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Multiple Sex Pheromone Genes Are Expressed in the Abdominal Glands of the Smooth Newt (*Lissotriton vulgaris*) and Montandon's Newt (*L. montandoni*) (Salamandridae)

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The smooth newt (*Lissotriton "Triturus" vulgaris*) and Montandon's newt (*L."T." montandoni*) are sister species exhibiting pronounced differences in male secondary sexual traits but nevertheless hybridizing and producing fertile hybrids in nature. Since pheromonal communication is an important aspect of the reproductive biology of urodeles, structural differentiation of peptide pheromones and their receptors may contribute to incipient reproductive isolation. The aim of the study was the identification of genes encoding putative courtship pheromone precursors in two newt species and the reconstruction of phylogenetic relationships among them. Our analyses were based on cDNA obtained from the transcripts from the abdominal glands of male newts. We identified five unique cDNA sequences encoding the putative pheromone precursors in *L. vulgaris* and three additional unique sequences in *L. montandoni*. The results indicate that in the abdominal glands of *Lissotriton* newts more than one pheromone-encoding gene is expressed and that these loci form a gene family. Phylogenetic analysis indicates that the divergence of at least some of these genes predates the radiation of European newts.

Key words: abdominal glands, *Lissotriton*, newts, peptide pheromones, sodefrin

INTRODUCTION

Peptide pheromones produced by specialized glands are thought to be the most important factor in mate recognition during courtship in tailed amphibians (Caudata) (Houck, 1986; Rollmann et al., 1999; Toyoda et al., 2004). The first peptide pheromone in vertebrates, sodefrin, was isolated from the abdominal glands of the male red-bellied newt (*Cynops pyrrhogaster*, Family Salamandridae) (Kikuyama et al., 1995). Isolation and identification of this pheromone was preceded by experiments showing that water in which males were kept attracted females, while water in which abdominal gland–ablated males were kept did not attract females (Toyoda et al., 1994). Sodefrin is a decapeptide, with amino-acid sequence SIPSKDALLK, and shows no similarity to any other known peptide (Kikuyama et al., 1995). Subsequently, a sodefrin-like peptide sex pheromone called silefrin was identified in a closely related species, *C. ensicauda* (Yamamoto et al., 2000). Silefrin differs from sodefrin by two amino-acid substitutions (SILSKDAQLK).

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Biological tests with natural and synthesized pheromones have shown that sodefrin and silefrin attract only conspecific females, suggesting that differences in the amino-acid composition of peptide pheromones may play a crucial role in species recognition during mating in newts. Further studies in *C. pyrrhogaster* brought about the discovery of a regional variant of sodefrin, [Val^8]sodefrin (also called aonirin), isolated from males from a local population of *C. pyrrhogaster*, and differing from sodefrin by only one amino-acid substitution (valine for lysine at position 8) (Nakada et al., 2007b). Aonirin attracts the females from this local population but does not attract females from other populations (in which aonirin has not been found in male abdominal glands). On the other hand, the gene encoding the common pheromone, sodefrin, is expressed in all populations tested, and the females from all locations react positively to this common form of the peptide (Nakada et al., 2007b). This observation supports previous conclusions that even minor differences in the amino-acid sequence of peptide pheromones in newts may lead to reproductive isolation and speciation (Kikuyama et al., 1995; Yamamoto et al., 2000).

Molecular cloning of cDNA synthesized on the basis of mRNA encoding sodefrin revealed that in *Cynops* the biologically active pheromone is situated at amino-acid positions 177–186 of the precursor, which consists of 189

amino-acid residues (Iwata et al., 1999). cDNA showing 81% sequence similarity to the cDNA encoding the sodefrin precursor has also been produced from the abdominal gland extract of *Triturus carnifex* (Salamandridae) (GeneBank accession no. AF446080). The sequence of the biologically active pheromone in this species has not yet been identified.

