RESEARCH ARTICLE

Molecular Cloning of VIP and Distribution of VIP/VPACR System in the Testis of *Podarcis sicula*

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ABSTRACT

Using molecular, biochemical, and cytological tools, we studied the nucleotide and the deduced amino acid sequence of PHI/VIP and the distribution of VIP/VPAC receptor system in the testis of the Italian wall lizard *Podarcis sicula* to evaluate the involvement of such a neuropeptide in the spermatogenesis control. We demonstrated that (1) *Podarcis sicula* VIP had a high identity with other vertebrate VIP sequences, (2) differently from mammals, VIP was synthesized directly in the testis, and (3) VIP and its receptor $VPAC_2$ were widely distributed in germ and somatic cells, while the $VPAC_1R$ had a distribution limited to Leydig cells. Our results demonstrated that in *Podarcis sicula* the VIP sequence is highly preserved and that this neuropeptide is involved in lizard spermatogenesis and steroidogenesis. *J. Exp. Zool. 321A:334–347, 2014.* © 2014 Wiley Periodicals, Inc.

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Vasoactive Intestinal Peptide (VIP) is a 28 amino acid neuropeptide (Mutt and Said, '74), originally isolated from the porcine ileum (Said and Mutt, '70); it belongs to the glucagon/secretin superfamily (Vaudry et al., 2000, 2009) and is characterized by high sequence conservation among vertebrates (Sherwood et al., 2000; Cardoso et al., 2007, 2010). As with other members of the superfamily, it is produced as a precursor, a 170 amino acid preprohormone, namely prepro-VIP, and is then cleaved in its mature form in two neuropeptides (Fahrenkrug, 2010): the VIP and a VIP-related peptide, reported as peptide histidine-methionine amide (PHM) in human (Itoh et al., '83) or peptide histidine-isoleucine amide (PHI) in other vertebrates (Cardoso et al., 2007). Complementary DNA (cDNA) cloning and peptide characterization have shown that the VIP sequence is highly conserved: the only amino acid substitutions in the sequence occur in the central and C-terminal regions; conversely, the N-terminal portion is the most conserved, as it is important for the biological function of VIP and for the selectivity for the receptors compared to that of another member of the VIP superfamily, the PACAP (Pituitary Adenylate Cyclase Activating Polypeptide) (Dickson and Finlayson, 2009). In this regard, it is worth noting that VIP acts by the interaction with two G-protein coupled receptors: $VPAC_1$ (VIP/PACAP receptor, subtype 1) and $VPAC_2$ (VIP/PACAP receptor, subtype 2), that are coupled in the adenylate cyclase pathway (Dickson and Finlayson, 2009). The PACAP also binds to these receptors, in addition to a PACAP-specific receptor (PAC_1R). The N-terminal region of PACAP and VIP is responsible for the receptor selectivity, thus it is the most conserved portion of both the neuropeptides (Onoue et al., 2004, 2008). Although VIP was originally considered to be only a gut hormone, it is now well-known that it has numerous functions since VIP and its receptors show a widespread distribution. Indeed, it is a well-established neurotransmitter in central and peripheral nervous systems (Larsson et al., '76a,b; Fahrenkrug, '93), and it is involved in functions of many other peripheral districts (Bryant et al., '76; Duckles and Said, '82; Fahrenkrug and Hannibal, 2004; Van

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Geldre and Lefebvre, 2004), as in the testis (Matsumoto et al., '86; Vaudry et al., 2009). The studies about the presence and the functions of VIP in the testis are limited to mammals, where it has been shown that VIP is not locally synthesized, but it is produced in the nervous system (Zhu et al., '95). VIP regulates testicular functions by the interaction with $VPAC_2R$, localized in spermatocytes (Csaba et al., '97) and in Leydig cells (Hueso et al., '89; Krempels et al., '95). Differently, the $VPAC_1R$ is poorly represented in the mammalian testis: it is located only in human spermatozoa (Li and Arimura, 2003), or in vasa deferentia (Moretti et al., 2002). Several studies have reported that VIP influences testicular functions, by regulating the protein synthesis (West et al., '95) and the testosterone production (Kasson et al., '86; El-Gehani et al., '98a,b). The activity of this neuropeptide seems to be essential for spermatogenesis, as VIP-deficient males rats show a testosterone dramatic decrease and a testicular degeneration (Lacombe et al., 2007). Few information is available about the expression and function of VIP and its receptors in the testis of non-mammalian vertebrates: we have previously demonstrated the distribution of VIP and its receptors in the cartilaginous fish *Torpedo marmorata* (Agnese et al., 2012) and the presence of VIP receptors has been demonstrated by PCR in zebrafish (Fradinger et al., 2005). No information is available about the presence of the VIP in the testis of terrestrial non-mammalian vertebrates. So we investigated the involvement of VIP in the spermatogenesis of the Italian wall lizard *Podarcis sicula*, a terrestrial reptile and seasonal breeder characterized by a tubular testis (Galgano and D'Amore, '53; Angelini and Botte, '92).

In particular, using molecular, biochemical, and cytological tools, we studied the nucleotide and the deduced amino acid sequence of PHI/VIP and the distribution of VIP/VPAC receptor system in the testis of the wall lizard *Podarcis sicula*. Our results show that the nucleotide and amino acid sequences of *Podarcis sicula* VIP are highly conserved when compared with those of other vertebrates and that VIP is synthesized directly in the testis, where, together with its receptor $VPAC_2$, it shows a widespread localization, suggesting that the action of this neuropeptide is critical for the regular proceeding of spermatogenesis in *Podarcis sicula*.

MATERIALS AND METHODS

Animals

Sexually mature male wall lizards, *Podarcis sicula*, were collected in the region Campania (Campagna, SA, 40°39′58″68 N; 15° 6′24″12 E) during the breeding season (May). *Podarcis* males were maintained in a soil-filled terrarium and fed ad libitum with *Tenebrio* larvae. The experiments were approved by institutional committees (Ministry of Health) and organized to minimize the number of animals used. Lizards were killed by decapitation after deep anesthesia with ketamine hydrochloride (Parke-Davis, Berlin, Germany) 325 μg/g of body weight. The testes were then excised using sterile RNase-free dissecting equipment and, once removed, were frozen at −80°C or fixed for 24 hr in Bouin's solution and then dehydrated with graded ethanol and embedded in paraffin wax. After microtome sectioning to 5 μm, sections were placed on Superfrost Plus or Polylisine glass slides (Menzel-Glaser, Braunschweig, Germany), for in situ hybridization and immunohistochemistry analysis, respectively.

Total RNA Isolation and RT-PCR

Total RNA was extracted from testis using TRI-Reagent (Sigma-Aldrich, St. Louis, MI) according to the manufacturer's instructions and was evaluated by 1% agarose gel electrophoresis; the RNA was then reverse transcribed with SuperScript II Reverse Transcriptase (Invitrogen) using oligo$(dT)_{15-18}$ as primers. In order to amplify cDNA of PHI/VIP, we designed a pair of primers (VIPfor3Ac, 5′-TAGGAAACAGAATGCCCTTTGA-3′; Viprev1Ac, 5′-AACTCTGTCTTAACTGGGAA-3) on the sequence of *Anolis carolinensis* VIP (ENSACAT00000005611) to cover the whole coding region of PHI and VIP. PCR amplification was performed with 2 μL of cDNA, 1 U Taq DNA polimerase (Invitrogen), 0.4 mM of each dNTP, 2.5 mM $MgCl_2$ and 0.4 μM of each primer. The PCR was carried out for 35 cycles: 1 min at 94°C, 45 sec at 48°C and 45 sec at 72°C. A negative control, without cDNA, was performed. The PCR product was analyzed on a 1.8% agarose gel stained with ethidium bromide for visualization; the amplified fragment was purified using QIAquick gel extraction kit (QIAGEN), following the manufacturer's instruction; steril water was added to elute DNA from the spin column. The fragment was then sequenced with BigDye Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA) and run on the ABI PRISM 310 Genetic Analyzer (Applied Biosystems).

