component produced by the mental gland.

This article focuses on a second pheromone component, structurally unrelated to PRF, that was discovered in the mental gland of plethodontid salamanders. We name this component sodefrin precursor-like factor (SPF) in reference to its biochemical affinities. SPF is a protein of ~23 kDa, which has a putative 18 amino acid secretion signal, carries a strong negative charge at neutral pH, and is structurally related to the sodefrin precursor protein of aquatic newts. This component was first discovered in terrestrial salamanders as a rare element in a cDNA library made from mental glands of sexually active male *Plethodon shermani.* We later discovered that SPF is highly expressed in the mental glands of male *Desmognathus ocoee,* a member of a plethodontid clade distantly related to *Plethodon* (Fig. 1). To determine whether SPF is biologically active, we conducted behavioral assays with female *D. ocoee*. These bioassays showed that a pheromone extract enriched for SPF shortened the time to insemination during courtship (Watts et al. 2007). Furthermore, a survey for SPF (detailed in this article) revealed that this pheromone component is present throughout the family Plethodontidae, indicating that SPF is an ancestral pheromone in plethodontids. Thus, a primary goal of this article is to determine whether the presence of two pheromone components in eastern *Plethodon* affected the evolutionary dynamics of these proteins. The alternative possibilities of evolutionary coexistence or replacement are suggested by recent models of sexual selection.

Traits that are elaborated and maintained by sexual selection in one lineage may be lost in another (Wiens 2001), a possibility long recognized in the theoretical literature (Lande 1981; Mead and Arnold 2004). One reason that sexually selected traits are lost is antagonism in the evolutionary dynamics (Pomiankowski and Iwasa 1993; Iwasa and Pomiankowski 1994). For example, when two different male traits affect mate choice, two kinds of evolutionary outcomes are possible: coexistence of both traits or replacement of one trait by the other. Whether a newly evolved trait (e.g., PRF) coexists with an ancestral trait (e.g., SPF) or replaces it depends on how the two traits interact in their effects on female receptivity. An additive interaction promotes coexistence, whereas a nonadditive interaction can lead to replacement (Pomiankowski and Iwasa 1993; Iwasa and Pomiankowski 1994).

Although we do not know how PRF and SPF together interact in their effects on female mating behavior, we can ask whether these two pheromone components show an evolutionary pattern of coexistence or replacement. In particular, the scenario

of evolutionary replacement leads to the prediction that the evolutionary origin of PRF in the eastern *Plethodon* clade should affect the evolutionary dynamics of SPF. Specifically, if SPF normally shows the same evolutionary mode as PRF, we expect to see a less rapid pace of evolution and less evidence for positive selection acting on SPF in the eastern *Plethodon* clade. Conversely, the *P. welleri* clade, which deviates away from the normal, incessant mode of PRF evolution (Palmer et al. 2005), should show a reversion toward rapid evolution of SPF.

In summary, this article has two major aims. First, we aim to determine whether SPF, an ancient pheromone component in plethodontid salamanders, shows the same pattern and mode of evolution as PRF, an unrelated pheromone component that originated more recently. Second, we will test the hypothesis that the origin and subsequent incessant evolution of PRF interfered with the evolution and persistence of SPF, producing a pattern of evolutionary replacement.

Materials and Methods

DATABASE SUBMISSION

The sequences reported in this manuscript have been deposited in GenBank under the accession numbers DQ097016 through DQ097074 and DQ384536 through DQ384570.

TAXON SAMPLING AND RNA/DNA ISOLATION

Gene sequences were obtained from mRNA expressed in male mental glands. Male salamanders with enlarged mental glands were collected during the breeding season from the localities listed in Table 1. In addition, a single male/female breeding pair from each of two species, *P. shermani* (Macon Co., NC) and *D. ocoee* (Clay Co., NC), was used to determine if SPF is expressed in tissues other than the mental gland. Skin was obtained from the chins of females and from the venter and dorsal tail base regions from both sexes. Livers were also taken from the *P. shermani* breeding pair. The animals were anesthetized, tissues were surgically removed, and total RNA was extracted as described in Palmer et al. (2005).

Genes were amplified by PCR from cDNA derived from mental glands. A primer pair (forward: SPF-O-F 5′-TGA ACG CCT TCC TTA CTG GTG TC-3′; reverse: SPF-O-R 5′-AGT TGG CAG CCC GCT GTC AT-3′) was designed from the untranslated regions of a SPF sequence obtained from a *P. shermani* cDNA library. A 50.0 μl PCR reaction was prepared with 1.0 μl template cDNA, 2.5 units PfuUltra™ High-Fidelity DNA Polymerase (Stratagene [La Jolla, CA] #600382), 5.0 μl 10X reaction buffer, 0.2 mM of each DNTP, and 100.0 pmol of each primer. Amplification was carried out using initial denaturation at 95°C for 1 min, followed by 40 cycles each of (1) denaturation at 95°C for 30 sec, (2) primer annealing at 58°C for 30 sec, and (3) an extension at 68°C for 1 min. Amplification was finalized with a single extension at 68°C for 10 min. To avoid random PCR amplification error, a proofreading DNA polymerase (*PfuUltra* High-Fidelity, Stratagene [La Jolla, CA] #600382) was used. PCR products were visualized and excised from a 1.5% agarose gel, purified (QIAquick gel extraction kit; Qiagen [Valencia, CA] #28706), and cloned using the Zero Blunt® Topo® PCR cloning kit (Invitrogen [Carlsbad, CA] #K2800-20). Five to 10 SPF clones per individual were purified (QIAprep spin miniprep kit, Qiagen [Valencia, CA] #27106) and sent for sequencing in both the forward and reverse directions using universal T3 and T7 primers.

The cDNA libraries were constructed from *P. shermani* ($N = 10$) and *D. ocoee* ($N = 20$) mental glands as described in Palmer et al. (2005). Three hundred clones were randomly sequenced from the libraries of each of these two species.

