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Rapid Evolution of Plethodontid Modulating Factor, a Hypervariable Salamander Courtship Pheromone, is Driven by Positive Selection

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Abstract Sexual communication in plethodontid salamanders is mediated by a proteinaceous pheromone that a male delivers to a female during courtship, boosting her receptivity. The pheromone consists of three proteins from three unrelated protein families. These proteins are among a small group of pheromones known to affect female receptivity in vertebrates. Previously, we showed that the genes of two of these proteins (PRF and SPF) are prone to incessant evolution driven by positive selection, presumably as a consequence of coevolution with female receptors. In this report, we focus on the evolution of the third pheromone protein gene family, plethodontid modulating factor (PMF), to determine whether it shows the same pattern of diversification. We used RT-PCR in mental gland cDNA to survey PMF sequences from three genera of plethodontid salamanders (27 spp.) to measure rates of evolution, level of gene diversification, modes of selection, and types of amino acid substitution. Like PRF and SPF, PMF is produced by a multigene family characterized by gene duplication and high levels of polymorphism. PMF evolution is rapid, incessant, and driven by positive selection. PMF is more extreme in these dimensions than both PRF and SPF. Nestled within this extraordinary variation, however, is a signature of purifying selection, acting to preserve important structural and biochemical features of the PMF protein (i.e., secretion signal, cysteine residues, and p*I*). Although a pattern of persistent diversification exists at the molecular level, the morphological and behavioral aspects of the pheromone delivery system show evolutionary stasis over millions of years.

Keywords Pheromone gene · Rapid evolution · Positive selection · Reproductive protein · Courtship signal · Sexual communication

Introduction

Pheromones were first described by Karlson and Luscher (1959) as chemical signals released by an individual that elicit a specific behavioral or physiological response in a conspecific. Since then, hundreds of different pheromones have been identified in insects, and several dozen have been characterized in vertebrates. Behavioral effects of pheromones include: mate attraction (Kikuyama et al. 1999; Wabnitz et al. 1999; Maxwell et al. 2010), repulsion of rival males (Christensen et al. 1991; Park and Propper 2001), alarm signaling and predator avoidance (Smith 1992; Chivers et al. 1997), kin recognition (Brown and Brown 1992), mate assessment (review by Johansson and Jones 2007), territorial defense (review by Mathis et al. 1995), and synchronization of reproductive physiologies

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and behavioral patterns (Dulka et al. 1987; Mason et al. 1989; Sorensen et al. 1989; Houck and Reagan 1990; Rollmann et al. 1999; Vitazka et al. 2009).

While it is well established that chemical signals are used to mediate mating between conspecifics in many species, less clear is the fashion by which these signals evolve. Traditional views propose that strong stabilizing selection on mating signals (and receivers) is necessary to prevent mating among heterospecifics (Dobzhansky 1937) or to promote mating between conspecifics (Paterson 1985), and rapid changes in pheromone composition appear to have a role in reproductive isolation and speciation (Löfstedt et al. 1991; Roelofs and Rooney 2003; Shirangi et al. 2009). On the other hand, chemical signals involved later in the courtship sequence, for example in mate assessment, sexual persuasion, and/or coordination of courtship behaviors, are likely shaped by sexual selection (see for example Houck et al. 1985; Gershman and Verrel 2002). Signals influenced by sexual selection may experience especially rapid, adaptive evolution, and this might lead to significant intraspecific variation (Houck et al. 1985; Andersson 1994; Phelan 1997).

Courtship interactions between male and female plethodontid salamanders include a courtship pheromone delivery phase that utilizes a functional complex of molecular, behavioral, and morphological characters in the male (Watts et al. 2004). Plethodontids are a 110–140 My-old radiation comprising more than 300 aquatic and terrestrial species in four major lineages (Chippindale et al. 2004; Macey 2005; Min et al. 2005; Wiens et al. 2006; Zhang and Wake 2009). Throughout this time period, despite an extensive ecological radiation, courtship of a female by a male has been accomplished in the same basic way (Houck and Arnold 2003). During a sexual encounter, the male pursues the female and engages her in a tail-straddling walk (Noble 1929). This walk, in which the female straddles the male's tail with her forelimbs, aligns the sexual partners for an intricate process of sperm transfer via a spermatophore deposited on the substrate (Stebbins 1949). A female can terminate courtship at any time, including after spermatophore deposition by the male. As a consequence, there is a premium on the male capacity to persuade the female to complete courtship, and variation in this male capacity exposes it to sexual selection (Houck et al. 1985; Gershman and Verrel 2002).

The tail-straddling walk provides the venue for courtship pheromone delivery (Gershman and Verrel 2002). As the female straddles the male's tail and follows him as he marches forward, the male turns around and scratches the female with his hypertrophied premaxillary teeth. While scratching, the male effectively swabs the abraded site with secretions from a specialized gland on his chin, the mental gland (Arnold 1977; Houck and Arnold 2003). Experiments have shown that these mental gland secretions affect the female's receptivity, as measured by a shortening of the time between the initiation of a courtship encounter and successful insemination of the female (Houck and Reagan 1990). Biochemical and genetic analyses have shown that the secretions of the mental gland consist primarily of proteins in three distinct families, each of which affects the duration of courtship (Rollmann et al. 1999; Houck et al. 2007a, b, 2008; Kiemnec-Tyburczy et al. 2009).

Different evolutionary processes prevail at the molecular, behavioral, and morphological levels of organization of the salamander pheromone delivery functional complex. The functional complex consists of male pheromone delivery behavior (e.g., scratching), morphology (specialized teeth and mental gland), and a proteinaceous pheromone blend (Watts et al. 2004). Stasis is the primary evolutionary pattern at the behavioral and morphological levels. Scratching delivery of pheromones via protruding premaxillary teeth and small mental glands is the ancestral delivery mode, and has been retained in each of the four major plethodontid lineages (Houck and Arnold 2003). Elsewhere we have argued that stabilizing selection is the most likely explanation for this 110–140 My pattern of stasis (Watts et al. 2004). However, departures from this picture of stasis have occurred in some lineages. In the genus *Plethodon*, for example, males of one monophyletic species group (the large eastern *Plethodon*) lack protruding premaxillary teeth and deliver courtship pheromone by 'slapping' their large mental glands across the female's nares (Arnold 1977; Highton and Larson 1979; Highton 1989; Houck and Arnold 2003). In these species, the pheromone enters the nasal cavity, activates neurons in the vomeronasal organ, and shortens the duration of courtship (Rollmann et al. 1999; Wirsig-Wiechmann et al. 2002, 2006; Houck et al. 2007a, b). The transition from scratching to olfactory delivery was not instantaneous, as indicated by the fact that species in the *P. welleri* and *P. wehrlei* species groups show intermediacy in delivery behavior (Organ 1960; Picard 2005; Dyal 2006). Once the evolutionary transition was accomplished, however, behavioral and morphological stasis resumed. Thus, the 28 species of large eastern *Plethodon* are remarkably uniform in mental gland morphology and slapping behavior (Highton 1962; Marvin and Hutchison 1996).

