

Lineage-Specific Differences in Evolutionary Mode in a Salamander Courtship Pheromone

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Functionally equivalent genes may evolve heterogeneously across closely related taxa as a consequence of lineage-specific selective pressures. Such disparate evolutionary modes are especially prevalent in genes that encode postcopulatory reproductive proteins, presumably as a result of sexual selection. We might therefore expect genes that mediate reproduction prior to insemination to evolve in a similar manner. Plethodontid receptivity factor (PRF), a proteinaceous salamander pheromone produced by the male, increases female receptivity during courtship interactions. To test for lineage-specific differences in PRF's evolution, we intensively sampled PRF genes across the eastern *Plethodon* phylogeny (27 spp.; 34 populations) to compare gene diversification, rates of evolution, modes of selection, and types of amino acid substitution. Our analyses indicate that PRF evolutionary dynamics vary considerably from lineage to lineage. Underlying this heterogeneity, however, are two well-defined transitions in evolutionary mode. The first mode is representative of a typical protein profile, wherein neutral divergence and purifying selection are the dominant features. The second mode is characterized by incessant, cyclical evolution driven by positive selection. In this mode, the positively selected sites are bound by a limited assortment of acceptable amino acids that appear to evolve independently of other sites, resulting in a tremendous number of unique PRF alleles. Several of these selected sites are implicated in receptor binding. These sites are apparently involved in a molecular tango in which the male signal and female receptors coevolve within a confined molecular space. PRF's lineage-specific evolutionary dynamics, in combination with evidence of a molecular tango, highlight the molecular action of sexual selection on a chemical signal that is used during courtship.

Introduction

Selection pressures vary greatly among genes and among different regions of a gene as a consequence of dissimilar functional and structural constraints. Genes involved in core cellular processes (i.e., translational regulation, protein biosynthesis, and mRNA splicing) evolve slowly and evolutionary changes are governed primarily by functional dynamics (Zhang and Li 2004). For this reason, functionally similar "housekeeping" genes may exhibit homogenous patterns of selection across highly disparate taxa (Castillo-Davis et al. 2004). In contrast, genes involved in coevolutionary interactions (both passive and active) can experience rapid, adaptively driven evolution (Bishop, Dean, and Mitchell-Olds 2000; Palumbi 2000; Shaw et al. 2002; Swanson and Vacquier 2002; Wang et al. 2003). The evolutionary forces acting on these coevolving genes include lineage-specific selective pressures that produce heterogeneous patterns of evolution among distant taxa (Castillo-Davis et al. 2004) or even between closely related species (Metz and Palumbi 1996; Yang, Swanson, and Vacquier 2000).

Genes that encode proteins involved in reproduction can experience especially rapid, adaptive evolution (review by Swanson and Vacquier 2002). Because reproductive proteins have a direct effect on fertilization success, adaptive change in these molecules is probably often driven by sexual selection within populations or selection favoring reproductive isolation between species (Swanson and Vacquier 2002; Torgerson, Kulathinal, and Singh 2002;

Kingan, Tatar, and Rand 2003; McCartney and Lessios 2004). Both processes can account for the discordant patterns of selection that have been characterized in reproductive genes of closely related taxa (Swanson and Vacquier 2002; Haygood 2004). Similar evolutionary dynamics should also be pervasive earlier in mating, during preinsemination processes such as sexual persuasion and/or coordination of courtship behaviors (Swanson and Vacquier 2002; Ryan and Rand 2003). Indeed, recent studies indicate that evolutionary changes in pheromones that influence mating responses, as well as their putative receptor genes, have been shaped by positive selection (Mundy and Cook 2003; Emes et al. 2004; Watts et al. 2004).

The present study details the extraordinary molecular evolution that has occurred in the pheromone signaling system of *Plethodon* salamanders with the aim of achieving a better characterization of the evolutionary processes at the molecular level. Our focus is on the pheromone component plethodontid receptivity factor (PRF), a 22-kDa cytokine-like protein that increases female receptivity during courtship (Rollmann, Houck, and Feldhoff 1999). The plethodontid courtship pheromone system is a functional complex characterized by discordant modes of evolution at different levels of organization (Watts et al. 2004). At the molecular level, the complex consists of the proteinaceous pheromone component, PRF. This component is produced in a gland on the male's chin (the mental gland) and delivered directly to the female during courtship (Houck and Arnold 2003). At morphological and behavior levels, the system consists of the mental gland, specialized male premaxillary teeth, and two stereotyped male behaviors (scratching and slapping). A male using the ancestral vaccination (scratching) mode of delivery administers the

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pheromone by wiping his mental gland across the skin on the female's dorsum while scraping her skin with his enlarged premaxillary teeth (Arnold 1972). Mental gland secretions delivered by this method likely diffuse directly into the female's circulatory system (Arnold 1977). A male, having the derived olfactory delivery mode, no longer possesses enlarged premaxillary teeth and instead "slaps" his mental gland directly across the female's nares (Organ 1958; Arnold 1976, 1977). Pheromones delivered to the female's nares enter the nasal cavity by capillary action and are directed laterally to chemoreceptors in the highly specialized epithelium of the vomeronasal organ (Dawley and Bass 1989; Wirsig-Wiechmann et al. 2002). Pheromones delivered by either mode (vaccination or olfactory) effectively increase female responsiveness to the male (Houck and Reagan 1990; Rollmann, Houck, and Feldhoff 1999).

Although morphological and behavioral stasis arising from stabilizing selection has prevailed over millions of years within each of the two delivery modes (vaccination and olfactory), molecular evolution of PRF within delivery modes has been driven by positive selection (Watts et al. 2004). In pursuing our overall goal of a better characterization of the evolutionary dynamics at the molecular level, we had several objectives. First, we wished to determine if PRF was expressed in epidermal sites other than the mental gland. Such extramental gland expression bears on the issue of whether PRF is exclusively a sexual signal. Second, we wished to determine whether PRF is expressed only in the mental glands of an eastern lineage of *Plethodon*. We pursued this objective by testing for the presence of PRF in a nested series of out-group taxa, both within and outside the family Plethodontidae. Finally, we wanted to follow up on hints from an earlier study (Watts et al. 2004) that PRF experiences different modes of evolution in different lineages. To test for lineage-specific differences in evolution, we compared diversification, rates of evolution, modes of selection, and kinds of amino acid substitution in different lineages of the PRF gene tree.

In a previous report, Watts et al. (2004) surveyed PRF genes from 12 *Plethodon* species from two distinct lineages. To test for different modes of evolution in different lineages, we intensively sampled PRF from four major lineages of *Plethodon* that are found in eastern North America (27 spp.; 34 populations). These lineages include the *Plethodon cinereus* species group, the *Plethodon wehrlei* group, the *Plethodon welleri* group, and the *Plethodon glutinosus* group (fig. 1; Highton and Larson 1979; Highton 1989; Highton and Peabody 2000; Larson, Weisrock, and Kozak 2003). All members of the *P. cinereus* group develop enlarged premaxillary teeth during the breeding season and use the ancestral vaccination mode of pheromone delivery. Members of the *P. glutinosus* species group no longer possess enlarged premaxillary teeth and have evolved olfactory delivery ("slapping"; Houck and Arnold 2003). Analysis of male secondary sex characteristics and recent behavioral observations for the *P. welleri* and *P. wehrlei* species groups ("intermediate group") indicate that these species have an intermediate mode of pheromone delivery. Although these species lack premaxillary teeth and have the round, posteriorly positioned mental glands characteristic of olfactory delivery (Highton 1962; Coss

1974), pheromone delivery by way of slapping has rarely been observed (Organ 1960; Arnold 1972; Picard 2005). Instead, pheromones are likely delivered to the female's nares during subtle behaviors, such as male head rubbing and head sliding (Arnold 1972; Picard 2005). Furthermore, males of the intermediate species group transfer pheromones early in the courtship sequence, in a temporal context similar to species using vaccination delivery (Picard 2005).

Analyses of PRF show that both positively selected and conserved variation in the signal is widespread, that there is overall heterogeneity in the evolution of PRF across the *Plethodon* phylogeny, and that underlying this heterogeneity are two well-defined transitions in evolutionary mode.