The smooth newt (*Lissotriton "Triturus" vulgaris*) and Montandon's newt (*L. "T." montandoni*) (Family Salamandridae) are sister species separated by a moderate genetic distance (D_N=0.41; Rafiński and Arntzen, 1987). Despite substantial morphological differences in male epigamic traits, these two species readily hybridize under natural and laboratory conditions (Michalak et al., 1997) and show extensive mtDNA introgression (Babik et al., 2003; 2005; Babik and Rafiński, 2004). The most apparent features distinguishing the males of the two species are a large dorsal skin crest, toe flaps, and large black spots on the flanks and the belly of *L. vulgaris* males, whereas *L. montandoni* males lack these characters but possess a long filament at the end of the tail and black hind feet. Moreover, *L. vulgaris* males are larger than females, an unusual situation in urodeles. The moderate genetic distance along with high morphological differentiation meet the requirements listed by Butlin and Tregenza (1997) that characterize potential model species pairs for investigations into the process of speciation. For these reasons, various behavioral (e.g. Michalak et al., 1997) as well as morphological and genetic (Babik et al., 2003, 2005) aspects of *L. vulgaris* and *L. montandoni* hybridization have been investigated.

The elaborate and complex mating behavior of European newts of the former genus *Triturus* has been described in detail from the evolutionary perspective (Halliday 1976; 1977; 1990; Pecio and Rafiński, 1985). Mating takes place in the spring in small water bodies. Male courtship involves sequences of visual, chemical, and tactile stimuli (Halliday, 1977). In the culminating moment of courtship, the male deposits a spermatophore later picked up by the female's cloacal lips. The sperm is then stored in the female sperm storage organ, the spermatheca, and used for fertilization of eggs, laid one-by-one during a prolonged egg-laying period.

During courtship the male newts vibrate their tails, driving water from the proximity of their cloacas in the direction of the female. Similar behavior is also part of the courtship of *Cynops* males (Toyoda and Kikuyama, 2000). The most probable explanation for this behavior is the creation of a stream carrying pheromones produced by male abdominal glands towards the female (Halliday, 1990; Toyoda et al., 2004). Pecio and Rafiński (1985) confirmed that the rate of tail vibration differs between the smooth and Montandon's newts. This difference may contribute to species recognition as a tactile stimulus and/or influence the intensity of pheromone delivery to the female (Michalak et al., 1997).

Despite hybridization in nature, *L. vulgaris* and *L. montandoni* show a high level of prezygotic reproductive isolation. Only 4% of heterospecific sexual encounters progress to successful sperm transfer, in contrast to 34% in homospecific pairs (Michalak et al., 1997). The influence of various aspects of courtship on this phenomenon was not tested in this study, but pheromonal communication is proposed to be one of the major factors affecting strong reproductive isolation. Behavioral experiments under laboratory and semi-natural conditions have shown that sexual isolation between these species is asymmetric during heterospecific encounters, i.e., *L. montandoni* females appear to be more discriminating than *L. vulgaris* females (Michalak et al., 1997). The differences in epigamic traits between *L. vulgaris* and *L. montandoni* males may significantly contribute to prezygotic barriers and imply strong disruptive sexual selection on male traits. These predictions have been confirmed by the low frequencies of morphologically intermediate hybrid males in hybrid zones (Babik et al., 2003; Babik and Rafiński, 2004). It is likely that divergent chemosensory signals play a crucial role in species recognition during courtship in newts (Michalak et al., 1997), as reported for *Cynops* (Kikuyama et al., 1995; Yamamoto et al., 2000; Nakada et al., 2007b). This prediction is supported by the observation that the major differences in courtship between *L. montandoni* and *L. vulgaris* concern the frequency and types of tail vibrations (Pecio and Rafiński 1985).

Direct investigation of the amino-acid structure of peptide pheromones in newts is difficult and requires sacrificing many animals to obtain enough material for analysis (Kikuyama et al., 1995; Yamamoto et al., 2000; Nakada et al., 2007b). Therefore we established the putative sequences of peptide pheromones in *L. vulgaris* and *L. montandoni* through an analysis of mRNA extracted from the abdominal glands of males of these species. The obtained sequences show high similarity with known precursors of sodefrin-like peptide pheromones. We present the variation in cDNA sequences encoding putative sodefrin-like pheromone precursors and show the phylogenetic relationships among the precursor variants of *Lissotriton* and other salamandrids. We also discuss the potential influence of pheromone differentiation on the reproductive barrier between *L. vulgaris* and *L. montandoni*.

