Stabilizing Selection on Behavior and Morphology Masks Positive Selection on the Signal in a Salamander Pheromone Signaling Complex

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Natural selection maintains the integration and coordination of sets of phenotypic characters that collectively perform a task. In functional complexes in which characters span molecular to behavioral levels of organization, we might then expect similar modes of selection to produce similar patterns in evolutionary divergence at each level. To test this expectation, we diagnosed selection at behavioral, morphological, and molecular levels for courtship pheromone signaling by plethodontid salamanders. At the levels of morphology and behavior tens of millions of years of stasis (stabilizing selection) occur on each side of a transition from vaccination to olfactory delivery modes. As a proxy for the molecular level, we used plethodontid receptivity factor (PRF), a protein that is an active component of the pheromone. We cloned PRF from 12 *Plethodon spp*. spanning the delivery transition and obtained multiple alleles from each individual surveyed. Analyses of 61 alleles for PRF identified elevated nonsynonymous over synonymous substitution rates along lineages in a molecular phylogeny, and at 8% of sites in the protein, indicating that positive (directional) selection has acted on this vertebrate pheromone gene. Structural models showed PRF is in a family of cytokines characterized by a four–α-helix bundle. Positive selection in PRF was associated with receptor binding sites that are under purifying selection in other cytokines of that family. The evolutionary dynamics of the plethodontid pheromone delivery complex consists of stabilizing selection on morphological and behavioral aspects of signal delivery but positive selection on the signal mediated by receptors. Thus, different selection modes prevail at different levels in this reproductive functional complex. Evolutionary studies of integrated sets of characters therefore require separate analyses of selective action at each level.

Introduction

Functional complexes are sets of characters that collectively perform a distinct function. Such complexes occur in all animals that perform intricate tasks such as web spinning, insemination, venom production and delivery, and byssal thread attachment (Klauber 1956; Clarke and McMahon 1996; Olivera 1999; Opell 1999). A typical complex spans molecular to behavioral levels of organization, and there will be a selective premium on integration and coordination of the parts that compose it. This premium must apply across levels of organization, as well as within them. We might then expect the same mode of selection to percolate from level to level, with the consequence that similar patterns of evolutionary divergence will occur at each level. For example, if stasis prevails at the morphological level, we might expect stasis at the molecular level.

The concordance of evolutionary processes at different levels in functional complexes is an unresolved issue. Although a neutral/purifying selection mode commonly prevails at the molecular level (Kimura 1983; Endo, Ikeo, and Gojobori 1996; Barrier et al. 2003), and a stabilizing selection mode prevails at the morphological level (Charlesworth, Lande, and Slatkin 1982), significant departures from these selective modes have been found at each level (Boag and Grant 1981; Schubart, Diesel, and Hedges 1998; Stahl and Bishop 2000; Yang and Bielawski 2000; Miller and Pitnick 2002; Swanson and Vacquier 2002). When such a departure occurs at one level of organization in a functional complex, does it cause shifts in selection at others? To answer this question, we must diagnose modes of selection at multiple levels in a single complex.

Positive (directional) selection on reproductive aspects of morphology is widespread (Kingsolver et al. 2001). At the molecular level, positive selection has also been demonstrated to occur in proteins that mediate postcopulatory processes and may also occur earlier in the courtship phase (Willett 2000; Swanson and Vacquier 2002; Mundy and Cook 2003). Despite this widespread occurrence of positive selection, mechanistic details of mating are often conserved over tens of millions of years. In such conserved reproductive functional complexes, constraints at one level might constrain evolution at other levels. Alternatively, the mode of selection at one level might be uncoupled from that at another. Here, we use salamander pheromone delivery as a test case for dissecting the evolutionary dynamics at multiple levels in a functional complex.

Diagnosis of Selection in a Pheromone Delivery Complex

About 100 MYA, plethodontid salamanders evolved a stylized courtship during which the male delivers a pheromone produced by a pad of glandular tissue on his chin (the mental gland) while the female straddles his tail (Houck and Sever 1994; Houck and Arnold 2003). In their subsequent radiation, the diverse tribes of plethodontids have retained this system of chemical communication.

Key words: positive selection, pheromone, plethodontid receptivity factor, cytokine, pheromone delivery.

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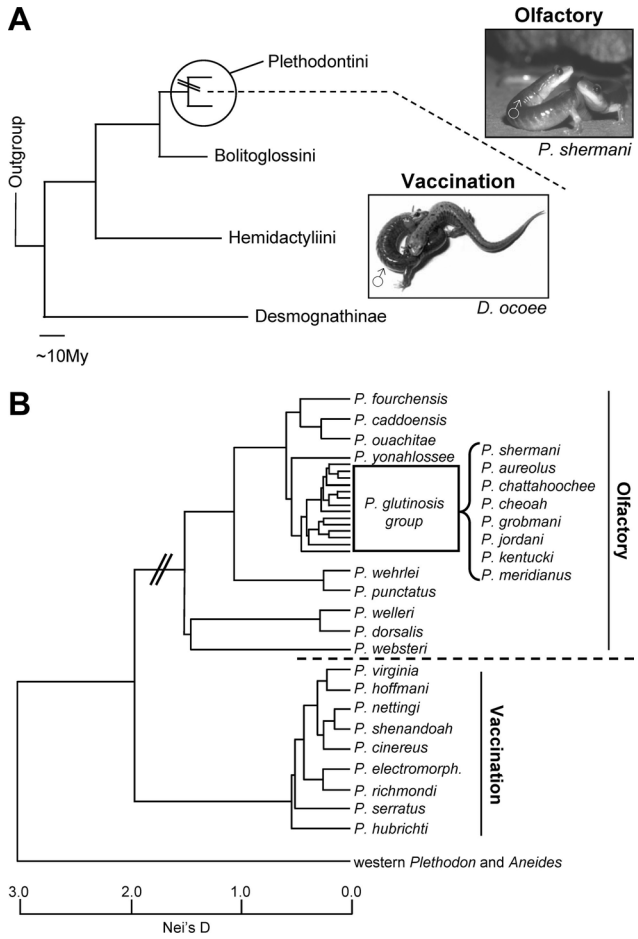

FIG. 1.—Transition in pheromone delivery mode in plethodontid salamanders. (*A*) Phylogeny of plethodontid salamanders showing the transition from vaccination to olfactory delivery modes that has occurred in *Plethodon spp*. (Houck and Sever 1994). The upper photo shows a male *Plethodon shermani* (olfactory delivery) turned back towards a female in tail-straddling walk as he prepares to slap her nares with his mental gland. In the lower photo, a male *Desmognathus ocoee* is vaccinating pheromone into a female. (*B*) Phylogeny of eastern *Plethodon spp*. that span the delivery mode transition.

The characters used for pheromone signaling during plethodontid courtship form a typical functional complex consisting of a mental gland, specialized teeth, delivery behaviors, and a chemical signal.