Materials and Methods

Collection of Animal Tissues

Mature males with enlarged mental glands were collected during the breeding season (table 1). Mental glands were obtained from four eastern *Plethodon* lineages: the *P. glutinosus*, *P. wehrlei*, *P. welleri*, and *P. cinereus* species groups (27 spp.; 34 populations). To determine if PRF is expressed in the epidermis in regions other than the mental gland, a single male/female breeding pair of *Plethodon shermani* was collected from Macon County, North Carolina (table 1) in August 2003. Skin samples were obtained from the chin of the female, as well as from the cloacal and dorsal regions of the tail base in both sexes. To establish the evolutionary origin of PRF, mental glands were taken from a western lineage of *Plethodon* (*Plethodon dumni*, *Plethodon stormi*, and *Plethodon vehiculum*) as well as from seven additional plethodontid species (*Aneides ferreus*, *Batrachoceps wrighti*, *Eurycea guttolineata*, *Eurycea bislineata*, *Desmognathus ocoee*, *Desmognathus monticola*, and *Desmognathus quadramaculatus*). The relationship of these taxa to eastern *Plethodon* is discussed by Mueller et al. (2004) and Chippindale et al. (2004). Tissue samples were also taken from two more distantly related salamanders that lack mental glands: *Rhyacotriton variegatus* (family Rhyacotritonidae) and *Taricha granulosa* (family Salamandridae). These tissues included epidermis from the chin of sexually active males and the cloacal glands, the latter of which are known to produce pheromones in other salamandrids (Kikuyama et al. 1997).

Isolation and Sequencing of PRF Alleles

Animals were anesthetized and then tissues for analysis were surgically removed and immediately frozen at -80°C . Total RNA was extracted from the tissues using Trizol reagent (Invitrogen [Carlsbad, Calif.] #15596-026) and resuspended in 30 μl RNase-free dH_2O . Single-stranded cDNA was synthesized from 1 μl total RNA using oligo-dT and the ImProm-II reverse transcriptase system (Promega [Madison, Wisc.] #A3800). Primers designed from the 3' and 5' untranslated regions (UTRs) were used to amplify a 757- to 805-bp region that included the PRF coding sequence (Watts et al. 2004). The following polymerase chain reaction (PCR) conditions were used for

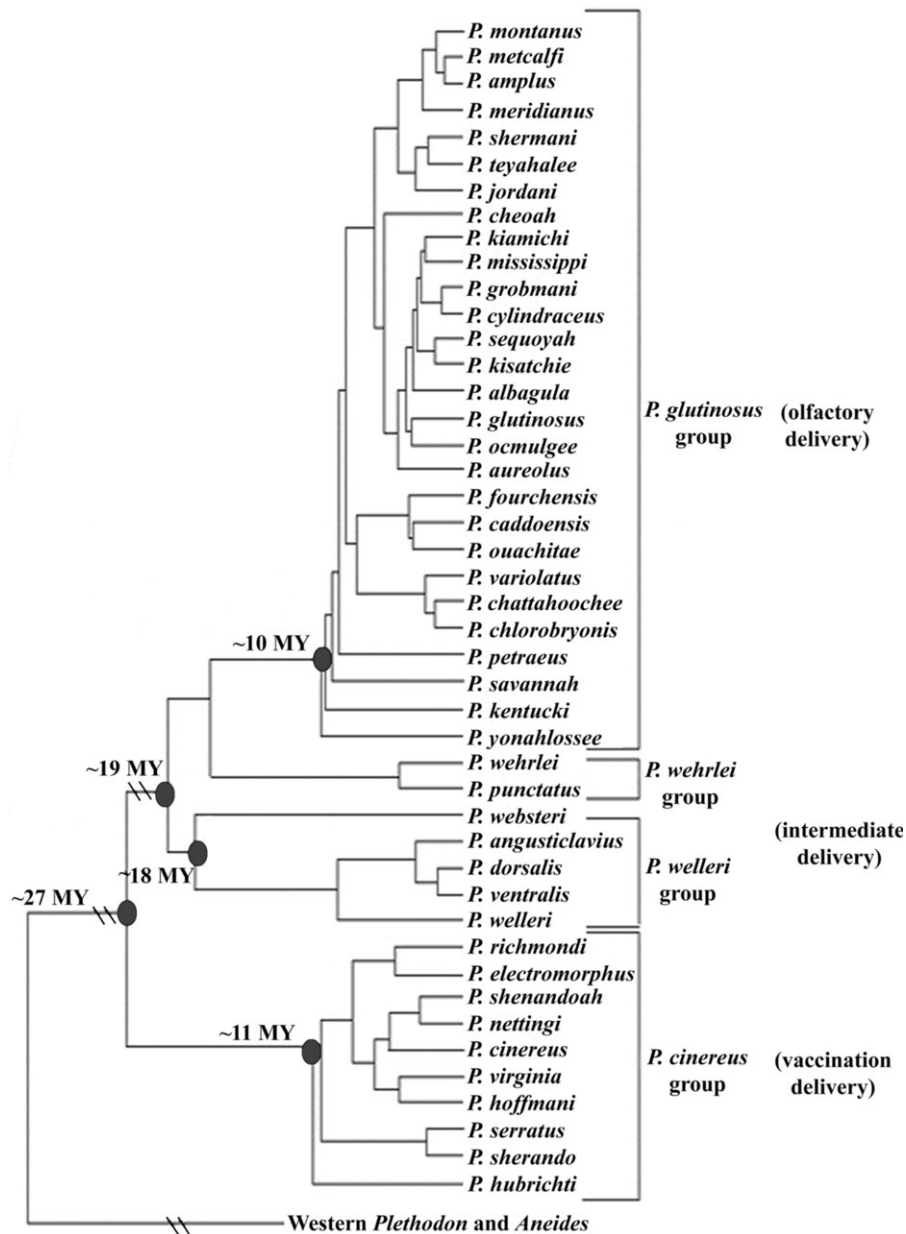


FIG. 1.—Phylogenetic relationships of the four eastern *Plethodon* species groups based on divergence in allozymes (adapted from UPGMA trees of Larson and Highton 1978; Highton 1989; Highton and Peabody 2000; unpublished data). Estimated times of divergence based on albumin immunology (Maxson, Highton, and Wake 1979; Maxson, Highton, and Ondrula 1984; Hass, Highton, and Maxson 1992; review by Larson, Weisrock, and Kozak 2003) (\ = branch is not to scale).

amplification: 95°C for 3 min, then 40 cycles of 95°C for 1 min, 53°C for 45 s, and 72°C for 1.5 min, and a final extension time of 10 min at 72°C. To avoid random PCR amplification error, a proofreading DNA polymerase (*PfuUltra* High-Fidelity, Stratagene [La Jolla, Calif.] #600382) was used in all PCR reactions. PCR products were visualized and excised from a 1.5% agarose gel, purified (QIAquick gel extraction kit; Qiagen [Valencia, Calif.] #28706), and cloned using the Zero Blunt Topo PCR cloning kit (Invitrogen #K2800-20). Ten PRF clones per individual were purified (QIAprep spin miniprep kit, Qiagen #27106) and sequenced in both the forward and reverse

directions using universal T3 and T7 primers. PRF sequences obtained by Watts et al. (2004) also were incorporated into our analyses (GenBank accession numbers AY499347–AY499404).