Nucleotide and Amino Acid Sequence Analysis

The sequence was analyzed for similarity with BLASTn program (http://www.ncbi.nlm.nih.gov). Protein translation was done using Genamics Expression 1.1 software (Valiante et al., 2007, 2009). Putative protein molecular weight was analyzed using the tools from ExPASy Molecular Biology Server (http://www.expasy.org/).

The phylogenetic and molecular evolutionary analyses were conducted on the nucleotide sequences using the software MEGA version 4.0. The nucleotide and the amino acid sequences of the *Podarcis sicula* VIP were aligned with the VIP sequences of different vertebrates. The number of conserved and variable sites, of parsimony-informative (sites that contain at least two types of nucleotides (or amino acids), and at least two of them occur with a minimum frequency of two) and singleton (sites that contain at least two types of nucleotides (or amino acids) with, at most, one occurring multiple times) were calculated. Furthermore, for the nucleotide sequence, the number of 0-fold degenerated sites (in which all changes are nonsynonymous), of twofold degenerated sites (in which one out of three changes is synonymous) and of fourfold degenerated sites (in which all changes are synonymous) were calculated.

For the nucleotide sequence, the evolutionary distances were estimated using Kumar method that is a modified Pamilo Bianchi Li's method (Li, '93; Pamilo and Bianchi, '93), which resolves the treatment of some difficult codons such arginine and isoleucine (Nei and Kumar, 2000). Particularly, the number of synonymous substitutions (in which a nucleotide change does not cause the changing in amino acid coding sequence) per synonymous sites (sites where one or more changes are synonymous) (d_S), the number of nonsynonymous substitutions (in which a nucleotide change causes the changing in amino acid coding sequence) per nonsynonymous sites (sites where one or more changes are nonsynonymous) (d_N), the number of substitutions at the fourfold degenerate sites (d_4f), sites where substitutions produce synonymous changes exclusively, and the number of substitutions at the 0-fold degenerate sites (d_0f), in which substitutions generate only nonsynonymous changes, were calculated between *Podarcis sicula* and other species. To evaluate the kind of selection acting on VIP sequence during the evolution, the codon-based one-tailed Z-test of selection (which compares the relative abundance of synonymous and nonsynonymous substitutions that occurred in the gene sequences) and the one-tailed Fisher's exact test (which is more fitting when a small number of differences in sequences occurs) were carried out. For the amino acid sequence, the evolutionary distances were estimated using the Dayhoff Matrix method, computing both the pairwise distance and the distance between the species grouped in classes; the Dayhoff Matrix Method calculates the proportion of amino acid sites at which the two compared sequences are different; it takes into account the correction for multiple substitutions at the same site, assuming uniform rates of variation among sites.

The phylogenetic reconstructions were carried out using the Maximum Parsimony method, where only the minimum number of changes required to produce the observed variation was considered; this analysis was carried out assuming 1,000 bootstrap replicates as reliability test.

In Situ Hybridization (ISH)

In situ hybridization was carried out as described elsewhere (Agnese et al., 2012). Dig-labeled *Podarcis sicula* PHI/VIP (gi|380450150) cDNA probe was generated by PCR using the Dig-DNA labeling mix (Roche). The probe was used at the concentration of 1 ng/μL at the temperature of 42°C. For negative control, the hybridization mix did not contain *PHI/VIP* cDNA probe. Hybridization signal was observed on a Zeiss Axioskop microscope; images were acquired with an AxioCam MRc5 camera and analyzed with AxioVision 4.7 software (Zeiss, Oberkochen, Germany).

Antibodies and Dilutions

The primary antibodies used in immunohistochemistry were: rabbit anti-human VIP (Phoenix Pharmaceuticals, Belmont, CA, USA), rabbit anti-human $VPAC_1R$, and $VPAC_2R$ (Santa Cruz Biotechnology, Santa Cruz, CA, USA).

The dilutions used were: 1:500 for anti-VIP and 1:300 for anti-$VPAC_1R$ and anti-$VPAC_2R$.

Protein Extraction and Immunoprecipitation

Testes were homogenized in a lysis buffer (10 mM Tris-HCl pH 7.4, 150 mM NaCl, 1 mM EDTA, 1%Tryton X-100, 10% glicerol), containing protease inhibitor cocktail (Sigma-Aldrich) and then centrifuged at 7,000*g*. The protein concentration was determined by Pierce method.

The proteins were then used for immunoprecipitation with the anti-VIP antibody, using the ExactaCruz Matrix (Santa Cruz Biotechnology) according to the manufacturer's instructions. The immunoprecipitate was loaded onto 15% polyacrilamide gel; prestained molecular weight markers (ovalbumine 45 kDa, carbonic anidrase 30 kDa, tripsine inhibitor 20.1 kDa, lysozime 14.3 kDa, aprotinine 6.5 kDa, insulin, β chain, 3.5 kDa; GE Healthcare) were also run. After the electrophoresis, the gel was stained with Coomassie Blue.

Immunohistochemistry

Immunohistochemistry was carried out as described elsewhere (Agnese et al., 2012, 2013). After inactivation of endogenous peroxidase and reducing of non-specific background, the sections were incubated over night at 4°C with the primary antibodies diluted in normal goat serum. The reaction was revealed with a biotin-conjugated goat anti-rabbit secondary antibody and an avidin-biotin-peroxidase complex (ABC immunoperoxidase Kit, Pierce), using DAB as chromogen. Finally, sections were counterstained with Mayer's hemalum. Negative controls were carried out omitting primary antibodies. Immunohistochemical signal was observed on a Zeiss Axioskop microscope; images were acquired with an AxioCam MRc5 camera and analyzed with AxioVision 4.7 software (Zeiss).

RESULTS

VIP

RT-PCR. The amplification experiments produced a cDNA fragment of about 350 bp in length (Fig. 1, T). Control showed no band amplification (Fig. 1, K).

VIP Sequence Analysis. The nucleotide sequence was published in the NCBI web site (http://www.ncbi.nlm.nih.gov/genbank/) with the GenBank accession no. JQ517276.1. The nucleotide and deduced amino acid sequences of the *Podarcis sicula* PHI/VIP were presented in Figure 2. The nucleotide sequence of the cloned cDNA was 350 bp in length with an ORF of 312 bp. The deduced mature protein consisted of 104 amino acids including part of the propeptide, the entire PHI (27 amino acids, with a MW of 3 kDa) and the entire VIP (28 amino acids, with a MW of about 3.3 kDa) (ExPASy: http://www.expasy.ch/).

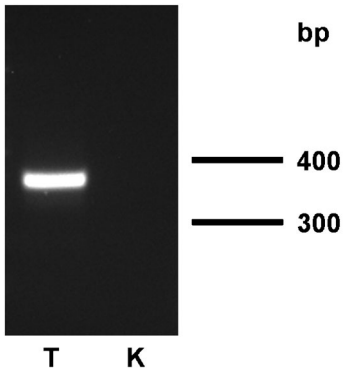

Figure 1. PCR for *Podarcis sicula PHI/VIP*. The amplification product of about 350 bp is evident in the testis of *Podarcis sicula* (T). No band is evident in the negative control obtained by no RT in the PCR reaction (K).