This picture of stasis at the behavioral and morphological levels, before and after occasional periods of evolutionary transition, is completely different from the picture at the molecular level. The pheromone proteins of plethodontids are engaged in incessant evolutionary change rather than stasis (Watts et al. 2004; Palmer et al. 2005, 2007a). The evolution of two previously studied pheromone components (PRF and SPF) can be characterized as a

molecular tango (Palmer et al. 2005, 2007a). These two components represent two, unrelated protein families. Plethodontid receptivity factor (PRF) is a 22 kDa protein structurally related to IL-6 type cytokines (Rollmann et al. 1999; Watts et al. 2004). Sodefrin precursor-like factor (SPF) is ~23 kDa protein structurally related to phospholipase A2 inhibitors (Palmer et al. 2007a). Rapid diversification of both proteins is driven by positive selection (Watts et al. 2004; Palmer et al. 2005, 2007a). Compared to most proteins, a relatively high proportion of amino acid sites bear the signature of positive selection (30% in PRF, 16–19% in SPF), and in the case of PRF, many positively selected sites correspond to putative binding sites for receptors (Watts et al. 2004). Convergent and parallel amino acid substitutions are often observed. These characteristics suggest that both proteins have actively coevolved with their receptors. Instead of a runaway coevolutionary process that would result in rapid, progressive divergence, the molecular tango occurs within a constrained molecular space, with limits imposed on change, perhaps by functional constraints. In other words, the coevolution of each pheromone component with its receptor is a dance that occurs on a dance floor with defined limits; a molecular tango. Detailed studies of the evolution of PRF and SPF indicate that the tempo of the molecular tangos of these proteins varies from lineage to lineage (Palmer et al. 2005, 2007a). Furthermore, these two proteins also show a pattern of evolutionary replacement (Pomiankowski and Iwasa 1993; Iwasa and Pomiankowski 1994), such that one—but not both proteins—evolves at a rapid tempo in any particular lineage (Palmer et al. 2007a).

The evolutionary pattern of a third pheromone component has not been fully characterized. This component, plethodontid modulating factor (PMF), is a 7 kDa protein, structurally related the snake toxin like superfamily of proteins (Palmer et al. 2007b). Unlike PRF and SPF, PMF lengthens the duration of courtship when it alone is experimentally applied to the female (Houck et al. 2007a). Based on this experimental result, it has been argued that PMF synergistically interacts with other pheromone components to increase female sexual receptivity, since the native pheromone blend (which includes PRF, SPF, and PMF) increases receptivity (Rollmann et al. 1999). Sequence variation in PMF has been studied in only a single population of one species (*P. shermani*), and that study (Palmer et al. 2007b) revealed extraordinary sequence divergence (range 1–60%, average 35%) across the gene family. Individuals were found to express up to 26 isoforms, with sequence variation greatly exceeding indel variation, suggesting that the large number of isoforms arises from numerous duplications of PMF genes (Palmer et al. 2007b) rather than transcriptional processes such as alternative splicing.

The overarching goal of this article is to determine whether the evolution of PMF is decoupled from the pattern of stasis that prevails at the behavioral and morphological levels in the functional complex. Our immediate aims are to determine whether PMF shows rapid sequence diversification driven by positive selection, and if PMF is involved in evolutionary replacement with PRF and SPF. We also focus on the issue of whether certain structural and biochemical features of the PMF protein (i.e., cysteine residues and p*I*) provide evidence for purifying selection as predicted from the constrained dance floor of the molecular tango. The launching point for our analyses is to sample PMF sequences in 26 additional species of plethodontid salamanders.

Materials and Methods

Males representing 27 species of plethodontid, and possessing enlarged mental glands, were collected during the breeding season (Table 1). Usually a single male was sampled from a single population of each species; a few exceptions are noted in Table 1. The genera in this sample (*Aneides, Desmognathus, Plethodon*) represent three of the four major clades of plethodontids (Chippindale et al. 2004; Macey 2005; Min et al. 2005). Animals were anesthetized in the laboratory and their mental glands were surgically removed and stored individually at −80°C (cf Palmer et al. 2005). Total RNA was extracted from the gland tissue and reverse transcribed into first strand cDNA using oligo-dT following the protocol of Palmer et al. (2005). A 50 µl PCR reaction was prepared with 1 µl cDNA, 2.5 units TAQ polymerase, 5 µl 10× buffer, 0.2 mM of each dNTP, 2.5 mM $MgCl_2$, and 100 pmol each of PMF specific primer. The primer pair (P7NF: 5′-CACC TGGAATCCAGAATGA-3′ and P7NR: 5′-AAGAGTGT GTGACTAGTTGCAGA-3′) was designed from the conserved untranslated regions of PMF sequences taken from a cDNA library (Palmer et al. 2007b). Amplification was carried out using initial denaturation at 95°C for 3 min, 40 cycles of denaturation at 95°C for 1 min, primer annealing at 51°C for 45 s, an extension at 72°C for 1 min and one final extension at 72°C for 10 min. Based on previous research (Palmer et al. 2007b), PMF sequences were expected to range in size from 231 to 258 bps. Amplified PCR product was visualized and excised from a 1.5% gel, purified (QIAquick gel extraction kit; Qiagen [Valencia, CA] # 28706), and cloned using the Topo® TA cloning kit (Invitrogen [Carlsbad, CA] # K4575-01). Twenty PMF clones per individual were purified (QIAprep spin miniprep kit, Qiagen [Valencia, CA] # 27106) and sequenced in both the forward and reverse directions using universal T3 and T7 primers. To obtain a more complete picture of PMF expression, cDNA libraries were constructed from

Table 1 Summary of collection sites (all in the USA) and PMF sequences isolated from RNA in the mental gland of an individual male salamander from each species listed[a]