MATERIALS AND METHODS

Samples

Mature males of *L. vulgaris* and *L. montandoni* were collected by dip-netting during the peak of the breeding season (May) from two allopatric populations in southern Poland. Two individuals of *L. vulgaris* (Lv-1 and Lv-2) were taken from Spytkowice near Oświęcim (49°59'N, 19°29'E), and two *L. montandoni* males (Lm-1 and Lm-2) were collected from Pucułowski Stawek (Pond) (Gorce Mountains, Carpathians, 49°29'N, 20°08'E).

The individuals were sacrificed in a 1% solution of MS-222, and the abdominal glands were dissected and stored in RNALater (Sigma) overnight at 4°C. The RNALater was then removed and the glands were stored at –75°C for further procedures.

PCR and cloning

Total RNA was extracted using NucleoSpin® RNA II (Macherey-Nagel) including DNase treatment. cDNA was synthesized by using the 3'/5' RACE KIT (Roche); 5' and 3' RACE were performed by using sets of primers designed on the basis of known *Cynops* and *T. cristatus* cDNA encoding pheromone precursor sequences and partial *L. vulgaris* sequences obtained from genomic DNA. On the basis of these results, we designed PCR primers amplifying about 1000-bp fragments of cDNA containing the complete peptide-encoding sequence. PCR reactions were performed using primers Sod8F (5'-CATCACCCTACTCCTTACTCTCC-3') and Sod8R (5'-GGAATGGCTGTTCATGGCTAC-3'), which anneal to the 3' and 5'

UTR regions of the cDNA encoding pheromone precursors. Thus the cloned PCR products consisted of the complete precursor sequence and fragments of its 5' and 3' UTR sequences. PCR products were purified (CleanUp Kit, A & A Biotechnology), cloned with pGEM®-T Easy Vector System II (Promega), and plated onto LB agar plates with ampicillin (100 µg/ml). Recombinant clones were identified by blue/white colony screening. Plasmid DNA mini-preps were prepared with the PlasmidMini Kit (A & A Biotechnology). Cloned DNA fragments were sequenced in both the forward and reverse directions using standard T7 and SP6 primers and the Big–Dye Terminator Kit (ABI). After removing terminators using ExTerminator columns (A & A Biotechnology), sequencing products were separated on an ABI Prism 3100 Avant Genetic Analyser (Department of Comparative Anatomy, Jagiellonian University).

We employed conservative criteria for allele identification in order to avoid PCR and cloning-generated errors. Twenty-five clones from each of the four individuals were sequenced. Only sequences found in at least two clones were treated as confirmed, unique sequences. Unconfirmed singleton sequences were excluded from the analysis. Only the pheromone precursor-coding fragments were used for further analyses; UTR fragments were excluded.

Phylogenetic analysis

BioEdit software (Hall, 1999) was used for sequence alignment, editing, and translation. Phylogenetic analyses were conducted using *MEGA* version 4 (Tamura et al., 2007).

Average pairwise nucleotide genetic distances were calculated using the Kimura 2-parameter (*K2P*) model. Amino-acid distances were computed with the Poisson correction for multiple substitutions. Cladograms were constructed using the neighbor-joining method. Standard errors were obtained through 1000 bootstrap replicates. Alignments of *L. montandoni*, *L. vulgaris*, *Triturus carnifex*, and two *Cynops* sequences were possible only for a 360 bp cDNA fragment and 119 amino-acids; thus, genetic distances and phylogenetic relationships were assessed on the basis of alignments of these partial sequences (Fig. 1A).