Courtship pheromone delivery by plethodontids presents a remarkable picture of morphological and behavioral stasis (Houck and Sever 1994). Species in all four major plethodontid lineages share a vaccination mode of delivery (fig. 1). In the courtship season, a male's premaxillary teeth and mental gland hypertrophy. During courtship, the male abrades the female's skin with his teeth and rubs secretions from his gland into the abraded site (Arnold 1977). These secretions shorten the time to sperm transfer (Houck and Reagan 1990). Vaccination occurs in all major plethodontid lineages but no other salamander, and so it was almost certainly present in the ancestral plethodontid. The family is approximately 100 Myr old (Ruben et al. 1993), so the morphological (glands and teeth) and behavioral elements (tail-straddling walk and vaccination) of this delivery system have been conserved over that entire period. The behavioral and morphological conservation includes many small details of histology and sexual choreography. Charlesworth, Lande, and Slatkin (1982) persuasively argued that such long-term stasis must be a consequence of stabilizing selection. Other mechanisms, such as evolutionary inertia or developmental constraint, may produce short-term stasis but cannot account for long-term stasis.

A rapid transition in pheromone delivery mode has occurred within the genus *Plethodon* (fig. 1). The forty-one species of this genus found in eastern North America form a monophyletic group with two subdivisions (Highton and Larson 1979; Larson et al. 1981; Highton and Peabody 2000). One clade retains the ancestral vaccination delivery mode, whereas members of the other clade share a derived olfactory mode (Houck and Sever 1994). Males in this second clade lack protruding premaxillary teeth and have a greatly enlarged mental gland, which they slap on the snout of the female during courtship in a highly stereotyped behavior pattern (Highton 1962; Arnold 1977). The pheromone then acts on the female's vomeronasal system to promote receptivity (Houck and Reagan 1990; Wirsig-Wiechmann et al. 2002). The clade within the genus *Plethodon* that employs the olfactory delivery mode arose about 19 MYA (Larson, Weisrock, and Kozak 2003) and have subsequently retained a unique combination of morphological and behavioral traits. By the same argument as above, stasis in the olfactory delivery mode is probably a consequence of stabilizing selection maintained over this period (Houck and Arnold 2003).

The implausibility of behavioral and morphological stasis over a 19-Myr period arising from genetic drift can be assessed using a mode of analysis for phenotypic characters described by Lynch (1990). Consider divergence in the size (diameter) of the mental gland, the most rapidly evolving character in the behavioral-morphological aspect of the functional complex, which among species with olfactory delivery ranges from about 2 mm in *P. dorsalis* to about 6 mm in *P. yonahlossee* (Highton 1962). In the Wayah population of *P. shermani* (Macon County, NC [table 1]) the gland averages 3.361 mm in diameter with a coefficient of variation (CV) of 0.196 ($n = 20$ males). Assuming that this CV is characteristic of *Plethodon* and that the average generation time is 5 years, using Lynch's (1990) methods, we obtain a per generation rate of squared character change of 4.14×10^{-6}, which is more than an order of magnitude slower than the minimum rate we would expect under neutrality (5×10^{-5}). This result indicates that some evolutionary force retards the rate of divergence, compared with neutral expectation. In this and other similar analyses of phenotypic evolution, the most likely retarding force is stabilizing selection (Lande 1976; Charlesworth, Lande, and Slatkin 1982; Lynch 1990).

Prediction of Selection on Salamander Pheromone Genes

Patterns of evolution in morphology and behavior reveal that the salamander pheromone delivery complex has undergone long periods of stabilizing selection on each

Table 1
***Plethodon spp.* from Which Plethodontid Receptivity Factor (PRF) Sequences Were Obtained and PRF Allelic Diversity Found**

	Collection Site				Number of PRF Alleles		
Plethodon sp.	State	County	Latitude (°N)	Longitude (°W)	N	Total	Unique Translation
Olfactory delivery							
P. aureolus	TN	Monroe	35 27.490	84 1.400	2	4	3
P. chattahoochee	GA	Towns	34 52.481	83 48.676	1	1	1
P. cheoah	NC	Swain	35 21.300	83 43.040	1	3	3
P. grobmani[a]	GA	Toombs	32 20.400	82 32.200	1	2	2
P. jordani	TN	Sevier	35 36.637	83 26.274	1	1	1
P. kentucki	VA	Buchanan	37 03.167	82 02.417	1	3	3
P. meridianus	NC	Burke	35 36.050	81 37.430	1	2	2
P. shermani	NC	Macon	36 10.720	83 33.740	10	12	11
P. yonahlossee	NC	McDowell	35 42.975	82 11.326	2	4	3
Vaccination delivery							
P. cinereus	VA	Giles	37 22.030	80 31.560	8	14	12
P. hoffmani	WV	Pocahontas	38 13.717	79 47.967	1	3	3
P. richmondi	VA	Wise	36 53.700	82 37.967	3	12	10
Total						61	54

NOTE.—Pheromone delivery modes, collection localities, and number of specimens sampled (N) are also shown.
[a] Frozen specimen provided by Dr Richard Highton, University of Maryland.

side of a rapid transition in delivery mode that was presumably driven by directional (positive) selection. If the mode of selection at one level in a complex predicts the mode of selection at another level, then this pattern of selection should also characterize molecular evolution. An active component of a plethodontid courtship pheromone has been identified from *Plethodon shermani*, a species with olfactory delivery. The *P. shermani* pheromone is a protein mixture with two major components (Feldhoff, Rollmann, and Houck 1999). Delivery of one of these components, plethodontid receptivity factor (PRF), is sufficient to make females more receptive during courtship (Rollmann, Houck, and Feldhoff 1999). Sequence homology places PRF in the same cytokine family as interleukin-6 (IL-6). These cytokines act as soluble ligands that sequentially bind extracellular domains of at least two transmembrane receptors and so bring together cytoplasmic domains capable of signal transduction (Bravo and Heath 2000). A four–α-helix bundle forms a structural core of the proteins to which receptors bind at conserved positions (Kallen et al. 1999; Bravo and Heath 2000; Hill, Morea, and Chothia 2002).

We compared patterns of selection on the PRF gene with the predictive framework derived from morphology and behavior. We detected significant positive selection within delivery modes, rejecting the molecular analog of stabilizing selection. Contrary to expectation, different evolutionary processes prevail at different levels of organization in this functional complex. We suggest that this uncoupling of modes of selection at each level of organization may be a general feature of functional complexes.

Materials and Methods

Mental Gland Collection

For each study species, males with enlarged premaxillary teeth and/or a visible mental gland were collected from the field during the courtship season (table 1). A single (point) locality was sampled for each species. Mental glands were taken from sedated animals as described by Rollmann, Houck, and Feldhoff (1999), and the animals were sacrificed.