Species name	Collection site				PMF isoforms				
	County	State	Lat (°N)	Long (°W)	# of clones	# of unique sequences	% Nucleotide dissimilarity	# of unique translations	% Amino acid dissimilarity
Genus *Desmognathus*					35	7	3.7 ± 0.8	4	6.7 ± 2.1
D. ocoee[b]	Clay	NC	35 02′20″	083 33′08″	15	5[a]	0.8 ± 0.4	2	0.7 ± 0.7
D. monticola[b]	Macon	NC	35 01′50″	083 08′49″	20	2	0.4 ± 0.4	2	1.2 ± 1.1
Genus *Aneides*									
A. ferreus[b]	Lane	OR	43 47′15″	123 49′06″	20	4	53.1 ± 6.4	4	70.6 ± 9.8
Genus *Plethodon*									
Western *Plethodon*					38	3	0.3 ± 0.3	3	0.8 ± 0.8
P. dunni[b]	Lane	OR	43 49′51″	123 45′40″	18	2[a]	0.4 ± 0.4	2	1.2 ± 1.1
P. vehiculum[b]	Benton	OR	44 30′38″	123 28′02″	20	1	–	1	–
Eastern *Plethodon*									
P. cinereus group[b]					64	31	18.4 ± 2.2	27	38.2 ± 5.4
P. cinereus	Giles	VA	37 22′02″	080 31′34″	20	5	0.6 ± 0.3	5	1.9 ± 1.0
P. hoffmani	Bath/Pocahontas	VA/WV	38 15′50″	079 48′03″	12	5	13.8 ± 2.1	5	26.2 ± 4.8
P. richmondi	Wise	VA	36 53′42″	082 37′58″	20	14	17.5 ± 1.9	10	25.8 ± 5.5
P. serratus	Henry	GA	33 29′59″	084 10′58″	12	7	17.2 ± 2.1	7	34.0 ± 4.9
P. welleri group[c]					51	35	16.5 ± 1.7	30	32.0 ± 5.0
P. dorsalis	Parke	IN	39 53′14″	087 11′20″	8	7	11.4 ± 1.7	7	24.8 ± 4.6
P. ventralis	Jefferson	AL	33 43′32″	086 49′20″	12	11	18.8 ± 2.2	11	36.2 ± 5.8
P. websteri	Jefferson	AL	33 43′32″	086 49′20″	19	13	18.3 ± 2.1	8	32.2 ± 4.9
P. welleri	Madison/Unicoi	NC/TN	36 06′36″	082 21′40″	12	4	9.2 ± 1.4	4	17.2 ± 3.6
P. wehrlei group[c]					30	11	22.9 ± 2.6	11	47.5 ± 7.0
P. wehrlei	Floyd	VA	36 47′37″	080 27′58″	12	5	15.0 ± 2.0	5	32.1 ± 5.2
P. wehrlei	Pocahontas	WV	38 27′10″	080 00′12″	18	6	25.2 ± 2.9	6	50.4 ± 7.3
P. glutinosus group[d]					271	145	16.6 ± 1.75	117	34.9 ± 5.3
P. aureolus	Monroe	TN	35 27′29″	084 01′24″	12	8	16.4 ± 2.0	8	33.6 ± 6.0
P. chattahoochee	Towns	GA	34 52′21″	083 48′31″	11	9	15.0 ± 1.8	9	31.9 ± 4.9
P. cheoah	Graham	NC	35 21′30″	083 43′04″	20	10	17.9 ± 2.1	10	39.6 ± 5.5
P. cylindraceus	Johnson	TN	36 23′58″	081 57′55″	12	5	13.9 ± 2.0	5	29.0 ± 5.0
P. jordani	Sevier	TN	35 36′34″	083 26′50″	17	9	24.2 ± 2.4	9	49.2 ± 6.3
P. kentucki	Wise	VA	36 53′42″	082 37′58″	12	8	16.0 ± 2.1	7	35.7 ± 5.6
P. metcalfi	Macon	NC	35 19′40″	083 20′10″	19	9	17.5 ± 2.2	6	36.4 ± 5.2
P. mississippi	Scott	MS	32 24′37″	089 29′02″	12	9	20.1 ± 2.3	8	41.1 ± 5.5
P. montanus	Madison	NC	35 50′24″	082 57′11″	20	5	26.5 ± 2.9	4	52.5 ± 7.6
P. ouachitae	Le Flore	OK	34 47′50″	094 54′29″	20	5	10.8 ± 1.7	5	24.2 ± 4.8
P. shermani	Macon	NC	35 10′48″	083 33′38″	79	45[a]	18.9 ± 1.9	30	38.2 ± 5.3
P. teyahalee	Madison	NC	35 50′24″	082 57′11″	17	11	17.5 ± 1.9	9	36.0 ± 5.8
P. yonahlossee	Yancey	NC	35 44′38″	082 12′51″	20	12	15.4 ± 1.9	10	33.4 ± 5.2
All plethodontids					509	217	20.2 ± 1.9	180	39.3 ± 5.9

Species groups within the genus *Plethodon* are monophyletic and are based on allozyme and nuclear gene sequence data (Larson and Highton 1978; Highton 1989; Highton and Peabody 2000; Wiens et al. 2006). The placement of *P. websteri*, however, remains problematic (Wiens et al. 2006)

[a] The unique sequences for these species include sequences obtained from cDNA libraries constructed from the mental glands of multiple males and from sequencing reported by Palmer et al. 2007b (*P. shermani*)

[b] Species with scratching delivery mode

[c] Species with intermediate delivery mode

[d] Species with olfactory delivery mode

P. shermani ($N = 10$), *P. dunni* ($N = 1$), and *D. ocoee* ($N = 20$) mental glands as described in Palmer et al. (2005). Up to three hundred clones were randomly sequenced from the libraries of these species.

Because of the many copies of PMF genes in plethodontid genomes (see Palmer et al. 2007b), we did not employ locus-specific primers in our amplification of PMF sequences. Instead, throughout this paper we treat the entire family of gene sequences en masse. Consequently, we use the term 'polymorphic' loosely to refer to multiple sequences, both within and among loci.

Gene Tree Reconstruction

The BioEdit Sequence Alignment Editor program was used for sequence alignment and editing (Hall 1999). Nucleotide sequences were translated to amino acid sequences and then aligned using the ClustalW algorithm. Minor adjustments were made manually prior to back-translation.

Gene trees representing all the sequences as well as subsets of sequences were analyzed. A master tree based on all of the 236 sequences (from 27 species) was estimated using the methods described below. Because of the low branch support, we generated four independent versions of this master tree and conducted separate PAML analyses on each version to determine whether results were robust to differences in topology. We also independently estimated trees for seven different groups recognized as monophyletic in systematic studies. In those analyses we used only sequences from each group in turn. The following procedures were used in all of these tree building exercises.

Datasets were built from unique cDNA haplotypes and maximum likelihood trees were reconstructed from the aligned cDNA 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 inference was performed using the codon model with rates specified as gamma-distributed across sites. A molecular clock was not enforced. Four Monte Carlo Markov chains were run simultaneously for 1,000,000 generations with a random tree assigned at the start of each chain. Trees were sampled every 100 generations, for a total of 10,000 trees generated. Log-likelihood scores converged on a stable value at approximately 400,000 generations and all trees that were generated prior to the stationary likelihood value were discarded (as "burn-in"). The remaining 6,000 trees were used to generate a 50% majority rule consensus tree on PAUP* (Version 4.0b10; Swofford 2003), which depicted the posterior probability values of the observed clades (Huelsenbeck and Ronquist 2001). For all analyses, the xenoxin gene (accession X72673) of *Xenopus laevis* was used as the outgroup.

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 on 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 number of synonymous changes per synonymous site (d_S) and non-synonymous substitutions per nonsynonymous site (d_N) was 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 non-synonymous-to-synonymous rate ratio (d_N/d_S) was averaged across PMF genes for each lineage to determine the dominant mode of selection that has acted on the gene family. 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 selection statistic, $Z = (d_N - d_S)/\sqrt{\mathrm{Var}(d_N) + \mathrm{Var}(d_S)}$, was computed in MEGA (Version 3.0; Kumar et al. 2004) with 500 bootstrap replicates 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 4; Yang 1997, 2007). The nucleotide alignment and maximum likelihood tree were used as input for each of the following six datasets: (1) *Desmognathus* PMF (2 spp.; 7 sequences; scratching delivery), and PMF from eastern *Plethodon* of the: (2) *P. glutinosus* group (13 spp.; 145 sequences; olfactory delivery), (3) *P. welleri* group (4 spp.; 35 sequences; intermediate delivery), (4) *P. wehrlei* group (1 spp.; 11 sequences; intermediate delivery), (5) *P. cinereus* group (4 spp.; 31 sequences; scratching delivery), and (6) PMF from all 27 plethodontid species studied (236 sequences; all delivery types). Each dataset (1–5) includes PMF sequences from a single species group, comprised of several closely related species having the same pheromone delivery mode, across three distinct genera of plethodontid salamanders. See Table 1 for the justification and composition of these species groups. The final data set included PMF sequences from all 27 species, representing species from three genera and all existing pheromone delivery modes. To test for robustness, PAML analyses were performed on four independent trees built from all 236 PMF sequences.

