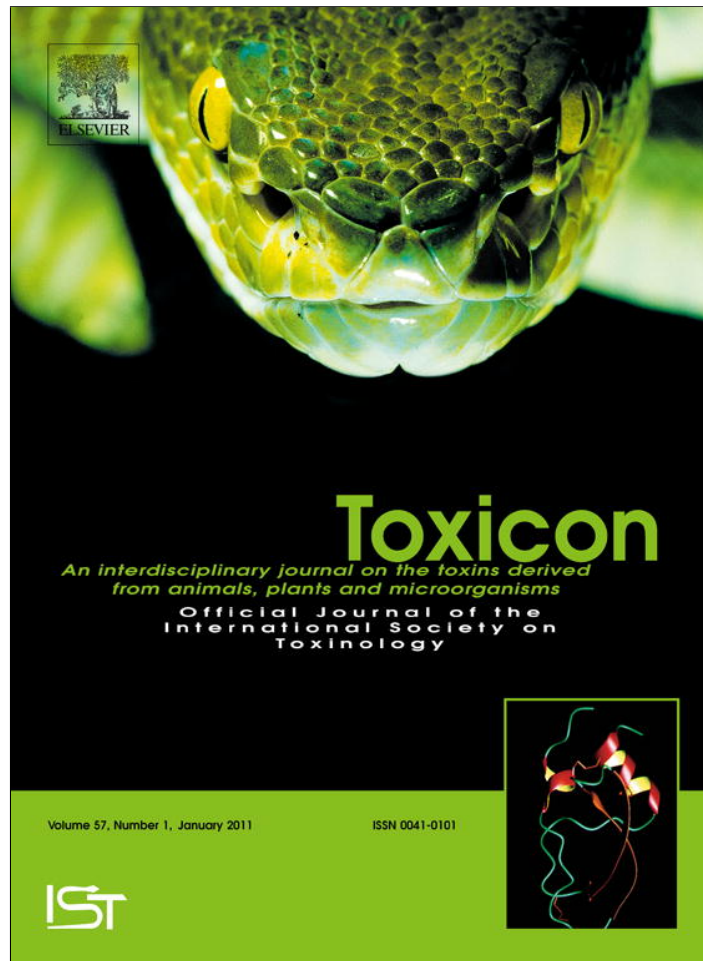


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Variability of tetrodotoxin and of its analogues in the red-spotted newt, *Notophthalmus viridescens* (Amphibia: Urodela: Salamandridae)

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ABSTRACT

Efts and adult specimens ($n = 142$) of the red-spotted newt *Notophthalmus viridescens* from various locations in Canada and USA were analyzed for the presence of tetrodotoxin (TTX) and of its analogues 6-epitetrodotoxin and 11-oxotetrodotoxin. Considerable individual variations in toxin levels were found within and among populations from New Hampshire, New York, Pennsylvania, and Virginia ranging from non-detectable to 69 μg TTX per g newt. TTX and its analogues were absent in efts and adults from various locations in the Canadian province Nova Scotia, the northernmost distribution of the newt, and in adults from Florida. Newts kept in captivity for several years and reared on toxin-free diet lost their toxicity. Bayesian and maximum likelihood phylogenetic analysis of specimens from the various populations using three phylogenetic markers (COI, ND2 and 16S RNA) revealed that populations from the northern states of the USA and Canada are genetically homogenous, whereas the newts from Florida exhibited a much higher level of genetic divergence. An exogenous source of TTX in the newts either via the food chain or by synthesis of symbiotic bacteria is suggested to explain the high variability and lack of TTX in certain populations.

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1. Introduction

Tetrodotoxin (abbreviated TTX) is a specific blocker of voltage-gated sodium channels in excitable membranes (Narahashi, 2008) and occurs in a wide range of unrelated marine organisms such as fish, clams, worms and an octopus (Yasumoto et al., 1988; Khora and Yasumoto, 1989;

Yasumoto and Yotsu-Yamashita, 1996; Noguchi et al., 2006; Noguchi and Arakawa, 2008). Moreover TTX and its analogues 6-epiTTX and 11-oxoTTX have been detected in terrestrial animals, particularly in some species of newts and salamanders (*Taricha*, *Notophthalmus*, *Cynops*, *Triturus* spp.; Wakely et al., 1966; Yasumoto et al., 1988; Hanifin et al., 1999; Yotsu-Yamashita and Mebs, 2001, 2003; Yotsu-Yamashita et al., 2007; Hanifin, 2010), frogs and toads (*Atelopus*, *Brachycephalus*, *Colostethus*, *Polypedates* spp.; Kim et al., 1975; Yotsu-Yamashita et al., 1992; Daly et al., 1994; Mebs et al., 1995; Tanu et al., 2001; Pires et al., 2005). Its role in defence of newts (*Taricha* spp.)