PHYLOGENY RECONSTRUCTION

The set of programs in the Lasergene package (DNASTAR, Madison, WI) was used for sequence alignment and editing. Alignments were performed using the Clustal˙W algorithm with a gap penalty of 15.0 and a gap length penalty of 5.0 with minor adjustments made manually. Datasets were built from unique DNA haplotypes and maximum likelihood trees were reconstructed from the aligned DNA sequences using Bayesian inference (MrBayes Version 3.0b4; Huelsenbeck and Ronquist 2001). Gapped positions were considered ambiguous and treated as missing data during phylogeny reconstruction. Bayesian analyses were performed using the codon model with rates specified as gamma-distributed across sites. Four Monte Carlo Markov chains were run simultaneously for 1,000,000 generations with a random tree assigned at the start of each chain. A molecular clock was not enforced. Trees were sampled every 100 generations, for a total of 10,000 trees generated. The first 2000 gene trees were discarded as "burn-in" (Huelsenbeck and Ronquist 2001) and the remaining 8000 trees were used to generate a 50% majority rule consensus tree in PAUP* (Version 4.0b10; Swofford 2003), which depicted the posterior probability values of the observed clades (Huelsenbeck and Ronquist 2001).

ESTIMATING GENE DISSIMILARITY AND PATTERNS OF SELECTION

Average nucleotide sequence dissimilarity was measured as the number of unique substitutions per nucleotide site for a pair of sequences with a correction for multiple hits (Tamura-Nei method in MEGA, Version 3.0; Kumar et al. 2004). Average dissimilarity at the protein level was estimated using the Poisson correction distance, which accounts for multiple substitutions at the same site (MEGA, Version 3.0; Kumar et al. 2004). The average numbers of synonymous changes per synonymous site (d_S) and nonsynonymous substitutions per nonsynonymous site (d_N) were

Table 1. Locality data for 28 plethodontid species from which sodefrin precursor-like (SPF) sequences were obtained and description of nucleotide and amino acid sequence diversity.

Species name	Collection site in the U.S.A.				SPF isoforms				
	County	State	Lat (°N)	Long (°W)	Number of clones	Number of unique sequences	Nucleotide dissimilarity(%)	Number of unique translations	Amino acid dissimilarity (%)
Genus *Eurycea*					20	2	8.99±1.22	2	20.76±3.23
E. wilderae[1]	Swain	NC	35 10′43″	083 33′44″	10	1	n/a	1	n/a
E. guttolineata [1]	Graham	NC	35 02′36″	083 09′37″	10	1	n/a	1	n/a
Genus *Desmognathus*					16	9	8.23±0.89	8	20.11±2.43
D. ocoee[1]	Clay	NC	35 02′20″	083 33′08″	10	4	9.56±1.06	3	22.8±2.96
D. monticola[1]	Macon	NC	35 01′50″	083 08′49″	6	5	8.05±0.90	5	19.23±2.52
Genus *Aneides*									
A. ferreus[1]	Lane	OR	43 47′15″	123 49′06″	9	2	8.36±1.19	2	20.6±3.57
Genus *Plethodon*									
Western *Plethodon*									
P. stormi[1]	Jackson	OR	42 06′23″	123 01′18″	9	2	6.46±1.01	2	17.50±3.57
Eastern *Plethodon*									
P. cinereus group[1]					31	12	1.55±0.32	9	3.44±0.86
P. cinereus	Giles	VA	37 22′02″	080 31′34″	9	3	0.23±0.16	3	0.69±0.48
P. hoffmani[4]	Bath/Pocahontas	VA/WV	38 15′50″	079 48′03″	6	2	0.17±0.16	1	n/a
P. richmondi[4]	Wise	VA	36 53′42″	082 37′58″	10	6	1.25±0.37	4	2.86±0.93
P. serratus	Henry	GA	33 29′59″	084 10′58″	6	1	n/a	1	n/a
P. welleri group[2]					26	12	5.47±0.62	12	13.6±1.72
P. dorsalis	Parke	IN	39 53′14″	087 11′20″	6	1	n/a	1	n/a
P. ventralis	Jefferson	AL	33 43′32″	086 49′20″	6	5	5.17±0.7	5	14.08±2.04
P. websteri	Jefferson	AL	33 43′32″	086 49′20″	8	3	5.07±0.74	3	12.05±2.20
P. welleri	Madison/Unicoi	NC/TN	36 06′36″	082 21′40″	6	3	5.96±0.81	3	14.87±2.26
P. wehrlei group[2]					15	4	0.58±0.26	4	1.02±0.56
P. wehrlei[4]	Floyd	VA	36 47′37″	080 27′58″	10	2	0.53±0.21	2	1.00±0.54
P. wehrlei	Pocahontas	WV	38 27′10″	080 00′12″	5	2	0.17±0.16	2	0.51±0.47
P. glutinosus group[3]					103	51	4.05±0.53	43	8.89±1.44
P. aureolus	Monroe	TN	35 27′29″	084 01′24	7	5	2.13±0.38	3	4.15±0.90
P. chattahoochee[4]	Towns	GA	34 52′21″	083 48′31″	7	7	0.86±0.26	7	1.44±0.54
P. cheoah	Graham	NC	35 21′30″	083 43′04″	8	4	4.20±0.68	3	9.18±1.71
P. cylindraceus	Johnson	TN	36 23′58″	081 57′55″	5	1	n/a	1	n/a
P. jordani	Sevier	TN	35 36′34″	083 26′50″	9	4	4.01±0.65	3	9.29±1.72
P. kentucki[4]	Wise	VA	36 53′42″	082 37′58″	6	2	3.29±0.73	2	7.92±2.02
P. metcalfi	Macon	NC	35 19′40″	083 20′10″	10	5	3.93±0.65	5	9.16±1.71
P. mississippi	Scott	MS	32 24′37″	089 29′02″	7	6	0.37±0.15	6	1.12±0.45