Data were fitted in PAML to site-specific codon-substitution models that allowed ω to vary among sites, with the parameters of the model estimated using maximum likelihood (Yang and Bielawski 2000). Two pairs of

models were compared: the discrete models M1a (nearly neutral) versus M2a (selection), and the non-discrete models M7 (beta) versus M8 (beta and ω). M1a assumed two ω site classes with $\omega_0 < 1$ estimated from the data and $\omega_1 = 1$, while M2a allowed a third class of sites to be estimated from the data which may be under positive selection, $\omega_2 > 1$ (Wong et al. 2004; Yang et al. 2005). M7 assumed a beta distribution and fit ω to 10 site classes in the interval [0,1]. M8 added an extra site class to M7 with a free ω value larger than 1 estimated from the data. The two parameters estimated for the beta distribution (p and q) can be used to visualize the shape of the distribution (PAML ver. 4 User Guide, p. 45). 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 2 degrees of freedom (Yang 1998). Specific codon sites subjected to positive selection were predicted with the Bayes empirical Bayes (BeB) calculation of posterior probabilities for site classes (Yang 1998; Yang et al. 2005) employed in CodeML (PAML, models M2a and M8). Sites showing a signature of positive selection according to the LRT were also tested using the Naive empirical Bays method (Nielsen and Yang 1998; Wong et al. 2004) to verify $\omega > 1$ and for comparison with our earlier studies that used Naive empirical Bayes rather than BeB. Sites that were identified as undergoing positive selection were significant in both tests and the posterior probabilities under both M2a and M8 models were $\geq$0.95.

Estimation of p*I* Values

The charge of a polypeptide of comparable length to PMF at pH 7.0 was estimated from the amino acid sequence by summing over all ionizable groups and the N- and C-termini the fractional charge for each group at pH 7.0. The fractional charge for each group was estimated from the Henderson–Hasselbalch equation on the assumption of fixed pK_a values for all ionizable groups, as given by Stryer (1995). The random 65 amino acid polypeptides were generated by mapping each of the 20 standard amino acids onto one of 20 equal sized partitions of the interval [0,1], and then using this mapping to translate a string of 65 random numbers sampled from the interval into an amino acid sequence.

Results

PMF is an Ancient and Persistent Pheromone Component

We surveyed 27 species, representing three genera (*Aneides, Desmognathus* and *Plethodon*, see Table 1) and two plethodontid salamander tribes (desmognathines and plethodontines) for the presence of PMF transcript in the mental gland. PMF was expressed in the mental gland of every species that we sampled; suggesting that the pheromone function of this gene originated at least 50–85 Mya (Wiens et al. 2006). Sequencing of 509 PMF clones resulted in 217 unique cDNA sequences that encoded 180 unique amino acid sequences. cDNA sequences varied in length from 216–273 bps (72–91 amino acids) and included 167 polymorphic base pair sites. Variation in sequence length resulted from several deletions and/or insertions (ranging from 3 to 27 bps in length) throughout the PMF gene (Fig. 1).

PMF Gene Tree Topology Reveals Numerous Instances of Homoplasy and Gene Duplication

The PMF master gene trees had a much different topology than allozyme trees (see Watts et al. 2004 for a summary) or trees based on the sequences of house-keeping genes (Wiens et al. 2006). Familiar monophyletic groups that are routinely recovered in the genus *Plethodon* with allozyme and nuclear gene sequence data (Larson and Highton 1978; Highton 1989; Highton and Peabody 2000; Wiens et al. 2006), as well as higher order relationships that are consensus features in recent nuclear and mitochondrial gene trees (Chippindale et al. 2004; Macey 2005; Min et al. 2005; Wiens et al. 2006), were not apparent in the PMF master gene trees (Fig. 2). Instead the PMF trees showed a recurrent pattern of gene duplication. Within each of the gene clades that include the descendants of one of these gene copies are alleles from distantly related congeneric species and genera, a pattern consistent with long-term maintenance of polymorphism or sequence convergence. In addition, rates of evolution are heterogeneous. In one especially vivid case, three haplotypes in *P. shermani* have shown especially rapid evolution (long branches at a tip of the tree) and have apparently converged on a haplotype found in *A. ferreus*.

PMF Shows Extraordinary Sequence Variation

Average nucleotide dissimilarity across all plethodontid PMF sequences surveyed was 20%. Within genera and within major lineages of *Plethodon*, PMF nucleotide dissimilarity ranged from 0.3 to 53%. Intraspecific diversity (in most cases, determined from a single individual) also varied considerably, ranging from a low of only 0.4% in *Desmognathus monticola* to a high of 53% in *Aneides ferreus* (Table 1). Most males expressed numerous PMF sequences in the mental gland, and this resulted in high levels of sequence diversity within individuals (Table 1). For example, sequencing of 40 clones from a single

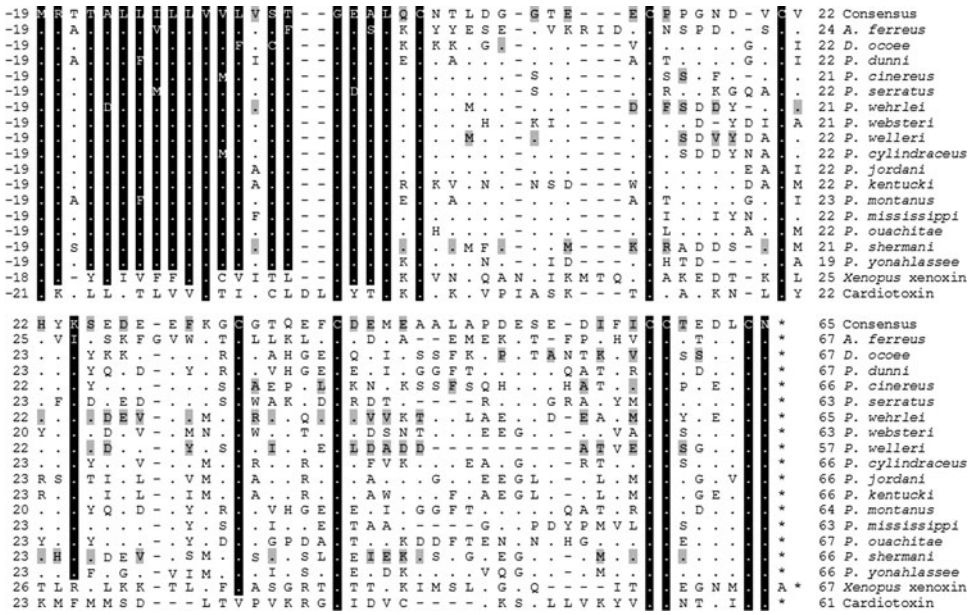

Fig. 1 Representative amino acid sequences of plethodontid modulating factor (PMF). The *dots* represent amino acid residues that are identical to the consensus sequence, whereas *dashes* represent gaps in the sequences. Highly conserved residues (>95%) are given against a *black* background. The secretion signal (−1 through −18) is shown against a *light gray* background. Residues predicted to have experienced positive selection (>95% BeB probability) are highlighted in *dark gray*. Sites under positive selection were identified by PAML analyses conducted separately on different clades (Table 3), hence diagnoses of selection vary from row to row in the alignment. The GenBank accession number for the comparative xenoxin sequence from *Xenopus laevis* is CAA51225