PRF Cloning

PRF sequences were obtained by reverse-transcriptase PCR on cDNA (ImProm II system: Promega) synthesized from mental gland total RNA (Trizol: Invitrogen). PCR primers (5′-AGC ATC AAC GGA GGC AAG AG-3′ and 5′-CCC AAT GCA AGA TAG CTC-3′) were used that anneal to the 5′ and 3′ untranslated regions of *P. shermani* PRF mRNA. *Pfu* polymerase (Stratagene) was used to avoid nucleotide incorporation errors. Amplicons were cloned into TOPO4Blunt (Invitrogen) and sequenced in both directions. This approach identified extensive polymorphism within species. To confirm that this polymorphism was not a PCR artifact, a *P. shermani* mental gland cDNA library was constructed (Lambda-ZapII: Stratagene), and 300 random clones sequenced. The same extensive polymorphism in PRF was found using this non-PCR approach.

Sequence Analyses and Database Searches

Sequences were analyzed with GCG version 10 (Genetics Computer Group, Madison, Wis.). PsiBlast searches of GenBank and of the Conserved Domain Database (www.ncbi.nlm.nih.gov) were used to confirm amplicon homology to PRF and to find related sequences. Protein structure predictions were made with PredictProtein (www.embl-heidelberg.de/predictprotein). Sequences used in selection analyses of IL-6 (26 taxa) and leukemia inhibitory factor (LIF; seven taxa) were obtained from public databases using PsiBlast and key word searches. IL-6 sequences were *Aotus spp.* (AF097323.1, AF014510.1,

AF097322.1, and AF014505.1), *Bos taurus* (X57317.1), *Canis familiaris* (U12234.1 and AF275796.1), *Capra hircus* (D86569.1), *Cercocebus torquatus* (L26032.1), *Delphinapterus leucas* (AF076643.1), *Enhydra lutris* (L46804.1), *Equus caballus* (U64794.1 AF041975.1 AF005227.1), *Felis catus* (L16914.1), *Gallus gallus* (AJ309540.1), *Homo sapiens* (M54894.1 NM_000600.1), *Macaca spp*. (AB000554.1 and L26028.1), *Marmota monax* (AF012908.1 Y14139.1), *Mus musculus* (NM_031168.1), *Orcinus orca* (L46803.1), *Oryctolagus cuniculus* (AF169176.1), *Ovis aries* (X62501.1 and X68723.1), *Phoca vitulina* (L46802.1), *Rattus norvegicus* (NM_012589.1), *Sigmodon hispidus* (AF421389.1), *Saimiri sciureus* (AF294757.1), *Sus scrofa* (AF309651.1, AF493992.1, M86722.1, and M80258.1). LIF sequences were *Bos taurus* (D50337), *Homo sapiens* (NM_002309), *Mus musculus* (NM_008501), *Mustela vison* (AF048827), *Rattus norvegicus* (NM_022196), *Sus scrofa* (AJ296176), *Trichosurus vulpecula* (AF303448).

Phylogenetic Reconstruction

Molecular phylogenies were constructed using maximum-parsimony analyses of nucleotide sequence alignments. Gapped positions were excluded from the data before phylogeny reconstruction. A majority-rule consensus tree (100 random additions) was found using PAUP* version 4.0b10 with the heuristic search mode and random starting seeds. Bootstrap (250 pseudoreplicates) analyses of the alignments were completed, and branches with less than 60% support were collapsed. Other optional parameters were set to the defaults. Outgroups were mouse cardiotrophin-2 (NM-178885.8) for PRF phylogeny reconstructions and *P. shermani* PRF isoform 1 (AAF01025) for analyses of IL-6 and CNTF.

Analyses of Selection

Modes of selection at amino acid sites in proteins and along lineages in molecular phylogenies were identified from estimates of the ratio (ω) of nonsynonymous to synonymous substitution rates (Li 1997). In this test, $\omega = 1$ at neutral sites, whereas $\omega < 1$ or $\omega > 1$ identify purifying or positive selection, respectively. We estimated ω using the maximum-likelihood method implemented by the PAML version 3.13d software package (Yang 1997; Yang et al. 2000; Yang and Nielsen 2002). Analyses of selection were performed on nucleotide sequence alignments and majority-rule consensus trees obtained during phylogenetic reconstructions. Equilibrium codon frequencies were estimated from average nucleotide frequencies at each codon position and transition-transversion rate ratios were estimated from the data. Tests for differences in selection along lineages compared three models: (1) a model with a single ω for all lineages; (2) a model in which a separate ω was estimated for each lineage; and (3) a model in which two different ω values are allowed, one value for the branch leading to the change in delivery mode and a second value for the remaining branches that have a stable delivery mode. Tests for variation in selection among sites compared models described in detail by Yang et al. (2000). Briefly, these were a null model M1, in which ω at each site was forced to be either 0 or 1, corresponding to a strict interpretation of Kimura's neutral theory of protein evolution, and M3, in which sites are assigned to one of three discrete ω value categories estimated from the data. M3 permits $\omega > 1$ and so allows for positive selection. Models were compared using log-likelihood values in a chi-square test with 2 degrees of freedom (Yang 1998). We also compared continuous distribution models (M5 to M10) described by Yang et al. (2000) but found no significant differences between M1 or M3 and equivalent continuous models, so results for M5 to M10 are not reported.

Results

PRF Is Maintained Across a Delivery Mode Transition and Is Polyallelic

We constructed a composite phylogeny of *Plethodon spp*. (fig. 1) from published allozyme studies (Highton and Larson 1979; Larson et al. 1981; Highton and Peabody 2000). Mapping courtship behavior (known or inferred from mental gland size and presence/absence of premaxillary teeth) onto this phylogeny (fig. 1) supports the Houck and Sever (1994) inference of a single evolutionary transition from vaccination to olfactory delivery modes. We then collected 12 *Plethodon* species spanning this transition, restricting our choice of species to those in which pheromone delivery mode is unambiguously known. We obtained complete open reading frames for PRF from each of the 12 species. This survey identified 58 PRF sequences, including four known *P. shermani* isoforms (Rollmann, Houck, and Feldhoff 1999), with considerable nonsynonymous polymorphism (49 derived primary sequences [table 1]). Each species had a unique complement of alleles, but three sequences occurred in more than one species, for a total of 61 alleles.

Some variation in PRF has arisen from gene duplication. Species having olfactory delivery yielded up to five alleles per individual, so they must express three or more PRF genes in the mental gland. Sequence variation among all alleles from these species was less than 5%, and many differ by only a single nucleotide replacement. In every species, strongly conserved untranslated regions flank the variable coding regions. PRF sequences from species having vaccination delivery were of two types that differed by about 15%. Variation within each type was similar to that among sequences from species having olfactory delivery. Each individual provided up to two discrete sequences for each type, suggesting vaccinating species express two divergent PRF genes in the mental gland.