Analysis of selection patterns

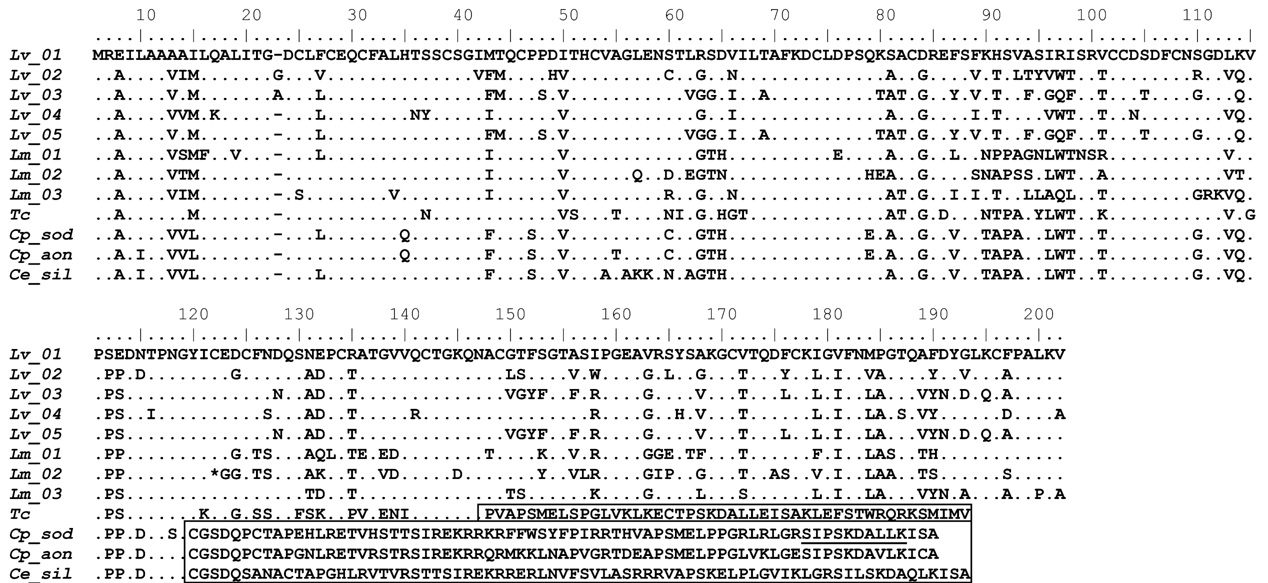
To test if selection shaped the evolution of the surveyed sequences (the full peptide-coding fragment of the cDNA molecule), we compared the likelihoods of three codon-based models of sequence evolution available in PAML 3.15 (Yang 1997): 1) M0, which assumes one ω (dN/dS ratio); 2) M7, which assumes that some sites evolve neutrally (ω=1) and some under purifying selec-

Table 1. Characteristics of the sodefrin precursor-like cDNA sequences found in *Lissotriton vulgaris* (Lv-1 and Lv-2) and *L. montandoni* (Lm-1 and Lm-2) males.

Sequence variant	Individual	Precursor-encoding sequence length (bp)	Precursor length (amino-acids)
Lv_01	Lv-1	603	201
Lv_02	Lv-1	606	202
Lv_03	Lv-2	606	202
Lv_04	Lv-2	603	201
Lv_05	Lv-2	603	201
Lm_01	Lm-1 & Lm-2	603	201
Lm_02	Lm-1	603	201*
Lm_03	Lm-2	603	201

* Stop codon at nucleotide position 360.

A



B

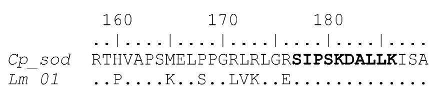


Fig. 1. (A) Alignment of sodefrin precursor-like amino-acid sequences of *Lissotriton montandoni*, *L. vulgaris*, *Triturus carnifex*, *Cynops pyrrhogaster*, and *C. ensicauda*. The unalignable regions between (*Cynops, Triturus*) and *Lissotriton* are boxed. The sequence of the biologically active sodefrin decapeptide in *Cynops* is underlined. **(B)** Partial alignment of the deduced sodefrin precursor-like sequence of the *Lm_01* variant, obtained by a 1-bp shift in the ORF, and the sodefrin precursor (*Cynops pyrrhogaster*). Both sequences contain the sodefrin sequence, SIPSKDALLK (underlined).

Table 2. *K2P* distances between nucleotide (above the diagonal) and deduced amino-acid (below the diagonal) sequences of pheromone genes in *Lissotriton vulgaris* (*Lv*), *L. montandoni* (*Lm*), *Triturus carnifex* (*Tc*), *Cynops pyrrhogaster* (*Cp*) and *C. ensicauda* (*Ce*). Nucleotide distances were calculated for a partial 360 bp precursor cDNA fragment, whereas peptide distances for partial, 119 amino-acid sequence.