RT-PCR reaction on the mental gland of one male *P. shermani* resulted in 26 unique PMF haplotypes, suggesting that at least 13 PMF genes were expressed by this individual. Nucleotide sequence diversity was 20.5% for this individual, compared to an average of only 16.6% interspecific variation across the entire *P. glutinosus* species group. In contrast, sampling of *Desmognathus* and western *Plethodon* species yielded a maximum of only three unique alleles per individual, suggesting that only two PMF genes were present (Table 1). Differences in the number of PMF sequences obtained per species are probably not due to primer bias or polymerase error during PCR amplification. The variation we observed far exceeds normal levels of polymerase error and was present when a proof-reading enzyme was employed during preliminary experiments (data not shown). Primer bias might arise from mutations accumulating within the primer binding sites in some species. However, PCR independent approaches yielded similar results to PCR-based cloning strategies. For example, random sequencing of a *D. ocoee* cDNA library revealed only two additional unique PMF sequences in this species, and a *P. dunni* cDNA library (~200 clones, 1 gland) revealed none. Thirty-seven percent of the cDNA transcript sequenced from the library of *P. shermani* (~300 clones, 10 glands) encoded for PMF, and provided 10 unique sequences that were not revealed by RT-PCR. It is likely that we have not yet identified all PMF alleles even in this intensively sampled population.

The occurrence of multiple amino acid sequences across species suggests that polymorphism is actively maintained. Twelve different PMF sequences were conserved (100% identical) across several species within the *P. glutinosus* group over a time period of approximately 12–20 My (Highton and Larson 1979; Maxson et al. 1979; Wiens et al. 2006). However, several of the species we sampled in this group have histories that involve hybridization with one another (Highton 1995; Highton and Peabody 2000; Weisrock et al. 2005; Wiens et al. 2006), which complicates interpretation. One PMF sequence, however, was conserved across two species (*P. ventralis* and *P. dorsalis*) within the *P. welleri* group that do not have a history of hybridization (Highton 1995). In other cases, sporadic occurrences of the same sequences in two or more distantly related, non-hybridizing taxa probably represent instances of either convergence or reversion. For example, a PMF sequence found in western *Plethodon* species (*P. vehiculum*) was identical to a cDNA sequence recovered in *P. montanus*, a representative of the distantly related eastern *Plethodon* group.

PMF sequence variation was higher at the amino acid level than it was at the nucleotide level, suggesting that selection has favored functional diversification. Average PMF amino acid sequence dissimilarity was 39.3% across all taxa sampled. PMF amino acid sequence dissimilarity ranged from 0.7 to 71% within species and was lowest within the ancient *Desmognathus* and western *Plethodon*

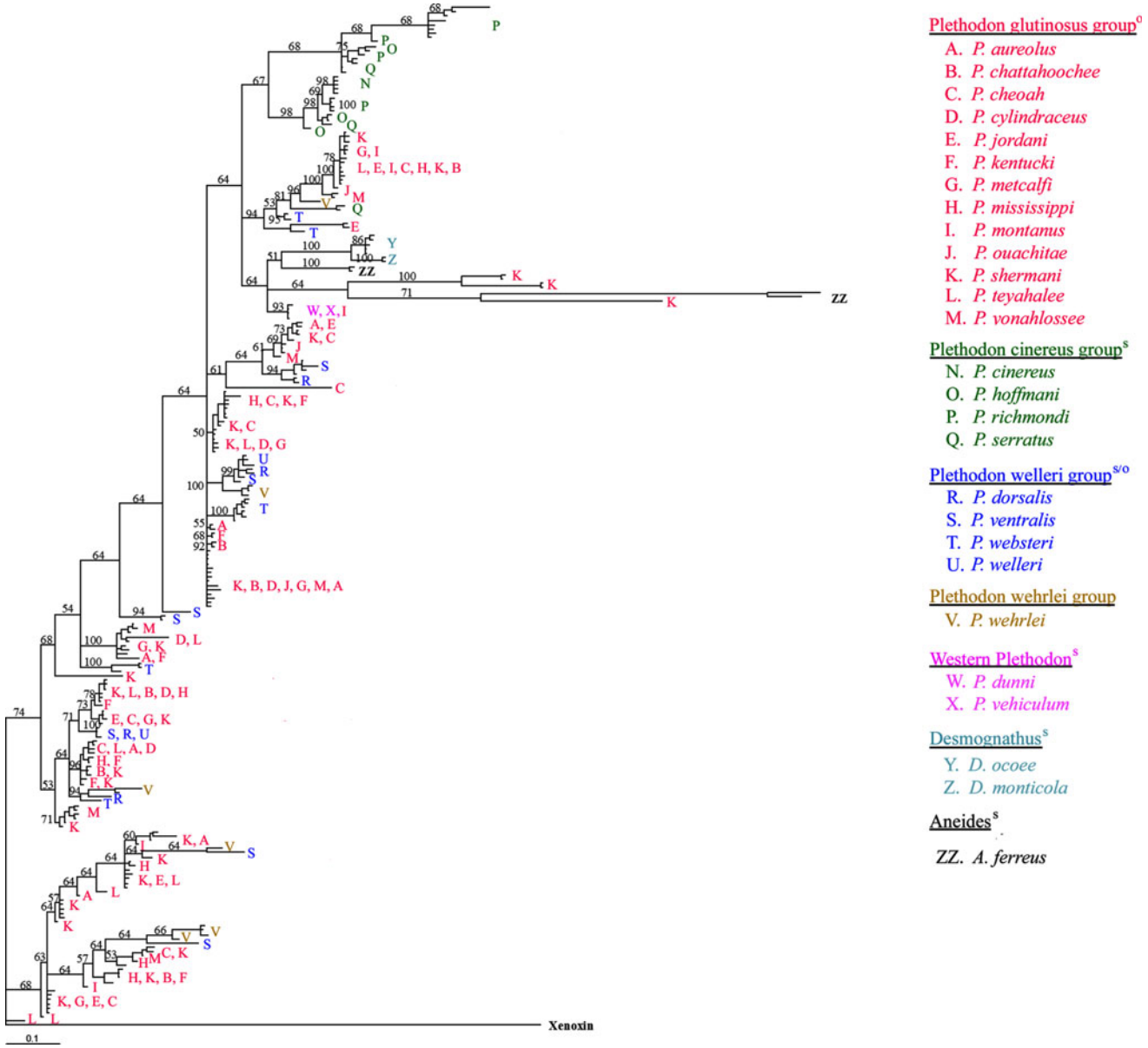

Fig. 2 Maximum likelihood tree of plethodontid modulating factor (PMF) sequences from 27 salamander species. Bayesian posterior probabilities are labeled at nodes having at least 50% support. The *distance bar* represents 0.1 nucleotide substitutions per site. The gene phylogeny was rooted with a *Xenopus* xenoxin sequence (accession CAA51225)

lineages (Table 1). Much of the amino acid sequence dissimilarity across PMF genes arises from gene comparisons within single individuals rather than between closely related species.