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against its major predator, garter snakes (*Thamnophis* spp.), has been studied by Brodie and his coworkers (Brodie and Brodie, 1991; Brodie et al., 2005; Feldman et al., 2009). They demonstrated that a coevolutionary relationship of newts with the snake predator exists leading to a high toxin concentration in newts and to elevated TTX-resistance snakes. Although symbiotic bacteria have been shown to be involved in the biosynthesis of TTX in marine organisms, the question, whether it is of exogenous origin or synthesized *de novo* by the amphibians is still a matter of discussion.

In young specimens, called efts, and adults of the red-spotted newt, *Notophthalmus viridescens*, from eastern North-America (Virginia), remarkably high levels of TTX and its analogue 11-oxoTTX were assayed beside 6-*epi*TTX as a minor component (Yotsu-Yamashita and Mebs, 2001, 2003). The newt's distribution extends from Florida to the eastern provinces of Canada reaching the north-eastern extremity of its range in Nova Scotia (Petranka, 1998). In the present study, we report the results of TTX-analyses of newts from various locations in Canada and along the east-coast of the USA. Bayesian and maximum likelihood phylogenetic analysis of red-spotted newt populations was performed to elucidate their genetic relationships.

2. Materials and methods

2.1. Geographic sampling

Efts and adults of the red-spotted newts, *Notophthalmus viridescens*, were collected at various locations as listed in Table 1 (Fig. 1). Newts from the Virginia population were kept in aquaria for 3 and 6 years. They were reared with Dipteran larvae, copepods and artificial turtle food (Sera, Heinsberg, Germany).

2.2. Toxin analysis

The newts were killed by freezing and placed in 70% ethanol containing about 0.1% acetic acid. The alcoholic extracts were evaporated to dryness at 45 °C in a stream of nitrogen. Each dry residue was dissolved in 0.05 M acetic

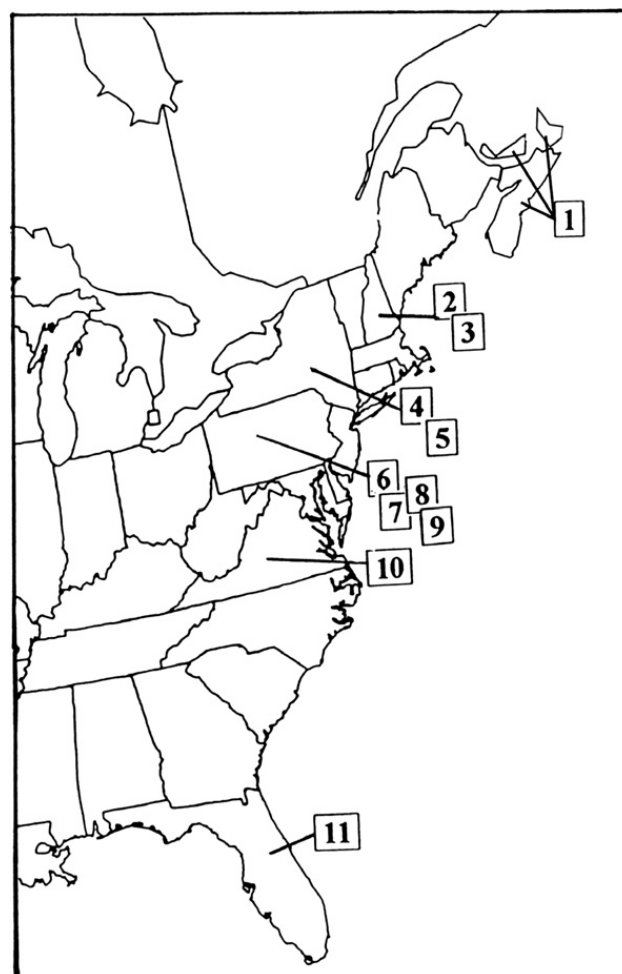


Fig. 1. Map of the collection sites of *Notophthalmus viridescens*. The location numbers correspond to those in Table 1 where geographic details are listed.

acid (1.0 ml/g newt), centrifuged and a part of the supernatant (50 μ l) was adjusted to pH 6 with 1 M NaOH and applied to charcoal (200 μ l) packed in a glass pipette equilibrated with water. Then, the charcoal was washed with water (200 μ l) and the toxins were eluted with acetic acid-ethanol-water 1:49:50 (500 μ l). Aliquots of the eluate

Table 1
Collection sites of efts and adults of *Notophthalmus viridescens*.