Construction of Mental Gland cDNA Libraries and Expressed Sequence Tag Analysis

A mental gland cDNA library was constructed for each of the following three species: *P. shermani* WA ($N = 10$ glands pooled; Macon County, North Carolina table 1); *D. ocoee* ($N = 20$ glands pooled; Clay County, North Carolina, 35°02'20"N, 083°33'08"W); and *P. dumni* (single

Table 1
***Plethodon* Species from Which PRF Sequences Were Obtained and Description of Allelic Diversity Found**

<i>Plethodon</i> Species	Collection Site					PRF Alleles			
	N	County	State	Latitude (N)	Longitude (W)	Clones Sequenced	Unique Sequences	Unique Translations	% Sequence Dissimilarity
Olfactory delivery									
<i>P. amplus</i>	4	Buncombe	NC	35°27'40"	082°22'09"	39	9	9	0.42
<i>P. aureolus</i>	2	Monroe	TN	35°27'29"	084°01'24"	10	5	3	0.56
<i>P. chatahoochee</i>	6	Towns	GA	34°52'28"	083°48'41"	16	8	8	0.71
<i>P. cheoah</i>	4	Graham	NC	35°21'30"	083°43'04"	39	13	13	1.51
<i>P. cylindraceus</i>	1	Johnson	TN	36°23'58"	081°57'55"	10	5	4	0.41
<i>P. fourchensis</i>	1	Scott	AR	34°39'20"	093°57'00"	4	2	2	0.16
<i>P. grobmani</i>	1	Toombs	GA	32°20'24"	082°32'12"	10	2	2	0.31
<i>P. jordani</i>	5	Sevier	TN	35°36'34"	083°26'50"	44	3	2	0.21
<i>P. kentuckyi</i>	1	Wise	VA	36°53'25"	082°37'35"	7	3	3	2.47
<i>P. metcalfi</i> HBS	5	Macon	NC	35°01'50"	083°08'49"	46	4	3	0.26
<i>P. metcalfi</i> CW	6	Macon	NC	35°19'40"	083°20'10"	35	17	15	1.24
<i>P. meridianus</i>	5	Burke	NC	35°36'05"	081°37'43"	43	3	3	0.21
<i>P. mississippi</i>	1	Scott	MS	32°24'37"	089°29'02"	7	5	3	0.50
<i>P. montanus</i>	4	Madison	NC	35°50'24"	082°57'11"	39	17	15	1.55
<i>P. ouachitae</i>	1	Le Flore	OK	34°40'59"	094°22'16"	6	2	2	0.31
<i>P. ouachitae</i>	1	Le Flore	OK	34°43'10"	094°32'33"	9	3	3	0.31
<i>P. savannah</i>	1	Richmond	GA	33°19'48"	082°03'49"	8	5	3	0.88
<i>P. shermani</i> WA	4	Macon	NC	35°10'48"	083°33'38"	37	16	13	2.04
<i>P. shermani</i> DG	4	Clay	NC	35°02'26"	083°33'10"	39	16	16	0.77
<i>P. sher.Xp.teyah</i> ^a	4	Macon	NC	35°02'20"	083°27'33"	34	16	14	1.40
<i>P. teyahalee</i> MP	4	Madison	NC	35°50'24"	082°57'11"	31	4	2	0.36
<i>P. teyahalee</i> SG	3	Graham	NC	35°21'30"	083°43'04"	23	6	4	0.52
<i>P. yonahlossee</i>	2	McDowell	NC	35°42'58"	082°19'56"	19	3	3	0.31
Intermediate delivery									
<i>P. angusticlavius</i>	1	Madison	AR	36°14'25"	093°40'27"	10	2	2	0.16
<i>P. websteri</i>	1	Jefferson	AL	33°43'32"	086°49'20"	9	2	2	0.80
<i>P. wehrlei</i>	2	Pocahontas	WV	38°27'10"	080°00'12"	11	3	3	0.21
<i>P. wehrlei</i>	1	Floyd	VA	36°47'22"	080°27'34"	10	2	2	0.16
<i>P. welleri</i>	1	Unicoi	NC/TN	36°06'36"	082°21'40"	5	3	3	0.53
<i>P. ventralis</i>	1	Jefferson	AL	33°43'32"	086°49'20"	5	1	1	n/a
Vaccination delivery									
<i>P. cinereus</i>	5	Giles	VA	37°22'02"	080°31'34"	27	14	13	9.85
<i>P. hoffmani</i>	1	Pocahontas	WV	38°13'43"	079°47'58"	8	3	3	12.79
<i>P. richmondi</i>	3	Wise	VA	36°53'00"	082°37'58"	16	13	11	10.13
<i>P. serratus</i>	1	Henry	GA	33°29'59"	084°10'58"	9	4	4	12.32
<i>P. serratus</i>	1	Stoddard	MO	36°57'02"	090°09'31"	10	3	2	0.52
Total	88					675	217	191	8.61

NOTE.—Pheromone delivery mode, collection locality, and number of individual males (*N*) are also depicted. NC, North Carolina; TN, Tennessee; GA, Georgia; AR, Arkansas; VA, Virginia; MS, Mississippi; OK, Oklahoma; AL, Alabama; WV, West Virginia; and MO, Missouri.

^a Hybrids between *P. shermani* and *P. teyahalee* (Highton and Peabody 2000).

mental gland; Lane County, Oregon, 43°49'51"N, 123°45'40"W). Gland removal and extraction of total RNA from mental gland tissue were performed as above. The *P. shermani* library was constructed in Lambda-ZAP vector (uni-zap XR; Stratagene) with the ZAP Express XR Library Construction Kit (Stratagene #200451). mRNA starting material was prepared using the Stratagene mRNA isolation kit (#200347) with the optional enrichment protocol. The *D. ocoee* and *P. dunnii* mental gland cDNA libraries were prepared using the SMART cDNA library construction kit (BD Biosciences Clontech [Mountain View, Calif.] #K1053-1). Inserts were generated from 50 ng total RNA by the long-distance PCR protocol. Small amplification products were removed by gel purification followed by CHROMA SPIN-400 column size fractionation. The inserts were ligated into the plasmid vector and primary transformants were generated as per the supplied protocol. The primary libraries were plated, and primary clones (*P. shermani* = 300;

D. ocoee = 450; and *P. dunnii* = 200) were cored, excised, and sequenced from the 5' end by automated dye-terminator sequencing using standard procedures.

Phylogenetic Analysis

Nucleotide sequences were translated into amino acid sequences using the program MegAlign (DNASTar version 5.0). Amino acid sequences were aligned using ClustalX (version 1.83; Thompson, Higgins, and Gibson 1994), and minor refinements were made by eye. Nucleotide sequences were then aligned accordingly. Data sets were built from unique DNA haplotypes at the following levels: (1) for all species having olfactory delivery gene "A" (*N* = 167 sequences), (2) for species having vaccination delivery gene "A" (*N* = 23 sequences), (3) for species having vaccination delivery gene "B" (*N* = 13 sequences), (4) for species with vaccination delivery genes A and B combined (*N* = 36 sequences), (5) for the *P. welleri* and *P. wehrlei*

species groups (intermediate group gene “A”; $N = 13$ sequences), and (6) for 27 species of eastern *Plethodon* ($N = 217$ sequences).

Maximum likelihood trees were reconstructed using Bayesian inference (MrBayes version 3.0b4; Huelsenbeck and Ronquist 2001), with a random start tree and without the use of a molecular clock. The general time reversible model was used, with rates specified as gamma distributed across sites. Four Monte Carlo Markov chains were run simultaneously for 1,000,000 generations with one heated chain at the default setting of 0.2. Sample trees were acquired every 100 generations and the first 2,000 trees were discarded as “burn-in” (trees generated before likelihood values reached stationary). The remaining 8,000 trees were used to construct a 50% consensus tree on PAUP* version 4.0b10 (Swofford 2003).

Estimating Gene Divergence and Evolutionary Change

Sequence dissimilarity was measured as the number of unique nucleotide changes per nucleotide site for a pair of sequences following a correction for multiple hits (Tamura-Nei method on MEGA version 2.1; Kumar et al. 2001). The hypothesis of equal evolutionary rates across lineages was tested by estimating the average number of synonymous and nonsynonymous substitutions per site using the method of Nei and Gojobori (1986) with the Jukes-Cantor correction for multiple hits (MEGA version 2.1; Kumar et al. 2001). In these tests, the rate of evolution is estimated as the average number of changes per nonsynonymous or per synonymous site that has accumulated in pairs of homologous sequences since their divergence. Standard errors were determined using 500 bootstrap replicates. Divergence times (absolute rates) for eastern *Plethodon* and three of the four species groups (*P. glutinosus*, *P. welleri*, and *P. cinereus*) were based on time intervals estimated from albumin (microcomplement fixation) studies (Highton and Larson 1979; Maxson, Highton, and Wake 1979; Maxson, Highton, and Ondrula 1984; Hass, Highton, and Maxson 1992; Larson, Weisrock, and Kozak 2003). The time of the eastern *Plethodon* radiation (~27 MYA; Maxson, Highton, and Wake 1979; Larson, Weisrock, and Kozak 2003) used to estimate d_S and d_N in our analyses may be an overestimation. A recent analysis implementing a penalized likelihood approach contends that eastern *Plethodon* radiated as little as 9–17 MYA (Chippindale et al. 2004). Despite this, we use the divergence times estimated from albumin data because the study of Chippindale et al. (2004) does not provide ages for the major species groups within eastern *Plethodon*. We acknowledge, however, that the d_S and d_N that we calculate may actually be 1.5–3 times greater than the rates reported here. This correction should apply across all taxonomic groups and therefore should not affect our overall conclusions concerning rate comparison across lineages.