Nucleotide analysis. The comparison of the vertebrate nucleotide PHI/VIP sequence with that of *Podarcis sicula* was reported in Figure 3. BLASTn analysis revealed that the *Podarcis sicula* PHI/VIP sequence was highly conserved; particularly, the nucleotide sequence showed an identity of 82% when compared with that of *Anolis carolinensis* (ENSACAT00000005611) and an identity of at least 70% compared with other vertebrates (Fig. 3). By the comparison of the overall PHI/VIP obtained sequence with that of the other vertebrates here reported, it merged that 44% nucleotides were conserved; however, the conserved nucleotides increased to 51% and 61%, when we considered the PHI or the VIP sequence separately. As regards PHI, *Podarcis sicula* PHI sequence had 91% identity with that of *Anolis carolinensis*, 78% with *Takifugu rubripes* (gi|163954954), 77% with *Xenopus laevis* (gi|148232101), 73% with *Canis familiaris* (gi|73946149), and 72% with *Gallus gallus* (gi|487632).

The alignment of *Podarcis sicula* VIP sequence with those of other vertebrates was reported in Figure 4A: for instance, *Podarcis sicula* VIP had 94% identity when compared with *Anolis carolinensis*. The results from the comparison of the *Podarcis sicula* VIP nucleotide sequence with VIP sequences of other vertebrates were presented in Figure 4B: 59.5% were conserved sites, 36.9% were parsimony-informative and 3.6% were singleton sites; the 0-fold degenerated sites (d_{0f}) were the 63.1%, while the twofold (d_{2f}) and the fourfold (d_{4f}) degenerated sites were the 15.5% and the 8.3%, respectively. The evolutionary distances, calculated with the Kumar method, were presented in Figure 5A: the synonymous (d_S) and nonsynonymous (d_N) distances and the ratio d_N/d_S were calculated; particularly, the ratio between synonymous and nonsynonymous distances was greatly smaller than 1 in all lineages considered. Furthermore, the number of substitutions at both d_{0f} and d_{4f} sites was reported (Fig. 5A). The results of the Z-test and the Fisher's exact test of selection were shown: in the Z-test, the probability P of rejecting the null hypothesis of neutral selection in favor of the alternative hypothesis of purifying selection was shown: the results were statistically significant, pointing out the presence of purifying selection that works against changes in VIP sequence during the evolution. For the Fisher's exact test, the probability P to reject the null hypothesis of neutral selection in favor of the alternative hypothesis of positive selection was shown. The inferred phylogenetic tree was reported in Figure 5B.

Amino acid analysis. The amino acid sequence of *Podarcis sicula* PHI/VIP was reported in Figure 6. BLASTx analysis showed that *Podarcis sicula* PHI/VIP deduced amino acid sequence had an identity of at least 82% compared with that of *Anolis carolinensis*, 76% with *Xenopus laevis*, 69% with *Gallus gallus*, 68% with *Canis familiaris*, and 64% with *Takifugu rubripes*

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aaatctgaggctgacattttgcaaactacactacctgagaatgacaagttctattatgat
 K  S  E  A  D  I  L  Q  T  T  L  P  E  N  D  K  F  Y  Y  D
gtgtacagagctatggataggaacacaagacatgccgatggactcttcacaagcggctac
 V  Y  R  A  M  D  R  N  T  R  H  A  D  G  L  F  T  S  G  Y
agcaaacttctgggtcaaatttcggcaagaaaatatttggaatcgcttataggaaaaaga
 S  K  L  L  G  Q  I  S  A  R  K  Y  L  E  S  L  I  G  K  R
gttggaaataacactccccttgatgaacagacaccgccagtcaaacgccattcagatgct
 V  G  N  N  T  P  L  D  E  Q  T  P  P  V  K  R  H  S  D  A
gtctttactgacaactacagtcgctttcgaaagcagatggctgtgaagaaatatttgaac
 V  F  T  D  N  Y  S  R  F  R  K  Q  M  A  V  K  K  Y  L  N
tctgtcttaactga
 S  V  L  T
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Figure 2. Nucleotide and deduced amino acid sequences of *Podarcis sicula* PHI/VIP (ExPASy: http://www.expasy.ch/).

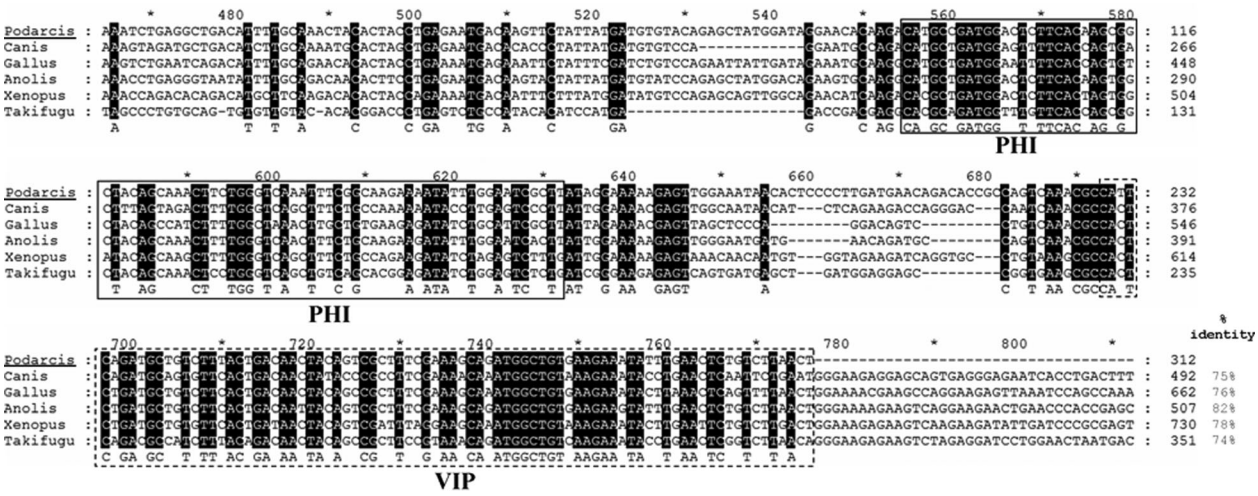

Figure 3. Alignment of *Podarcis sicula PHI/VIP* nucleotide sequence (underlined) with those of other vertebrates: the obtained sequence corresponds to part of the region coding the propetide, to the region coding PHI (continuous box), VIP (dotted box), and the propeptide between PHI and VIP. The conserved sites are highlighted in black: the highest identity is evident in the region coding PHI and VIP. The identity percentage is reported.

