SALAMANDRA **52**(3) | 255–260 | 30 October 2016 | ISSN 0036–3375

Tetrodotoxin concentrations in rough-skinned newts, *Taricha granulosa*, from populations of their northern distribution range

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Manuscript received: 30 December 2014
Accepted: 19 April 2015 by Stefan Lötters

Abstract. Tetrodotoxin (TTX), which is one of the most potent neurotoxins blocking voltage-gated sodium channels of excitable membranes, is present in newts of the genus *Taricha* in varying concentrations. Whether there is a genetic basis of that variability was tested by maximum likelihood phylogenetic analysis of *T. granulosa* specimens from the most northern distribution range in southeastern Alaska (USA), British Columbia (Canada), and from Oregon (USA), using COI and 16S RNA as markers. TTX was quantitatively assayed in methanol extracts of the newts. Whereas very low TTX concentrations were detected in newts from Alaska, high individual variations in toxin levels were found in populations from British Columbia and Oregon, ranging from non-detectable amounts to 0.838 mg per specimen. Phylogenetic analysis revealed that theses populations are genetically homogenous. Therefore variation in TTX levels is not based on genetically distinct newt phenotypes, but may be the result of unknown endogenous mechanisms and/or exogenous, e.g., environmental, influences.

Key words. Amphibia, Urodela, *Taricha granulosa*, Alaska, British Columbia, Oregon, tetrodotoxin.

Introduction

Newts of the genus *Taricha* are well known to contain tetrodotoxin (TTX) (Mosher et al. 1964, Brodie 1968), one of the most potent neurotoxins specifically blocking voltage-gated sodium channels (Kao 1966, Narahashi et al. 1967, Moczydlowski 2013). High concentrations of TTX were found to be present in the skin, ovaries, and eggs, and minor amounts were detected in muscle and blood (Wakely et al. 1966, Hanifin et al. 1999). However, a high variability of TTX levels within and between populations throughout their range of distribution was observed (Hanifin et al. 1999, 2008). Along the west coast of the United States and Canada, TTX in the skin of *Taricha* specimens varied from zero to 4.695 mg (Hanifin et al. 2008). This has been considered to be the result of co-evolution with the newts' main predators, i.e., garter snakes (*Thamnophis* spp.) (Brodie & Brodie 1990, Hanifin et al. 1999, 2008, Brodie et al. 2005, Feldman et al. 2009, 2012, Williams et al. 2010). In areas where the newts exhibit high toxicity, remarkable resistance to TTX is observed in these snakes, which enables them to predate upon *Taricha* species (Feldman et al. 2012). Structural changes in their voltage-gated sodium channel $Na_v1.4$ by amino acid replacements dramatically reduce the binding affinity of TTX to the channel (Geffeney et al. 2002, Feldman et al. 2012). On the other hand, garter snakes living in habitats of newts with low toxicity were found to possess TTX-sensitive sodium channels.

The distribution of the rough-skinned newt *Taricha granulosa* ranges from the San Francisco Bay in west-central California to southeastern Alaska, including the coastline and various islands of British Columbia (BC), Canada (Nussbaum et al. 1983, Petranka 1998). Very low TTX concentrations have been assayed in specimens from Vancouver and Texada Island, and no TTX was detected in those from the Inland Lake (Provincial Park, BC), but toxicity seemed to increase in southern populations with the highest toxin levels being found in specimens from Oregon (USA) (Hanifin et al. 2008).

Brodie et al. (2005) and Hanifin et al. (2010) suggested that TTX levels and the high spatial variability of toxicity amongst populations are under genetic control in *Taricha* species. This raises two questions: (1) Do genetically distinct populations exist along the wide range of their geographic distribution, and (2) do low or high TTX level traits characterise particular newt phenotypes, i.e., subspe-

cies? To answer these questions, phylogenetic analysis and assays of TTX concentrations were performed using specimens of *T. granulosa* populations from their most northern distribution range, i.e., southeastern Alaska (USA), British Columbia (Texada Island, Canada), and Oregon (USA).