Using the gene tree generated by MrBayes and the PRF protein sequence data as input in MacClade (version 4.0; W. P. Maddison and D. R. Maddison 2000), we reconstructed the pattern of character (amino acid) change within each clade and calculated the number of parallel and convergent evolutionary events across gene lineages.

Physiochemical changes at amino acid sites were scored according to Grantham’s (1974) index, which considers composition of the side chain, polarity, and molecular volume. Amino acid replacements were deemed “conservative” if the score was less than 100 and “radical” otherwise.

Tests for Selection

Tests of adaptive molecular evolution were performed using phylogeny-based maximum likelihood models of codon evolution implemented by the program CODEML (PAML version 3.12; Yang 2002). The nucleotide alignment and maximum likelihood tree for each of the data sets were used as inputs. The PAML models allow the nonsynonymous/synonymous substitution rate ratio (d_N/d_S) to vary among amino acid sites or among lineages in a phylogeny. A d_N/d_S ratio of 1 ($\omega = 1$) indicates neutrality, whereas $\omega < 1$ indicates purifying selection and $\omega > 1$ indicates positive selection. Five site-specific models were implemented: M0 (the “one-ratio” model with a single ω averaged among sites), M1 (“neutral” model which assumes two site classes, $\omega_0 = 0$ and $\omega_1 = 1$), M3 (“discrete” model that has three class sites, ω_0 , ω_1 , and ω_2 , all estimated from the data), M7 (“beta” model which assumes a beta distribution with a continuous distribution of ω values limited to the interval 0–1), and M8 (“beta plus omega” model which adds an extra site class to M7 with a free ω value estimated from the data). In addition, three lineage-specific models were applied to a reduced data set ($N = 57$ sequences) to determine if selective pressures varied among branches in the tree. The one-ratio model assumes one site class for all lineages, the “free-ratio” model estimates ω from the data for each lineage, and the “three-ratio” model estimates a separate ω for branches leading to the groups distinguished by their mode of pheromone delivery (vaccination, “intermediate,” and olfactory). The model that provided the best fit to the data was determined by comparing the likelihood ratio test statistic to a chi-square distribution with two degrees of freedom (Yang 1998). The empirical Bayes approach was used to determine the posterior probability that a particular codon site was positively selected (Nielsen and Yang 1998; Yang et al. 2000).

Results

PRF Expression Is Restricted to the Mental Glands of Eastern *Plethodon*

To determine if PRF was expressed in the mental gland, we used reverse transcriptase–polymerase chain reaction to survey multiple species and genera of North American salamanders. While PRF was amplified from the mental gland of every eastern *Plethodon* species surveyed, it was neither found in the nonmental gland epidermis of *P. shermani* of either sex nor was it detected in the glands or skin of any species that we sampled outside of the eastern *Plethodon* clade (*A. ferreus*, *B. wrighti*, *D. ocoee*, *D. monticola*, *D. quadramaculatus*, *E. guttolineata*, *E. bislineata*, *P. dunni*, *P. stormi*, *P. vehiculum*, *R. variegatus*, and *T. granulosa*). Because this survey used a PCR approach, it is possible that PRF is present in the mental glands of these species but is sufficiently divergent that primers designed against eastern *Plethodon* failed to anneal

and amplify the DNA. To examine this possibility, we performed random sequencing of mental gland cDNA libraries from *P. shermani* (300 clones), *P. dunni* (~200 clones), and *D. ocoee* (~450 clones). Approximately 20% of clones from *P. shermani*, but none from the other two species, encoded for PRF. It appears that PRF is either not expressed or is expressed at very low levels in mental glands and submandibular regions of salamanders other than eastern *Plethodon* and so is probably not a major courtship pheromone component in those species.

PRF Diversity Among All Eastern *Plethodon*

PRF was expressed in the mental glands of each of the 27 eastern *Plethodon* species that were surveyed. The 34 sampled populations yielded 191 unique haplotypes. Average PRF sequence dissimilarity across all eastern *Plethodon* species was 8.61%, with variable levels of intraspecific diversity (table 1). Our phylogenetic analysis revealed two highly divergent PRF genes in all members of the *P. cinereus* species group (vaccination delivery; fig. 2), which presumably resulted from an ancient gene-duplication event (Watts et al. 2004). The exception was a single *Plethodon serratus* individual from Missouri, which had only one form of the gene (PRF gene A; table 1). In accordance with the previous results (Watts et al. 2004), sequence comparisons across delivery modes (vaccination to olfactory) indicate that intermediate group and “olfactory” PRF sequence types are more similar to vaccination gene A than to vaccination gene B (fig. 2 and table 2). Species within the intermediate group have a form of the PRF gene that is nearly equally divergent from olfactory-type and vaccination gene A-type PRF genes (fig. 2). PRF variation at the amino acid level is high, ranging from 25.6% to 58% sequence dissimilarity across eastern *Plethodon* species. Only 61 amino acid residues are conserved across all delivery modes, representing 29.1% of the molecule (fig. 3). Although the coding region of the PRF gene is variable, the 5' and 3' UTRs are strongly conserved across species and across delivery modes. For example, a 60-bp region flanking the primer on the 5' end shares 90%–100% sequence identity within lineages and 86%–92% identity across lineages. A 56-bp region of the 3' UTR occurs as one of two distinct sequence types, irrespective of species, and these two sequences share only 25% identity. Within each flanking sequence type, however, there is a minimum of 80% sequence identity across lineages and up to 100% identity within lineages.

PRF Diversity Varies Across Lineages

Variation Within the P. glutinosus Group

The PRF gene in species with olfactory delivery consisted of 648 bp (216 amino acids) and had 542 monomorphic and 106 polymorphic sites. Sampling from 23 populations (18 species) yielded a total of 167 distinct PRF sequences. Interspecific sequence dissimilarity varied from 0.40% to 4.9%, with an average level of divergence of 2.24%. Average intraspecific sequence dissimilarity ranged from 0.16% to 2.47% (table 1). Some individuals possessed up to seven variant alleles, indicating that at least four PRF genes are expressed in the mental gland of these species.

An array of diverse PRF sequences obtained from the *P. shermani* cDNA library confirms that variation in this pheromone gene is not a PCR artifact. Each population had a unique complement of sequences, but 12 sequences were conserved (100% identity) across populations and species, reducing the total number of unique haplotypes to 148. These conserved sequences were restricted to members of the *Plethodon jordani* and *P. glutinosus* species complexes (Highton 1989; Highton and Peabody 2000). PRF sequence conservation across these species suggests that many haplotypes originated before the divergence of these two species complexes and so have been maintained for approximately 8 Myr (Highton and Peabody 2000).

The gene tree (Supplementary Fig. 1 in Supplementary Material online) for 167 olfactory-type PRF sequences revealed groups of gene clusters with short branches, low branch support, and numerous polytomies. The topology of the gene tree indicates at least six possible gene-duplication events for the olfactory-type PRF gene. Amino acid residue changes were detected at 62 of the 216 shared sites. While most of these changes were conservative, 17 radical substitutions occurred which may have important functional consequences (olfactory PRF residues –20, 10, 15, 64, 72, 86, 95, 107, 109, 126, 139, 158, 170, 179, and 182; fig. 3). A total of 60% of these radical substitutions occur at sites that are of known importance for receptor binding in closely related IL-6-type cytokines (cf. fig. 4 in Watts et al. 2004). Olfactory-type PRF amino acid sequences have a 6-aa deletion at the N-terminus and a 5- to 8-aa deletion at the C-terminus that are absent in both of the vaccination gene types (fig. 3). Average sequence dissimilarity for species with olfactory delivery compared to vaccination gene A and gene B is 17.3% and 24.5%, respectively (table 2).