To estimate total allelic variation for PRF in a single population, 10 *P. shermani* (olfactory delivery) and eight *P. cinereus* (vaccination delivery) individuals were surveyed. Eleven and 14 alleles were identified, respectively, in these two species. A continuing yield of new sequences with every individual shows we had not yet identified all alleles. PRF occurs with many slight variants and is highly variable within populations.

Phylogenetic Associations Among PRF Sequences

Relationships among PRF sequences were explored by maximum-parsimony cladistic analyses (fig. 2). This analysis identified two major clades of PRF sequences, each with bootstrap support greater than 95%. One major clade (type A) contained all PRF sequences identified from species having olfactory delivery, as well as sequences from one of the two PRF types from vaccinating species. The second major clade (type B) contained the other sequence type from vaccinating species. Within the two major clades, PRF sequences from vaccinating species cluster together (fig. 2). Sequences from species having olfactory delivery appear to cluster at random with many unresolved branch points. Apparently, two genes arising from an ancient duplication event are expressed in mental glands of vaccinating species. Olfactory species now express one of these ancestral gene types in the gland, but this gene has also been duplicated.

PRF Is Under Positive Selection Within Delivery Modes

Evolutionary analyses of morphology and behavior show that the plethodontid pheromone delivery complex has undergone long periods of stabilizing selection either side of a transition from vaccination to olfactory delivery. We compared this result to patterns of selection on the signal molecule. An estimate of ω for each branch in a PRF phylogeny rejected stabilizing (purifying) selection within delivery modes ($P < 0.001$), in favor of models in which multiple lineages are under positive selection (fig. 2). In contrast, we could not reject neutrality for the branch joining delivery modes, despite free ratio models identifying it as under weak positive selection ($\omega = 1.54$), and in two-rate models, there was no support for models in which selection over that branch was greater than for the branches within delivery modes ($P = 0.48$).

Analyses of variation in selection over sites (amino acid positions) in PRF also rejected neutral models ($P < 0.001$) in favor of a model in which 8% of sites have undergone positive selection ($\omega = 5.45$), with 62% of remaining sites neutral and 30% under purifying selection (table 2). Analyzing sequences from within the vaccination or olfactory delivery modes separately showed variation at 5% of sites in PRF delivered by vaccination and up to 25% in PRF delivered by olfaction, is explained by positive selection (table 2). We can reject the hypothesis of concordance between selection modes at the levels of morphology or behavior and at the molecular level in this functional complex.

Positive Selection in PRF Occurs at Receptor Binding Epitopes

From our analyses of selection, we identified the positions of amino acid sites (codons) in PRF that have undergone positive selection (fig. 3) and then used sequence similarity between PRF and the IL-6 cytokine family to form hypotheses about functions for the positively selected sites (Ciapponi et al. 1995; Clackson and Wells 1995; Panayotatos et al. 1995; Hudson, Vernallis, and Heath

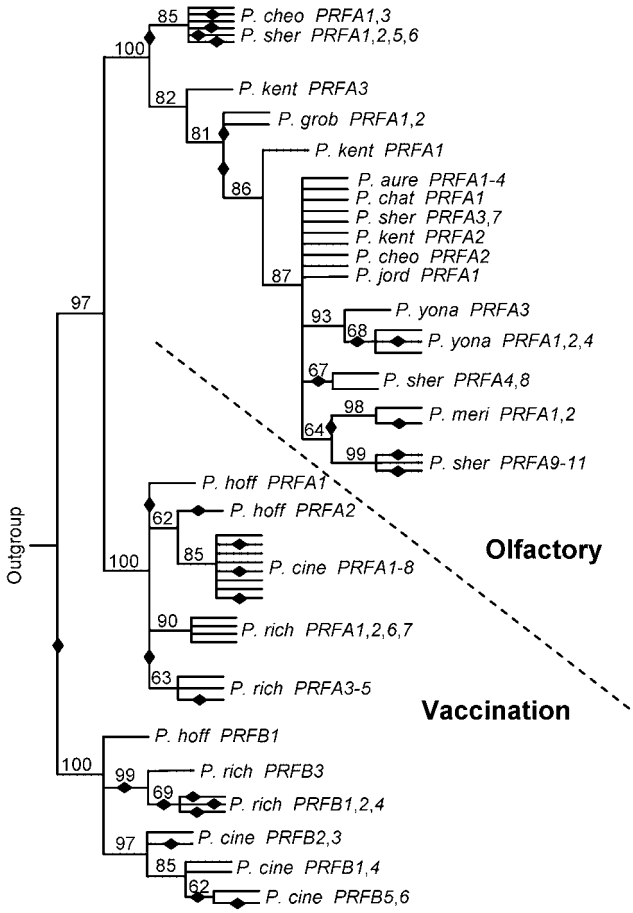

FIG. 2.—Maximum-parsimony tree of 61 plethodontid receptivity factor (PRF) sequences from 12 *Plethodon spp*. and relationship to delivery mode. Diamonds identify lineages under positive selection ($\omega > 2.0$). Bootstrap values are given for nodes having over 60% support; all other nodes are drawn as polytomies. Species are identified by the first four letters of the species name (cf. table 1). PRF sequences were denoted type A or B according to which of two major clades they fall into and then given sequential numerical designators. Phylogeny was rooted with mouse CNTF–like sequence XM_146055.

1996; Wells 1996; Behncken et al. 1997; Kallen et al. 1999; Bravo and Heath 2000; Hill, Morea, and Chothia 2002). PRF is most similar to neurotrophin-1, followed by cardiotrophin-1, CNTF, leukemia inhibitory factor (LIF), oncostatin-M, and IL-6. These four–α-helix cytokines all bind a shared receptor, gp130 at binding site II. PRF is predicted to have four α-helices and a long-short-long loop pattern that maps onto the structure of these cytokines, and this was used to align PRF with related cytokines (fig. 4). From this alignment, PRF has a F-X-X-K motif at the N-terminal end of the fourth α-helix and a Pro residue in the central loop that are conserved in cytokines that bind the LIF signaling receptor (LIF-R) at binding site III. PRF lacks a G-X-X-X-N site II motif conserved in the third helix of LIF-R binding cytokines that bind gp130 with high affinity. A model for PRF-receptor interactions is then that a non-signaling receptor binds at site I, promoting gp130 binding at site II. An LIF-R–related signaling receptor then binds at site III to generate the active signaling complex (Bravo and Heath 2000).