A

Species	Sequence	% identity	Reference
Podarcis sicula	**CAT TCA GAT GCT GTC TTT ACT GAC AAC TAC AGT CGC TTT CGA AAG CAG ATG GCT GTG AAG AAA TAT TTG AAC TCT GTC TTA ACT**		g1|380450150
Homo sapiens	..C ... .A ... ..C ... ...T .CC ... C.. A.. ..A ..A ... .A ... ...A A.T C.G .A.	80%	g1|37588851
Rattus norvegicus	..C ..T ... ..A ... ..C ..A ..T ... ... .CC ... C.. A.. ... ..A ... ...C ... .C A.T C.. .A.	80%	g1|209870056
Mus musculus	..C ..T ... ..C ... ..C ..A ..T ... ... .CC ... C.C A.. ... ..A ... ...C C.. ... ..C A.. C.G .A.	77%	g1|142365020
Bos taurus	..C ... ...C ... ... .CA ... C.. ... ..A ..A ... ...C C.. ... ..A A.T C.. .A.	83%	g1|112181314
Ovis aries	..C ... ...C ... ... .CA ... C.. ... ..A ..A ... ...C ... ..T ..A A.T C.. .A.	83%	g1|187607662
Canis familiaris	..C ... .A ..G ..C ... ...T .CC ... C.. ... ..A ..A ... .A ... .C C.. ... ..A A.T C.G .A.	77%	g1|73946149
Sus scrofa	..C ..G ... ..A ... ..C ... ... .CC ... C.. ..C ..A ..A ... .C ... ..G ..C ... .C A.T C.. .A.	79%	g1|305377067
Gallus gallus	..C ..T ... ...C ... ...C ... ...A ... ...C ..A ... ..A ..T	89%	g1|487632
Taenopygia guttata	..C ..T ... ...C ... ...C ... ...C ..A ... ..A ..T	90%	ENSTGUT00000011658
Anolis carolinensis	..C ..T ... ...C ... .T ... ...G	94%	ENSACAT00000005611
Xenopus laevis	..C ..T ... .G ..C ... ..T ... ...A ... A.G ... ..A ... .A ... .C ... ..T ... .G ...	84%	g1|148232101
Danio rerio	... ...A A.A ..C ..A ... ...C ... ...G ... ... C.. ... .T C.C ..A	86%	g1|167621465
Oryzias latipes	..C ... ..C ..C A.. ..C ..A ... ...C ... ..C ..C ..A ... .A ..C ... ..G ..C C.. ... ..A ... .A	80%	ENSORLT00000004877
Takifugu rubripes	..C ... ..C ..C A.. ... ..A ... ...C ... ..C ..T ..A ... ...C ... .C C.. ... ..G ... .A	83%	g1|163954954

B

Conserved sites	Variable sites	Parsimony Informative Sites	Singleton	0-fold degenerated sites	2-fold degenerated sites	4-fold degenerated sites
50/84	34/84	31/84	3/84	53/84	13/84	7/84
59.5%	40.5%	36.9%	3.6%	63.1%	15.5%	8.3%

Figure 4. **A**: Alignment of *Podarcis sicula VIP* nucleotide sequence (in bold) with those of other vertebrates: the percentage of identity between *Podarcis sicula* sequence and each other vertebrate sequence and the accession number are reported. **B**: Analysis of *Podarcis sicula* nucleotide sequence in comparison with the sequences of other vertebrates.

A

Species	d_N	d_S	d_N/d_S	d_{4f}	d_{0f}	Z-test	F-Fisher
Homo sapiens	0.06699	0.97786	0.068507	0.75646	0.07701	0.03813	1.000
Rattus norvegicus	0.06699	1.05092	0.063744	0.83084	0.07701	0.03989	1.000
Mus musculus	0.06719	1.37543	0.04885	1.06938	0.07701	0.01544	1.000
Bos taurus	0.07453	0.72742	0.102458	0.17094	0.07701	0.03322	1.000
Ovis aries	0.07428	0.70171	0.105856	0.17863	0.07701	0.04131	1.000
Canis familiaris	n/c	n/c	n/c	n/c	n/c	n/c	1.000
Sus scrofa	0.06717	1.21316	0.055368	0.88068	0.07663	0.02998	1.000
Gallus gallus	0	0.56032	0	0.31559	0	0.03214	1.000
Taenopygia guttata	0	0.48073	0	0.31559	0	0.03369	1.000
Anolis carolinensus	0	0.26347	0	0.08909	0	0.06598	1.000
Xenopus laevis	0	1.07540	0	0.75719	0	0.03809	1.000
Danio rerio	n/c	n/c	n/c	n/c	n/c	n/c	1.000
Oryzias latipes	n/c	n/c	n/c	n/c	n/c	n/c	1.000
Takifugu rubripes	0.01835	1.34226	0.013671	0.90419	0.01835	0.02644	1.000

B

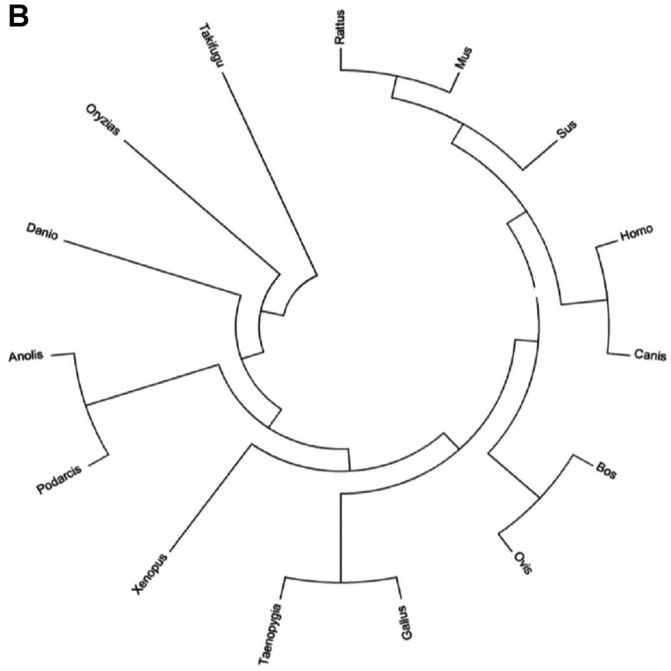

Figure 5. **A**: Evolutionary distances, calculated with the Kumar method between *Podarcis sicula* VIP sequence and other vertebrate sequences. Z-test of selection: the probability P of rejecting the null hypothesis (H_0: neutral selection, $d_N = d_S$) in favor of the alternative hypothesis (H_A: purifying selection: $d_N < d_S$) is shown ($P < 0.05$). Fisher's exact test: the probability P to reject the null hypothesis of neutral selection in favor of the alternative hypothesis of positive selection is shown; if the observed number of synonymous differences per synonymous site exceeds the number of nonsynonymous differences per nonsynonymous site then *MEGA* sets $P = 1$ to indicate purifying selection, rather than positive selection. **B**: Inferred phylogenetic tree on *VIP* nucleotide sequences, using MEGA 4.0 software with Maximum Parsimony Method: note that *Podarcis sicula* VIP sequence is strictly related to that of *Anolis carolinensis*.

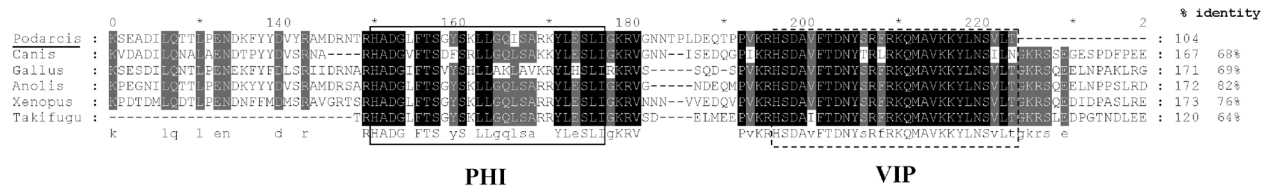

Figure 6. Alignment of *Podarcis sicula* PHI/VIP deduced amino acid sequence (underlined) with those of other vertebrates: the obtained sequence corresponds to part of the propeptide, to PHI (continuous box), VIP (dotted box), and the propeptide between PHI and VIP. The conserved sites are highlighted in black; the sites differing only in one species are in highlighted in gray; the highest identity is evident in the region coding PHI and VIP. The identity percentage is reported.