Materials and methods

Geographic sampling and toxin analysis

Adults of rough-skinned newts were collected with dip net at the locations listed in Table 1 and shown in Figure 1. A total of 50 specimens of adult *T. granulosa* from the extreme northern distribution, i.e., southeastern Alaska (19 specimens, 5 from the Stikine River were combined in one sample) (Figs 2, 3) and British Columbia, Canada (12 specimens from Texada Island), and from a southern location, Woahink Lake in Oregon, USA (19 specimens), were analysed for the presence of TTX (Fig. 4). The newts were killed either by freezing or lidocaine injection and placed in 80% methanol containing about 0.1% acetic acid. The methanolic extracts were evaporated to dryness at 25°C in a jet of compressed air. Each dry residue was dissolved in 0.05 M acetic acid (0.5 mµl/g newt), centrifuged, and a part of the supernatant (1 ml) was applied to post-column LC-fluorescent detection (LC-FLD) (Yasumoto and Michishita, 1985, Shoji et al. 2001) to test for the presence of TTX. The lowest detection level of TTX was 0.00015 mg/g.

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

DNA was isolated from tissue samples (tip of the tail) of 13 *Taricha granulosa* specimens and one *T. torosa* (from Edgewood, CA) by proteinase-K digestion and standard phenol-chloroform extraction. For amplification and sequencing of COI fragments, either the primer pair Chmf4 (5'-TYTCWACWAAYCAYAAAGAYATCG G-3'), Chmr4 (5'-ACYTCRGGRTGRCCRAARAATCA-3') and/or COI-

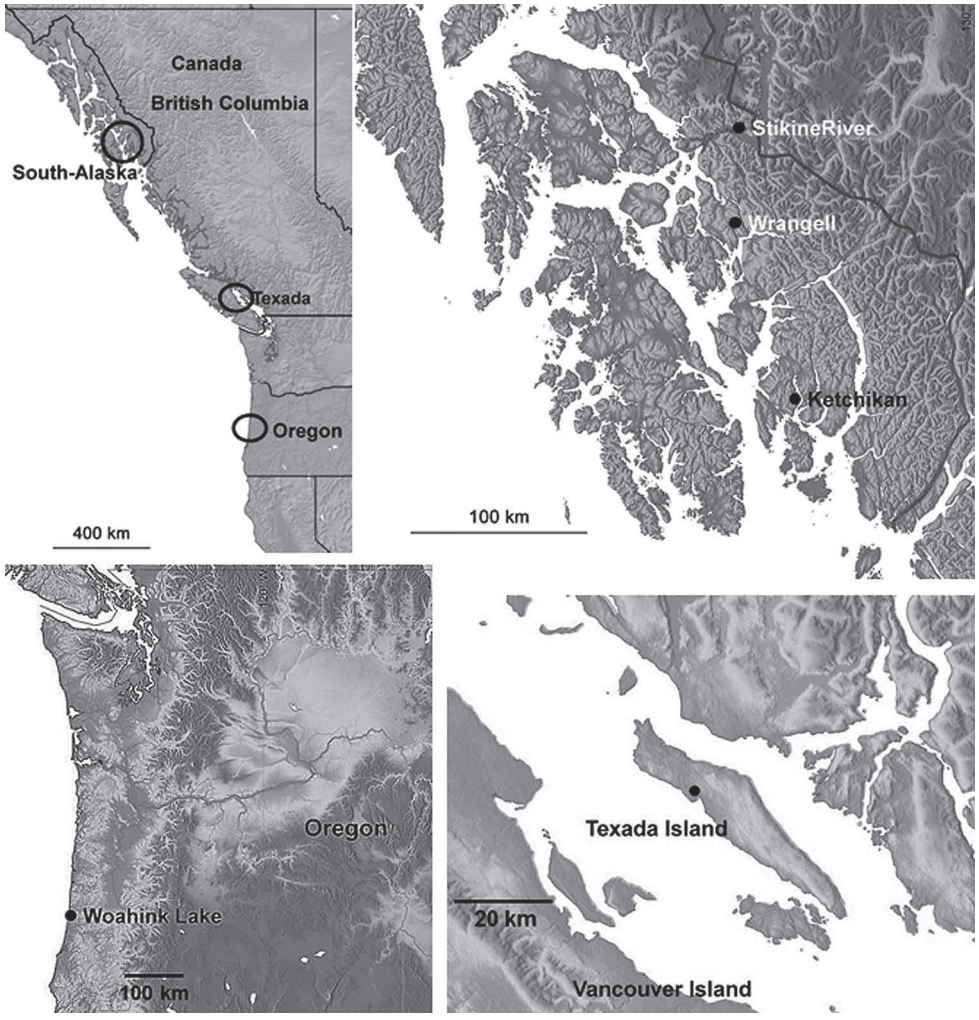

Figure 1. Maps of the collection sites of *Taricha granulosa* in southeastern Alaska, British Columbia (Texada Isl., Canada), and Oregon (USA). For details see Table 1.