Variation Within the P. cinereus Group

The PRF gene in species with vaccination delivery consisted of 681–690 bp (227–230 aa), with 526 invariant and 155 polymorphic sites. Thirty-seven unique sequences were obtained from the four species sampled. A single individual within this group had up to eight unique sequences, indicating that at least four PRF genes are expressed in the mental gland. Only one sequence was conserved across species (100% identity in *P. cinereus* and *Plethodon richmondi* gene B), yielding a total of 36 unique haplotypes. Average intraspecific sequence dissimilarity ranged from 0.52% to 12.8% (table 1), whereas average interspecific sequence dissimilarity was 9.7%. Of 229 shared residues, there were 88 sites across the protein that differed in amino acid composition and 18 of these substitutions were radical changes (vaccination PRF sites –15, –16, 5, 16, 23, 49, 59, 60, 64, 74, 112, 127, 150, 155, 158, 185, 201, 202; fig. 3). Approximately 67% of these radical substitutions occur at, or very near, sites with known receptor-binding function in related IL-6-type cytokines (cf. fig. 4 in Watts et al. 2004).

Twenty-three of the 36 unique vaccination-type PRF haplotypes were of the “gene A” type, whereas the remaining 13 sequences were “gene B.” Sequence dissimilarity in gene A was only 1.3% compared to 2.7% in gene B. The amino acid sequences of gene A versus gene B were variable at 60 sites, and seven of these sites had radical changes.

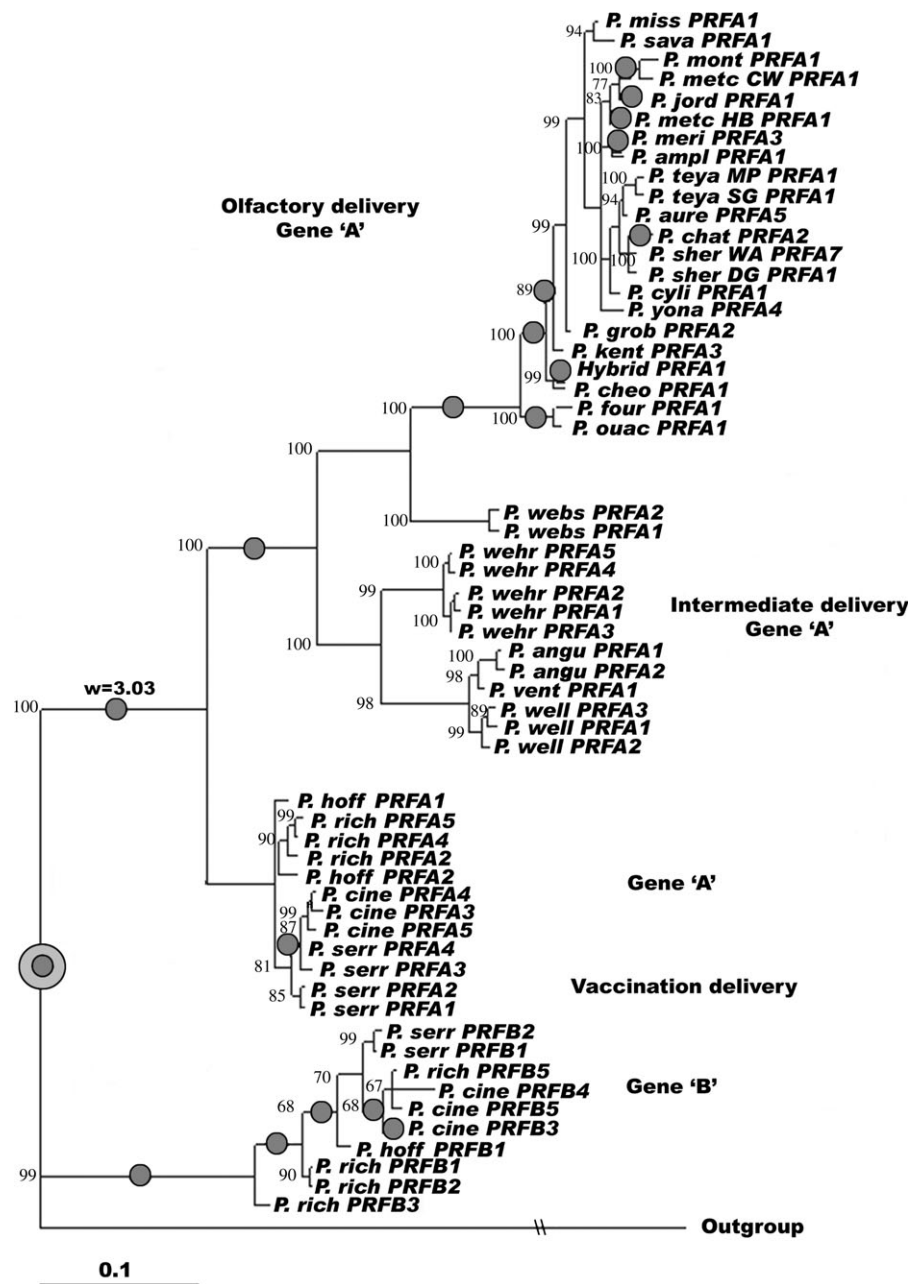


FIG. 2.—Maximum likelihood tree of a subset of PRF sequences from eastern *Plethodon* (27 spp.). Bayesian posterior probabilities are labeled to the left of branches having over 60% support. The distance bar represents 0.1 nucleotide substitutions per site. The gene phylogeny was rooted with a mouse cardiostrophin-2 sequence (cytokine; NM_198858). The large circle indicates an ancient gene-duplication event and the small circles mark branches predicted to have experienced positive selection.

Within gene types, gene A had 26 sites that varied in amino acid composition (five radical changes), whereas gene B had 39 variable sites (eight radical changes). The 12 amino acids prior to the stop codon (C-terminus) were relatively well conserved within gene types but differed in residue composition and length between the two gene types (fig. 3). Exceptions included two alleles of gene B (*P. cinereus* 1D and *P. richmondi* 2C; fig. 2), which had a C-terminal sequence (~20 residues) similar to gene A. A recombinant event, such as gene conversion, would explain the resemblance to gene A sequences at the C-terminus in these two sequences.

Variation Within the *P. welleri* and *P. wehrlei* Species Groups

Eight unique haplotypes (537 monomorphic and 96 polymorphic sites) were obtained from the four species sampled from the *P. welleri* group. A single *P. welleri* individual had up to three divergent PRF sequences, indicating that at least two PRF genes are expressed in the mental gland of this species. Two separate populations of *P. wehrlei* yielded five unique haplotypes with 676 monomorphic and 5 polymorphic sites. Overall, average intraspecific

Table 2
PRF Sequence Dissimilarity (Above the Main Diagonal) and Number of Nucleotide Differences Out of 630 bp (Below Main Diagonal) Between Eastern *Plethodon* Lineages

	Vaccination			Intermediate				
	Olfactory	Gene A	Gene B	<i>P. wehrlei</i>	<i>P. websteri</i>	<i>P. welleri</i>	<i>P. angust.</i>	<i>P. ventralis</i>
Olfactory		17.3	24.5	14.2	12.8	14.6	14.8	14.3
Gene A	96.8		18.1	15.5	13.7	16.9	17.8	16.7
Gene B	130.5	100		23.6	23.2	23.3	24.2	23
<i>P. wehrlei</i>	81.2	87.3	126.2		12.3	8.7	9.4	8.6
<i>P. websteri</i>	73.8	78.5	124.6	70.7		14.1	14.8	14
<i>P. welleri</i>	83.5	94.7	125.4	51.8	81		2	1.3
<i>P. angusticlavius</i>	84.6	99.2	129.4	55.5	84.5	12.5		1
<i>P. ventralis</i>	82	93.7	123.9	51	80	8	6.5	

NOTE.—*P. angust.* = *P. angusticlavius*.

sequence dissimilarity was rather low for this “intermediate” group, varying from 0.16% to 0.80% (table 1), whereas average across species/population sequence dissimilarity was relatively high in the *P. welleri* complex (~6.9%) and low between *P. wehrlei* populations (~0.38%). PRF amino acid sequence comparisons across the *P. welleri* species group revealed 56 amino acid substitutions (out of 222 residues), 6 of which were radical changes (*P. welleri* PRF sites 31, 67, 78, 108, 139, and 175; fig. 3). Four of these radical changes were unique only to *Plethodon websteri* PRF sequences. Amino acid sequence comparisons across unique *P. wehrlei* haplotypes revealed five substitutions (out of 228 residues), none of which were radical changes.

Unlike the olfactory- and vaccination-type PRF genes, the gene tree for species within the intermediate group showed no evidence of gene duplication. The PRF gene of *P. websteri* was the outlier of this group (fig. 2).