Location no.	State	Collection site	No. of specimens	Collection time
1	Nova Scotia Canada Prince Edward Island, Canada	Colpton, Lunenburg Co, Otter Lake, Goffs, Antrim, Elderbank, East River/Sheet Harbour, all Halifax Co.,Kingsville, Inverness Co.Cape Breton Island, Stellarton, Tidnish, Beach Point	27 efts, 8 adults	June/July 2007
2	New Hampshire, NH, USA	Forests, Stratham Co	11 efts	July 2009
3	NH, USA	Forests, Rockingham Co.	5 efts	July 2009
4	New York, NY, USA	Huntington Wildlife Forest, Newcomb, Essex Co.	6 efts	August 2009
5		Seneca Co., forest	12 efts	July 2009
6	Pennsylvania, PA, USA	Rector, Westmoreland Co. Powdermill Res. Station	12 adults	May 2009
7		Cranberry Swamp, Clinton Co	6 adults	May 2009
8		Big Tree Conservation Area, Dauphin Co.	12 adults	May 2009
9		Pond, Hawley, Wayne Co.	6 adults	August 2009
10	Virginia VA, USA	Mt. Lake Biological Station, Giles Co.	10 efts, 12 adults	August 2001
11	Florida FL, USA	Alachua, Jefferson, Okaloosa, Putnam Co.	6 adults	February/April 2008

(100 μ l) were concentrated to dryness in vacuo. The residue obtained was dissolved in 10 μ l of 0.05 M acetic acid and 2.5 μ l was applied to a post-column LC-fluorescent detection (LC-FLD) (Yasumoto and Michishita, 1985; Shoji et al., 2001) for the analysis of the presence of TTX and of its analogues. This charcoal purification procedure was effective to avoid matrix interference. For calibration of the LC-FLD method, authentic TTX, 6-*epi*TTX and 11-oxoTTX which were prepared from natural source or synthesized and quantified using ^1H NMR spectroscopy as previously described (Yotsu-Yamashita et al., 1999) was used.

2.3. DNA isolation, amplification and sequence analysis of 16S rRNA, ND2 and COI

Genomic DNA was isolated from tissue samples of 102 specimens of *Notophthalmus viridescens* by proteinase-K digestion and standard phenol-chloroform extraction. A DNA sequence region coding for the ribosomal RNA subunit 16S was amplified using the primers 16Sa-L and 16Sb-H from Palumbi (1996). For amplification ND2 and COI fragments the ND2-specific primers L3780 and H5018 from Babik et al. (2005) and the COI-specific primers LCO1490 and HCO2198 of Folmer et al. (1994) were employed. PCR conditions were 94 °C (15sec) –45 °C (45sec) –72 °C (90sec) for 5 cycles followed by 30 cycles with 94 °C (15sec) –48 °C (45sec) –72 °C (90sec) (16S); 94 °C (30sec) –56 °C (45sec) –72 °C (90sec) for 35–40 cycles (ND2) and 94 °C (60sec) –45 °C (60sec) –72 °C (60sec) for 40 cycles (COI). These primers were also utilized as sequencing primers. For DNA sequence determination, the PCR products obtained were further amplified by employing the BigDye[®] Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems; Foster City, CA, USA) according to the manufacturer's instructions. Then, the amplified DNA samples were purified by utilizing the DyeEx[®] 2.0 Spin Kit (Qiagen; Hilden, Germany) according to the manufacturer's instructions, denatured for 2 min at 95 °C and sequenced on an ABI 3130 Genetic Analyzer or an ABI 3730 DNA Analyzer. Finally, the obtained DNA sequences specific for 16S rRNA, ND2 and COI were aligned and analyzed using BioEdit (Hall, 1999) and MEGA 4 (Tamura et al., 2007) resulting in fragment lengths of 548–549 bp for 16S, 626 bp for COI and 937bp for ND2. The 16S rRNA sequences were aligned using Clustal W2 (Larkin et al., 2007).