continued

Table 1. continued

Species name	Collection site in the U.S.A.				SPF isoforms				
	County	State	Lat (°N)	Long (°W)	Number of clones	Number of unique sequences	Nucleotide dissimilarity(%)	Number of unique translations	Amino acid dissimilarity (%)
P. montanus	Madison	NC	35 50′24″	082 57′11″	8	3	4.94±0.78	3	11.64±2.09
P. ouachitae[4]	Le Flore	OK	34 47′50″	094 54′29″	9	3	0.23±0.16	2	0.35±0.32
P. shermani	Macon	NC	35 10′48″	083 33′38″	10	2	7.05±1.16	2	15.07±2.90
P. teyahalee	Madison	NC	35 50′24″	082 57′11″	10	7	3.01±0.46	5	6.55±1.18
P. yonahlossee[4]	Yancey	NC	35 44′38″	082 12′51″	7	2	0.17±0.17	1	n/a
Total					229	93	11.36±0.74	82	23.65±2.1

[1]Species having scratching delivery mode.
[2]Species having intermediate delivery mode.
[3]Species having olfactory delivery mode.
[4]These species are from the same localities reported by Palmer et al. (2005) but minor corrections have been made to the coordinate data.

calculated using the modified Nei and Gojobori method with the Jukes–Cantor correction for multiple hits (MEGA Version 3.0; Kumar et al. 2004). Standard errors were determined using 500 bootstrap replicates. The nonsynonymous-to-synonymous rate ratio (d_N/d_S) was averaged across SPF genes for each lineage to determine the dominant mode of selection that has acted on the gene. A d_N/d_S ratio of 1 ($\omega = 1$) indicates neutrality, whereas $\omega < 1$ indicates purifying (stabilizing) selection and $\omega > 1$ indicates positive selection. The Z test of selection ($Z = (d_N - d_S)/\text{SQRT}(\text{Var}(d_S) + \text{Var}(d_N))$; MEGA, Version 3.0; Kumar et al. 2004; 500 bootstrap replicates) was then used to test the hypothesis that $d_N > d_S$. In addition, tests of adaptive molecular evolution were carried out using phylogeny-based maximum likelihood models of codon evolution implemented by the PAML computer program CODEML (Version 3.12; Yang 2002). The nucleotide alignment and maximum likelihood tree were used as input for each of the following five datasets: (1) desmognathine SPF (2 spp; 9 sequences), (2) SPF from eastern *Plethodon* of the *P. glutinosus* group (13 spp; 51 sequences), (3) SPF from eastern *Plethodon* of the *P. welleri* group (4 spp; 12 sequences), (4) SPF from eastern *Plethodon* of the *P. cinereus* group (4 spp; 12 sequences), and (5) SPF from all 28 plethodontid species studied (94 sequences).

Codon substitution models, implemented in PAML, were used to estimate nonsynonymous/synonymous substitution rate ratios (d_N/d_S) in different lineages in the SPF gene tree and among individual amino acid sites. Four models of variable selective pressures among branches were implemented (Yang 1998; Yang and Nielsen 2002). These models estimate d_N/d_S (ω) by averaging over all sites. The one-ratio model assumed one site class for all branches, the free-ratio model estimated ω from the data for each branch of the topology, the two-ratios model assumed two ω ratios: one for the branch leading to the transition in pheromone delivery mode in eastern *Plethodon* and one for all other branches in the tree, and the three-ratios model, which assumed three separate ω ratios: one for *Desmognathus* SPF, one for *Eurycea* SPF, and one for all other branches in the tree.

In addition, six site-specific models were implemented: M0 (one-ratio model, with a single ω averaged among sites), M1 (neutral model, which assumed two site classes, $\omega_0 = 0$ and $\omega_1 = 1$), M2 (selection model, which assumed two site classes, $\omega_0 = 0$ and $\omega_1 = 1$, and a third site class estimated from the data), M3 (discrete model that had three class sites, ω_0, ω_1, and ω_2 all estimated from the data), M7 (beta model, which assumed a beta distribution with a continuous distribution of ω values limited to the interval 0-1), and M8 (beta plus omega model, which added an extra site class to M7 with a free ω value estimated from the data). These models average ω for each site over all branches. The model that provided the best fit to the data was determined by comparing the likelihood ratio test (LRT) statistic to a chi-square distribution with the appropriate degrees of freedom (Yang 1998). The empirical Baye's

approach was used to determine the probability that a particular codon site was positively selected (Nielsen and Yang 1998; Yang et al. 2000).

Results

THE SPF GENE HAS AN ANCIENT ORIGIN

We surveyed 28 species from four genera of plethodontid salamanders (*Aneides*, *Desmognathus*, *Eurycea*, and *Plethodon*, see Table 1) for the presence of the SPF transcript in the mental gland. SPF was present in the mental gland of every species sampled, suggesting that the pheromone function of this gene originated at least 50–100 million years ago. The recruitment of SPF to a pheromone function thus predates that of the pheromone gene PRF, which appears to have originated within eastern *Plethodon* (∼27 million years ago; Palmer et al. 2005; Fig. 1).

GENE DUPLICATION AND SEQUENCE DIVERSITY ARE CHARACTERISTIC OF PLETHODONTID PHEROMONE GENES

Comparisons of SPF and PRF (Palmer et al. 2005) suggest that gene duplication and nucleotide sequence diversity are common features of plethodontid pheromone genes. Sequencing of 229 SPF clones resulted in 94 unique nucleotide sequences that encoded 93 unique amino acid sequences. Two closely related, allopatric eastern *Plethodon* species (*P. metcalfi* and *P. jordani*) shared a single SPF allele (100% identity). SPF nucleotide sequence length ranged from 573 to 618 bps (191–206 amino acids) with 318 variable sites. Sequence length variation in SPF was due to a six amino acid insertion in eastern *Plethodon*, a two residue deletion in *Eurycea*, and two separate deletions (three and five amino acids) in *Aneides* SPF (Fig. 2). As in PRF (Palmer et al. 2005), SPF transcript was not detected in the liver or epidermal tissues from the male/female breeding pairs of *Plethodon* and *Desmognathus,* and is presumably not expressed outside of the male mental gland.