P. websteri is a member of the *P. welleri* species group (fig. 1), yet average PRF sequence dissimilarity was 14.3% when compared to PRF sequences from other members of this group (i.e., *Plethodon angusticlavius*, *Plethodon ventralis*, and *P. welleri*) and only 12.8% compared with olfactory-type PRF (table 2). In contrast, *P. wehrlei* (a member of the *P. wehrlei* species group) PRF sequence dissimilarity was only 8.6%–9.4% when compared to sequences from *P. welleri*, *P. angusticlavius*, and *P. ventralis* (table 2). PRF genes from *P. welleri*, *P. angusticlavius*, and *P. ventralis* formed a tight cluster with only 1%–2% sequence dissimilarity among them (table 2 and fig. 2). These three species shared a unique, 2-aa deletion in the signal sequence. In addition, PRF sequences for these three species had a 6-aa deletion near the N-terminus and an 8-aa deletion at the C-terminus of the protein. This deletion pattern at both ends of the molecule was also present

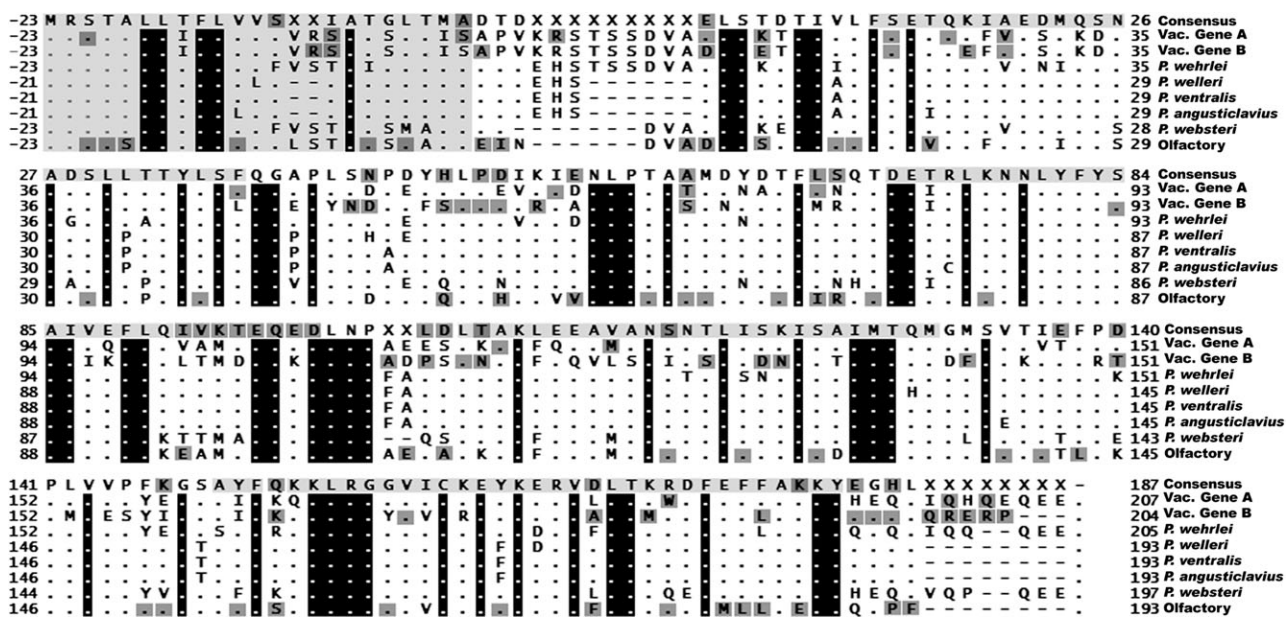


FIG. 3.—Alignment of PRF amino acid sequences from eastern *Plethodon* across all delivery modes. Representative vaccination (Vac.) gene A and gene B sequences are from *Plethodon cinereus*, and the olfactory sequence is a *Plethodon shermani* isoform. The dots represent amino acid residues that are identical to the consensus sequence. Unique deletions are represented by dashes. The protein signal sequence and putative α -helices are highlighted in light gray whereas dark gray boxes highlight positively selected sites with posterior probabilities $\geq 60\%$. Black vertical bars indicate residues that are conserved across all delivery modes.

in olfactory-type PRF sequences (fig. 3). *Plethodon wehrlei* PRF sequences, in contrast, were more similar to vaccination-type PRF in that they lacked these characteristic deletions (fig. 3). Average PRF sequence dissimilarity for all species in the intermediate group versus species with vaccination gene A, vaccination gene B, and olfactory delivery was 16%, 23.5%, and 14.2%, respectively.

Evolutionary Rates Vary Among PRF Sequence Types

An estimate of the rate of evolution, approximated as the number of substitutions per site per year, suggests that the rates of both nonsynonymous and synonymous substitutions in the vaccination type A gene have occurred at a faster rate than in any other forms of the gene (table 3). Similarly, the synonymous substitution rate was higher in the intermediate species group than it was in vaccination gene B or in species with olfactory delivery (table 3). Overall, our rate comparisons indicate that the vaccination PRF gene A evolved approximately 2.5 times faster than vaccination PRF gene B, three times faster than olfactory PRF, and 1.5 times faster than PRF of intermediate species.

Overall ω values for PRF across the entire *Plethodon* phylogeny and for species with olfactory delivery indicate nonneutral evolution driven by positive selection ($\omega = 1.16$ and 1.30, respectively; table 3), even though positive selection is usually difficult to detect when substitution rates are averaged across all sites in a protein (Yang 1998). In contrast, overall ω values for PRF genes for species in the intermediate group ($\omega = 0.78$) and for species with vaccination gene A ($\omega = 0.78$) and B ($\omega = 0.89$) are consistent with neutrality/purifying selection (table 3). At this level of analysis, however, we cannot reject the hypothesis that positive selection operated on specific residues in all of the PRF gene types but was masked by averaging.

Olfactory and Vaccination PRF Genes Show Different Patterns of Positive Selection

To determine whether the pattern of selection is similar for all gene types, codon-based models of molecular evolution (Nielsen and Yang 1998; Yang et al. 2000) were used to investigate the pattern of selection at each amino acid residue across the PRF gene. Models M3 and M8, which allow for positive selection, provided the best fit(s) to five of the six data sets (table 4). For tests across all plethodontid lineages, 217 unique PRF sequences were analyzed. The results indicate that approximately 30.3% of the sites are under strong positive selection with $\omega = 1.59$ and $\omega = 7.12$ (95% probability; table 4). The selection analysis for species with olfactory delivery included 167 PRF alleles from 18 species and 23 populations. Model estimates suggest that the majority of sites are under purifying selection ($\omega = 0.07$), but approximately 16.3% of the sites have experienced strong diversifying selection ($\omega = 1.77$ and 9.56; 95% probability; table 4). The parameter estimates for analysis of the 36 vaccination-type PRF sequences (four spp.) indicate that 22.2% (95% probability) of the PRF molecule has experienced positive selection ($\omega = 2.74$ and $\omega = 24.84$), while the remainder of the protein has been maintained by purifying selection ($\omega = 0.09$; table 4). Only 23.4% of the positively selected residues occur at

the same sites in both the olfactory and vaccination gene types (fig. 3). This discordant pattern of selection across PRF gene types may reflect functional differences in the signal related to differences in pheromone delivery.

Two additional data sets for species with vaccination-type delivery were tested for adaptive evolution in vaccination-type PRF genes A and B separately. In both analyses, models which allowed for positive selection (M3, M8) provided the best fit to the data and detected 4.4% and 17.3% positively selected sites (95% probability) in gene A ($\omega = 6.3$) and gene B ($\omega = 3.8$ and $\omega = 37.8$; table 4), respectively. Only a small number of positively selected residues were located at equivalent codon sites between genes A and B (~16.3%; fig. 3). In addition, only 9.8% of the selected sites in gene A and 14% of the sites in gene B corresponded to positively selected residues in the olfactory-type PRF gene (fig. 3).