A

Species	Sequence	% identity
Podarcis sicula	**HSDAVFTDNY SRFRKQMAVK KYLNSVLT**	
Homo sapiens	..... T.L..... .....I.N	86%
Rattus norvegicus	..... T.L..... .....I.N	86%
Mus musculus	..... T.L..... .....I.N	86%
Bos taurus	..... T.L..... .....I.N	86%
Ovis aries	..... T.L..... .....I.N	86%
Canis familiaris	..... T.L..... .....I.N	86%
Sus scrofa	..... T.L..... .....I.N	86%
Gallus gallus	....	100%
Taenopygia guttata	....	100%
Anolis carolinensis	....	100%
Alligator mississipiensis	....	100%
Rana ridibunda	....	100%
Xenopus laevis	....	100%
Danio rerio	....I.....	96%
Oryzias latipes	....I.....	96%
Takifugu rubripes	....I.....	96%
Oncorhynchus mykiss	....I.....	96%
Sparus aurata	....I.....	96%
Scyliorhinus canicula	....I..... ..I..L.A	86%

B

Conserved sites	Variable sites	Parsimony Informative Sites	Singleton
22/28	6/28	5/28	1/28
78.6%	21.4%	17.6%	3.6%

Figure 7. **A**: Alignment of *Podarcis sicula* VIP amino acid sequence (in bold) with those of other vertebrates: the percentage of identity between *Podarcis sicula* sequence and each other vertebrate sequence is reported. **B**: Analysis of *Podarcis sicula* amino acid sequence in comparison with the sequences of other vertebrates.

A

Species	Distances from *Podarcis sicula*
Homo sapiens	0.140801
Rattus norvegicus	0.140801
Mus musculus	0.140801
Bos taurus	0.140801
Ovis aries	0.140801
Canis familiaris	0.140801
Sus scrofa	0.140801
Gallus gallus	0.000001
Taenopygia guttata	0.000001
Anolis carolinensis	0.000001
Alligator mississipiensis	0.000001
Rana ridibunda	0.000001
Xenopus laevis	0.000001
Danio rerio	0.033403
Oryzias latipes	0.033403
Takifugu rubripes	0.033403
Oncorhyncus mykiss	0.033403
Sparus aurata	0.033403
Scyliorhinus canicula	0.139530

B

Classes	Distances from *Podarcis*
Mammals	0.140801
Birds	0.000001
Reptiles	0.000001
Amphibians	0.000001
Teleosts	0.033403
Elasmobranchs	0.139530

C

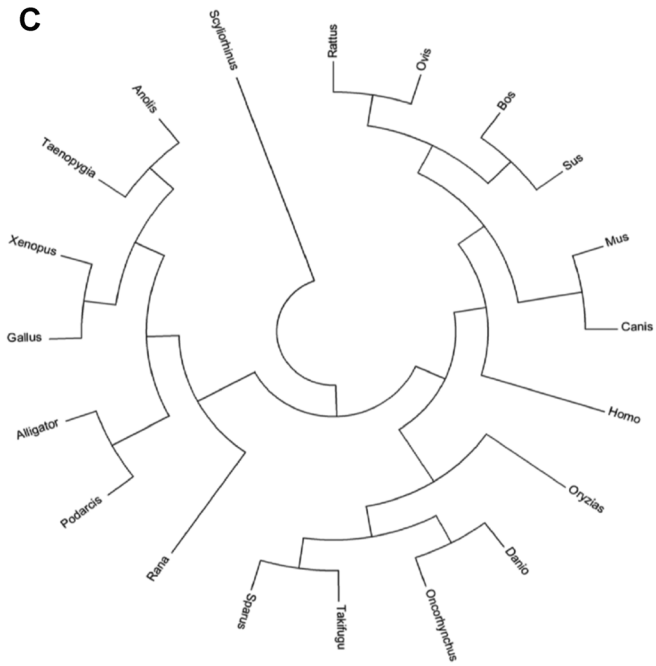

Figure 8. **A** and **B**: Pairwise distance values between amino acid sequences of *Podarcis sicula* and other species (**A**) and between *Podarcis sicula* and the other species grouped in classes (**B**) evaluated with the Dayoff Matrix method. C: Inferred phylogenetic tree on VIP amino acid sequences, using MEGA 4.0 software with Maximum Parsimony Method. Note that *Podarcis sicula* VIP sequence is related to that of other reptiles, amphibians, and birds, as they show 100% identity.

(Fig. 6). The results from the comparison of the *Podarcis sicula* PHI/VIP amino acid sequence with PHI/VIP sequences of other vertebrates showed that 49% were conserved sites, but the percentage increased to 54% and 82%, when the sequences of PHI and VIP were considered separately. As regards to PHI, *Podarcis sicula* PHI had 92% identity with *Anolis carolinensis*, *Xenopus laevis* and *Takifugu rubripes*, 77% with *Canis familiaris* and 58% with *Gallus gallus*.

Particularly, the comparison of VIP sequences was reported in Figure 7A; for instance, *Podarcis sicula* VIP had 100% identity when compared with *Gallus gallus*, *Taenopygia guttata*, *Anolis carolinensis*, *Alligator mississipiensis*, *Rana ridibunda*, and *Xenopus laevis*. The results from the analysis of the *Podarcis sicula* VIP amino acid sites with VIP of other vertebrates showed that 78.6% were conserved sites (most of which were in the N-terminal region), 17.6% were parsimony-informative (most of