Some Sites in PMF Have Experienced Strong Positive Selection, but the Pattern is Lineage Specific

The ratio d_N/d_S (ω) was used to determine the dominant mode of selection that has acted on the PMF genes of each salamander lineage. High ω-values, averaged across all sites in PMF, suggest evolution driven by positive selection in the eastern *Plethodon* species groups (*P. cinereus* group ($\omega = 1.90$; $Z = 2.81$, $P < 0.01$), *P. welleri* group ($\omega = 1.91$; $Z = 3.0$, $P < 0.01$), *P. wehrlei* group ($\omega = 2.86$; $Z = 4.19$, $P < 0.001$), and *P. glutinosus* group ($\omega = 2.05$; $Z = 3.83$, $P < 0.001$)) (MEGA results, Table 2). In contrast, average ω-values are lower for *Aneides* ($\omega = 1.11$; $Z = 0.40$, $P > 0.05$) and *Desmognathus* ($\omega = 1.72$; $Z = 0.95$, $P > 0.05$) and provide no statistical support for positive selection (i.e., the hypothesis that $\omega = 1$ cannot be rejected) (MEGA results, Table 2).

Codon-substitution models were used to determine the mode of selection that has acted at each amino acid residue

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 PMF gene family, with *P*-values for the *Z* selection statistic, for plethodontid PMF genes based on analyses with MEGA

Gene	# species	# sequences	d_S	d_N	d_N/d_S PMF	*Z* statistic	d_N/d_S PRF	d_N/d_S SPF
PMF plethodontid[a]	27	236	0.128 ± 0.019	0.237 ± 0.036	1.85**	3.92		1.51*
PMF *Desmognathus*[b]	2	7	0.025 ± 0.012	0.043 ± 0.017	1.72	0.95	Not present	3.06**
PMF *Aneides*[b]	1	4	0.412 ± 0.110	0.457 ± 0.089	1.11	0.40	Not present	1.96
PMF Western *Plethodon*[b]	2	3	0	0.005 ± 0.005	–	1.07	Not present	3.65**
PMF *Plethodon cinereus* group[b]	4	31	0.090 ± 0.023	0.171 ± 0.029	1.90*	2.81	1.19	0.87
PMF *Plethodon welleri* group[c]	4	35	0.090 ± 0.020	0.172 ± 0.033	1.91*	3.00	0.98	2.72**
PMF *Plethodon wehrlei* group[c]	1	11	0.085 ± 0.025	0.243 ± 0.039	2.86**	4.19	–	0.56
PMF *Plethodon glutinosus* group[d]	13	145	0.101 ± 0.018	0.207 ± 0.032	2.05**	3.83	1.70	1.21

** $P < 0.001$; * $P < 0.01$. The absence of ** or * indicates $P > 0.05$

d_N/d_S ratios for Plethodontid Receptivity Factor (PRF) and Sodefrin Precursor-like Factor (SPF) are from Palmer et al. 2007a. A (–) for the d_N/d_S ratio denotes a missing value due to a lack of synonymous substitutions in the genes

[a] The gene tree analyzed here corresponds to tree 1 in Table 3

[b] Species having scratching delivery mode

[c] Species having intermediate delivery mode

[d] Species having olfactory delivery mode

in the PMF genes. Models that allow for positive selection (M2a and M8) provided the best fit(s) to all of the data sets (PAML results, Table 3). In particular, the superior fit of M8 (compared with M7) indicates that a substantial subset of sites have experienced positive selection (ω^+). The ω values for this subset of ω^+ sites, representing 15–56% of all sites, ranged from 2.70 to 24.28, depending on lineage (see Table 3). The number of sites predicted to have experienced positive selection is reduced to 6.5–22.8% when only sites with ≥95% probability (naïve empirical Bayes and BeB results) for both the M2 and M8 models are considered (Fig. 1). In either case, the high levels of sequence diversity appear to have been driven by strong positive selection acting on the PMF genes in every salamander lineage considered in this study. Beta distributions fitted to the ω values in the range 0–1 under model M8 revealed two patterns. The distribution for the entire set of plethodontid sequences, as well as for most lineages, was strongly bimodal (U-shaped) with strong peaks near $\omega = 0$ (purifying selection) and $\omega = 1$ (neutrality). *Desmognathus* and the *Plethodon wehrlei* groups were the exceptions. In both of those lineages, the ω distribution (in the range $\omega = 0$–1) was L-shaped with a single peak near $\omega = 0$ (purifying selection). The failure of these two groups to show a bimodal distribution of ω values (due to the absence of a neutral ($\omega = 0$)) is probably a consequence of small sample size. Overall, a smaller proportion of sites was predicted to have experienced positive selection in *Desmognathus* and the *Plethodon cinereus* group than in other lineages ($P = 0.15$ and 0.26, respectively, versus $P = 0.31$–0.56 in other groups). Nevertheless, 6.5% of sites in desmognathine PMF gene sequences presented a picture of very strong positive selection ($\omega = 24.28$). Whether the divergence in strength and pattern of selection across the PMF gene tree of these diverse salamander lineages is associated with differences in protein function is a question for future study.

Some Sites in PMF Are Under Purifying Selection

Conservatism at some sites apparently arises as a consequence of functional constraints. An alignment of PMF protein sequences from 16 plethodontid species is depicted in Fig. 1. Of 77 shared residues, 28 amino acid sites (36% of the gene sequence) were conserved across 95–100% of all plethodontid PMF sequences. Seventeen of the 28 conserved residues were located at the N-terminus, along a portion of the gene sequence that corresponds to the putative secretion signal (residues M^{-1} to A^{-19}; Fig. 1). Eight additional sites were cysteine residues that likely provide protein conformational stability via the formation of disulfide bonds. The three remaining conserved residues included L^1, K^{24}, and N^{64} (Fig. 1, consensus).

A comparison of charge at pH 7.0 for 217 unique PMF sequences, 26 three-finger protein sequences and 10,000 random-sequence 65 amino acid proteins suggests that selection has constrained the charge profile of PMF (Fig. 3). The PMF charge distribution is shifted towards negative net charge (mean = −9.53, SD = 3.93) compared to other three-finger proteins (mean = 0.60, SD = 3.58), which are predominately positively charged and have a distribution similar to the random distribution (mean = 1.75, SD = 3.83, Fig. 3). Over 85% of PMF sequences have a net charge between −7 and −15.