2.4. Phylogenetic analysis of red-spotted newt populations on the basis of three concatenated phylogenetic markers (COI, ND2 and 16S RNA)

Three concatenated phylogenetic markers (COI, ND2 and 16S RNA) were used to construct the phylogenetic trees using the Bayesian and maximum likelihood (ML) methods implemented in the TOPALi software (Milne et al., 2009). Maximum likelihood-based phylogenetic reconstruction was performed with the PhyML 3.0 program (Guindon et al., 2010) and Bayesian phylogeny was inferred with MrBayes 3 program (Ronquist and Huelsenbeck, 2003). HKY + G was identified as the best-fit DNA substitution model both for MrBayes and PhyML. Reliability of the internal branches was assessed using the the 500 bootstrap

replications in PhyML. *Taricha granulosa* was used as an outgroup.

3. Results

3.1. Toxin analysis

A total of 142 specimens of the red-spotted newt *Notophthalmus viridescens* from the northern extreme distribution such as from Nova Scotia and Prince Edward Island, Canada, and from various locations in the United States, i.e. New Hampshire, New York, Pennsylvania, Virginia and Florida, were analysed for the presence of TTX and of its analogues 6-*epi*TTX and 11-oxoTTX. A high individual variability of the toxin levels was observed among the newt populations. TTX was ranging from <0.17 (non-detectable) to 69 μ g/g (Table 2; Fig. 2). Efts and adults exhibited similar toxin levels as demonstrated previously (Yotsu-Yamashita and Mebs, 2001). 11-OxoTTX was a major analogue in the newts from Pennsylvania and Virginia which is in accordance with previous analyses (Yotsu-Yamashita and Mebs, 2001, 2003). However, 6-*epi*TTX, instead of 11-oxoTTX, was the major analogue in the newts from New Hampshire, indicating that the ratio of TTX analogues is also highly variable among the populations. Surprisingly, all specimens from Canada as well as from Florida were entirely toxin-free.

Five specimens from the Virginia population (Mt. Lake Biological Station) were kept in aquaria and reared with toxin-free diet over a period of three (2 specimens) and six years (3 specimens), respectively. Whereas low toxin levels were identified in the newts after 3 years, no traces of toxins were detected in the specimens kept for 6 years in captivity (Table 3).

3.2. Phylogenetic analysis of the newt populations

The Bayesian and ML phylogenies of all the three concatenated markers used (COI, ND2 and 16S RNA) revealed very low levels of genetic diversity among the north-eastern populations of *N. viridescens* from Canada, New Hampshire, New York, Pennsylvania and Virginia, which in principle are genetically identical (Fig. 3). In

Table 2

Mean values and standard deviation of TTX and of its analogues 6-*epi*TTX and 11-oxoTTX in *Notophthalmus viridescens* newts from the various collection sites, expressed in μ g/g (s. Table 1).

Location no	No of specimens	TTX	6- <i>epi</i> TTX	11-oxoTTX
1	35	n.d.	n.d.	n.d.
2	11	19.6 \pm 13.5	25.8 \pm 16.6	5.8 \pm 4.2
3	5	7.6 \pm 3.0	12.5 \pm 3.6	3.9 \pm 2.3
4	5	6.3 \pm 6.6	3.0 \pm 2.5	2.2 \pm 2.5
5	12	7.7 \pm 9.3	1.8 \pm 1.5	4.8 \pm 4.4
6	12	7.8 \pm 4.0	4.3 \pm 3.8	5.5 \pm 6.4
7	6	24.6 \pm 22.4	6.9 \pm 5.3	13.0 \pm 11.4
8	12	19.8 \pm 18.1	6.8 \pm 6.3	7.7 \pm 8.6
9	6	11.3 \pm 3.7	9.5 \pm 3.5	0.6 \pm 1.34
10	22	13.6 \pm 7.2	1.1 \pm 1.1	8.2 \pm 6.8
11	6	n.d.	n.d.	n.d.

n.d. – not detectable (TTX:<0.17 μ g/g, 6-*epi*TTX:<0.016 μ g/g, 11-oxoTTX:<0.26 μ g/g).