Table 1. Details of sites where *Taricha granulosa* was sampled. Altitude in metres above sea level is abbreviated m a.s.l.

Location	Collection site	No. of specimens	Collection date
Ketchikan Island, AL, USA	Small pond next to road to Silvis Lake N 55.2286°N, 131.2978°W 246 m a.s.l.	5	June 4, 2013
	Same area, 2nd pond 55.2287°N, 131.2948°W 211 m a.s.l.	8	June 4, 2013
Wrangell Island, AL, USA	Peat bog, near road 6265 56.1963°N, 132.0928°W 125 m a.s.l.	1	June 5, 2013
Stikine River, AL, USA	Warm spring near Figure-Eight Lakes, Stikine River 56.7012°N, 132.2835°W 18 m a.s.l.	5	June 5, 2013
Texada Island, BC, Canada	Small pond near golf course 49.4613°N, 124.3644°W 22 m a.s.l.	12	June 6, 2012
Woahink Lake, Florence, OR, USA	Lake shores 43.5560°N, 124.0600°W 23 m a.s.l.	19	June 9, 2013

COI (5'-TYTCWACWAAYCAYAAAGAYATTGG-3'), COI-CO3 (5'-ACYTCYGGRTGACCAAARAAYCA-3') (Che et al. 2012) were employed. PCR conditions were 95°C (5 min) followed by 35 cycles at 94°C (1 min), 46°C (1 min) for either primer set or 72°C (1 min), completed by a subsequent elongation step at 72°C for 10 min. A region of 16S rRNA was amplified using the primers LO2510 (5'-CGCCTGTTTATCAAAAACAT-3') and HO3063 (5'-CTCCGGTTTGAACTCAGATC-3') (Palumbi et al. 1996), which were also used as sequencing primers. PCR conditions were 94°C (3 min) followed by 35 cycles at 94°C (30 sec), 53°C (30 sec), and 72°C (40 sec), completed by a subsequent elongation step at 72°C for 7 min. 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. Finally, the obtained DNA sequences specific for 16S rRNA were aligned using CodonCodeAligner 5.0 and analysed applying the MEGA 6 nucleotide substitution model HKY (Hasegawa et al. 1985, Tamura et al. 2007), resulting in fragment lengths of 440 to 506 bp.

Figure 2. Rough-skinned newt, *Taricha granulosa*, from Ketchikan Island, Alaska (USA). Specimen not collected.

Phylogenetic analysis

The evolutionary history was inferred by using the Maximum Likelihood method based on the Hasegawa-Kishino-Yano model (Hasegawa et al. 1985). Initial tree(s) for the heuristic search were obtained by applying the Neighbor-Join and BioNJ algorithms to a matrix of estimated pairwise distances using the Maximum Composite Likelihood (MCL) approach and then selecting the topology with a superior log likelihood value.

Results

Toxin analysis

Whereas the newts from southeastern Alaska exhibited rather low TTX values, high individual variability in toxin concentration was observed in a population from Texada

Figure 3. Typical habitat of *T. granulosa* on Wrangell Island, southeastern Alaska, where a specimen (listed Tab. 1) was found. Forest peatland (muskeg, an acidic soil type in boreal Alaska) with dense moss cover (*Sphagnum*), shore pines (*Pinus contorta*), spruce (*Picea* sp.), and stagnant pools (water temperature 10°C in June of 2013).

Island (BC, Canada), ranging from 0.0069 to 0.839 mg per specimen (Fig. 4). Similar values were detected in newts from a southern population (Woahink Lake, OR), ranging from below detectable level to 0.6628 mg/specimen. In all samples TTX was the major toxin, and only traces of its analogue 6-epiTTX were detected.

Phylogenetic analysis of the newt populations

Since COI sequences showed complete identity among the *T. granulosa* populations, using 16S RNA as marker, maximum likelihood phylogeny analysis confirmed a very low level of genetic diversity among the populations (Fig. 5).