Table 3 Likelihood ratio tests for positive selection using PAML site-specific models for plethodontid modulating factor (PMF)

Gene family (# spp./# sequences)	d_N/d_S	2δ M2 vs. M1	2δ M8 vs. M7	Parameter estimates under M2	Parameter estimates under M8 (beta & ω)
Plethodontid PMF (27/236)—tree 1	1.33	226.54**	210.15**	$p_0 = 0.29$, $\omega_0 = 0.08$	
				$p_1 = 0.34$, $\omega_1 = 1.00$	$p_1 = 0.38$, $\omega_1 =$ **3.16**
				$p_2 = 0.37$, $\omega_2 =$ **3.49**	$p_0 = 0.62$, B(0.27, 0.28)
Plethodontid PMF (27/236)—tree 2	1.37	231.59**	214.55**	$p_0 = 0.28$, $\omega_0 = 0.08$	
				$p_1 = 0.33$, $\omega_1 = 1.00$	$p_1 = 0.39$, $\omega_1 =$ **3.27**
				$p_2 = 0.39$, $\omega_2 =$ **3.58**	$p_0 = 0.61$, B(0.26, 0.27)
Plethodontid PMF (27/236)—tree 3	1.31	252.02**	210.14**	$p_0 = 0.30$, $\omega_0 = 0.09$	
				$p_1 = 0.29$, $\omega_1 = 1.00$	$p_1 = 0.41$, $\omega_1 =$ **3.58**
				$p_2 = 0.41$, $\omega_2 =$ **3.60**	$p_0 = 0.60$, B(0.26, 0.27)
Plethodontid PMF (27/236)—tree 4	1.39	423.22**	352.52**	$p_0 = 0.41$, $\omega_0 = 0.11$	
				$p_1 = 0.30$, $\omega_1 = 1.00$	$p_1 = 0.29$, $\omega_1 =$ **3.23**
				$p_2 = 0.29$, $\omega_2 =$ **3.43**	$p_0 = 0.71$, B(0.22, 0.24)
Desmognathus PMF (2/7)	1.29	17.28**	17.33**	$p_0 = 0.85$, $\omega_0 = 0.00$	
				$p_1 = 0.00$, $\omega_1 = 1.00$	$p_1 = 0.15$, $\omega_1 =$ **24.28**
				$p_2 = 0.15$, $\omega_2 =$ **24.28**	$p_0 = 0.85$, B(0.005, 99.0)
Plethodon glutinosus group PMF (13/145)	1.22	69.01**	72.69**	$p_0 = 0.37$, $\omega_0 = 0.08$	
				$p_1 = 0.25$, $\omega_1 = 1.00$	$p_1 = 0.40$, $\omega_1 =$ **2.70**
				$p_2 = 0.38$, $\omega_2 =$ **2.75**	$p_0 = 0.60$, B(0.22, 0.29)
Plethodon welleri group PMF (4/35)	1.95	88.61**	98.10**	$p_0 = 0.32$, $\omega_0 = 0.01$	
				$p_1 = 0.37$, $\omega_1 = 1.00$	$p = 0.31$, $\omega =$ **5.54**
				$p_2 = 0.31$, $\omega_2 =$ **5.63**	$p_0 = 0.69$, B(0.02, 0.02)
Plethodon wehrlei group PMF (1/11)	2.53	30.48**	41.69**	$p_0 = 0.44$, $\omega_0 = 0.29$	
				$p_1 = 0.00$, $\omega_1 = 1.00$	$p = 0.56$, $\omega =$ **5.23**
				$p_2 = 0.56$, $\omega_2 =$ **5.22**	$p_0 = 0.44$, B(41.13, 99.0)
Plethodon cinereus group PMF (4/31)	1.70	24.86**	24.88**	$p_0 = 0.08$, $\omega_0 = 0.00$	
				$p_1 = 0.66$, $\omega_1 = 1.00$	$p = 0.26$, $\omega =$ **4.42**
				$p_2 = 0.26$, $\omega_2 =$ **4.38**	$p_0 = 0.74$, B(0.05, 0.005)

d_N/d_S is the average ω across all sites and branches calculated under M0. '2δ' indicates twice the log-likelihood difference between the two models. p_i denotes the estimated proportion of sites with a given value of ω_i. The parameters p and q for the beta distribution, B(p, q), are given for M8. Parameters indicating positive selection are in bold. ** $P \leq 0.001$ and * $P \leq 0.01$ with d.f. = 2

Nevertheless, eight PMF sequences are positively charged. These positively charged sequences are represented by seven *Desmognathus* sequences and one *Aneides* PMF sequence, which occur in one small clade on the gene tree (Fig. 2). The unusual negative charge distribution in PMF is maintained in the face of extraordinary sequence variation, suggesting that this characteristic of PMF is under stabilizing selection for important but unknown functional reasons.

Discussion

Evolutionary Dynamics of the Functional Complex

Like other salamander courtship pheromones, PMF shows ongoing molecular diversification and widespread positive selection within both the scratching and olfactory delivery modes. In addition, PMF shares with other pheromone components the characteristic of evolutionary decoupling from stasis that prevails at the behavioral and morphological levels of the pheromone delivery functional complex (Watts et al. 2004; Palmer et al. 2007a). At the same time, PMF does not participate in the pattern of evolutionary replacement previously reported for PRF and SPF (Palmer et al. 2007a). The expectation of replacement is based on quantitative genetic models for sexual selection, which predict that only one male signal at a time will be involved in rapid coevolution with female receptors if preferences for multiple signals incur an interactive (joint) cost to the female (Pomiankowski and Iwasa 1993; Iwasa and Pomiankowski 1994). In support of this replacement model, a slowdown in SPF evolution coincides with the origin and subsequent rapid evolution of PRF in the eastern *Plethodon* (Palmer et al. 2007a). The replacement model is further supported by a reversal of patterns in the *P. welleri* species

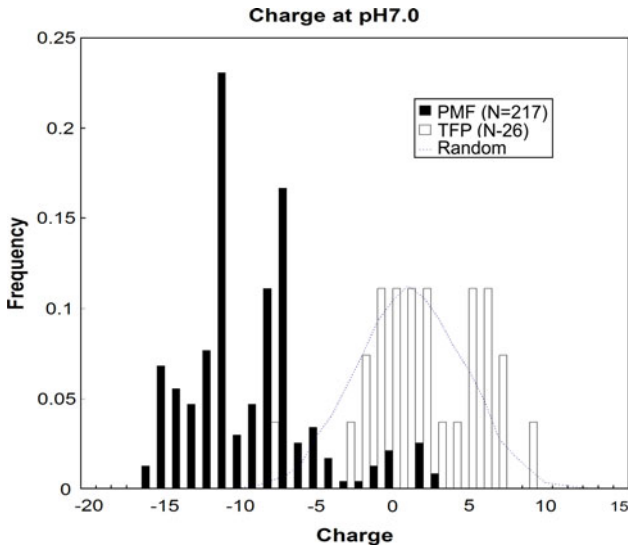

Fig. 3 Frequency distribution of the net charge of PMF (mean = −9.53, SD = 3.93) and other three-finger proteins (mean = 0.60, SD = 3.58) at neutral pH compared to neutral expectation. The *dashed line* represents the frequency distribution of the net charge at pH 7.0 for 10,000 random 65 amino acid proteins (mean = 1.75, SD = 3.83). For all proteins, the known or predicted signal peptide was removed from the sequences prior to computation

group: i.e., rapid evolution of SPF but neutral evolution of PRF. Superimposing PMF on this picture, we see a uniform pattern of rapid, selection-driven diversification in PMF (Table 2) and no evidence that the evolution of PMF was impeded by rapid evolution of PRF or SPF, as would be expected if evolutionary replacement was occurring.

