New Microsatellite Markers for Examining Genetic Variation in Peripheral and Core Populations of the Coastal Giant Salamander (*Dicamptodon tenebrosus*)

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Abstract

The Coastal Giant Salamander (*Dicamptodon tenebrosus*) is classified as threatened at the northern periphery of its range in British Columbia (BC), Canada, primarily due to forestry practices and habitat fragmentation. Characterising dispersal behaviour and population connectivity is therefore a priority for this region, while genetic differentiation in core versus peripheral locations remains unstudied in this wide-ranging species. We present seven new polymorphic microsatellite markers for use in population genetic analyses of *D. tenebrosus*. We examine locus characteristics and genetic variation in 12 streams at the species’ northern range limit in BC, and within two regions representing sub-peripheral (North Cascades) and core localities (South Cascade) in Washington State, United States. In BC, the number of alleles per locus ranged from 2–5 and observed heterozygosity ranged from 0.044–0.825. Genetic differentiation was highest between BC and the South Cascades, and intermediate between BC and the North Cascades. Across loci, mean allelic richness was similar across regions, while private allelic richness was highest in the core locality (corrected for sample size). These new microsatellite loci will be a valuable addition to existing markers for detailed landscape and population genetic analyses of *D. tenebrosus* across its range.

Introduction

Species at the periphery of their range may show reduced genetic diversity that can limit microsatellite variation and the potential for detailed genetic analyses that are relevant for conservation [1]. The stream-breeding Coastal Giant Salamander (*Dicamptodon tenebrosus*) is endemic to the Pacific North-west coast of North America, from northern California up to an approximately 100 km² area in the Chilliwack Valley of southern British Columbia (BC), Canada. In Canada, *D. tenebrosus* is designated as Threatened and is on the provincial Red List in BC (COSWC 2000: http://www.sararegistry.gc.ca/species/speciesDetails), due to high susceptibility to decline from habitat degradation owing to forestry practices [1,2]. Such peripheral populations are uniquely positioned to aid conservation management throughout a species’ range, as they can provide information regarding adaptive potential and local adaptation at environmental margins [1]. We describe seven new polymorphic loci specific for *D. tenebrosus*, complementing existing microsatellite loci developed for *D. tenebrosus* [2,3] and those previously cross-amplified from *D. copei* [4]. We examine locus characteristics in populations of *D. tenebrosus* at their northern range limit to evaluate genetic variation at these new microsatellite markers. We also provide locus characteristics for two regions in Washington State (WA) to examine for differences in allelic diversity, heterozygosity, and genetic differentiation between peripheral populations in BC, with sub-peripheral (North Cascades) and core (South Cascades) regions of the species’ range. These new loci have potential use in other *Dicamptodon* species and combined with existing loci, will enable range-wide genetic analyses using high-resolution microsatellite data.

