

# Genetic structure in a montane ranid frog: restricted gene flow and nuclear–mitochondrial discordance

KIRSTEN J. MONSEN\* and MICHAEL S. BLOUIN

Department of Zoology, Oregon State University, 3029 Cordley Hall, Corvallis, OR 97331, USA

## Abstract

There is substantial debate over the criteria that should be used to group populations of a species into distinct units for conservation (e.g. evolutionarily significant units, management units, distinct population segments). However, in practice molecular genetic differentiation is often the only or main criterion used to identify such units. Most genetic studies attempting to define conservation units in animals use a single molecular marker, most often mitochondrial, and use samples from a limited number of populations throughout the species' range. Although there are many benefits to using mtDNA, certain features can cause it to show patterns of differentiation among populations that do not reflect the history of differentiation at the nuclear genome where loci controlling traits of adaptive significance presumably occur. Here we illustrate an example of such mitochondrial–nuclear discordance in a ranid frog, and show how using mtDNA or nuclear loci alone could have led to very different conservation recommendations. We also found very high genetic differentiation among populations on a local scale, and discuss the conservation implications of our results.

*Keywords:* amphibian conservation, discordant molecular markers, DPS, gene flow, ranidae

*Received 25 July 2003; revision received 3 September 2003; accepted 3 September 2003*

## Introduction

A fundamental tenet of conservation biology is to identify and protect the evolutionary heritage and future evolutionary potential in threatened species. How to group populations into cohesive conservation units that contribute significantly to the evolution of a species has been debated for many years (Ryder 1986; Waples 1991, 1995, 1998; Moritz 1994; Pennock & Dimnick 1997; Bowen 1998; Paetkau 1999; Taylor & Dizon 1999). In order to be considered for protection under the United States Endangered Species Act, a vertebrate population must be considered a Distinct Population Segment (DPS). A DPS is defined as a population that is discrete, biologically or ecologically significant, and threatened or endangered regardless of the status of other populations in the same species (US Department of the Interior *et al.* 1996). Although most authors emphasize that it is important to avoid making

these divisions solely on the basis of information from genetic markers (Ryder 1986; Paetkau 1999; Taylor & Dizon 1999), in practice molecular genetic differentiation is often the only or main criterion used to identify threatened or endangered populations that conform to these classifications.

Most genetic studies attempting to define conservation groups in animals use a single type of molecular marker, and most often it is mitochondrial. For example, of 35 papers published over the last 8 years that attempted to classify vertebrate populations specifically into conservation units, 29 used a single type of marker and 22 of those 29 used mtDNA. Although there are numerous benefits to using mtDNA for intraspecific studies (Avise *et al.* 1987), there can be discordance between genetic patterns seen in mtDNA and nuclear loci (Melnick & Hoelzer 1992; Jack *et al.* 1995; Hare & Avise 1998; Buonaccorsi *et al.* 1999; Nyakaana & Arcander 1999; Franck *et al.* 2001). Owing to the expected fourfold reduction in effective population size ( $N_e$ ) (Birky *et al.* 1983), one would expect mitochondrial DNA to reach reciprocal monophyly faster than nuclear DNA, but given enough time after separation of two populations, both markers should produce the same signal (Avise *et al.* 1987; Moore 1995). However, differences

Correspondence: Kirsten Monsen. \*Present address: Department of Biology, Western Washington University, 516 High Street, Bellingham, WA 98225–9160, USA. Fax: 360 650 3148; E-mail: monsenk@biol.wvu.edu

in sex ratio and mating system can alter this relationship significantly (Hoelzer 1997). Additionally, fluctuating effective population size can have profound and different effects on the population genetic patterns at nuclear and mitochondrial genomes (Birky *et al.* 1983; Avise *et al.* 1984; Hoelzer 1997). In this study, we used mitochondrial and microsatellite data to test for major subdivisions within the species-wide range of a montane frog, *Rana cascadae*. We also used these loci to assess levels of genetic connection between populations on a local scale (nearest neighbours separated by approximately 50 km). At the species-wide scale we found an interesting discrepancy between patterns of phylogeographical subdivision suggested by nuclear and mtDNA markers. We show how decisions on the conservation status of disjunct populations of *R. cascadae* could depend heavily on which type of marker was examined. At the local scale we found patterns consistent with extremely limited gene flow. We discuss the implications of our results for conservation of *R. cascadae*.

## Materials and methods

### Study species

The Cascades frog, *Rana cascadae*, is endemic to the Pacific Northwest of North America. It occurs at elevations between 800 and 2740 m in three disjunct regions: the Olympic Mountains of Washington, the Cascades Mountains of Oregon and Washington and the Klamath–Siskiyou Mountains in Northern California (Fig. 1). Although population declines have been observed in the Klamath–Siskiyou region (Fellers & Drost 1993), the species appears to be healthy in Oregon and Washington.