PRF Genes from Intermediate Species Have Experienced Purifying Neutral Selection

The PRF alleles for the intermediate group species (*P. welleri*, *P. angusticlavius*, *P. ventralis*, *P. websteri*, and *P. wehrlei*) were combined for tests of positive selection. Results from the discrete model (M3) indicate that 9.1% of the sites have slightly elevated ω values ($\omega = 1.17$; 95% probability), but this model did not provide a statistically better fit to the data than the one-ratio model ($P = 0.40$). The one-ratio model predicted that 29% of the intermediate-type PRF gene is under purifying selection and 71% of the molecule is free of selective pressures (table 4). In a second test for selection, the selection model (M8) did not provide better fit to the data set than the beta model (M7) ($P = 0.30$). These data suggest that the intermediate-type PRF gene has experienced a history of neutrality/purifying selection, in contrast to the purifying/positive selection histories of both vaccination- and olfactory-type PRFs.

Positive Selection on PRF Has Occurred at Many Branches Within Delivery Modes

In all lineage-specific tests, an estimate of ω for each branch in the PRF phylogeny rejected purifying selection within delivery modes ($P < 0.001$) in favor of models in which multiple lineages are under positive selection (fig. 2). Several branches for olfactory and vaccination gene B PRF types have experienced strong positive selection. In contrast, the ω values for branches leading to the intermediate and vaccination gene A PRF groups indicate a history of purifying selection (fig. 2). The highest ω value ($\omega = 3.03$) occurs at the branch separating the vaccination-type and intermediate/olfactory-type PRF (fig. 2). In the three-ratio models, however, there was no support for models in which selection at this branch is greater than for branches within delivery modes ($P = 0.40$).

Parallel and Convergent Evolution Occur Within Delivery Modes

Phylogeny-based codon substitution models detected several positively selected residues in both olfactory- and vaccination-type PRF genes but are not informative about

Table 3
Numbers of Synonymous Substitutions Per Synonymous Site (d_S) and Nonsynonymous Substitutions Per Nonsynonymous Site (d_N) for PRF Within Each Eastern *Plethodon* Lineage

PRF Gene	Age (Myr)	d_S	d_S Rate	d_N	d_N Rate	d_N/d_S (ω)
Vaccination species gene A	11	0.074 ± 0.011	3.36	0.066 ± 0.007	2.99	0.89
Vaccination species gene B	11	0.033 ± 0.010	1.51	0.026 ± 0.004	1.18	0.78
Olfactory species	10	0.018 ± 0.006	0.92	0.024 ± 0.005	1.20	1.30
Intermediate species	18	0.083 ± 0.017	2.30	0.065 ± 0.009	1.80	0.78
All eastern <i>Plethodon</i>	27	0.073 ± 0.010	1.35	0.085 ± 0.008	1.57	1.16

NOTE.—Rates are in units of substitutions per site per 10^9 years. $\omega < 1$ indicates purifying selection, $\omega = 1$ denotes neutrality, and $\omega > 1$ signifies positive selection.

the direction or magnitude of these changes. A closer examination of variation at positively selected sites suggests that these sites do not accumulate a random assortment of nucleotide changes. Instead, a limited number of substitutions occur at each site. Most positively selected sites show a fluctuating pattern of substitutions involving just two or three different amino acids. This continual cycling of amino acids leads to a tremendous number of unique alleles because sites appear to evolve independently of the others. Notable exceptions to this pattern of restricted substitutions are three sites in the olfactory type (residues 64, 109, and 184; fig. 3), which have up to five different amino acids. All of these sites have experienced radical amino acid changes. Structural comparisons to related proteins (see Watts et al. 2004) indicate that these highly variable sites are not associated with residues known to have important functional roles, thus it is difficult to determine the significance of polymorphisms at these positions. Overall, however, many of the fluctuating sites (59% for olfactory type and 55% for vaccination type) map onto or very near residues associated with receptor binding (using fig. 4 in Watts et al. 2004). The changes are either parallel (e.g., amino acid A changes to

amino acid B in both lineages) or convergent (e.g., amino acids A and C both change to amino acid B; Nei and Kumar 2000) substitutions. Our results indicate that 28 amino acid sites in olfactory-type PRF evolved in parallel, and two additional sites show evidence of convergent evolution (data not shown). Similarly, in the vaccination form of the gene, 30 amino acid residues evolved parallel changes across the two gene lineages. Both convergent and parallel evolution imply positive selection, thus this analysis lends additional support to the patterns of selection that were identified using codon substitution models. Nevertheless, an outstanding issue is to determine what factors in the molecular environment have driven adaptive changes in this pheromone protein.

Discussion

Variable Modes of Evolution of PRF Across *Plethodon*

A private sexual signal that is retained across closely related species with similar courtship and life histories may be expected to evolve uniformly across all taxa if broad-spectrum structural and functional constraints govern

Table 4
Nonsynonymous to Synonymous Substitution Rate Ratios (ω), Prior Probabilities (P [ω]), and Model Significance (χ^2 P value) for Selection Models Fitted to PRF Genes

Gene	Number of Sequences	Selection Model		χ^2	(+ Selection \geq 95% Probability)
		M1 (neutral)	M3 (discrete)		
Within delivery mode					
Intermediate delivery	13	$P(\omega = 0) = 0.29$	$P(\omega = 0.11) = 0.49$	=0.4	9.1
		$P(\omega = 1) = 0.71$	$P(\omega = 0.73) = 0.18$		
Olfactory delivery	167	$P(\omega = 0) = 0.68$	$P(\omega = 1.17) = 0.33$	<0.001	16.3
		$P(\omega = 1) = 0.32$	$P(\omega = 0.07) = 0.72$		
			$P(\omega = 1.77) = 0.20$		
Vaccination delivery	36	$P(\omega = 0) = 0.47$	$P(\omega = 9.56) = 0.08$	<0.001	22.2
		$P(\omega = 1) = 0.53$	$P(\omega = 0.09) = 0.72$		
			$P(\omega = 2.74) = 0.20$		
Vaccination gene A	22	$P(\omega = 0) = 0.66$	$P(\omega = 24.84) = 0.08$	<0.001	4.4
		$P(\omega = 1) = 0.34$	$P(\omega = 0.13) = 0.72$		
			$P(\omega = 0.13) = 0.20$		
Vaccination gene B	14	$P(\omega = 0) = 0.64$	$P(\omega = 6.34) = 0.08$	<0.001	17.3
		$P(\omega = 1) = 0.36$	$P(\omega = 0) = 0.72$		
			$P(\omega = 3.81) = 0.25$		
Across delivery modes					
All PRF	217	$P(\omega = 0) = 0.41$	$P(\omega = 37.76) = 0.03$	<0.001	30.3
		$P(\omega = 1) = 0.59$	$P(\omega = 0.21) = 0.54$		
			$P(\omega = 1.58) = 0.37$		
		$P(\omega = 7.12) = 0.09$			

NOTE.—M8 (not shown) estimates of positively selected sites are in very close agreement with the results of the discrete model.

evolutionary change. On the other hand, because a courtship signal that affects reproductive success is a candidate target for sexual selection (Arnold and Houck 1982), we might expect rapid evolution of the signal as well as heterogeneous evolutionary patterns across closely related taxa (Lande 1981; Mead and Arnold 2004). This second expectation is clearly supported in the case of PRF. Multiple PRF alleles and positive selection commonly occur, but there are lineage-specific differences in magnitude of selection, distributions of sites under positive selection, rate of evolution, and within-population allelic diversity. The evolutionary characteristics of this signal component vary from lineage to lineage.

Overall, however, two distinct evolutionary modes underlie this heterogeneity in evolutionary patterns. The first mode occurs among olfactory A and vaccination B PRF genes and is characterized by a complex tree topology (non-linear divergence), a slow synonymous substitution rate ($d_S \approx 0.9\text{--}1.5$), and a high proportion of positively selected sites across the protein (16%–17% of sites with $\omega > 1$). Only a small percentage of these positively selected sites map onto the same amino acid position in both gene types, suggesting that the underlying selective pressures differ for the two genes. The second evolutionary mode occurs among the intermediate type and vaccination type A PRF genes, which show clock-like regularity in sequence divergence such that the gene tree has similar topology as the allozyme tree, faster evolutionary rates ($d_S \approx 2.3\text{--}3.6$), and a low proportion of sites in the protein bearing the signature of positive selection (0%–4% of sites with $\omega > 1$). We found no evidence that PRF was expressed in epidermal sites other than the mental gland, suggesting that PRF is exclusively a sexual signal. As a private signal that is delivered to the female at close range it is unlikely that natural selection processes govern the evolution of the molecule. Instead, these two dominant modes of evolution likely result from different sexual selection regimes prevailing in different parts of the *Plethodon* phylogeny.