The topology of the SPF gene tree suggests that gene duplications occurred both in *Desmognathus* and in eastern *Plethodon* (Fig. 3). Thus, both species of *Desmognathus* occur in each of the major branches for that genus in the gene tree. Likewise, the same set of *Plethodon* species (*P. teyahalee, P. montanus, P. metcalfi, P. jordani, P. cheoah, P. shermani, P. kentucki, P. welleri, P. ventralis*, and *P. websteri*) is represented in both of the major branches for that genus. Average interspecific nucleotide sequence dissimilarity across all plethodontid SPF sequences was 11.4%. Within each lineage, nucleotide dissimilarity was highly variable, ranging from 0.6 to 9.0% (Table 1). The level of intraspecific sequence dissimilarity also varied considerably, ranging from only 0.17% in *P. yonahlossee, P. wehrlei*, and *P. hoffmani* to 9.56% in *D. ocoee* (Table 1). In *Desmognathus*, each SPF gene sequence had a unique pattern of variation, and the level of dissimilarity within each species (8.1–9.6%) was comparable to that between species (8.2%). In contrast, many species within the *P. glutinosus* group expressed two distinct forms of the gene, SPF A and SPF B, which elevated the overall levels of intra- and interspecific variation. Within these two gene types, however, interspecific variation was much lower (1.13% ± 0.22% for SPF "A" and 1.43% ± 0.23% for SPF "B"). Most SPF "B" gene isoforms were readily identified by the presence of a six amino acid insertion at position(s) 139–144 (Fig. 2). This gene duplication of SPF in eastern *Plethodon*, including the emergence of the six amino acid insertion, appears to have occurred in the *P. welleri* species group (Fig. 3).

Like PRF, the number of unique SPF nucleotide sequences isolated from individual glands varied considerably between species (Table 1). In most cases, a gland yielded between two and seven unique sequences, suggesting that as many as four unique SPF genes are expressed in the mental glands of some species (Table 1). *Plethodon dorsalis, P. serratus, Eurycea guttolineata,* and *E. wilderae*, however, each yielded only a single SPF allele out of the 5–10 clones sequenced. Putative pseudogenes were isolated from *P. teyahalee* (deletions in the coding region 268 bps and 415 bps long) and *P. montanus* (90 bp insertion that resulted in a nonsense frameshift) that were not included in the evolutionary analyses.

THE SPF AND PRF PROTEINS ARE STRUCTURALLY UNRELATED, BUT BOTH ARE HIGHLY VARIABLE

SPF is similar to PRF in that sequence variation was higher at the amino acid level than it was at the nucleotide level (Table 1). Average SPF amino acid sequence dissimilarity was 23.7% across all taxa. Within species, SPF sequence dissimilarity ranged from 0.4 to 23.0% (Table 1). As in PRF, much of the amino acid sequence dissimilarity across SPF genes arises from gene comparisons within a single individual rather than between closely related species. An alignment of SPF amino acid sequences from 18 plethodontid species is shown in Figure 2. Of 190 shared amino acid residues, 55 residues (29% of the gene) were conserved across the SPF genes of all plethodontid species sampled. Fifteen of the 55 conserved residues are located within a putative signal sequence (residues M^{-1} to G^{-18}), and 16 additional conserved sites are cysteine residues that likely provide protein conformational stability via the formation of disulfide bonds. The remaining 24 conserved residues are scattered throughout the protein (consensus; Fig. 2).

SPF shares no sequence or structural similarities to PRF. BLASTX searches on GenBank (www.ncbi.nlm.nih.gov/BLAST) were used to find sequences related to SPF. Our database search indicated that approximately 22% of the SPF amino acid sequence is identical with the sodefrin precursor protein from the aquatic breeding newt, *Cynops pyrrhogaster*. SPF amino acid sequences also share approximately 25% identity to snake γ-type phospholipase A2 inhibitor proteins.