Table 2
Nonsynonymous to Synonymous Substitution Rate Ratios (ω), Prior Probabilities ($p[\omega]$), and Log-Likelihood Values (lnL) for Selection Models Fitted to Plethodontid Receptivity Factor (PRF) and Two Cytokines in the Same Structural Family

Cytokine	Sample Size: N_{seqs} (N_{taxa})	Selection Model		
		M1 (neutral)	M3	χ^2
PRF (both delivery modes)	61 (12)	$p(\omega = 0) = 0.33$ $p(\omega = 1) = 0.66$	$p(\omega = 0.00) = 0.30$ $p(\omega = 1.15) = 0.62$ **$p(\omega = 5.45) = 0.08$**	
		lnL = −2650.3	lnL = −2630.4	<0.01
PRF (olfactory delivery only)	32 (9)	$p(\omega = 0) = 0.74$ $p(\omega = 1) = 0.26$	$p(\omega = 0.00) = 0.75$ **$p(\omega = 2.99) = 0.21$** **$p(\omega = 11.38) = 0.04$**	
		lnL = −1589.2	lnL = −1454.6	<0.01
PRF (vaccination delivery only)	29 (3)	$p(\omega = 0) = 0.47$ $p(\omega = 1) = 0.53$	$p(\omega = 0.00) = 0.50$ $p(\omega = 1.73) = 0.45$ **$p(\omega = 7.82) = 0.05$**	
		lnL = −2018.7	lnL = −1454.6	<0.01
Interleukin-6 (IL-6)	35 (26)	$p(\omega = 0) = 0.12$ $p(\omega = 1) = 0.88$	$p(\omega = 0.13) = 0.25$ $p(\omega = 0.63) = 0.44$ $p(\omega = 1.23) = 0.30$	
		lnL = −4563.6	lnL = −4528.6	<0.01
Leukemia Inhibitory Factor (LIF)	7 (7)	$p(\omega = 0) = 0.5$ $p(\omega = 1) = 0.5$	$p(\omega = 0.026) = 0.33$ $p(\omega = 0.026) = 0.34$ $p(\omega = 0.26) = 0.32$	
		lnL = −2240.4	lnL = −2147.8	<0.01

NOTE.—Signatures of strong positive selection are shown in bold.

The 21 sites in PRF predicted to be under positive selection (mean $\omega > 2.5$) are distributed across the PRF primary sequence in a pattern that varies slightly between delivery modes (fig. 3). About half of these sites associate with known receptor-binding epitopes (fig. 4). Seven sites align with residues in the first, second, and fourth helices and first interhelix loop that significantly affect receptor binding at the site I epitope of related cytokines, and others are in close proximity to such residues. Three positively selected sites are near residues that affect binding at the site II epitope where gp130 binds. Two others are close to residues that affect binding at the site III epitope where an LIF-R like receptor may bind. Roughly one-third of positively selected sites do not align with known receptor-binding epitope residues. These may be false positives or residues not yet identified as important for receptor binding that indirectly modulate the receptor-binding surface (Clackson and Wells 1995; Boulanger et al. 2003). The association of many positively selected sites in PRF with receptor-binding epitopes suggests that receptor variation is a significant source of selection on this signal.

Evolution of Two Related Cytokines Is Nearly Neutral

To test whether PRF is evolving in a manner different from similar proteins, we analyzed some other four–α-helix cytokines for positive selection. PRF is the only amphibian cytokine known from this family, so we tested mammalian members. Growth hormone has previously been analyzed (Liu et al. 2001) and does not have sites under strong positive selection, although in primates, receptor-binding sites have more substitutions than other sites, implying positive selection. Only IL-6 (26 taxa) and LIF (seven taxa) provided large enough data sets for analysis. Phylogenies for these proteins in our analyses were essentially as described previously (King et al. 1996). Analyses of selection revealed that all sites in these two proteins are under moderate to strong purifying selection or are nearly neutral (table 2). For PRF, we obtained ω values in the range 5.45 to 11.38, whereas IL-6 and LIF yielded ω values less than 1.23. This absence of positive selection implies that the evolutionary change from internal signaling to two-party pheromone signaling has altered the way in which PRF evolves.

Discussion

The evolutionary dynamics of the plethodontid pheromone signaling complex has three characteristics: (1) stabilizing selection on behavioral and morphological aspects of signal delivery; (2) heterogeneity of the signal within contemporary populations; and (3) positive selection on the signal. Strong positive selection at the molecular level presents a striking contrast with long-term stasis at the morphological and behavioral levels in this functional complex. In our plethodontid system, stasis in behavior and morphology apparently is a consequence of intricate functional coupling between males and females (Houck and Arnold 2003). During courtship, the behavior of the male and female is dynamically adjusted to the behavioral responses of the mating partner (Arnold 1976). Pheromone delivery is embedded in a larger courtship ritual that includes a complicated tail-straddling walk (Houck and Arnold 2003). Deviations in this two-party ritual apparently are opposed by stabilizing selection, resulting in stasis over tens of millions of years. These stabilizing aspects of selection do not extend to the molecular level. The pheromone signal appears to be

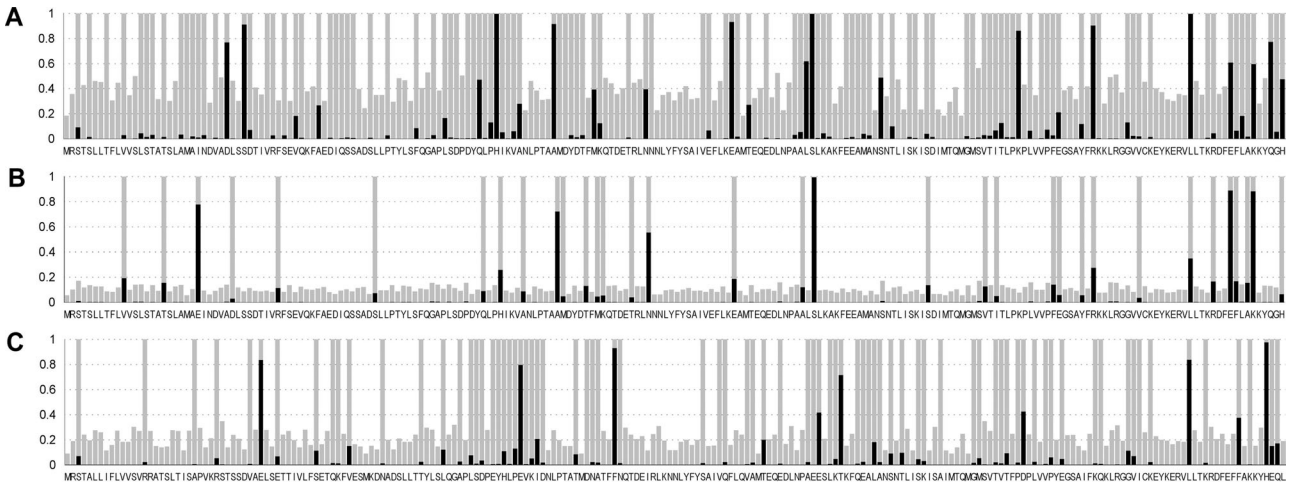

FIG. 3.—Maximum-likelihood identification of amino acid sites (codons) along plethodontid receptivity factor (PRF) under positive selection. A discrete model (M3) was used to fit three classes of sites (ω values) to the gene. Bars give the (posterior) probabilities that a given site is in each site class: white bars indicate $p(\omega_1)$, gray bars indicate $p(\omega_2)$, and black bars indicate $p(\omega_3)$. See table 2 for the estimated frequencies (prior probabilities) of each site class and Yang et al. (2000) for further description of the interpretation of the histogram. (*A*) Analysis of PRF across olfactory and vaccination delivery modes: $\omega_1 = 0.00$, $\omega_2 = 1.15$, and $\omega_3 = 5.45$. (*B*) Analysis of PRF within the olfactory delivery mode: $\omega_1 = 0.00$, $\omega_2 = 2.98$, and $\omega_3 = 11.38$. (*C*) Analysis of PRF within the vaccination delivery mode: $\omega_1 = 0.00$, $\omega_2 = 1.73$, and $\omega_3 = 7.82$.

uncoupled from the stabilizing influences of the two-party courtship ritual. As a consequence, the complex shows discordant evolutionary patterns at different levels of organization.