In terms of the Pomiankowski–Iwasa replacement model (Pomiankowski and Iwasa 1993; Iwasa and Pomiankowski 1994), it appears that the interactions between PMF and the other pheromone proteins are not substantially negative in females. PMF extends the duration of courtship in experimental trials (Houck et al. 2007a), whereas both PRF and SPF shorten courtship times (Rollmann et al. 1999; Houck et al. 2007b). These findings were interpreted as evidence that PMF acts synergistically with other pheromone components rather than directly to increase female receptivity (2007a). If so, the modality of PMF appears to be distinct from those of the primary pheromone components, PRF and SPF. Kiemnec-Tyburczy et al. (2009) explored models of independent modalities for courtship pheromone action, and assessing PMF from that perspective might be revealing.

The Tempo and Mode of Evolution is More Exaggerated in PMF than in PRF or SPF

The PMF gene tree (Fig. 2), the number of sequences expressed by individual salamanders, and the fact that sequence variation vastly exceeds indel variation in multiple sequence alignments, combine to suggest that PMF is a multigene family that has been produced by up to 12 instances of gene duplication. In contrast, the PRF gene family has experienced three duplications (Palmer et al. 2005), and the SPF gene family has experienced only two duplications (Palmer et al. 2007a). Restricting our attention to the large eastern species of *Plethodon*, because PRF is found only in this clade, the comparable figures for gene duplications are 12 for PMF, three for PRF and one for SPF. Thus, while all three pheromone families have proliferated by gene duplication, the process is most extreme in PMF.

PMF is also more hypervariable than PRF and SPF. Total sequence variation in a multigene family, both within populations and among closely related species, can be viewed as a balance between the opposing processes of birth and death as modified by genetic homogenization through concerted evolution (Nei et al. 1997; Nei and Rooney 2005). The fact that the balance in gene copy number is struck at a higher level in PMF indicates that the death process is more prolonged and/or the birth rate is higher, producing an exaggerated retention of sequence variants. A comparison of the molecular phylogeny of PMF (Fig. 2) with those for PRF and SPF reveals a greater presence of ancient gene copies and reduced evidence of species specific clustering patterns. From this, it appears that the death process may be prolonged, and that concerted evolution has had a minor role in PMF evolution when compared to the other pheromone components.

Positive selection also has played an exaggerated role in the diversification of PMF when compared to the other pheromone components. One sign of this role is the unusually high proportion of ω^+ sites in PMF (29–41% plethodontid PMF; Table 3). The comparable figures are 30% for PRF (Palmer et al. 2005) and 16–19% for SPF (Palmer et al. 2007a). All of these proportions are high in comparison to other proteins experiencing positive selection. For example, in a survey of 3019 male reproductive proteins in humans and chimpanzees by Clark and Swanson (2005) only one had a higher proportion of ω^+ sites than PMF, and only two had a proportion equal to or higher than PRF.

Rapid Evolution of PMF by Positive Selection Has Occurred in the Face of Structural/Chemical Constraints

In contrast to the picture of positive selection acting on a minority of sites in PMF, another set of sites has experienced strong purifying selection. Functional constraints are undoubtedly responsible for conservatism at these sites. The PMF protein is structurally related to members of the 'three-finger protein' superfamily (Tsetlin 1999) that

includes xenoxin, snake toxins (e.g., neurotoxins, cardiotoxins) and proteins in the CD59/Ly-6/uPAR protein family (Palmer et al. 2007a). Although members of this 'three-finger' superfamily differ substantially in function, they all maintain a conserved structural motif of eight to ten similarly spaced cysteine residues. This structural motif, as well as an N-terminal secretion signal, is highly conserved across all plethodontid PMF genes (Fig. 1). It is the 'fingers' of the PMF protein that are incredibly variable.

Another sign that purifying selection has acted on PMF is the restricted range of p*I*s shown by its variants (Fig. 3). If positive selection was acting to randomize the sequence of PMF then we would expect PMF to exhibit a similar p*I* distribution to that of a random series of polypeptides of similar length. Instead PMF variants are clustered in the extreme acid end of the random distribution, and are also more acidic than other three-finger proteins (Fig. 3). From this we can conclude that substitutions in PMF are not random but constrained by purifying selection to maintain functional attributes related to overall p*I*. Since three-finger proteins function by binding to other proteins (Tsetlin 1999) it is likely that that PMF is co-evolving with female receptors that are positively charged.

As in PRF and SPF, PMF Evolution Supports a Molecular Tango Model of Pheromone-Receptor Coevolution

PMF shares with other pheromone components a variety of characteristics that we have previously attributed to a coevolutionary molecular tango between pheromone signals and their receptors (Palmer et al. 2005, 2007a). Chief among these characteristics is the recurrent role that positive selection plays in driving the diversification of PMF in numerous lineages. The other characteristics of the tango include gene duplication, hyperexpression in the mental gland, and abundant polymorphism within populations arising from the tendency to both retain and reinvent sequence variants. The detailed considerations that lead us to propose the molecular tango model for PRF (Palmer et al. 2005) also apply to PMF.

The molecular tango model describes a co-evolutionary "dance" (*Sensu* Rice 1998) that takes place in a constrained molecular space, or "dance floor". While the source of the selective force driving the dance remains unidentified, it is likely driven by sexual selection (female preference or sexual conflict) given the context in which the pheromones act. There is a need to formalize the outcomes of sexual selection in the context of molecular evolution. However, models of phenotypic evolution have been constructed for sexual signals driven by sexual selection. Under various conditions these phenotypic models lead to continuous evolutionary chase, exaggerated diversification, or cyclical evolution (see for example Fisher 1958; Lande 1981; Mead and Arnold 2004; Gavrilets and Hayashi 2006). We would expect that when these outcomes occur in a constrained molecular space with the possibility of gene duplication, equivalent to males holding multiple character states for the selected trait, many of the characteristics of the salamander pheromone components would be reproduced. In this context the characteristics of PMF that we found are under stabilizing selection may be components of the constraints that tether PMF and its co-evolving partners to the "dance floor".

Conclusions

Beneath the evolutionary tranquility at the phenotypic level in this chemical signaling system, all three pheromone components have been engaged in repeated, periodic episodes of rapid molecular evolution driven by positive selection over many millions of years. Despite this, a substantial proportion of sites changed by neutral drift in each pheromone component, while change at another substantial proportion was restrained by purifying selection. Indeed, some features in PMF (cysteine bonds and p*I*) were restrained by very strong purifying selection. While these patterns and the other characteristics that we have attributed to co-evolution are consistent with our descriptive model of a molecular tango, this model needs to be formalized. Finally, a conclusive test of the molecular tango hypothesis requires that the evolution of each pheromone component be placed in the context of the evolution of their respective receptors.

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