Materials and Methods

Ethics statement

This research was conducted with approval of the Animal Care Committee of the University of British Columbia (permit A08-0241) in accordance with the Canadian Council on Animal Care. Samples were collected from *D. tenebrosus* throughout the Chilliwack Valley of British Columbia in Canada (∼70 km² in area), and from two regions in Washington State, United States (each ∼50 km² in area). All individuals were anesthetised in a 0.05% solution of MS-222 (0.5 g/L), before a 2–4 mm² sample of tail tissue was cut from the tail tip and preserved in 90% ethanol. Individuals were immediately recovered in a stream water bath for 10–20 minutes before being returned to their capture location. Genomic DNA was extracted from British Columbia samples using a standard phenol-chloroform extraction method [5] and from WA samples using a QIAGEN DNeasy 96 extraction kit. An
enriched library was made by Ecogenics GmbH (Zurich, Switzerland) from size-selected genomic DNA ligated into SNX forward/SNX reverse linker [6] and enriched by magnetic bead selection with biotin-labelled (CT)13, (GT)13, (GTAT)7 and (GATA)7 oligonucleotide repeats [7,8]. Of 528 recombinant colonies screened, 330 gave a positive signal after hybridization. Plasmids from 100 positive clones were sequenced and primers were designed for 33 microsatellite inserts, of which 25 were tested for polymorphism. Polymorphism at seven loci (Table 1) was established by preliminary testing undertaken by Ecogenics GmbH (Zurich, Switzerland) using 42 randomly selected individuals. We present locus characteristics and examine genetic diversity in a larger sample size from 12 streams (i.e. populations) sampled within the northern range limit of D. tenebrosus in British Columbia (Latitude: 49°4′10″; Longitude: -121°53′00″). We then compare BC locus characteristics with those from individuals collected within the North Cascades (NC) (Latitude: 48°42′00″; Longitude: -121°12′00″), and the South Cascades (SC) (Latitude: 45°41′00″; Longitude: -122°08′00″) of Washington State, which are located approximately 60 km and 350 km south of the Chilliwack Valley respectively. NC may therefore be regarded as sub-peripheral, and SC as core within the entire range of D. tenebrosus. Individuals with missing genetic data were excluded from the dataset. Genotypes from each region (BC, NC and SC) were screened for genetic relatedness in the program COLONY 2.0 [9] and full sibs were removed from each population to minimise the effect of relatedness on allele frequencies. This resulted in a total sample size of 291 individuals (16–32 per stream, for 12 streams) in BC, 22 for NC (4 streams) and nine for SC (two streams). Streams are analysed separately for BC, and are pooled by region in NC and SC. Streams sampled within the North Cascades (NC) (Latitude: 48°45′9″; Longitude: -121°5′59″) were collected within the North Cascades (NC) (Latitude: 48°45′9″; Longitude: -121°5′59″) and sampled within the northern range limit of D. tenebrosus, Dicten27 in one population (Table S1, Table 2). Dicten27 and Dicten11 deviated from HW equilibrium in 100% and 91.7% of the populations sampled respectively, while all other loci were in HW equilibrium across all BC populations except for Dicten29 in one population (Table S1, Table 2). Dicten27 and Dicten11 will therefore be of limited use in further studies of population substructuring. All loci in NC and SC were in keeping with HW expectations (Table 2). Evidence for null alleles was only found for Dicten27 in BC, as suggested by the excess of homozygotes for most allele size classes. Although similar among regions, observed heterozygosity (He) across loci increased with sample size of each region, with mean He (± s.e.) being highest in BC (0.321±0.11, n = 291), intermediate in SC (0.302±0.09, n = 22) and lowest in NC (0.266±0.09) (Table 2).

Results

None of the loci were in linkage disequilibrium within BC populations, NC or SC after correction for multiple comparisons. There was no evidence for scoring error due to stuttering or large allele dropout in any of the loci within any region. In BC, Dicten27 and Dicten11 deviated from HW equilibrium in 100% and 91.7% of the populations sampled respectively, while all other loci were in HW equilibrium across all BC populations except for Dicten29 in one population (Table S1, Table 2). Dicten27 and Dicten11 will therefore be of limited use in further studies of population substructuring. All loci in NC and SC were in keeping with HW expectations (Table 2). Evidence for null alleles was only found for Dicten27 in BC, as suggested by the excess of homozygotes for most allele size classes. Although similar among regions, observed heterozygosity (He) across loci increased with sample size of each region, with mean He (± s.e.) being highest in BC (0.321±0.11, n = 291), intermediate in SC (0.302±0.09, n = 22) and lowest in NC (0.266±0.09) (Table 2).

Table 1. Locus name, clone name, GenBank accession number, primer sequence with fluorescent dye label forward (F) and reverse (R), clone repeat unit and primer annealing temperature (T°a-C) for D. tenebrosus microsatellite loci.

| Locus  | Clone name | Genbank accession number | Primer sequence (5′-3′) | Clone repeat unit | T°a-C |
|--------|------------|--------------------------|-------------------------|-------------------|------|
| Dicten02 | 020048     | GU187896                 | F:NEDACCTCGTGTGAGGGAGTTTG RCATTCCCCGGTGCTGCAAC (GT)12 | (GT)12       | 57   |
| Dicten11 | 020320.2   | GU187905                 | F:FAMTCACCGGTGCTGCAAC RTGCCTGCTGGATACCTTGTGG (CATA)6    | (CATA)6      | 57   |
| Dicten18 | 020361     | GU187909                 | F:VICCTCGAGTGGACACATACACAGGRTTTCACAGGGTTGTGCAGTG (ATAC)12 | (ATAC)12     | 57   |
| Dicten20 | 020363     | GU187910                 | F:NEDACCTCGTGTGAGGGAGTTTG RCATTCCCCGGTGCTGCAAC (ATAC)12 | (ATAC)12     | 57   |
| Dicten25 | 020330     | GU187907                 | F:VICCTCGAGTGGACACATACACAGGRTTTCACAGGGTTGTGCAGTG (ATAC)12 | (ATAC)12     | 57   |
| Dicten27 | 020355     | GU187908                 | F:NEDACCTCGAGTGGACACATACACAGGRTTTCACAGGGTTGTGCAGTG (ATAC)12 | (ATAC)12     | 57   |
| Dicten29 | 020326     | GU187906                 | F:VICCTCGAGTGGACACATACACAGGRTTTCACAGGGTTGTGCAGTG (ATAC)12 | (ATAC)12     | 57   |