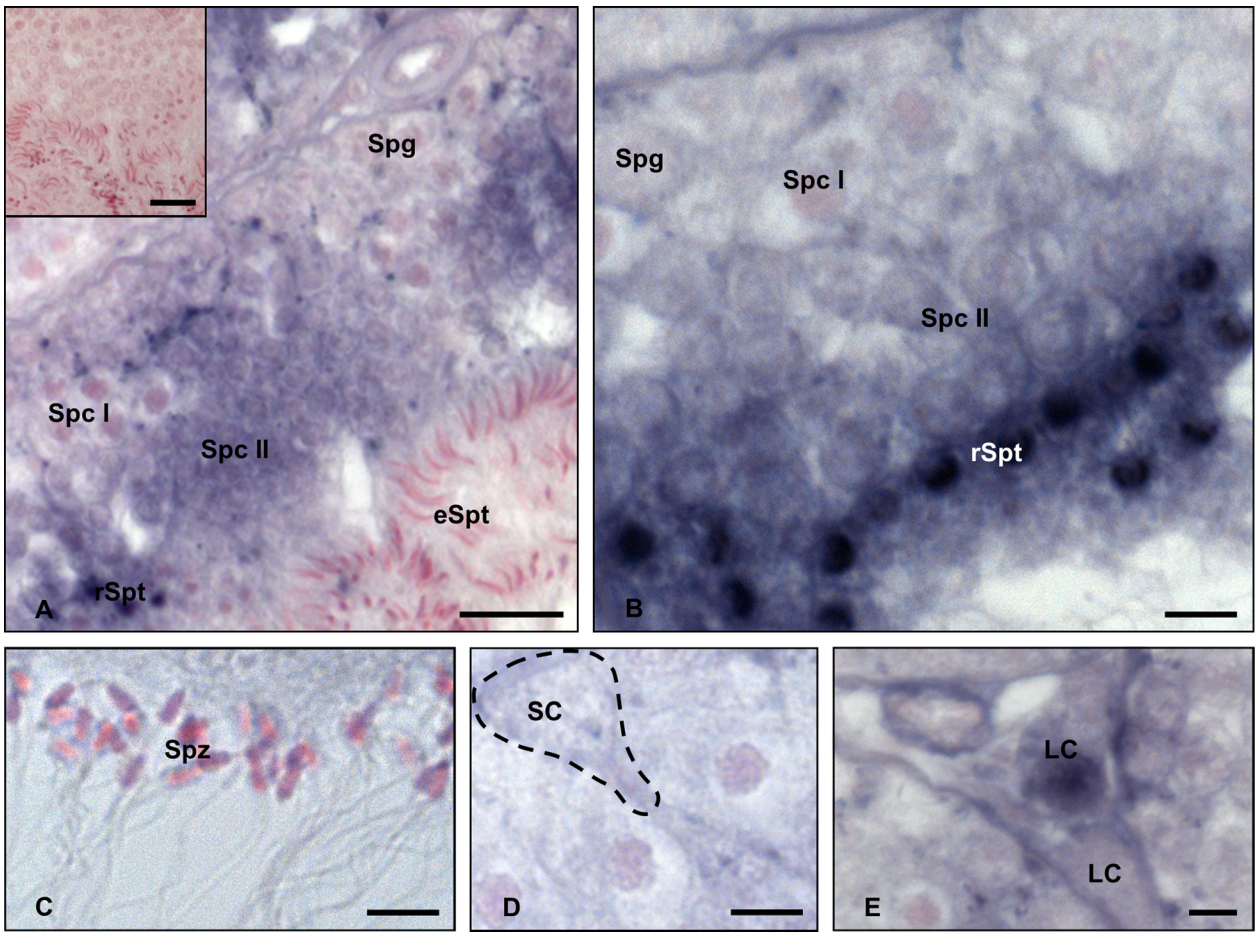

Figure 9. Expression of *PHI/VIP* in *Podarcis sicula* testis. Testis sections were incubated with Dig-labeled probe to detect *PHI/VIP* mRNA. The hybridization signal appears as blue areas in the cytoplasm of spermatogonia (Spg) (**A**, **B**), primary spermatocytes (Spc I) (**A**, **B**), secondary spermatocytes (Spc II) (**A**, **B**), and round spermatids (rSpt) (**A**, **B**); differently, elongated spermatids (eSpt) (**A**) and spermatozoa (Spz) (**C**) are not positive. The hybridization signal appears also in Sertoli cells (SC, dotted line) (**D**) and in Leydig cells (LC) (**E**). No hybridization signal is present in control sections (**A**, inset). Scale bars correspond to 50 μm in A and inset, and to 5 μm in **B**–**E**.

which were in the C-terminal region) and 3.6% were singleton (Fig. 7B). The pairwise distance values between *Podarcis sicula* and other species (Fig. 8A) and between *Podarcis sicula* and the other species grouped in classes (Fig. 8B) were evaluated with the Dayoff Matrix method. The inferred phylogenetic tree was shown in Figure 8C.

In Situ Hybridization for PHI/VIP mRNA. In situ hybridization revealed that *PHI/VIP* mRNA was widely distributed in germ and somatic cells (Fig. 9A–E). In germ cells, the positivity occurred in the spermatogonia, in the primary spermatocytes and, particularly, in secondary spermatocytes and round spermatids (Fig. 9A, B). Differently, no hybridization signal was found in elongated spermatids (Fig. 9A) and in spermatozoa (Fig. 9C). In somatic cells, a positive hybridization signal was found in Sertoli (Fig. 9D) and Leydig (Fig. 9E) cells. No hybridization signal was found in the control sections (Fig. 9A, inset).

Immunoprecipitation. The immunoprecipitation carried out on the testicular proteins showed a band of less than 3.5 kDa (Fig. 10A), demonstrating the presence of VIP in the testis of *Podarcis sicula*.

Immunohistochemistry for VIP. Immunohistochemistry revealed that VIP was widely distributed in germ and somatic cells (Fig. 10B–F). In germ cells, the immunoreactivity occurred in all the stages: spermatogonia (Figs. 10B and C), primary and secondary spermatocytes (Fig. 10B and C), round and elongated spermatids (Fig. 10B and C), and spermatozoa (Fig. 10D). An immunoreactive signal was also found in somatic cells, that is, Sertoli (Fig. 10B and E) and Leydig (Fig. 10B and F) cells. No

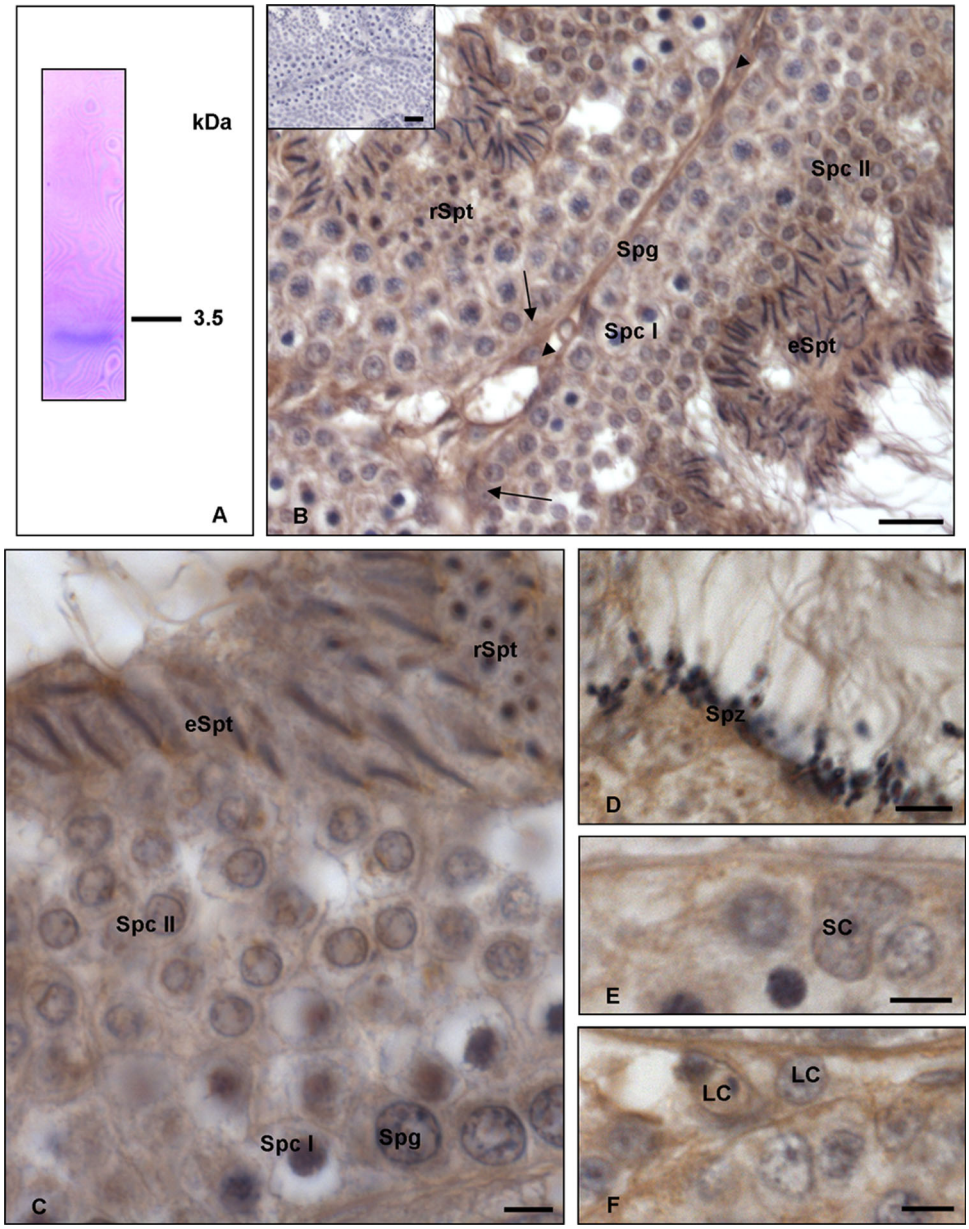

Figure 10. The VIP in the testis of *Podarcis sicula*. Testis proteins and sections were incubated with anti-VIP antibody. **A**: The anti-VIP antibody interacts with a band about 3.5 kDa. **B**: The immunolocalization signal appears as brown areas in all the germ cells, that is, spermatogonia (Spg) (**B, C**), primary (Spc I) and secondary (Spc II) spermatocytes (**B, C**), round (rSpt) and elongated (eSpt) spermatids (**B, C**), and spermatozoa (Spz) (**D**). Immunoreactivity occurs also in somatic cells, that is, Sertoli (**B**, arrows; **E**, SC) and Leydig (**B**, arrowheads; **F**, LC) cells. No signal is evident in control sections (**B**, inset). Scale bars correspond to 20 μm in B and inset, and to 5 μm in **C–F**.

immunoreactivity was evident in the control sections (Fig. 10B, inset).