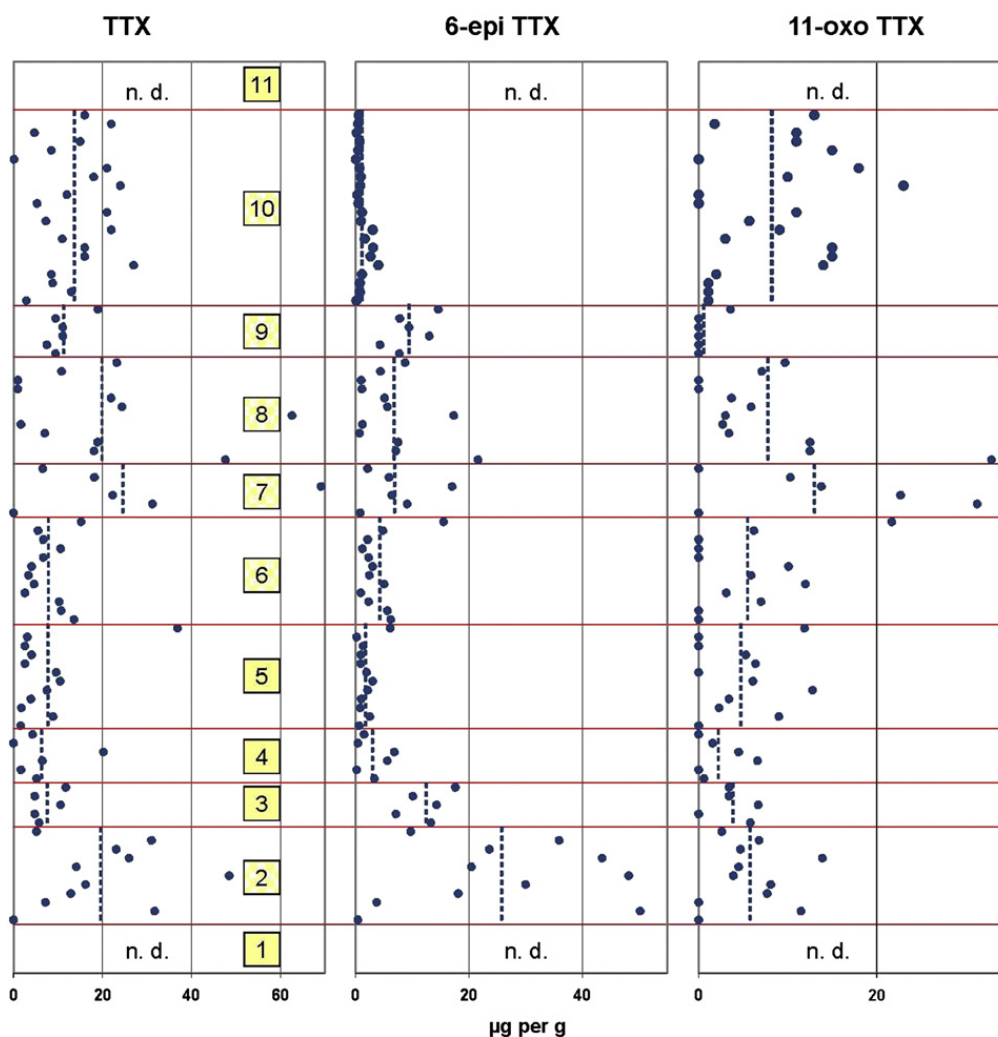


Fig. 2. Concentrations of TTX, 6-epiTTX and 11-oxoTTX in whole body extracts of *Notophthalmus viridescens* specimens from the locations listed in Table 1. The dotted lines represent mean values.

contrast to the north-eastern populations, the southern populations (from Florida) exhibited a higher level of genetic diversity. In the case of the 16S RNA, the level of genetic diversity was found to be rather small also between *N. viridescens* and *N. meridionalis* (distributed in southern Texas and northern Mexico).

4. Discussion

Variability of toxin levels, e.g. TTX and its analogues, has been reported for newts of the genus *Taricha* from the

Table 3

TTX and its analogues 6-epiTTX and 11-oxoTTX (expressed in µg/g) in 5 *Notophthalmus viridescens* newts from Virginia (location 10, Table 2) which had been kept in captivity and reared on toxin-free diet.