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The mean number of alleles across loci (± s.e.) showed an expected increase with sample size in each region (BC = 3.86±0.40; SC = 2.71±0.61; NC = 2.14±0.26). However, when corrected for sample size (n = 9 samples with 18 genes based on NC), allelic richness in BC was more similar to NC and SC (BC = 2.31±0.31; NC = 2.71±0.61; SC = 1.99±0.25), indicating that estimates of allelic richness in NC and SC may be underestimated due to sample size. Despite a higher number of private alleles in BC (as expected for a greater sample size), their mean frequency was very low (mean 0.04±0.002) compared to NC (0.295±0.0) and SC (0.22±0.15) (Table 2). Furthermore, when corrected for sample size, private allelic richness was highest in SC (0.92) compared to NC (0.25) and BC (0.53).

Pairwise Fst was calculated excluding Dicten 27 and Dicten 11 in all regions. Genetic differentiation (Fst) was significant (P<0.05) for all pairwise regional comparisons, and followed expectations of isolation by distance, with the lowest differentiation between the northern peripheral (BC) and sub-peripheral (NC) regions (Fst = 0.176) The highest genetic differentiation was between the most spatially distant BC and SC regions (Fst = 0.330) and was intermediate between NC and SC (Fst = 0.192). Genetic differentiation within BC populations was low to moderate and significant for ~51% of all pairwise comparisons, while all 12 populations differed significantly from NC and SC (Table 3), further emphasising the genetic distinctiveness of the three regions. There was highly significant genotypic differentiation (Fisher’s exact test = P<0.002, df = 14) within populations for all loci except Dicten18 and Dicten27 (Fisher’s exact test = P>0.1, df = 14).

**Discussion**

We present seven new polymorphic microsatellite loci for *D. tenebrosus*, and show variation in allelic richness and increasing genetic differentiation between peripheral (BC), sub-peripheral (NC) and core (SC) regions of the species’ range. Although our sample sizes restrict conclusions regarding differences in genetic variation between regions, our data indicate that allelic richness is comparable between regions, with rare alleles being more common in the range core. Previous studies at the species’ northern periphery have found monomorphism or low genetic diversity in loci for *D. tenebrosus* (range of *He* for three loci = 0.0-0.24) [2,3], compared to studies conducted in the South Cascades (range of *He* for nine loci

### Table 2. Summary of locus characteristics for *D. tenebrosus* by region (British Columbia, 12 populations pooled), North Cascades and South Cascades.

| Region                  | A | Allele size range | *He* | *Ho* | # Private alleles (frequency range) | Null | HW test       |
|-------------------------|---|-------------------|------|------|-------------------------------------|------|--------------|
| British Columbia (n = 291) |   |                   |      |      |                                     |      | # of streams not in HW (%) |
| Dicten02                | 5 | 168–190           | 0.374| 0.450| 4 (0.002–0.151)                     | −0.241| 0            |
| Dicten11                | 4 | 139–149           | 0.504| 0.825| 2 (0.009–0.009)                     | −0.444| 11.917       |
| Dicten18                | 3 | 118–126           | 0.044| 0.038| 0                                   | 0.038| 0            |
| Dicten20                | 4 | 211–225           | 0.130| 0.137| 1 (0.002)                          | −0.071| 0            |
| Dicten25                | 2 | 193–197           | 0.063| 0.058| 0                                   | 0.027| 0            |
| Dicten27                | 4 | 121–141           | 0.592| 0.343| 1 (0.138)                          | 0.227| 12 (100)     |
| Dicten29                | 5 | 158–174           | 0.332| 0.395| 1 (0.010)                          | −0.157| 1 (8.3)     |
| North Cascades (n = 22) |   |                   |      |      |                                     |      | P-value for HW test |
| Dicten02                | 2 | 178–188           | 0.416| 0.590| 1 (0.295)                          | −0.360| 0.121        |
| Dicten11                | 2 | 139–147           | 0.268| 0.320| 0                                   | −0.216| 1.0          |
| Dicten18                | 2 | 118–126           | 0.087| 0.090| 0                                   | −0.080| 1.0          |
| Dicten20                | 3 | 203–231           | 0.606| 0.500| 0                                   | 0.138| 0.101        |
| Dicten25                | 1 | 193               | 0.000| 0.000| 0                                   | 0.071| -            |
| Dicten27                | 3 | 133–141           | 0.492| 0.360| 0                                   | 0.175| 0.07         |
| Dicten29                | 2 | 158–182           | 0.087| 0.000| 0                                   | 0.238| 0.024*       |
| South Cascades (n = 9)  |   |                   |      |      |                                     |      |               |
| Dicten02                | 2 | 180–188           | 0.444| 0.444| 1 (0.667)                          | 0    | 1.0          |
| Dicten11                | 2 | 139–147           | 0.222| 0.198| 0                                   | −0.118| 1.0          |
| Dicten18                | 2 | 122–126           | 0.111| 0.105| 0                                   | −0.057| 0.860        |
| Dicten20                | 5 | 203–231           | 0.667| 0.710| 2 (0.056–0.111)                    | 0.053| 0.403        |
| Dicten25                | 2 | 193–197           | 0.222| 0.198| 0                                   | −0.118| 1.0          |
| Dicten27                | 1 | 133               | 0.000| 0.000| 0                                   | 0    | -            |
| Dicten29                | 5 | 158–182           | 0.444| 0.457| 1 (0.056)                          | 0.044| 0.548        |