	Lv_01	*Lv_02*	*Lv_03*	*Lv_04*	*Lv_05*	*Lm_01*	*Lm_02*	*Lm_03*	*Tc*	*Cp_sod*	*Cp_aon*	*Ce_sil*
Lv_01		47.2	47.2	25.1	29.6	30.8	29.6	29.6	28.5	32.0	32.0	35.5
Lv_02	29.3		24.0	30.8	38.0	43.1	41.8	34.3	44.5	35.5	38.0	41.8
Lv_03	29.3	22.7		35.5	12.7	51.4	48.6	35.5	51.4	45.8	48.6	49.9
Lv_04	24.9	18.6	21.7		21.9	25.1	24.0	19.8	26.2	24.0	26.2	27.4
Lv_05	29.3	22.7	0.0	21.7		33.1	30.8	21.9	34.3	29.6	32.0	32.0
Lm_01	30.5	27.1	32.8	24.9	32.8		19.8	30.8	25.1	20.8	23.0	24.0
Lm_02	29.3	24.9	30.5	23.8	30.5	19.6		28.5	26.2	20.8	20.8	23.0
Lm_03	29.3	20.6	21.7	19.6	21.7	30.5	28.2		29.6	29.6	29.6	33.1
Tc	28.2	28.2	34.0	26.0	34.0	24.9	26.0	29.3		28.5	26.2	30.8
Cp_sod	31.6	21.7	29.3	23.8	29.3	20.6	20.6	29.3	28.2		3.5	8.9
Cp_aon	31.6	22.7	31.6	26.0	31.6	22.7	20.6	29.3	26.0	3.4		8.9
Ce_sil	35.2	26.0	31.6	27.1	31.6	23.8	22.7	32.8	30.5	8.9	8.9	

tion (ω<1); and 3) M8, which assumes that positive selection influences the evolution of a fraction of codons (ω>1), in addition to neutral and purifying selection. The best fitting models were chosen on the basis of the value of the Akaike information criterion (AIC; Posada and Buckley, 2004; Sullivan and Joyce, 2005). Positively selected codons were identified through the Bayes empirical Bayes procedure (Zhang et al., 2005).

RESULTS

Cloning and sequencing of cDNA encoding pheromone precursors

We found five unique sequences in *L. vulgaris* males and three in *L. montandoni* males. The characteristics of the obtained cDNA sequences are shown in Table 1. The sequences are deposited in GeneBank (accession nos. EU526840–EU526847). One of the variants in *L. montandoni* (*Lm_01*) was present in both individuals tested, whereas the rest of the variants were present in single individuals only.

The putative pheromone precursors, deduced on the basis of an ORF, consisted of 201 or 202 amino-acid residues (Fig. 1A). In one sequence (*Lm_02*), a stop codon was detected at position 121, most likely representing a non-functional variant. In the *Lm_01* sequence, a 1-bp shift of the reading frame at position 360 in the cDNA gives a putative polypeptide with the sequence RTPVAPSKELSPGLVKLGE**SIPSKDALLK**ISA at amino-acid positions 145–176 (Fig. 1B). This peptide chain shows high similarity to the C-terminus of the sodefrin precursor of *C. pyrrhogaster* at positions 158–189 and contains the biologically active sodefrin sequence (highlighted in bold).

K2P distances between the *L. montandoni* and *L. vulgaris* nucleotide sequences varied from 19.8% to 51.4%, amino-acid distances ranged from 19.6% to 32.8%, respectively (Table 2). Distances between *Lissotriton* and *Cynops* variants ranged from 20.8% to 49.9% for nucleotide sequences and 20.6% to 35.2% for putative peptides (Table 2).

Phylogenetic analysis

The phylogenetic analysis of nucleotide cDNA sequences encoding putative pheromone precursors showed that variants from the two *Lissotriton* species do not form separate clades (Fig. 2). *Cynops* cDNA sequences form a distinct, clade with respect to *Lissotriton* and *Triturus* sequences. The *T. carnifex* sequence groups with two *L. montandoni* variants.