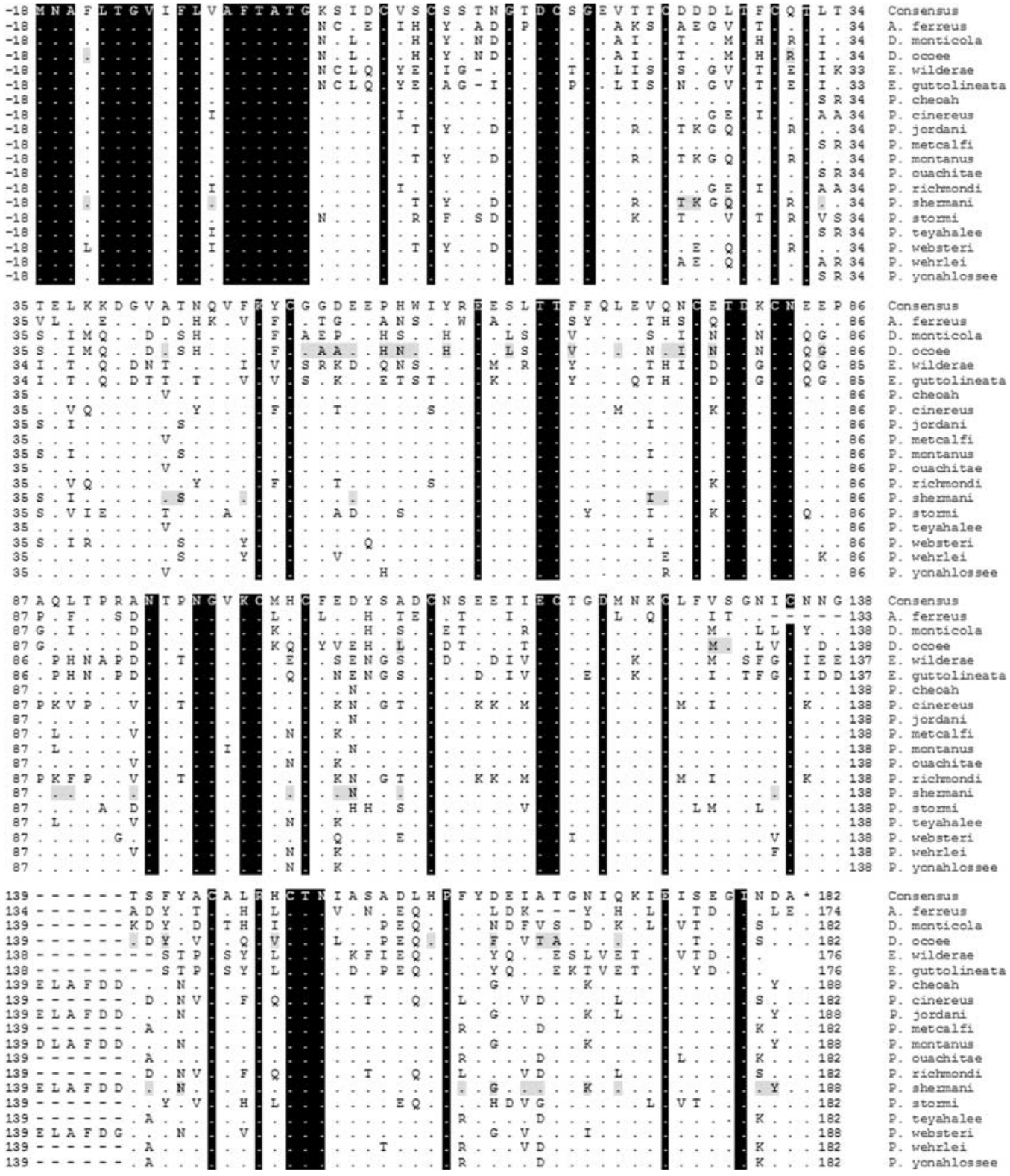

Figure 2. Alignment of sodefrin precursor-like factor (SPF) amino acid sequences from 18 species of plethodontid salamanders. The dots represent amino acid residues that are identical to the consensus sequence. The putative signal sequence includes the first 18 amino acid residues (positions ⁻1 through ⁻18). Unique deletions are shown as dashes and black vertical bars highlight residues that are identical across all sequences. Sites predicted to have experienced positive selection ($\geq$ 60% probability) are highlighted in gray on *P. shermani* for members of the *P. glutinosus* species group and on *D. ocoee* for desmognathines.