n = sample size of individuals, A = number of alleles, *He* = expected heterozygosity, *Ho* = observed heterozygosity, Null = estimated null allele frequency (Oosterhout method), and the number of private alleles per locus and their frequency range for each region. Results of Hardy-Weinberg (HW) equilibrium tests are presented for British Columbia as the number of populations with significant deviation from HW expectations (P<0.05 after Bonferroni correction). Within the North Cascades and the South Cascades, P-values of HW tests are presented for each locus.

* = significant deviation from HW equilibrium after Bonferroni correction.

# = evidence for null alleles at this locus.

See Table S1 for population-level locus characteristics in British Columbia.

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conduct these analyses, and ensure that genetic structure can be adequately tested the central-peripheral hypothesis in this species populations and more genetic markers will be necessary to different microsatellite markers. Clearly, a larger sample size of core = 0.18-0.85) [10], yet these studies were not concurrent and used different microsatellite markers. Clearly, a larger sample size of core populations and more genetic markers will be necessary to adequately test the central-peripheral hypothesis in this species [18]. The new markers we present will provide greater power to these new loci will complement existing loci [2,4] for conducting analyses of population genetic structure, and the effects of habitat degradation on core and threatened peripheral populations of this species. Not only will the new loci provide increased potential for it to broader questions relating to ecological adaptation in this stream amphibian.

### Supporting Information

**Table S1** Locus characteristics by population, British Columbia

| Locus | Population | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
|-------|------------|---|---|---|---|---|---|---|---|---|----|----|----|
| 1     |            | 0.089 |   |   |   |   |   |   |   |   |    |    |    |
| 2     |            | 0.113 | 0.007 |   |   |   |   |   |   |   |    |    |    |
| 4     |            | 0.065 | 0.020 | 0.025 |   |   |   |   |   |   |    |    |    |
| 5     |            | 0.066 | 0.006 | 0.004 | 0.014 |   |   |   |   |   |    |    |    |
| 6     |            | 0.202 | 0.149 | 0.118 | 0.175 | 0.114 |   |   |   |   |    |    |    |
| 7     |            | 0.066 | 0.032 | 0.047 | 0.002 | 0.022 | 0.249 |   |   |   |    |    |    |
| 8     |            | 0.013 | 0.079 | 0.104 | 0.038 | 0.059 | 0.219 | 0.028 |   |   |    |    |    |
| 9     |            | 0.166 | 0.029 | 0.017 | 0.087 | 0.035 | 0.080 | 0.132 | 0.167 |   |    |    |    |
| 10    |            | 0.157 | 0.047 | 0.045 | 0.058 | 0.052 | 0.158 | 0.092 | 0.124 | 0.068 |   |    |    |
| 11    |            | 0.091 | 0.030 | 0.025 | 0.066 | 0.020 | 0.042 | 0.103 | 0.110 | 0.010 | 0.062 |   |    |
| 12    |            | 0.193 | 0.027 | 0.011 | 0.090 | 0.039 | 0.123 | 0.132 | 0.184 | 0.006 | 0.071 | 0.043 |    |
| NC    |            | 0.260 | 0.172 | 0.169 | 0.167 | 0.176 | 0.256 | 0.214 | 0.221 | 0.157 | 0.076 | 0.160 | 0.174 |
| SC    |            | 0.395 | 0.286 | 0.283 | 0.320 | 0.278 | 0.479 | 0.268 | 0.316 | 0.322 | 0.168 | 0.290 | 0.356 | 0.192 |

Significant values are in bold after strict Bonferroni correction (P<0.05).

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### Author Contributions

Conceived and designed the experiments: RYD. Performed the experiments: RYD. Analyzed the data: RYD. Contributed reagents/materials/analysis tools: RYD AS SFS JSR. Wrote the paper: RYD.

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