Relaxed coupling between modes of selection at different levels of organization may be a general feature in functional complexes. For example, insect pheromone receptors can dynamically track shifts in signal characteristics despite stable morphology and behavior (Lofstedt 1993; Roelofs et al. 2002). Similarly, in predator envenomation of prey, stable behaviors can overlie dynamic adjustment of the venom component, and the converse may also be true (Klauber 1956; Downes and Shine 1998; Duda and Palumbi 1999; Olivera 1999). In a third example, web silk can be modified independently of web design (Clarke and McMahon 1996; Olivera 1999; Opell 1999). In intricate complexes such as these, modes of selection will probably be discordant across levels of organization. We must separately assess selection modes at each level to understand the evolution of such functional complexes.

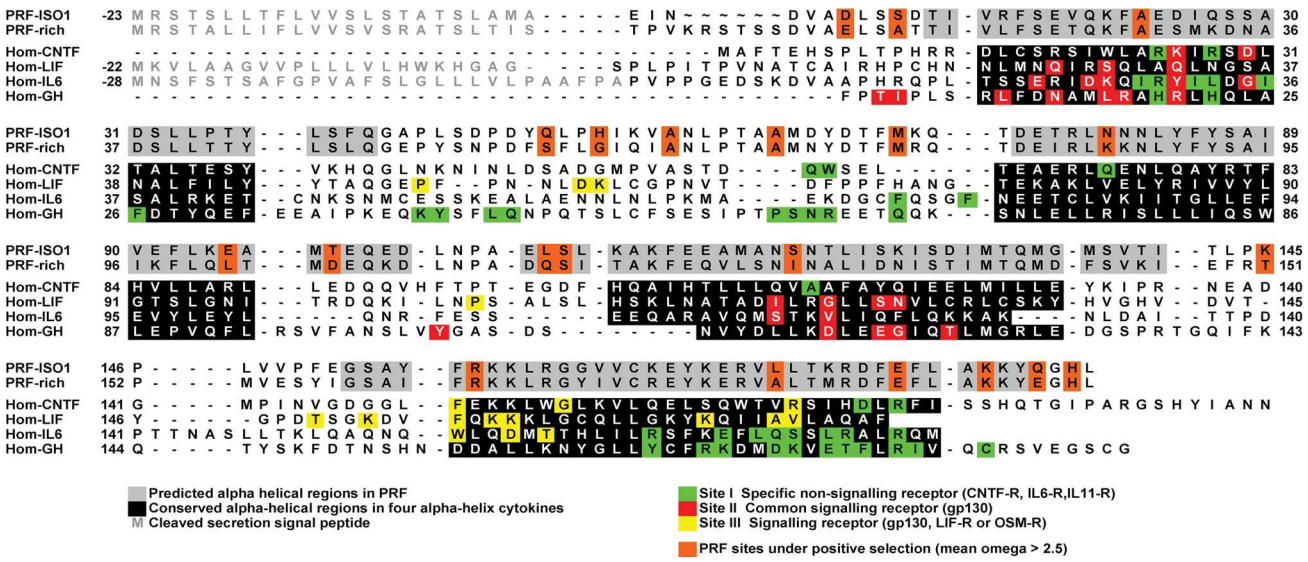

FIG. 4.—Structural alignment of PRF sequences from *P. shermani* and *P. richmondi* with some human four–α-helix cytokines. Putative α-helices in PRF (gray regions) are aligned with known human cytokine structures (based on Hill, Morea and Chothia [2002]). Black regions form a conserved four–α-helix bundle. The alignment is less reliable outside these regions. Residues in the human cytokines that give less than 50% reductions in receptor affinity after mutagenesis are green, red, or yellow, according to three conserved receptor recognition sites (sites I to III). Residues in PRF under positive selection (mean $\omega > 2.5$) are orange. Cytokines and GenBank accession numbers are PRF-ISO1 (*P. shermani* PRF isoform1: AAF01025), PRF-rich (*P. richmondi* PRF A1: this study), Hom-CNTF (ciliary neurotrophic factor: NP_000605), Hom-LIF (leukemia inhibitory factor: NP_002300), Hom-GH (growth hormone: NP_000506), and Hom-IL6 (interlukin-6: NP_000591).

Although positive selection on reproductive aspects of phenotype is widespread at multiple levels of organization (Kingsolver et al. 2001; Swanson and Vacquier 2002), mechanistic details of mating are frequently conserved over tens of millions of years. Our analysis of the salamander courtship functional complex shows that positive selection on molecular traits can underlie this conservation. This masking of positive selection is particularly likely to be true for chemical communication systems. The majority of identified cases of positive selection acting at the molecular level have been for proteins that mediate postinsemination processes. However, diversification of pheromone receptor genes in moths and in primates (Willett 2000; Mundy and Cook 2003) and the signal component of the salamander courtship pheromone communication system have now been found to be driven by positive selection. Pheromone proteins of several microorganisms also show extensive sequence variation. Tests for selection have yet to be completed on these proteins, but positive selection in pheromonal and other chemical communication systems is likely to be common (Swanson and Vacquier 2002).

Selection on pheromones may be the result of natural or sexual selection (Arnold and Houck 1982), and our salamander data cannot distinguish between these possibilities. Stabilizing selection on the delivery system seems to argue against sexual conflict, in which pheromone delivery or costs of mating reduce female fitness while increasing male fitness (Parker and Partridge 1998; Chapman et al. 2003). In this scenario, female resistance to males drives signal diversification, leading to perpetual coevolution of signals and receptors and to high levels of polymorphism within populations (Gavrilets 2000; Gavrilets and Waxman 2002). Sexual selection can produce an evolutionary pattern in which female receptors constantly change as a correlated response to the evolution of male signals (Lande 1981) or as a result of male exploitation of a female bias towards a complex signal. Natural selection, for example arising from virally encoded cytokine mimics (Moore et al. 1996), or drift acting in females might result in an ever-changing population of receptors that males must track (Lofstedt 1993). Additional observations are needed to distinguish between these selection scenarios.