VPAC Receptors

Immunohistochemistry for VPAC$_1$R. Immunohistochemistry revealed that the immunoreactivity to VPAC$_1$R occurred only in the Leydig cells (Fig. 11B); no immunoreactivity occurred in germ or in Sertoli cells (Fig. 11A), as in the control sections (Fig. 11C).

Immunohistochemistry for VPAC$_2$R. Immunohistochemical signal for VPAC$_2$R was evident in both germ and somatic cells (Fig. 12). In germ cells the immunoreactivity occurred in all the stages, that is,

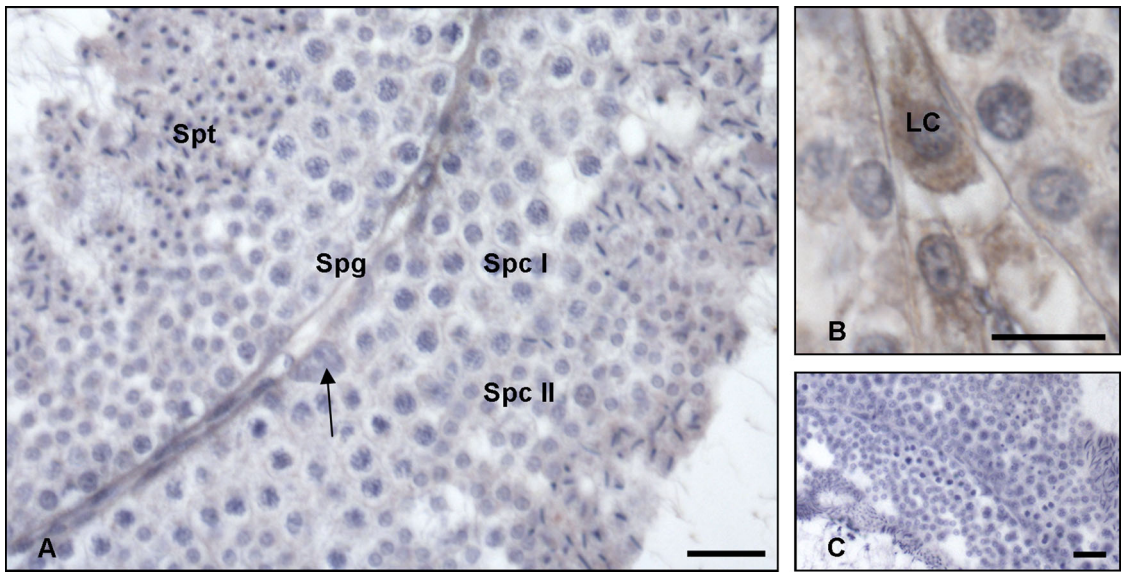

Figure 11. Distribution of $VPAC_1R$ in the testis of *Podarcis sicula*. **A** and **B**: Testis sections were incubated with anti-$VPAC_1R$ antibody. The immunolocalization signal that appears as brown areas occurs only in Leydig cells (LC, **B**), whereas no immunoreactivity is evident in germ and in Sertoli cells (arrow). **C**: No signal is evident in control sections. Scale bars correspond to 20 μm.

spermatogonia (Fig. 12A and B), primary and secondary spermatocytes (Fig. 12A and B), round and elongated spermatids (Fig. 12A and C), and spermatozoa (Fig. 12D). An immunoreactive signal was also found in somatic cells, that is, Sertoli and Leydig cells (Fig. 12A and B). No immunoreactivity was evident in the control sections (Fig. 12E).

DISCUSSION

Neuropeptides are not present only in the nervous system, but more and more studies are available about the presence of these molecules in peripheral organs, such as the testis (Lacombe et al., 2007; Moody et al., 2011). Neuropeptides are mainly involved in the regulation of cell communication, enabling the crosstalk among the cells. This principle is particularly true in the testis: in this organ germ and somatic cells communicate with each other in a paracrine manner, using peptides as signals; furthermore, a positive feedback is guaranteed by the autocrine communication, often mediated by neuropeptides (Vaudry et al., 2009). VIP is one of the numerous neuropeptides identified in the testis. At present, despite the knowledge about VIP and its receptors in the mammalian testis, the studies about the sequence of VIP and about the testicular expression and distribution of VIP and its receptors in non-mammalian vertebrates are still few (Fradinger et al., 2005; Agnese et al., 2012; Ng et al., 2012). So, we studied the sequence of PHI/VIP and the distribution of VIP/VPAC receptors system in the testis of the reptile *Podarcis sicula*. This is a useful experimental model, as it is characterized by a tubular testis, just as the mammals (Galgano and D'Amore, '53; Angelini and Botte, '92; Gribbins and Gist, 2003).

We clone a partial cDNA encoding for *Podarcis sicula* PHI/VIP, demonstrating that also in *Podarcis sicula* the VIP gene encodes also PHI, similarly to other non-mammalian vertebrates and differently from mammals, in which it encodes PHM (Yamagami et al., '88; Sherwood et al., 2000). The bioinformatic analysis shows that the sequence is highly conserved during the evolution: the region encoding VIP is the most conserved one, while the identity is a little less when considering PHI and propeptide sequences.

Particularly, *Podarcis sicula* VIP nucleotide sequence shows a high identity with *Anolis carolinensis* VIP (94%), although, when compared with other vertebrate sequences, the identity exceeds anyway 77%. Indeed, the comparison of the *Podarcis sicula* VIP nucleotide sequence with VIP sequences of other vertebrates shows that 59.5% are conserved sites; furthermore, among the variable sites, most of them are parsimony-informative, thus suggesting a conservation of the substitutions also in the variable sites. In this regard, it is worth noting that the 0-fold degenerated sites (d_{0f}) are more represented than the twofold (d_{2f}) and the fourfold (d_{4f}) degenerated sites: this indicates that the non-synonymous changes are more frequent than the synonymous ones. The evolutionary distances, calculated with Kumar's method, indicate again the high conservation of the sequence: the ratio between synonymous and non synonymous distances is smaller than one in all lineages considered, indicating that