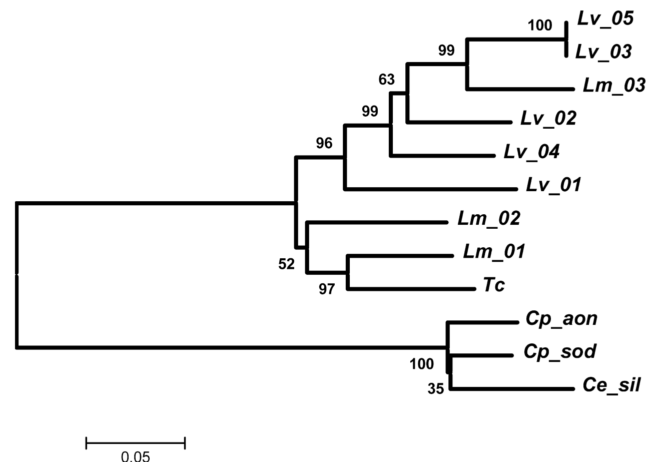


Fig. 2. Neighbor-joining tree showing the relationships among nucleotide sequences of partial cDNAs encoding sodefrin precursor-like peptides in *Lissotriton*, *Triturus*, and *Cynops*. Bootstrap support in percent is shown above respective branches.

Selection analysis

Model M8a, which assumes that a fraction of codons have evolved under positive selection, best explained our cDNA sequence data (δAIC compared to the second best nearly neutral model=13.0). The estimated proportion of sites identified as undergoing positive selection was 27.2%, with an average ω=2.13, in this class of codons. The Bayes empirical Bayes procedure identified only four codons (9, 88, 163, 166) with a posterior probability (PP) of being under positive selection>0.95, but as many as 34 codons with PP>0.5.

DISCUSSION

Variation in pheromone genes

We detected five unique cDNA sequences in two *L. vulgaris* males, and three in two *L. montandoni* males, encoding four unique putative polypeptides in *L. vulgaris* and three in *L. montandoni.* The large distances between these sequences indicate that more than one sodefrin

precursor-like coding locus is expressed in the abdominal glands of these species. The results clearly suggest that the genes encoding pheromones in *Lissotriton* newts form a gene family; however, an estimation of the number of genes is premature at this stage of research. Nonetheless, the expression in one *L. vulgaris* (Lv-2) male of three sodefrin precursor-like encoding variants (*Lv_03*, *Lv_04*, and *Lv_05*), differing by *K2P* distances between 12.7% and 35.5% (Table 2), indicates that the number of genes cannot be lower than three. The analysis of phylogenetic relationships among alignable, 360-bp sequence fragments of cDNAs from the *Lissotriton*, *Cynops*, and *Triturus* pheromone precursors showed that *Cynops* variants form a distinct clade related to those of *Triturus* and *Lissotriton*. However, the *L. vulgaris* and *L. montandoni* variants do not form separate clades, because *Lm_03* groups within *L. vulgaris*, not with the remaining *Lm* sequences. The *Triturus carnifex* variant does not form a distinct clade and groups with two *L. montandoni* variants. These results indicate that the divergence of at least some genes encoding the identified mRNA variants predates the divergence of *L. montandoni* and *L. vulgaris*. Another possible explanation is introgression of *L. montandoni* genes into the *L. vulgaris* gene pool.

Our findings are in accordance with the results obtained for *Cynops pyrrhogaster*. Iwata et al. (2004) found multiple structurally related peptides derived from post-translational processing of the sodefrin precursor. Analysis of partial cDNAs encoding C-terminal regions of the sodefrin precursor have also shown the presence of transcripts derived from more than one gene in the abdominal glands of *Cynops*.

Our results are similar to recent findings concerning pheromone gene variation in the most speciose family of tailed amphibians, the Plethodontidae. Expression of the gene encoding proteins similar to *Cynops* pheromone precursors, termed *SPF* (*sodefrin precursor-like factor*), has been found in the mental glands of males of various species across this family (Palmer et al., 2007). Surprisingly, the biologically active form of *SPF* in plethodontids is the long, uncleaved form. The differences between pheromones in Salamandridae and Plethodontidae also concern the organs in which pheromone genes are expressed. In salamandrids, sodefrin precursors are produced by the abdominal glands opening into the cloacal chamber, whereas in plethodontids pheromones are synthesized in the mental gland located on the male's chin. The structural similarity of the pheromone precursors in plethodontids indicates that the recruitment of this protein into pheromonal function occurred at least 50–100 millions years ago (Palmer et al., 2007). *SPF* shows a high level of polymorphism. Analysis of 229 cDNA clones from 28 species belonging to four genera (*Aneides*, *Desmognathus*, *Plethodon*, and *Eurycea*) revealed 94 unique nucleotide sequences encoding 93 unique amino-acid sequences (Palmer et al., 2007). The sequence dissimilarity among different variants varied from 0.4 to 23.0%, whereas the estimated number of SPF genes expressed in the mental gland of male plethodontids was not lower than four. Substantial polymorphism has been also found in other pheromone components in the family Plethodontidae, i.e., in PRF (*plethodontid receptivity factor*), structurally unrelated to sodefrin (Rollmann et al., 2000).