### Sampling

Tissue was collected from adult *Rana cascadae* by toe clipping during the summers of 1997 and 1998. Samples were collected from 11 populations throughout the species range (Fig. 1, Table 1). Sample sizes ranged from 18 to 73 individuals per population, with the exception of one site in California from which we could only obtain 11 individuals (Fig. 1, Table 1). Populations for collection were chosen in order to cover the entire range for a species-wide analysis. Additionally, six of these 11 populations were sampled at a scale of approximately 50 km apart in central Oregon to examine genetic differentiation at a finer geographical scale (Fig. 1).

### Molecular methods

Total genomic DNA was extracted from each toe using a standard phenol–chloroform protocol (Hillis *et al.* 1996). Three hundred and forty-eight *R. cascadae* individuals

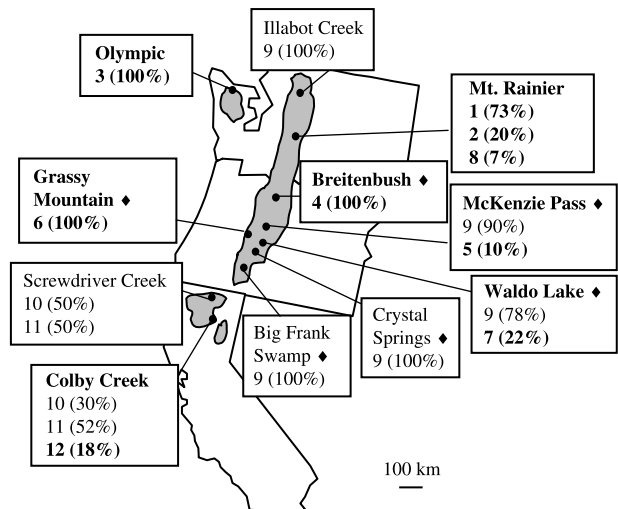


Fig. 1 Distribution of *R. cascadae* study sites and ND1/D-loop composite haplotypes. Shaded area indicates entire species distribution. Haplotypes are numbered 1–12, numbers in parentheses indicate percentage of individuals of a specific haplotype in each population. Notice seven of 11 populations have private alleles (bold text). Populations marked with ◆ were used in the isolation by distance analysis and migration estimates. Species distribution after R. Nauman and D. Olson (pers. comm.).

were genotyped at a 335-base pair (bp) fragment of the mitochondrial D-loop, and at a 347-bp fragment that includes 281 bp of the 5' end of the mitochondrial ND1 gene and 66 bp of the flanking tRNA leucine gene. For each fragment we sequenced initially 1–10 individuals from each population, and then screened the other individuals for the presence of new alleles by running polymerase chain reaction (PCR) product on single-strand conformation polymorphism gels (SSCP, Orita *et al.* 1989).

### D-loop fragment amplification

Primers MB75 and MB76 (D. Call, pers. comm. Table 2) were used to amplify a 335-bp fragment in *R. cascadae* individuals from 100 to 200 ng of genomic DNA in a 50- $\mu$ L PCR. The PCR components were: 50 mM KCl, 10 mM Tris-HCl pH 9, 0.1% Triton X-100, 1.5 mM MgCl<sub>2</sub>, 0.8  $\mu$ M of both the forward and reverse primers, 0.2 mM dNTPs, 2.5 units *Taq* polymerase and water to a final volume of 50  $\mu$ L. Amplification was carried out in a Perkin-Elmer 9600 thermocycler under the following conditions: 94 °C 3 min, followed by 30 cycles of 94 °C 45 s, 50 °C 30 s, 72 °C 30 s, and a final extension at 72 °C for 7 min.

### ND1/tRNA fragment amplification

Primers MB77 and MB129 (Table 2) were used to amplify a 347-bp fragment in *R. cascadae* individuals from 100 to