	TTX	6-epiTTX	11-oxoTTX
3 years captivity	5.6	0.6	n.d.
	2.0	0.3	2.6
6 years captivity 3 specimens	n.d.	n.d.	n.d.

n.d. – not detectable (TTX:<0.17 mg/g, 6-epiTTX:<0.016 mg/g, 11-oxoTTX:<0.26 mg/g).

western USA (Hanifin et al., 1999, 2008), *Cynops pyrrhogaster* from Japan (Yotsu et al., 1990; Tsuruda et al., 2001) and for *Triturus* species from Southern Germany (Yotsu-Yamashita et al., 2007). The present study on the red-spotted newt, *Notophthalmus viridescens*, revealed a similar pattern of toxin concentrations ranging from non-detectable to high levels of TTX in members of various populations from the eastern states of the USA, but newts from the northern (Nova Scotia, Canada) and southernmost part (Florida) of their distribution range were toxin-free. This includes efts as well as adult specimens from Canada, but adults only from Florida where the newt's larvae develop into aquatic juveniles omitting the eft stage.

The results of the Bayesian and ML phylogenetic analysis of *Notophthalmus viridescens* populations using three concatenated phylogenetic markers (COI, ND2 and 16S RNA), indicate that TTX-bearing populations are not genetically separated from those that lack TTX. Florida populations of *N. viridescens* showed a higher level of genetic diversity than the north-eastern populations. Surprisingly, TTX-negative specimens from Canada are genetically identical with those from locations in New

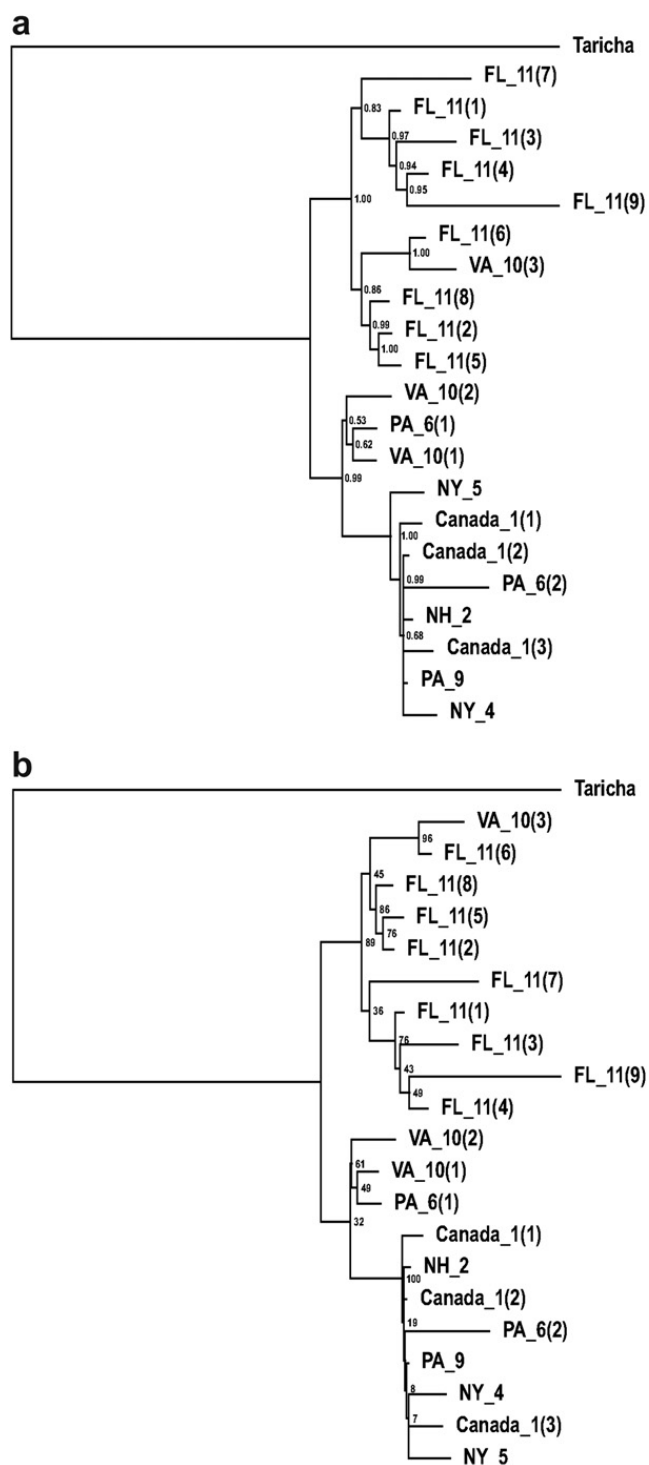


Fig. 3. Phylogenetic analysis of the newt populations. a) Bayesian phylogeny of the three concatenated phylogenetic markers used (COI, ND2 and 16S RNA). Bayesian phylogeny was generated with the MrBayes 3 program. *Taricha granulosa* has been used to root this tree. b) Maximum likelihood phylogenetic tree of the three concatenated phylogenetic markers used (COI, ND2 and 16S RNA). The ML phylogeny was generated with the PhyML 3.0 program. Reliability for the internal branches was assessed using the 500 bootstrap replications.