The analysis of selection on pheromone coding genes in *Lissotriton* newts did not produce clear-cut results. On the one hand, the model assuming that some fraction of codons evolved under positive selection explains the data better than the model assuming purifying selection only. On the other hand, only four codons were identified with high probability as having evolved under positive selection. More information on the structural and functional role of the different parts of the transcripts is needed for interpretation of these findings.

Amino-acid structure of biologically active pheromones in *Lissotriton*

Amino-acid sequences of putative pheromone precursors in *Lissotriton* newts are alignable with the sodefrin precursor of *Cynops* for the first 119 residues only (Fig. 1A). A lack of sequence similarity in the C terminus of the pheromone precursors in *Lissotriton* and *Cynops* newts precludes identification of the biologically active form of the pheromone by comparative analysis, since in *Cynops* this is situated at positions 177–186 of the precursor. In the case of clone *Lm_01*, found in both *L. montandoni* males, a 1-bp shift in reading frame at nucleotide position 360 results in a putative amino-acid sequence with high similarity to the sodefrin precursor (Fig. 1B). This result cannot be explained by PCR error, because all eight sequences identified in our research are alignable (Fig. 1A), and the results were confirmed by several independent PCRs. Since the pheromone precursors discovered here are longer than the sodefrin precursor (201/202 versus 189 amino acids), it is likely that either an insertion(s) occurred in the *Lissotriton* evolutionary lineage or a deletion(s) occurred in the *Cynops* line, shifting the reading frame in the center of the molecule. Thus it remains unclear which part of the pheromone precursor contains the biologically active peptide in *Lissotriton*.

In *Cynops*, the sodefrin sequence is sandwiched in the precursor between the monobasic amino acids arginin and lysine, and the biologically active pheromone is cleaved from the precursor by a protease (Iwata et al., 2004). The absence of dibasic amino acids flanking the sodefrin, silefrin and aonirin molecules suggests that the process of final precursor cleavage differs from the final processing of typical precursor proteins (Iwata et al., 1999). However, it has been shown that in some cases the cleavage of peptide precursors at monobasic amino acids may also occur (Schwartz, 1986; Devi, 1991), and this mechanism has probably evolved in *Cynops*. Nakada et al. (2007a) confirmed the presence of enzymes that may cleave the sodefrin precursor at monobasic sites flanking the sodefrin molecule in the abdominal glands of *Cynops*. In order to resolve this question in *Lissotriton*, an approach involving the isolation of the biologically active peptides from the abdominal glands of male newts is required.

Peptide pheromones and species recognition in newts

It has been shown that sodefrin attracts *C. pyrrhogaster* females but not females of the closely related species, *C. ensicauda*. On the other hand, silefrin, the pheromone produced by *C. ensicauda,* does not attract females of *C. pyrrhogaster* (Kikuyama et al., 1995; Yamamoto et al., 2000). Structural differentiation has also been found within *C. pyrrhogaster*. Aonirin, a sodefrin variant, has been iso-

lated from the abdominal glands of males from a local population of this species living in the proximity of the city of Nara (Nakada et al., 2007b). Aonirin differs from sodefrin by one amino-acid substitution. During behavioral tests, females from Nara reacted positively to 5 ng of aonirin in test tanks, while females from Niigata needed more than 100 ng of aonirin for a reaction to take place (Nakada et al., 2007b). Because of pronounced intraspecific differentiation within *L. vulgaris* in mtDNA and morphology, particularly in male epigamic traits (Babik et al., 2005), regional variants of peptide pheromones and regional differentiation in female preferences can be expected and deserve further research.

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