Our analysis assigned PRF to the group of four–α-helix cytokines that bind the gp130 receptor. Conservation of receptor-binding strategies in this protein family (Bravo and Heath 2000) means that we can expect PRF to use similar receptor-binding sites. Not all members of the gp130 binding class of cytokines bind receptors at site I, but on those most similar to PRF, a specificity-determining nonsignaling receptor binds there (Panayotatos et al. 1995; Bravo and Heath 2000). Many positively selected amino acid sites in PRF are likely to affect charge distributions at site I, so a receptor that determines specificity of action for PRF probably mediates this selection. It is likely that PRF also interacts with two shared signaling receptors: a LIF-R–like receptor at site III and gp130 at low affinity at site II. Fewer strongly selected amino acid sites in PRF are associated with those two sites. If, as in mammals, these sites also bind shared receptors in amphibians, they would be more constrained than site I. From the context in which PRF acts, the receptors it binds are likely to be in females. Selection on PRF arises from the pheromone signaling function placing a selective premium on males producing a signal that can be recognized by mixed and/or changing populations of female receptors. The nature of selection at the molecular level in our system warrants further study.

Supplementary Material

The sequences reported in this manuscript have been deposited in GenBank under accession numbers AY499347 to AY499404, inclusive.

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Literature Cited

Arnold, S. J. 1976. Sexual behavior, sexual interference, and sexual defense in the salamanders *Ambystoma maculatum*, *Ambystoma tigrinum* and *Plethodon jordani*. Z. Tierpsychol. **42**:247–300.

———. 1977. The courtship behavior of North American salamanders with some comments on Old World salamandrids. Pp. 141–83 *in* D. Taylor and S. Guttman, eds. The reproductive biology of amphibians. Plenum Press, New York.

Arnold, S. J., and L. D. Houck 1982. Courtship pheromones: evolution by natural and sexual selection. Pp. 173–211 *in* M. Nitecke, ed. Biochemical aspects of evolutionary biology. University of Chicago Press, Chicago.

Barrier, M., C. D. Bustamante, J. Yu, and M. D. Purugganan. 2003. Selection on rapidly evolving proteins in the Arabidopsis genome. Genetics **163**:723–733.

Behncken, S. N., S. W. Rowlinson, J. E. Rowland, B. L. Conway-Campbell, T. A. Monks, and M. J. Waters. 1997. Aspartate 171 is the major primate-specific determinant of human growth hormone: engineering porcine growth hormone to activate the human receptor. J. Biol. Chem. **272**:27077–27083.

Boag, P. T., and P. R. Grant. 1981. Intense natural selection in a population of Darwin's finches (Geospizinae) in the Galapagos. Science **214**:82–85.

Boulanger, M. J., A. J. Bankovich, T. Kortemme, D. Baker, and K. C. Garcia. 2003. Convergent mechanisms for recognition of divergent cytokines by the shared signaling receptor gp130. Mol Cell **12**:577–589.

Bravo, J., and J. K. Heath. 2000. Receptor recognition by gp130 cytokines. EMBO J. **19**:2399–2411.

Chapman, T., G. Arnqvist, J. Bangham, and L. Rowe. 2003. Sexual conflict. Trends Ecol. Evol. **18**:41–47.

Charlesworth, B. R., R. Lande, and M. Slatkin. 1982. A neo-Darwinian commentary on macroevolution. Evolution **36**:474–498.

Ciapponi, L., R. Graziani, G. Paonessa, A. Lahm, G. Ciliberto, and R. Savino. 1995. Definition of a composite binding site

for gp130 in human interleukin-6. J. Biol. Chem. **270**:31249–32154.

Clackson, T., and J. A. Wells. 1995. A hot spot of binding energy in a hormone-receptor interface. Science **267**:383–386.

Clarke, M., and R. F. McMahon. 1996. Comparison of byssal attachment in dreissenid and mytilid mussels: mechanisms, morphology, secretion, biochemistry, mechanics and environmental influences. Malacol. Rev. **29**:25.

Downes, S., and R. Shine. 1998. Sedentary snakes and gullible geckos: predator-prey coevolution in nocturnal rock-dwelling reptiles. Anim. Behav. **55**:1373–1385.

Duda, T. F., Jr., and S. R. Palumbi. 1999. Molecular genetics of ecological diversification: duplication and rapid evolution of toxin genes of the venomous gastropod *Conus*. Proc. Natl. Acad. Sci. USA **96**:6820–6823.

Endo, T., K. Ikeo, and T. Gojobori. 1996. Large-scale search for genes on which positive selection may operate. Mol Biol Evol **13**:685–690.

Feldhoff, R. C., S. M. Rollmann, and L. D. Houck. 1999. Chemical analyses of courtship pheromones in a plethodontid salamander. Pp. 117–125 *in* R. E. Johnston, D. Muller-Schwarze, and P. Sorensen, eds. Advances in chemical communication in vertebrates. Plenum Press, New York.

Gavrilets, S. 2000. Rapid evolution of reproductive barriers driven by sexual conflict. Nature **403**:886–889.

Gavrilets, S., and D. Waxman. 2002. Sympatric speciation by sexual conflict. Proc. Natl. Acad. Sci. USA **99**:10533–10538.

Highton, R. 1962. Revision of North American salamanders of the genus *Plethodon*. Bull. Florida State Mus. **6**:235–367.

Highton, R., and A. Larson. 1979. The genetic relationships of the salamanders of the genus *Plethodon*. Syst. Zool. **28**:579–599.

Highton, R., and R. B. Peabody. 2000. Geographic protein variation and speciation in salamanders of the *Plethodon jordani* and *Plethodon glutinosus* complexes in the southern Appalachian Mountains with the descriptions of four new species. Pp. 31–94 *in* R. C. Bruce, R. G. Jaeger, and L. D. Houck, eds. The biology of plethdontid salamanders. Plenum Press, New York.

Hill, E. E., V. Morea, and C. Chothia. 2002. Sequence conservation in families whose members have little or no sequence similarity: the four-helical cytokines and cytochromes. J. Mol. Biol. **322**:205–233.

Houck, L. D., and S. J. Arnold. 2003. Courtship and mating behavior. Pp. 383–424 *in* D. M. Sever, ed. Reproductive biology and phylogeny of Urodela. Science Publishers, Enfield, New Hampshire.

Houck, L. D., and N. L. Reagan. 1990. Male courtship pheromones increase female receptivity in a plethodontid salamander. Anim. Behav. **39**:729–734.

Houck, L. D., and D. M. Sever. 1994. Role of the skin in reproduction and behaviour. Pp. 351–381 *in* H. Heatwole and G. T. Barthalmus, eds. Amphibian biology, Vol. 1. The integument. Surrey Beatty & Sons, New South Wales, Australia.

Hudson, K. R., A. B. Vernallis, and J. K. Heath. 1996. Characterization of the receptor binding sites of human leukemia inhibitory factor and creation of antagonists. J. Biol. Chem. **271**:11971–11978.