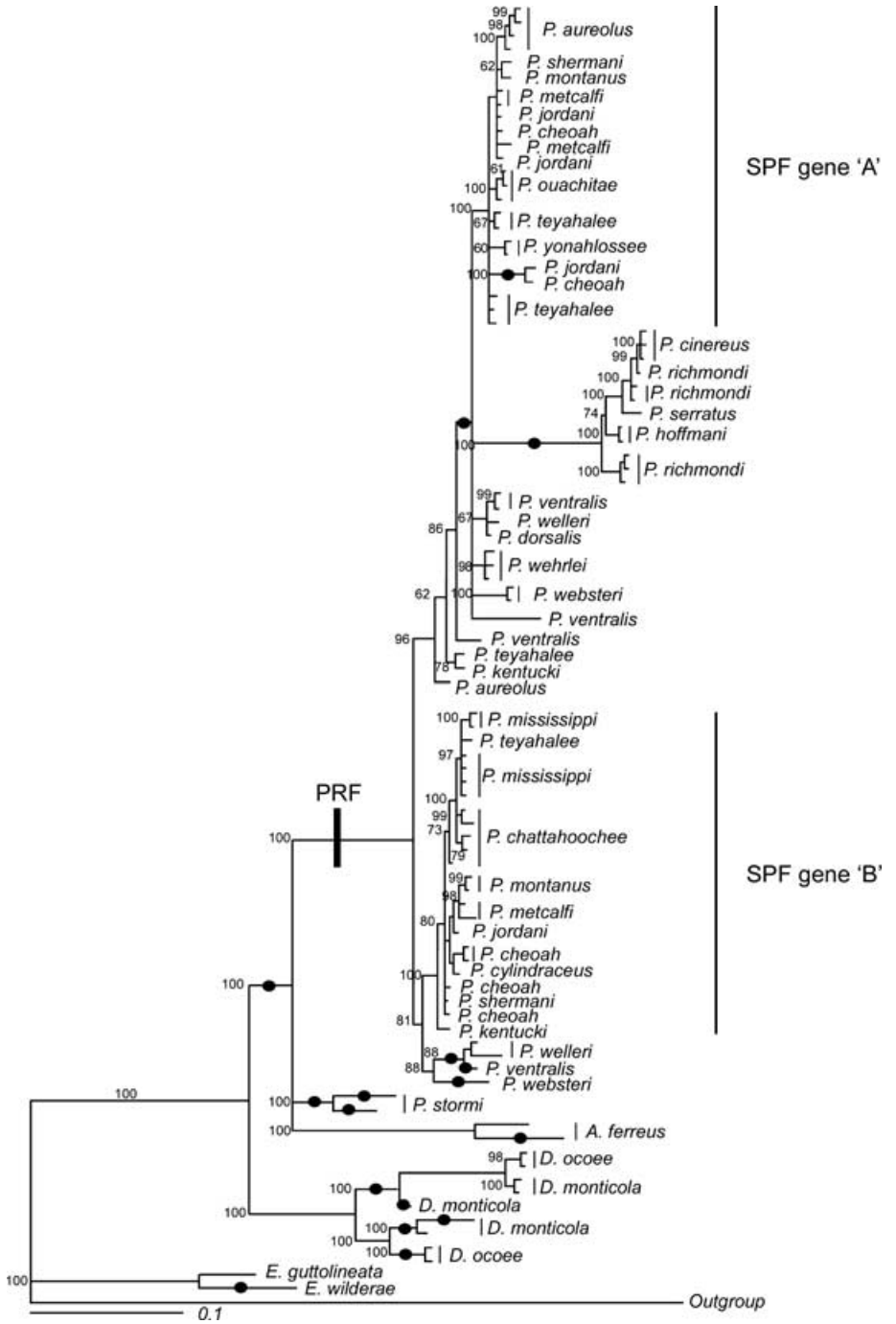

Figure 3. **Maximum likelihood tree of sodefrin precursor-like factor (SPF) genes for 28 plethodontid species. Posterior probabilities ≥ 60 are denoted at the left of each branch. Black circles mark branches where $\omega > 1.5$. The solid rectangular box shows the point of origin of plethodontid receptivity factor (PRF). SPF genes "A" and "B" are labeled for the *P. glutinosus* species group. The distance bar represents 0.1 nucleotide substitutions per site. The gene phylogeny was rooted with the *Cynops pyrrhogaster* sodefrin precursor sequence (accession CAB53093).**

AS IN PRF, SPF HAS EXPERIENCED AMONG-LINEAGE DIFFERENCES IN SELECTION

We used the ratio d_N/d_S (ω) to determine the dominant mode of selection that has acted on the SPF genes of each salamander lineage and compared the results to those of PRF. Omega (ω) values averaged across all sites in SPF genes from the *Plethodon cinereus* group ($\omega = 0.87$) and the *P. wehrlei* group ($\omega = 0.56$) were consistent with neutrality/purifying selection (Table 2). Overall ω values for SPF genes of *Desmognathus* ($\omega = 3.06, Z = 4.58, P < 0.001$), *Eurycea* ($\omega = 2.83$, $Z = 2.83$, $P = 0.009$), *Plethodon welleri*

Table 2. The average number of synonymous changes per synonymous site ($d_S \pm$ SE), nonsynonymous substitutions per nonsynonymous site ($d_N \pm$ SE), d_N/d_S rate ratio averaged across the gene and the *Z* test for selection statistic for plethodontid SPF genes. ** $P < 0.001$; * $P < 0.01$.

Gene	Number of species	Number of sequences	d_S	d_N/d_S	d_N/d_S	*Z* statistic	d_N/d_S PRF
SPF plethodontid	28	94	0.082±0.011	0.124±0.012	1.51	2.75*	
SPF *Eurycea*[1]	2	2	0.036±0.015	0.102±0.018	2.83	2.77*	Not present
SPF *Desmognathus*[1]	2	9	0.033±0.010	0.101±0.013	3.06	4.58**	Not present
SPF *Aneides*[1]	1	2	0.048±0.018	0.094±0.016	1.96	2.30	Not present
SPF Western *Plethodon*[1]	1	2	0.023±0.011	0.084±0.015	3.65	3.53**	Not present
SPF *Plethodon cinereus* group[1]	4	12	0.015±0.006	0.013±0.003	0.87	−0.15	1.19
SPF *Plethodon welleri* group[2]	4	12	0.025±0.008	0.068±0.009	2.72	3.50**	0.98
SPF *Plethodon wehrlei* group[2]	1	4	0.009±0.006	0.005±0.003	0.56	−0.59	n/a
SPF *Plethodon glutinosus* group[3]	14	51	0.034±0.009	0.041±0.007	1.21	0.61	1.70

[1]Species having scratching delivery mode, [2]species having intermediate delivery mode, [3]species having olfactory delivery mode. d_N/d_S ratios for plethodontid receptivity factor (PRF) were estimated from gene sequences obtained from GenBank, accession numbers AY926884- AY927045. Synonymous substitutions were not present in the PRF genes from members of the *P. wehrlei* group.

species group ($\omega = 2.72$, $Z = 3.50$, $P < 0.001$), and western *Plethodon* (*P. stormi*, $\omega = 3.65, Z = 3.53, P < 0.001$) revealed nonneutral evolution driven by positive selection (Table 2). Omega values for *Aneides* SPF sequences ($\omega = 1.96, Z = 2.30, P = 0.09$) and the *P. glutinosus* species group ($\omega = 1.21, Z = 0.61, P = 0.2$) were suggestive of positive selection, but the hypothesis that $d_N > d_S$ was not supported statistically. Omega values in excess of two for the SPF genes of *Desmognathus*, *Eurycea*, *P. welleri* species group and western *Plethodon* are striking because the method of averaging substitution rates across all sites in a protein rarely results in a $\omega > 1$ (Yang 1998).

A direct comparison of SPF and PRF evolutionary modes was not possible for many of these salamander lineages because PRF is present only in eastern *Plethodon* species (Table 2). Within eastern *Plethodon*, however, our results suggest that the PRF genes of the *P. cinereus* species group were shaped by strong positive selection, but SPF appears to have evolved neutrally (Table 2). Conversely, the PRF genes of the *P. welleri* species group showed evidence of neutral divergence, but SPF genes bear the signature of positive selection (Table 2). In all cases, there is a contrast in evolutionary mode between SPF and PRF where they co-occur.

Gene tree-based codon-substitution models (Nielsen and Yang 1998) were used to test for among-lineage differences in selective pressures. Four models were fitted by maximum likelihood to a dataset containing SPF sequences from 28 plethodontid species (94 sequences). The one-ratio model provided an average $\omega = 1.05$ across all codon sites and branches (lnL = −5435.46). The two- and three-ratio models, which tested for selection along branches leading to the major transition in delivery mode and to the division of three of the four genera (respectively), did not provide a significantly better fit to the data than the one-ratio model. The free-ratios model (lnL = −5342.44) assumed an independent ω ratio for each branch of the tree. This model provided a significantly better fit to the data than the one-ratio model ($2\delta = 186.04$, d.f = 150, $P = 0.02$) and detected positive selection at several branches scattered throughout the gene tree. The majority of these positively selected branches exist basal to eastern *Plethodon* (Fig. 3).