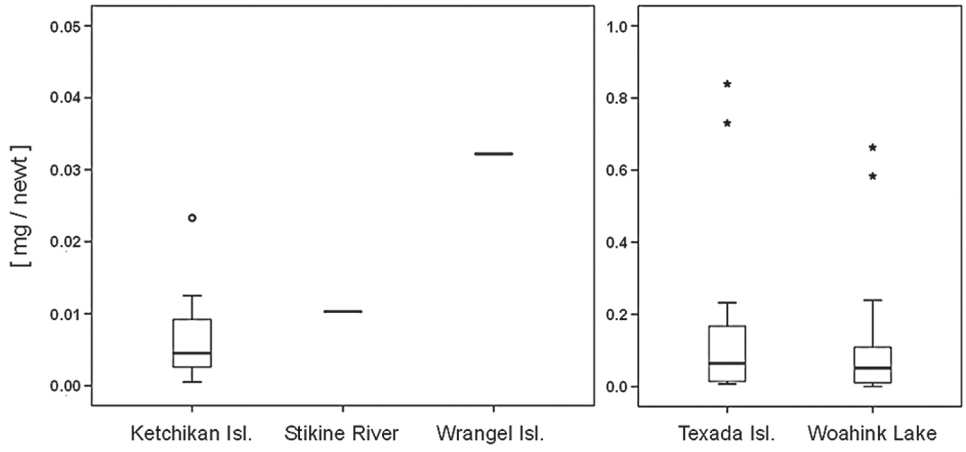

Figure 4. Tetrodotoxin concentrations of individual specimens (*T. granulosa*) from different locations: Alaska, USA (Ketchikan Isl., Wrangell Isl., Stikine River), British Columbia, Canada (Texada Island), and Oregon, USA (Woahink Lake). One specimen from Wrangell Isl. contained 0.0322 mg, and the mean value of 5 specimens from Stikine River was 0.0103 mg, respectively. Boxplots show the mean concentrations of TTX.

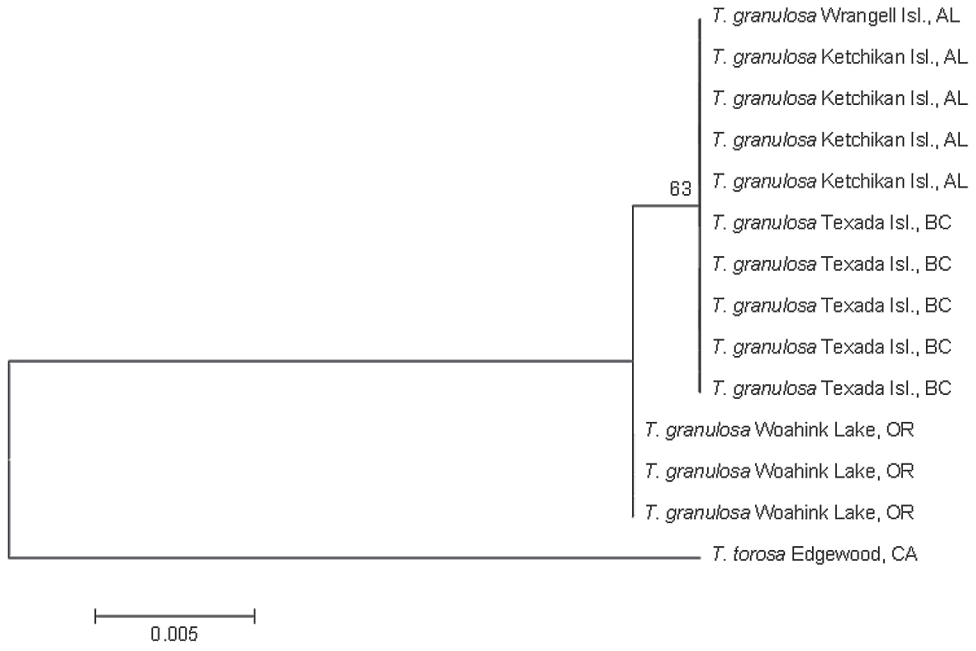

Figure 5. Maximum likelihood phylogenetic tree of the newt populations (*T. granulosa*) using 16S RNA as molecular marker. *Taricha torosa* was used to root the tree. The tree with the highest log likelihood (-790.3704) is shown. The percentage of trees, in which the associated taxa clustered together, is shown next to the branches. The tree is drawn to scale, with branch lengths being determined by the number of substitutions per site. The analysis involved 14 nucleotide sequences. All positions with less than 5% site coverage were eliminated, that is, fewer than 95% alignment gaps, missing data, and ambiguous bases were allowed at any position. There were a total of 506 positions in the final dataset. Evolutionary analyses were conducted applying the MEGA6 nucleotide substitution model: HKY (Hasegawa et al. 1985, Tamura et al. 2007).

Those from southeastern Alaska and British Columbia are genetically identical, and specimens from the Oregon population exhibited a single base substitution only (C to T).