Hampshire, New York, Pennsylvania and Virginia which generally contain high concentrations of TTX and of its analogues. The results of the Bayesian and ML phylogenetic analysis are therefore consistent with the Pleistocene

extinction and population expansion models that predict northern populations will have reduced genetic diversity and appear younger in evolutionary time due to their recent expansion (Hewitt, 2004). During Pleistocene, the north-eastern part of North America was glaciated between 90,000 and 18,000 years ago. Populations of plants and animals generally show lower levels of intra-specific variation in areas severely affected by the Pleistocene glaciations. Following the retreat of the Laurentide ice sheet, recolonization by animals including amphibians occurred from southern refugia (such as Florida), where populations of newts show higher genetic diversity. Animal populations in the province of Prince Edward Island and parts of Nova Scotia were isolated from the mainland by rising sea levels 5000 to 3000 years ago during the late Hypsithermal interval (Gilhen, 1984). After an expansion in the postglacial period, intraspecific diversity declined outside the refugia as a consequence of successive founder effects. These founder events may explain the low genetic diversity among the northern populations which colonized their present habitats in more recent time, e.g. 10,000 to 21,000 years ago (Hewitt, 2004; Zeisset and Beebe, 2008).

In marine organisms such as in red algae (Yasumoto et al., 1986), puffer fish (Noguchi et al., 1987, 2006; Noguchi and Arakawa, 2008) and in the blue-ringed octopus (Hwang et al., 1989), it has been demonstrated that bacteria are involved in the biogenesis of TTX. However, no evidence for the bacterial origin has been found in newts like *Taricha torosa* from the west-coast of North-America (Lehman et al., 2004; Cardall et al., 2004). Shimizu and Kobayashi (1983) showed that the newts were unable to synthesize TTX *de novo*. However, Hanifin et al. (2002) and Cardall et al. (2004) suggested that the newts are able to synthesize TTX, based on the observation that toxin levels in the dorsal skin of the newts increased under rearing with toxin-free, artificial diets and recovered after releasing substantial amounts of TTX by electric stimulation. Moreover, Brodie et al. (2005) proposed that the presence of TTX in salamandrids like *Taricha*, *Cynops* and *Notophthalmus* is ancestral and that variation in toxicity within and among populations of newts may have a genetic basis.

Biosynthesis of a complex molecule like TTX involves numerous steps and a complicated enzymatically driven process till an active compound is produced. Not one gene only, but a genetic machinery is necessary for a successful synthesis of TTX either by bacteria or higher organisms. Despite its long history and a thorough knowledge of its toxicity and pharmacology, neither the pathway to TTX nor even the biogenetic origin of TTX is known. The debate, whether TTX is derived from bacteria or it is of endogenous origin of the host animals is ongoing. The only study on substrates of TTX biosynthesis proved to be inconclusive (Chau et al., 2011). The central question concerning the chemical ecology and evolutionary biology of TTX in amphibians is still that of an endogenous versus exogenous origin of the compound (Hanifin, 2010).

If animals synthesize TTX *de novo* (endogenous TTX hypothesis), there is an energetic cost involved to produce the toxin. During evolution advantageous features, e.g.

genes, are rapidly fixed by selection, but will also be eliminated, if they are not beneficial. When toxicity is of advantage to the newts such as providing protection from predators, TTX synthesis is a survival factor. But when the predatory pressures are low, mutations may occur and will be fixed by selection ceasing the synthesis of the toxin. Due to the complexity of a TTX synthesis pathway, the loss of single enzymatic step in the pathway would be sufficient to disrupt the whole process.