**Table 1** Site location and sample size (*N*) information for *R. cascadae*. Nuclear *N* is the average sample size

| Site name              | Latitude  | Longitude   | mtDNA <i>N</i> | Nuclear <i>N</i> |
|------------------------|-----------|-------------|----------------|------------------|
| Olympic, WA            | 47.9163 N | -123.7814 W | 72             | 28               |
| Illabot Creek, WA      | 48.4402 N | -121.3876 W | 18             | 21               |
| Mt Rainier, WA*        | 46.9160 N | -121.6351 W | 46             | 30               |
| Breitenbush, OR        | 44.7716 N | -121.9495 W | 27             | 26               |
| McKenzie Pass, OR      | 44.2448 N | -121.8414 W | 21             | 29               |
| Grassy Mountain, OR*   | 44.2750 N | -122.7755 W | 21             | 14               |
| Waldo Lake, OR         | 43.7623 N | -122.0131 W | 60             | 28               |
| Crystal Springs, OR    | 43.3123 N | -122.1404 W | 24             | 22               |
| Big Frank Swamp, OR    | 42.4422 N | -122.2416 W | 20             | 18               |
| Screwdriver Creek, CA* | 40.9981 N | -121.7493 W | 11             | 11               |
| Colby Creek, CA*       | 40.1113 N | -121.4846 W | 33             | 30               |

\*Tissue collection conducted by others: Mt Rainier, WA, Robert Hoffman; Grassy Mt, OR, Marc Hayes; both CA sites, Gary Fellers.

**Table 2** Primer and product information for mtDNA D-loop and ND1/tRNA fragments; primer positions relative to published sequences of the bullfrog, *R. catesbeiana* are also given

| Locus                   | F primer<br>R primer  | Size (bp) | Anneal<br>(°C) | Position in <i>R. catesbeiana</i><br>mitochondrial genome                        |
|-------------------------|---|-----------|----------------|--|
| D-loop                  | MB75 5'gacgccatacatcagcc3'<br>MB76 5'acctgcaccgttagtccaa3'  | 335       | 50             | 1359 Yoneyama (1987)<br>1689 Yoneyama (1987)                                     |
| ND1/tRNA small fragment | MB77 5'tggcagagcttggtatgcaaaaga3'<br>MB129 5'gaaattggggtcatatratatttrg3'  | 349       | 52             | 2669 Nagae (1988)  |
| ND1/tRNA large fragment | MB74 5'ggatgagcccgatagctta3'<br>MB77 5'tggcagagcttggtatgcaaaaga3'<br>internal sequencing<br>MB130 5'gaaatgyraargaagagagggt3'<br>MB143 5'ggattcacctctctc3' | 1204      | 46             | 3853 Nagae (1988)<br>2669 Nagae (1988)<br>3227 Nagae (1988)<br>3221 Nagae (1988) |

200 ng of genomic DNA in a 25- $\mu$ L PCR. PCR components and conditions were the same as the D-loop fragment with a locus-specific annealing temperature (Table 2).

### SSCP

Six  $\mu$ L of PCR product was mixed with 4  $\mu$ L of loading buffer (95% formamide, 0.1 mg/mL xylene cyanol, 0.1 mg/mL bromophenol blue), heated at 94 °C for 3 min, and placed immediately on ice. Three  $\mu$ L of this mixture was loaded on a 0.5 $\times$  MDE (BioWhittaker Molecular Applications), 0.6 $\times$  TBE gel at 4 °C. Samples were run at 8 W for 16–18 h at 4 °C in 0.6 $\times$  TBE buffer. Bands were visualized by Sybr Gold staining (Molecular Probes), and photographed using a Polaroid camera. Unique banding patterns were scored as unique haplotypes. We found that D-loop reaction products were easier to visualize on SSCP gels if we first concentrated the product twofold by ethanol precipitation. This step was not necessary with the ND1/tRNA fragment. Two individuals of each putative new haplotype were sequenced in both directions to verify unique sequence.

### Sequencing

Products to be sequenced were first purified with Ultrafree-MC 30 000 NMWL spin columns (Millipore Corp.). Purified product was then sequenced on an automated ABI 377 sequencer (Applied Biosystems, Inc.). All sequences were aligned by eye in the program SEQED version 1.0.3 (Applied Biosystems, Inc.).

### Microsatellite loci

We used six microsatellite loci that were developed originally for *R. pretiosa* and *R. luteiventris* (RP17, RP193, SFC 139, SFC120, SFC128 and SFC134, Table 3; Blouin, unpubl. data), and one developed from *R. cascadae* (RC287). In addition, we used two bi-allelic loci that each showed size variation owing to a single indel (RP123 and RC174; Table 3). PCR amplifications were carried out in 25  $\mu$ L reactions using the same components and conditions as the D-loop fragment with locus-specific annealing temperatures and fluorescently labelled forward primers