Discussion

High variability in TTX levels is a common phenomenon in newts of the genus *Taricha* (Hanifin et al. 1999, 2008), in *Notophthalmus viridescens* from eastern North America (Yotsu-Yamashita et al. 2012, Bucciarelli et al. 2014), *Cynops pyrrhogaster* from Japan (Yotsu et al. 1990, Tsuruda et al. 2001), and *Triturus* species from southern Germany (Yotsu-Yamashita et al. 2007). A similar tendency in toxin concentration seems to exist in populations of *T. granulosa* as well as *N. viridescens*: No (*N. viridescens*) or very low levels (*T. granulosa*) of TTX are observed in the most northern populations of the newts.

It is noteworthy that specimens of *T. granulosa* from Alaska, British Columbia, and Oregon are genetically homogenous, as revealed by phylogenetic analysis using COI and 16S RNA as markers (Fig. 5). This confirms studies by Kuchta & Tan (2005) on populations from the states of Oregon, Washington, and Alaska. Based on data of allozyme variation at 45 loci and cytochrome-b sequences, they found that *T. granulosa* from these northern populations differed by only a single mutational step. Since the northern parts of North America were glaciated from 90,000 to 18,000 years ago (Hewitt 2004), this suggests a recent range expansion in the north following the retreat of the ice sheet 10,000 years ago and may explain the reduced genetic diversity (Zeisset & Beebee 2008). Similar results had been obtained for *N. viridescens* from various locations along the east coast of North America: newts from Canada (Nova Scotia, Prince Edward Island) were genetically identical to specimens from Virginia (Yotsu-Yamashita et al. 2012), which most probably is likewise a result of Pleistocene extinction followed by recolonisation of the present areas by animals from southern refugia.

The biogenetic origin of TTX is considered to be either endogenous (de novo synthesis of the toxin by the newt) or exogenous (bacterial) (Hanifin 2010). Whereas the latter has been demonstrated for marine animals (Noguchi et al. 2006, Noguchi & Arakawa 2008), no evidence proving a bacterial source has so far been found in *T. torosa* (Cardall et al. 2004, Lehman et al. 2004). On the other hand, increased toxin levels have been detected in newts when these were reared on an artificial, e.g., toxin-free, diet in long-term captivity (Hanifin et al. 2002). Releasing substantial amounts of TTX following electrical stimulation, the toxin is rapidly regenerated in the skin (Cardall et al. 2004). Moreover, female newts produce TTX-laden eggs even after long-term captivity (Gall et al. 2012). These results suggest that TTX is of endogenous origin, and that *Taricha* newts are able to synthesize TTX by means of still unknown metabolic pathways. It is also of interest that TTX is the main toxic component in *Taricha* species, and the analogue 6-epiTTX is only present in trace amounts, whereas in *N. viridescens* its concentration often exceeds that of TTX (Yotsu-Yamashita & Mebs 2001, Yotsu-Yamashita et al. 2012).

Co-evolution with a predator such as garter snakes (*Thamnophis* spp.) has been proposed to trigger toxin production in newts (Brodie & Brodie 1990, Brodie et al. 2005, Hanifin et al. 2008). In areas where *Taricha* species are highly toxic, sympatric garter snakes show remarkable resistance to TTX. Mutations in their genecoding the voltage-gated sodium channel $Na_v1.4$ in nerve and muscle caused amino acid substitutions in the channel's outer pore, which prevent the binding of TTX (Geffeney et al. 2002, Feldman et al. 2012). Interpreted as an "arms race" between garter snakes and their toxic prey, low levels of TTX in *Taricha* populations may suggest a low predatory pressure, which would be consistent with the present findings of very low toxin concentrations in newts from southeastern Alaska, where garter snakes are absent. Whereas the molecular basis of TTX resistance in *Thamnophis* snakes is well defined by gene mutations, the mechanisms mediating the adaptation of the newts to cope with predatory pressures are not understood. Provided that *Taricha* is able to synthesize TTX, an increase of toxin levels needs the activation of a complex biosynthetic pathway, involving a number of enzymes. This implies that high or low TTX levels, i.e., changes in the newt's toxicity, are not the result of mutations in a single gene, but are rather due to still unknown endogenous mechanisms or exogenous influences such as those of environmental factors like the availability of precursor molecules for TTX synthesis.

Acknowledgements

Collecting permits were issued by the Oregon Department of Fish and Wildlife (permit no. 028-12), the Ministry of Natural Resource Operations of British Columbia, Canada (no. VI11-73288), and the Department of Fish and Game, Alaska (no. SF 2013-071).

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