It may even be speculated that some populations of *N. viridescens* have been void of TTX. However, factors such as coevolution with a predator, as in the case of the *Taricha*-garter snake system (Hanifin et al., 2008), may have generated selection pressure which triggered TTX production. On the other hand, the low levels, absence or even loss of the toxin in specimens which belonged to a population, i.e. Virginia, exhibiting previously high toxin concentrations on average and which had been kept in captivity for several years contradicts a continuous or a rapid change in synthesis of TTX by the newts.

Environmental factors such as acquiring TTX from the diet may also be taken into account. For instance frogs of the families Dendrobatidae, Mantellidae and Myobatrachidae, but also toads from the genus *Melanophryniscus* accumulate alkaloids in their skin which originate from their diet like ants, termites and other arthropods (Daly et al., 1984, 2005, 2008; Garraffo et al., 1993; Smith et al., 2002; Mebs et al., 2005, 2007). Some of these alkaloids like the pumiliotoxins have been detected in formicine ants (Saporito et al., 2004) as well as in mites of the family Oribatidae (Takada et al., 2005; Saporito et al., 2007). *Notophthalmus* newts have a complex life cycle. In their eft stage they are terrestrial for several years, in some populations as long as 7 years (Forester and Lykens, 1991) or even longer. They feed on leaf litter which includes a wide variety of prey species where springtails (collembolans), mites, fly larvae and spiders are most abundant (MacNamara, 1977). It would be worth to investigate these food items for the presence of TTX or its analogues.

The implication of microbials as producers of the toxins, which are enriched and sequestered via the food chain, or even as symbionts in the newts is another option to explore. Storage and sequestration of TTX requires a special transport system in the newts as well as adaptation and resistance to the toxicity of this toxin. This seems to be a unique characteristic of *Notophthalmus* and *Taricha* species, which is not found in other sympatric salamanders.

The analysis of the TTX analogues showed that the stereoisomer 6-*epi*TTX is present in substantial concentrations in the newt extracts, but varies considerably among individuals as well as populations, particularly when the ratio TTX/6-*epi*TTX is concerned. Similar observations have been made with *Taricha* species (Hanifin et al., 1999; Hanifin, 2010). This toxin analogue is rarely associated with TTX in marine animals, but seems to be characteristic for amphibians (Yotsu et al., 1990; Yasumoto and Yotsu-Yamashita, 1996; Hanifin et al., 1999; Yotsu-Yamashita and Mebs, 2001). Although it appears to be difficult to interpret this phenomenon, it does not rule out a dietary or symbiotic origin of TTX and of its analogues.

Brodie and Brodie (1991) and Brodie et al. (2005) observed that in regions of western North America where newts of the genus *Taricha* exhibit high TTX values, the sympatric garter snake *Thamnophis* spp., which are predators of newts, are resistant to TTX. Amino acid substitutions in the pore-forming structures of the snake's sodium channel (Na_v 1.4) have been identified which prevent binding of TTX (Geffeney et al., 2002, 2005; Feldman et al., 2009). Studies on the phylogenetic relationships of *Thamnophis* species indicate that adaptive evolution of elevated TTX-resistance has occurred multiple times in these snakes via *de novo* acquisition of beneficial mutations (Feldman et al., 2009). Toxicity in *Taricha* species and the evolution of TTX-resistance in garter snakes are interpreted as a parallel arms race driven by coevolution, which may have occurred within a short time scale (Brodie et al., 2002; Williams et al., 2003; Hanifin et al., 2008). It is challenging to find out, whether there exists a similar arms race between *Notophthalmus* and predatory snakes like the local *Thamnophis* or other snake species along the east-coast of North-America.

Occasionally predation on *Notophthalmus* newts has been observed such as by bullfrogs, turtles and some snakes (Hurlbert, 1970; Brodie, 1968). Shure et al. (1989) had found some disemboweled newts suggesting that a predator, perhaps a bird, consumed only the internal organs avoiding contacts with the toxic skin. The bright red to yellow colouration of efts has been suggested to function as warning signal particularly for avian predators. Efts are considered to be part of a mimicry complex involving other salamanders such as *Pseudotriton ruber*, which is non-toxic (Howard and Brodie, 1971, 1973).

Further studies on additional populations of *Notophthalmus viridescens* distributed over mainland Canada (Quebec, Ontario) and the eastern USA south to Florida are required. This work will show whether TTX in this newt species is a common phenomenon with few exceptions or whether it occurs randomly in specific populations or individuals. Studies on newts kept and bred under controlled conditions are necessary to solve the central question of the biogenetic origin of the toxin.

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Conflict of interest

None declared.

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