AS IN PRF, SPF EXHIBITS VARIABLE SELECTIVE PRESSURES AMONG SITES

Positive selection was detected at approximately 30% of the amino acid sites in PRF (Palmer et al. 2005). For comparison, we used six codon-substitution models to assess the mode of selection acting on each amino acid residue in the SPF genes. Models M2, M3, and M8, which allow for positive selection, provided the best fit(s) to four of the five datasets (Table 3). For the dataset containing 28 plethodontid species, 94 SPF sequences were analyzed. M2, M3, and M8 parameter estimates indicate that 16–19% of the sites have experienced strong positive selection with $\omega = 2.96$–3.44 (95% probability; Table 3).

Tests for selection across the SPF genes in eastern *Plethodon* highlight a history of different selective pressures within the different species groups. In the *P. glutinosus* species group (13 species, 51 sequences), model estimates (M2 and M3) suggest that 2.1–3.2% of the sites have experienced positive selection ($\omega = 7.22$; 95% probability; Table 3). Selection model M8, however, did not fit the data significantly better than the null model (M7) for this group. In contrast, the results of all three selection models indicate that 12.2–27.4% of the sites have experienced strong positive selection within the *P. welleri* species group (4 species, 12 sequences) ($\omega = 2.38$–6.77; 95% probability; Table 3). For the

Table 3. Likelihood ratio tests of positive selection using PAML site-specific models for sodefrin precursor-like factor (SPF). d_N/d_S is the average omega (ω) across all sites and branches calculated under M0. "2δ" indicates twice the log-likelihood difference between the two models. "p" denotes the proportion of sites with a given estimated value of ω. The parameters *p* and *q* for the beta distribution B(*p*,*q*) are given for M8. The signature of positive selection was not detected in SPF from the *P. cinereus* species group and so parameter estimates for this group are not reported in the table. Parameters indicating positive selection are in bold. Asterisk denotes significance at 1% with d.f = 4 for M3 versus M0 and d.f = 2 for both M2 versus M1 and M8 versus M7. Percent positive selection indicates the proportion of sites across the gene predicted to have experienced positive selection with a 95% probability.

Gene (#spp/# sequences)	d_N/d_S	(2δ) M3 versus M0	(2δ) M2 versus M1	(2δ) M8 versus M7	Parameter estimates under M3	Parameter estimates under M8 (beta & ω)	Positive selection (%)
					p=0.28 ω=0.03		
					p_0=0.38 ω_0=0.75	p_1=0.30, ω_1=**2.96**	
Plethodontid SPF (28/94)	1.05	395.34*	145.70*	117.68*	p_1=0.33 ω_1=**2.85**	p=0.70, B(0.21, 0.21)	16.3 – 19.4
					p=0.81 ω=0.26		
					p_0=0.18 ω_0=**5.36**	p_1=0.17 ω_1=**14.42**	
Desmognathus SPF (2/9)	2.72	71.26*	64.20*	64.24*	p_1=0.01 ω_1=**10.53**	p=0.83 B(1.47, 0.10)	6.6 – 14.0
					p=0.53 ω=0		
					p_0=0.41 ω_0=0.82	p_1=0.58 ω_1=0	
Plethodon glutinosus SPF(13/51)	ω=0.64	109.84*	45.88*	1.02	p_1=0.06 ω_1=**7.22**	p=0.42 B(0.03, 0.003)	3.2
					p=0.64 ω=0		
					p_0=0.14 ω_0=**2.38**	p=0.33 ω=**5.45**	
Plethodon welleri SPF (4/12)	ω=1.56	87.94*	52.02*	52.22*	P_1=0.22 ω_1=**6.77**	p=0.67 B(0.001, 1.94)	12.2 – 27.4

P. cinereus species group (4 species, 12 sequences), the null model (M0) provided the best fit to the data, indicating that purifying selection and neutrality were the dominant modes of selection in SPF genes of this species group.

The results of all three selection models for the genus *Desmognathus* (2 species, 9 sequences) indicate that 6.6–14% of the sites across the SPF gene experienced positive selection ($\omega = 5.4$–14.4 with $\geq 95\%$ probability; Table 3). Only 16.5% of the positively selected residues exist at the same sites in both the eastern *Plethodon* and *Desmognathus* SPF genes (Fig. 2). This discordant pattern of selection across SPF gene lineages may reflect functional differences in the signal related to differences in olfactory versus scratching delivery. Overall, both SPF and PRF exhibited a high sequence diversity driven by positive selection at multiple amino acid sites in some salamander lineages but not others.

Discussion

SPF AND PRF EXHIBIT SIMILAR PATTERNS AND MODES OF EVOLUTION

Expression of both SPF and PRF is apparently limited to the male mental gland, and both proteins act as courtship pheromones that increase female sexual receptivity (Houck et al. 1998; Rollmann et al. 1999; Watts et al. 2007). In most lineages, SPF presents a strikingly similar picture of evolution to that of PRF in the eastern *Plethodon*. For example, SPF evolution in *Eurycea*, *Desmognathus*, *Aneides,* and western *Plethodon* resembles PRF evolution in most lineages of eastern *Plethodon*. In both proteins, a subset of sites is prone to perpetual, rapid change in amino acid composition that is driven by positive selection (Watts et al. 2004; Palmer et al. 2005). Gene duplication is common for both proteins, and occasionally functional genes are converted to pseudogenes (Palmer et al. 2005). We previously argued that PRF may be involved in a coevolutionary molecular tango with its receptors (Palmer et al. 2005). We adopted this metaphor because positively selected sites in PRF correspond with putative active sites identified using structural models of related cytokines and their receptors (Watts et al. 2004). Furthermore, these sites repeatedly converged on identical or functionally equivalent amino acid substitutions (Palmer et al. 2005). In other words, change was incessant but change was not cumulative. SPF presents a similar picture and likewise may be involved in a molecular tango with coevolving receptors.