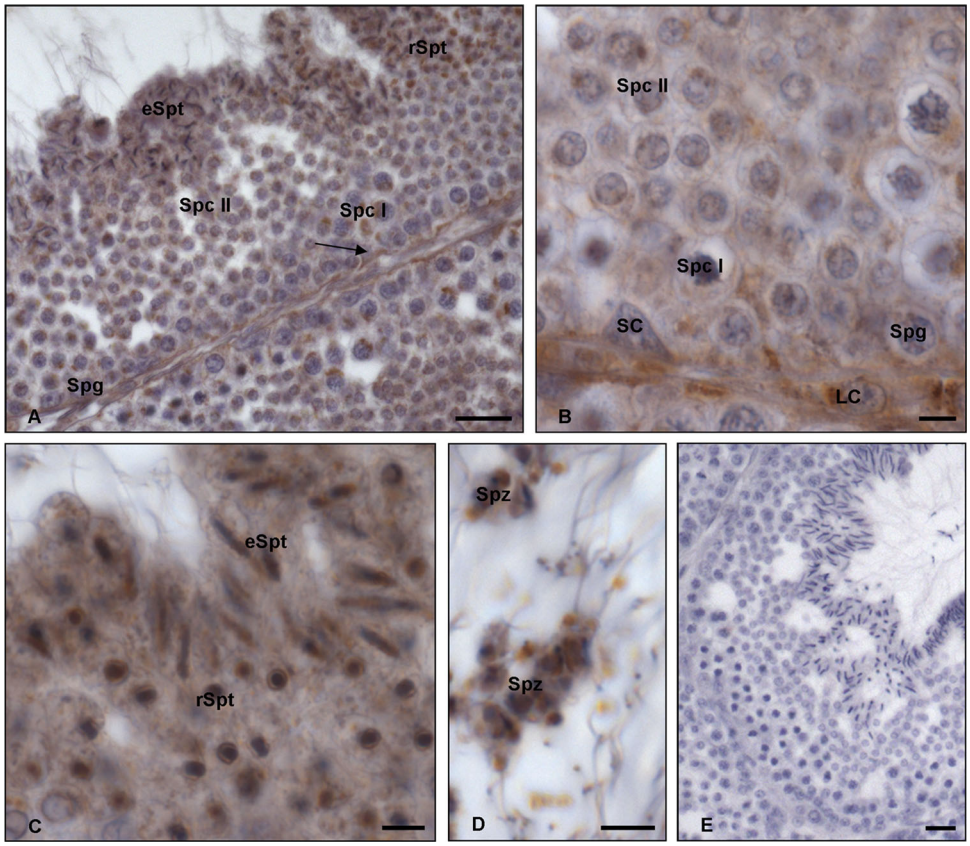

Figure 12. Distribution of $VPAC_2R$ in the testis of *Podarcis sicula*. The immunolocalization signal appears as brown areas in all the germ cells, that is, spermatogonia (Spg) (**A**, **B**), primary (Spc I) and secondary (Spc II) spermatocytes (**A**, **B**), round (rSpt) and elongated (eSpt) spermatids (**A**, **C**), and spermatozoa (Spz) (**D**). Immunoreactivity occurs also in somatic cells, that is, Sertoli (**A**, arrows; **B**, SC) and Leydig (**B**, LC) cells. No signal is evident in control sections (**E**). Scale bars correspond to 20 μm in **A** and **E**, and to 5 μm in **B**–**D**.

positive selection is unlikely (d_N/d_S ratio should be >1) and suggesting the action of a strong functional constraint, which limits the evolution of this peptide, leading to the overall conservation of the coding sites, probably because of its action with its receptor. The Z-test and the Fisher's exact test of selection, carried out to evaluate the kind of selection acting on VIP sequence, confirm the presence of a purifying selection that works against changes in VIP sequence during the evolution. The inferred phylogenetic tree suggests that *Podarcis sicula* VIP sequence is strictly related to that of the other reptile of which the nucleotide sequence is known, *Anolis carolinensis*.

Surprisingly, analyzing the VIP amino acid sequence, we observed that *Podarcis sicula* VIP has 100% identity not only with that of *Anolis carolinensis*, but also with those of *Gallus gallus* and *Xenopus laevis*, and about 90% with the other considered vertebrates. It is worth noting that the variable sites are generally in the C-terminal region and, particularly, that the variations usually involve conservative substitutions: for example, the valine in the fifth position, when substituted, is always substituted by an isoleucine. Differently, the N-terminal region is well preserved; in this regard, we have to remember that the differences between PACAP and VIP in this region determine the selectivity of PACAP for its PACAP-specific receptor (PAC_1R): the conservation of the amino acids in this region allows that VIP does not bind PAC_1R. Finally, on the basis of the phylogenetic consensus tree constructed on amino acid sequences, we conclude that, as the sequences have high identity among these vertebrates, VIP protein sequence has been preserved during the evolution, thus suggesting an involvement of the peptide in important biological functions.

Our data demonstrate that VIP is synthesized directly in the testis of *Podarcis sicula*, at the level of germ and somatic cells. Moreover we demonstrate that, as in mammals, $VPAC_1R$ is poorly represented, while $VPAC_2R$ distribution is wide and similar to VIP. In particular, we demonstrate that the distribution of VIP in the testis is wider than mammals, where it is limited to nerve fibers innerving the testis (Zhu et al., '95) and similar to the distribution

reported in the cartilaginous fish *Torpedo marmorata*, where almost all germ cells are positive to the neuropeptide (Agnese et al., 2012). Differently, the distribution of VIP receptors is similar to that reported in mammals (Hueso et al., '89; Csaba et al., '97; Krempels et al., '95) and in *Torpedo marmorata* (Agnese et al., 2012). In *Podarcis sicula* the distribution of VIP and its receptors strongly suggests an involvement of VIP in the regulation of spermatogenesis; in this regard, it is worth noting that also Sertoli cells, that support the germ cells in their differentiation, are positive to VIP and to its receptor $VPAC_2$. The regulation of spermatogenesis could be mediated trough the increase of cAMP levels after the binding to the $VPAC_2R$, as demonstrated in mammals, where it has been reported that VIP regulates protein synthesis (West et al., '95). Furthermore, as in mammals (Kasson et al., '86; El-Gehani et al., '98a,b; Lacombe et al., 2007), this neuropeptide could influence the regulation of steroidogenesis, as VIP and both its receptors were found in Leydig cells, responsible for testosterone production. The distribution of VIP/VPAC receptors system in *Podarcis sicula* testis is also similar to the localization reported in the non-mammalian vertebrate *Torpedo marmorata* (Agnese et al., 2012), where VIP and $VPAC_2R$ were present in the somatic and germ cells and in the cells with high steroidogenic activity (Prisco et al., 2002, 2008). Thus, in general, in these non-mammalian vertebrates VIP shows a broader tissue distribution than in mammals, while VPAC receptor localization is very similar. So this neuropeptide could have similar testicular functions in mammalian and non-mammalian vertebrates, as the receptors show a similar distribution; however, we could hypothesize that during evolution VIP synthesis sites have been transferred from the testis to other districts, as in the brain, since in mammals the testicular VIP is only of nervous origin. Furthermore, it is worth noting that the cell types positive to the VIP/VPAC receptors system were positive also to the VIP-associated system of PACAP/PACAP receptors (Agnese et al., 2010): indeed, these two local regulator neuropeptides act their functions by the interaction with common receptors, the VPACRs, although PACAP binds also to a PACAP-specific receptor (Dickson and Finlayson, 2009); in this regard, it is interesting that both PACAP and VIP in the non-mammalian vertebrates in which they have been studied (*Torpedo* and *Podarcis*) show a testicular distribution wider than in mammals, thus suggesting that in non-mammalian vertebrates the role of neuropeptides in regulating spermatogenesis could be more significant than in mammals. These two neuropeptides could also act sinergically in regulating testicular functions.

In conclusion, this work demonstrate for the first time the sequence of PHI/VIP in the wall lizard *Podarcis sicula* and the phylogenetic relations between *Podarcis sicula* and other vertebrate VIP sequences; furthermore, our localization data strongly suggest that VIP is an important regulator of *Podarcis sicula* spermatogenesis and, basing on the local production, of the steroidogenesis.

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