Perpetual Evolution of PRF

Among olfactory and vaccination type B genes, the characteristics of PRF evolution point to underlying processes in which positive selection as well as purifying and balancing selection play major roles. Heterogeneity across the PRF molecule in evolutionary rates and processes is another defining characteristic. Many PRF sites are conservative and bear the signature of purifying selection. In contrast, sites implicated in receptor binding are prone to rapid evolution driven by positive selection (Watts et al. 2004). Some of these receptor-associated sites show a fluctuating pattern of amino acid substitution, in which a small number of amino acids are prone to parallel and convergent substitutions. While many of these substitutions are conservative, some are radical and are likely to have had functional consequences (Hughes 1999). The process of positive selection acting on portions of the PRF protein did not produce steady divergence in PRF. Instead, some polymorphisms in PRF sequences are maintained within and among species for millions of years, a sign of balancing selection. Furthermore, on a timescale of 10 Myr or less,

distantly related as well as closely related species show many similarities in PRF, rather than clock-like regularity in sequence divergence. As a consequence, at this time-scale, the gene trees for olfactory and vaccination gene B PRF show a complex topology within delivery modes, rather than the simple topology of the allozyme tree. Pulling all these characteristics together in one picture, we see rapid, selection-driven evolution of receptor-associated sites that proceeds incessantly rather than episodically and results in cyclical rather than progressive change in the protein.

One candidate process that would account for many of these characteristics of PRF evolution is a molecular tango in which PRF coevolves with its receptors on a dance floor that corresponds to a confined molecular space. The tango is confined in the sense that only a few kinds of amino acid substitutions are apparently allowed at even the most rapidly evolving sites. Consequently, coevolution causes parallel and convergent substitutions. The resulting fluctuating pattern of substitutions means that the process is prone to reinvent the same amino acid sequences. The evidence for a dance partner is strong but indirect. In this context, our failure to detect PRF expression outside the mental gland is important because it restricts the field of possible candidates for a coevolving partner. In the first place, many of the positively selected PRF sites show good alignment with sites known to be involved in receptor binding in other cytokines (Watts et al. 2004). Secondly, we know that PRF, a male pheromone delivered to the female during sexual interactions, exerts its effects via the female's vomeronasal epithelium when delivered by the olfactory mode (Wirsig-Wiechmann et al. 2002). Thus, female receptors located in the vomeronasal epithelium of species with olfactory delivery are strongly implicated in PRF's mode of action. Our working hypothesis is that these uncharacterized, vomeronasal receptors are the dance partners in PRF's molecular tango. Only a small percentage of positively selected sites map onto the same amino acid position in both olfactory- and vaccination-delivered PRF. The location of PRF target receptors for species with vaccination delivery are currently unknown. It is possible that, although the evolutionary dynamics for olfactory gene A and vaccination gene B are similar, PRF interacts with different receptor populations when delivered by each of the two modes.

Although no formal models of molecular evolution have been devised for processes such as the molecular tango, models of phenotypic evolution have been constructed for sexual communication driven by sexual selection that have many of the same characteristics. In these models, a continuously distributed ornamental trait in males (e.g., tail size in peacocks) interacts with a continuously distributed behavioral trait in females (e.g., mating preference in peahens based on tail size) and produces rapid coevolution (Fisher 1958; Lande 1981). Such models can produce cyclical evolutionary outcomes in which the male ornament coevolves with female preference in a stable limit cycle under a variety of assumptions (Mead and Arnold 2004). Thus, phenotypic models of sexual coevolution can produce evolutionary dynamics with many of the characteristics of PRF evolution (e.g., incessant, perpetual evolution driven by positive selection arising from sexual interactions). By analogy, we might anticipate success in

producing these same characteristics in formal models of the molecular tango.

Neutrality/Purifying Evolution of PRF

In contrast to the molecular tango, the evolutionary mode of gene A sequences in intermediate and vaccinating species is dominated by random drift and purifying selection. The evolutionary mode for these sequences is the typical protein profile of neutral divergence superimposed on a process of purifying selection (Kimura 1983). Again, while there are no formal molecular sexual selection models that predict this pattern, phenotypic models with similar characteristics have been developed. In these models, the male trait and the female preference evolve to a stable point of equilibrium or a line of equilibria (Lande 1981; Mead and Arnold 2004). In the latter case, the male and female traits may drift along the line of equilibria. This shift from incessant evolution driven by positive selection to stability accompanied by drift does not signify a reduction in the importance of PRF as a courtship signal but rather implies a shift in the dynamics of sexual selection. Strong conservation of the signal sequence and other regions within the protein, as well as the retention of well-developed mental glands and pheromone delivery behaviors, suggest that PRF gene A in intermediate and vaccination species is actively expressed and conveyed to the female.

The ordinary evolution of gene A sequences in intermediate species occurs in a behavioral setting that has a mixture of the elements that characterize species with vaccinating and olfactory delivery. Most of the species within the intermediate group are morphologically more similar to species of the *P. cinereus* group (vaccination delivery), with the exception that they lack premaxillary teeth and have the round, posteriorly positioned mental glands characteristic of olfactory delivery (Highton 1962; Coss 1974). Species within this group are reluctant to mate in the laboratory, therefore only 19 courtship encounters have been observed over the last 25 years. Vaccination delivery behavior has never been witnessed in these encounters, and olfactory delivery has been reported in only 15% of the courtship trials (Organ 1960; Arnold 1972; Picard 2005). Unlike olfactory delivery, however, species in the intermediate group typically deliver pheromones early in the courtship sequence, in a temporal context similar to that of species with vaccination delivery (Picard 2005). Overall, courtship duration is relatively short for these species and pheromone delivery is likely accomplished using subtle behaviors such as head rubbing and head sliding (Arnold 1972; Picard 2005). These same subtle behaviors are present in both vaccinating and olfactory species and may represent the ancestral vehicle for effective delivery of type A pheromone.

Evolution of PRF in Relation to Other Reproductive Proteins

Recent studies indicate that the abalone sperm lysin gene (used for gamete recognition) and the sea urchin bindin gene (mediates sperm-egg attachment and fusion) also exhibit heterogeneous patterns of selection across lineages (Metz and Palumbi 1996; Yang, Swanson, and Vacquier 2000). Variation in these reproductive genes

differ from PRF in that genes of closely related, sympatric species bear the signature of strong diversifying selection, while purifying selection is the dominant mode of selection for genes of distantly related allopatric species (Metz and Palumbi 1996; Yang, Swanson, and Vacquier 2000; Zigler and Lessios 2003). In contrast, positive selection on PRF is detected both at the tips and deep within the *Plethodon* gene tree but is patchily distributed. The apparent correlation between mode of selection and geographical distribution in the reproductive proteins of broadcast spawners may indicate reinforcement to avoid hybridization in regions of sympatry (Yang, Swanson, and Vacquier 2000). More likely, however, this prezygotic isolating mechanism developed following divergence via sexual selection in allopatry (McCartney and Lessios 2004). The heterogeneous patterns of selection detected in most reproductive proteins as well as in PRF likely reflect a response to lineage-specific selective pressures governed by coevolutionary dynamics between the male and female (Yang, Swanson, and Vacquier 2000; Zigler and Lessios 2003; McCartney and Lessios 2004). To fully understand the origin of heterogeneous, lineage-specific selection regimes, we need to do more than simply reject null models of neutrality and purifying selection. Additionally, we need to test detailed models of molecular processes such as the molecular tango.

Supplementary Material

Supplementary Figure 1 is available at *Molecular Biology and Evolution* online (<http://www.mbe.oxfordjournals.org/>).

SUPPLEMENTARY FIG. 1.—Unrooted maximum likelihood tree of plethodontid receptivity factor (PRF) sequences from 23 populations of species having olfactory pheromone delivery. Bootstrap support of $\geq 60\%$ are labeled at nodes. Species represented by more than one population have locality designators (DG, HBS, MP, WA, and SG) following their names. Hybrid = *P. shermani* \times *P. teyahalee*. The distance bar represents 0.01 nucleotide substitutions per site. The sequences reported in the manuscript have been deposited in GenBank under the accession numbers AY926884–AY927045 inclusive.

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