The similarities between SPF and PRF in modes of evolution reinforce our earlier conclusion that evolution is decoupled at different levels in the functional complex that produces and delivers courtship pheromones (Watts et al. 2004). That earlier study focused on a comparison of molecular versus morphological/behavioral evolution over the 27 million year interval of diversification in the eastern *Plethodon* clade. During that diversification, the functional complex transitioned from scratching to olfactory delivery, a change that involved the loss of sexually dimorphic premaxillary teeth, enlargement of the mental gland, and a shift in behavior patterns used to apply the gland to the female. On either side of that transition, a remarkable degree of stasis prevailed in the morphological and behavioral details of the functional complex. Nevertheless, within each of the delivery modes, PRF evolved at a rapid pace, driven by positive selection (Watts et al. 2004; Palmer et al. 2005). The current investigation reveals a similar pattern of evolution in a second, more ancient pheromone component (SPF), across three clades of plethodontids (*Eurycea, Desmognathus, Plethodon*). Evidently SPF has persisted in this mode of evolution at least since the origin of plethodontids 50–100 million years ago. In contrast, at the morphological/behavior level in the functional complex, stasis is the predominant mode of evolution. The general picture is one of fast-paced evolution at the molecular level, decoupled from stasis at higher levels of organization in the functional complex.

EVIDENCE THAT PRF REPLACES SPF

When SPF and PRF coexist in the same clade, they show contrasting patterns of evolution. In particular, when one protein shows the syndrome of rapid evolution driven by positive selection, described above, the other shows neutral divergence, restrained by purifying selection. Specifically, a transition in SPF from a rapid to slow evolution coincides with the origin of PRF in the eastern *Plethodon* lineage. These roles are reversed in the *P. welleri* species group. Here SPF reverts to a rapid evolution (see Fig. 3, Tables 2, 3), whereas PRF evolves slowly without the impetus of positive selection (Palmer et al. 2005). Aside from these complementary patterns in evolutionary mode, evidence from cDNA libraries suggests that SPF has experienced a reduction in the level of expression in the *P. glutinosus* species group. In a cDNA library derived from *Desmognathus* mental glands, a large proportion of clones were SPF ($\sim$25%), but in a comparable *P. shermani* library only a small fraction of clones were SPF ($\sim$0.3%). Taken together, these various results suggest that the origin and evolution of PRF eclipsed both the evolution and expression of SPF.

Models of multiple traits evolving by sexual selection provide some insight into the complementary evolutionary patterns we have observed in SPF and PRF. According to a model developed by Pomiankowski and Iwasa (1993), evolutionary coexistence of pheromone components would be promoted by additive effects of the components on female receptivity. In contrast, the tendency toward replacement of SPF by PRF that we observed in the *P. glutinosus* species group could arise as a consequence of nonadditive effects between these two pheromone components, a physiological possibility that we have not explored. An outcome of this model is that the identity of the component that replaces the other is not arbitrary. Instead, the component that replaces the

other is expected to have a greater effect on female receptivity and to impose a smaller cost on female fitness. In other words, the model predicts that in the *P. glutinosus* species group, PRF was more effective in shortening courtship time and incurred less costs on females than SPF. These are predictions that are amenable to future empirical testing in our system.

THE ORIGIN OF SPF: THE SALAMANDRID CONNECTION

Sequence homology between plethodontid SPF and the sodefrin precursor gene of *Cynops* and *Triturus* (family Salamandridae) suggests that recruitment of this protein to a Pheromone function predates the divergence of the families Plethodontidae and Salamandridae. The sodefrin precursor gene of salamandrids consists of 189 amino acids and, prior to cleavage, has no reported pheromone function (Kikuyama et al. 1997). The active pheromone is a decapeptide (sodefrin) that is cleaved from the C-terminus of the sodefrin precursor protein by prohormone convertases and a proteolytic enzyme (Iwata et al. 2004). Sodefrin is produced by the abdominal gland in the cloaca of male *Cynops* and directed to the female by tail-fanning during aquatic courtship. Sodefrin stimulates the vomeronasal epithelium of the female mating partner (Toyoda and Kikuyama 2000) and can act as a sex attractant for nearby conspecific females (Iwata et al. 1999).

In contrast to the salamandrid system, plethodontid SPF functions as a pheromone in the uncleaved state. Although post-translational cleavage has not been examined experimentally in plethodontid SPF, convertase recognition sites at the C-terminal region of the protein sequence are absent. Furthermore, experimental application of the full length SPF protein directly to the dorsal skin of female *D. ocoee* during courtship resulted in a significant increase in female receptivity (Watts et al. 2007). These observations suggest that (1) the salamandrid sodefrin precursor molecule was co-opted to a pheromone function in plethodontids, (2) the pheromone function of the sodefrin precursor molecule was secondarily lost in salamandrids, or (3) the precursor molecule retained a pheromone function in salamandrids but its function has not yet been characterized.

Regardless of whether the full length protein (SPF) or a small segment of the protein (sodefrin) functions as the active pheromone, it is apparent that the sodefrin precursor gene has retained its role as a reproductive signal for at least 100 million years. The evolutionary persistence of this protein as a reproductive signal is remarkable given that the pheromone signaling systems of salamandrids and plethodontids are dramatically different (Houck and Sever 1994; Houck and Arnold 2003). SPF's long history of persistence highlights the significance of its replacement by PRF in eastern *Plethodon*. The replacement model does not, however, explain how two structurally different chemical signals converged on the same function. Whereas the SPF protein is structurally related to snake phospholipase A2 inhibitor proteins, PRF belongs to an unrelated protein family, the IL-6-type cytokines. Although SPF and PRF share no sequence or structural homology, they are both capable of increasing female receptivity during courtship. Future studies with a focus on female receptors will be necessary to determine the physiological mechanisms underlying this extraordinary evolutionary replacement.

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