Kallen, K. J., J. Grotzinger, E. Lelievre, P. Vollmer, D. Aasland, C. Renne, J. Mullberg, K. H. Myer zum Buschenfelde, H. Gascan, and S. Rose-John. 1999. Receptor recognition sites of cytokines are organized as exchangeable modules: transfer of the leukemia inhibitory factor receptor-binding site from ciliary neurotrophic factor to interleukin-6. J. Biol. Chem. **274**:11859–11867.

Kimura, M. 1983. The neutral theory of molecular evolution. Cambridge University Press, Cambridge, UK.

King, D. P., M. D. Schrenzel, M. L. McKnight, T. H. Reidarson, K. D. Hanni, J. L. Stott, and D. A. Ferrick. 1996. Molecular cloning and sequencing of interleukin 6 cDNA fragments from the harbor seal (*Phoca vitulina*), killer whale (*Orcinus orca*), and southern sea otter (*Enhydra lutris nereis*). Immunogenetics **43**:190–195.

Kingsolver, J. G., H. E. Hoekstra, J. M. Hoekstra, D. Berrigan, S. N. Vignieri, C. E. Hill, A. Hoang, P. Giber, and P. Beerli. 2001. The strength of phenotypic selection in natural populations. Am. Nat. **157**:245–261.

Klauber, L. M. 1956. Rattlesnakes: their habits, life histories, and influence on mankind. University of California Press, Berkeley, Calif.

Lande, R. 1976. Natural selection and random genetic drift in phenotypic evolution. Evolution **30**:314–334.

———. 1981. Models of speciation by sexual selection on polygenic traits. Proc. Natl. Acad. Sci. USA **78**:3721–3725.

Larson, A., D. B. Wake, L. R. Maxson, and R. Highton. 1981. A molecular phylogenetic perspective on the origins of morphological novelties in the salamanders of the tribe Plethodontini. (Amphibia, Plethodontidae). Evolution **35**:405–422.

Larson, A., D. W. Weisrock, and K. H. Kozak. 2003. Phylogenetic systematics of salamanders (Amphibia: Urodela): a review. Pp. 31–108 *in* D. M. Sever, ed. Reproductive biology and phylogeny of Urodela. Science Publishers, Enfield, New Hampshire.

Li, W.-H. 1997. Molecular evolution. Sinauer Associates, Sunderland, Mass.

Liu, J. C., K. D. Makova, R. M. Adkins, S. Gibson, and W. H. Li. 2001. Episodic evolution of growth hormone in primates and emergence of the species specificity of human growth hormone receptor. Mol. Biol. Evol. **18**:945–953.

Lofstedt, C. 1993. Moth pheromone genetics and evolution. Philos. Trans. R. Soc. Lond. B Biol. Sci. **340**:167–177.

Lynch, M. 1990. The rate of morphological evolution in mammals from the standpoint of the neutral expectation. Am. Nat. **136**:727–741.

Miller, G. T., and S. Pitnick. 2002. Sperm-female coevolution in *Drosophila*. Science **298**:1230–1233.

Moore, P. S., C. Boshoff, R. A. Weiss, and Y. Chang. 1996. Molecular mimicry of human cytokine and cytokine response pathway genes by KSHV. Science **274**:1739–1744.

Mundy, N. I., and S. Cook. 2003. Positive selection during the diversification of class I vomeronasal receptor-like (V1RL) genes, putative pheromone receptor genes, in human and primate evolution. Mol. Biol. Evol. **20**:1805–1810.

Olivera, B. M. 1999. *Conus* venom peptides: correlating chemistry and behavior. J. Comp. Physiol. A Sens. Neural. Behav. Physiol. **185**:353–359.

Opell, B. D. 1999. Changes in spinning anatomy and thread stickiness associated with the origin of orb-weaving spiders. Biol. J. Linn. Soc. **68**:593–612.

Panayotatos, N., E. Radziejewska, A. Acheson, R. Somogyi, A. Thadani, W. A. Hendrickson, and N. Q. McDonald. 1995. Localization of functional receptor epitopes on the structure of ciliary neurotrophic factor indicates a conserved, function-related epitope topography among helical cytokines. J Biol Chem **270**:14007–14014.

Parker, G. A., and L. Partridge. 1998. Sexual conflict and speciation. Philos. Trans. R. Soc. Lond. B Biol. Sci. **353**:261–274.

Roelofs, W. L., W. T. Liu, G. X. Hao, H. M. Jiao, A. P. Rooney, and C. E. Linn. 2002. Evolution of moth sex pheromones via ancestral genes. Proc. Natl. Acad. Sci. USA **99**:13621–13626.

Rollmann, S. M., L. D. Houck, and R. C. Feldhoff. 1999. Proteinaceous pheromone affecting female receptivity in a terrestrial salamander. Science **285**:1907–1909.

Ruben, J. A., N. L. Reagan, P. A. Verrell, and A. J. Boucot. 1993. Plethodontid salamander origins: a response to Beachy and Bruce. Am. Nat. **142**:1038–1051.

Schubart, C. D., R. Diesel, and S. B. Hedges. 1998. Rapid evolution to terrestrial life in Jamaican crabs. Nature **393**:363–365.

Stahl, E. A., and J. G. Bishop. 2000. Plant-pathogen arms races at the molecular level. Curr. Opin. Plant Biol. **3**:299–304.

Swanson, W. J., and V. D. Vacquier. 2002. The rapid evolution of reproductive proteins. Nat. Rev. Genet. **3**:137–144.

Wells, J. A. 1996. Binding in the growth hormone receptor complex. Proc. Natl. Acad. Sci. USA **93**:1–6.

Willett, C. S. 2000. Evidence for directional selection acting on pheromone-binding proteins in the genus *Choristoneura*. Mol. Biol. Evol. **17**:553–562.

Wirsig-Wiechmann, C. R., L. D. Houck, P. W. Feldhoff, and R. C. Feldhoff. 2002. Pheromonal activation of vomeronasal neurons in plethodontid salamanders. Brain Res. **952**:335–344.

Yang, Z. H. 1997. PAML: a program package for phylogenetic analysis by maximum likelihood. Comp. Appl. Biosci. **13**: 555–556.

———. 1998. Likelihood ratio tests for detecting positive selection and application to primate lysozyme evolution. Mol. Biol. Evol. **15**:568–573.

Yang, Z. H., and J. P. Bielawski. 2000. Statistical methods for detecting molecular adaptation. Trends Ecol. Evol. **15**:496–503.

Yang, Z. H., and R. Nielsen. 2002. Codon-substitution models for detecting molecular adaptation at individual sites along specific lineages. Mol. Biol. Evol. **19**:908–917.

Yang, Z. H., R. Nielsen, N. Goldman, and A. M. K. Pedersen. 2000. Codon-substitution models for heterogeneous selection pressure at amino acid sites. Genetics **155**:431–449.

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