**Table 3** Primer and product information for seven microsatellite loci and two size variant nuclear loci

| Locus  | F primer<br>R primer   | Clone size<br>(bp) | Repeat no. | Allele size<br>range (bp) | Total no.<br>alleles | Ave. no.<br>alleles per<br>population | Anneal<br>(°C) |
|--------|--|--------------------|------------|---------------------------|----------------------|---------------------------------------|----------------|
| SFC120 | 5'aaccttgtagtagatgaccaac3'<br>5'gtggaactccagttatgatcc3'        | 180                | 16         | 137–185                   | 13                   | 5.2                                   | 56             |
| SFC128 | 5'agaaaagcggactctgaaat3'<br>5'agccataatccctgttaaac3'           | 236                | 7          | 221–261                   | 10                   | 4.5                                   | 57             |
| SFC134 | 5'tgggaaaagactctgtgt3'<br>5'aggaaatgtgtggaagcat3'              | 240                | 11         | 225–273                   | 20                   | 6.0                                   | 57             |
| SFC139 | 5'ggcatggttaaagtggaaactc3'<br>5'tgcatgtctgtaatggacctc3'        | 277                | 17         | 230–278                   | 13                   | 5.4                                   | 58             |
| RP17   | 5'gtgtagacaacaaatgaaagtcag3'<br>5'gtcttacttccatccaaccattcc3'   | 118                | 7          | 111–119                   | 3                    | 1.8                                   | 50             |
| RP123  | 5'atgaaacaataaatctccagagacc3'<br>5'caaaataaagtggggaaggatgacg3' | 135                | NA         | 145 and 146               | 2                    | 1                                     | 52             |
| RP193  | 5'ccatttctctctgatgtgt3'<br>5'tgaagcagatcactggcaaac3'           | 183                | 21         | 143–203                   | 16                   | 6.5                                   | 49             |
| RC174  | 5'ggtcacacacaccgctgcccag3'<br>5'accatacatcacagttttccacc3'      | 140                | NA         | 86 and 140                | 2                    | 1                                     | 51             |
| RC287  | 5'atagggatgtggcggaagattga3'<br>5'caaggcactgtagatagatagaat3'    | 279                | 8          | 268–284                   | 4                    | 1.7                                   | 51             |

(Table 3). Microsatellite PCR product was run on an ABI 377 automated sequencer, and allele sizes were scored using the program GENOTYPER version 2.0.

#### mtDNA data analysis

Because mtDNA is a single nonrecombining molecule, the D-loop sequence and ND1/tRNA sequence were combined to create a single composite haplotype for each individual. A statistical parsimony network was created of all *R. cascadae* mtDNA alleles using the TCS software (Templeton *et al.* 1992; Clement *et al.* 2000). To assess genetic differentiation among the six Oregon populations we estimated Weir & Cockerham's (1984)  $F_{ST}$  using FSTAT version 2.9.3 (Goudet 1995), and obtained AMOVA estimates ( $\Phi_{ST}$ ; Excoffier *et al.* 1992) from the uncorrected number of nucleotide differences between haplotypes using ARLEQUIN version 2.0 (Schneider *et al.* 2000).

#### Nuclear DNA data analysis

Neighbour-joining trees based on Cavalli-Sforza & Edward's (1967) chord distance ( $D_C$ ) and Nei's 1972 genetic distance ( $D_N$ ) were constructed in the program POPULATIONS version 1.2.26 (© Olivier Langella 2000), and viewed in the program TREEVIEW version 1.6.6 (Page 1996). We used both genetic distances because  $D_N$  is thought to provide more accurate estimates of branch lengths while  $D_C$  provides a better estimate of tree topology (Takezaki & Nei 1996; Angers & Bernatchez 1998). These trees are based on the

two indel loci and only five of the seven microsatellite loci because loci SFC139 and RC287 would not amplify in the California populations. Also, owing to poor DNA quality we were unable to obtain clean data for indel locus RP123 in the Screwdriver Creek, CA population. Consequently, we did not include this population in the neighbour-joining trees. An estimate of Weir & Cockerham's (1984)  $F_{ST}$  among the six Oregon populations was made using GENEPOP version 3.2a (Raymond & Rousset 1995) and all seven microsatellite loci.

#### Estimated times of divergence between mtDNA alleles

Sequence analysis of the D-loop and ND1/tRNA variants suggested the presence of three very divergent groups of mtDNA alleles. In order to date the split between these three groups, we sequenced a 1204-bp mtDNA fragment that included the entire ND1 gene and four flanking tRNA genes (tRNA *Leu*, tRNA *Ile*, tRNA *Gln*, tRNA *Met*) using the primers MB74, MB77, MB130, and MB143 (Table 2) from the most common haplotype found in each of the three groups. Times of divergence between mtDNA alleles were based on a molecular clock of 1.4% sequence divergence per million years for anuran mtDNA that was based on the ND1 gene (Macey *et al.* 1998a). Similar rates of divergence have been shown in other anurans (A. Crawford, pers. comm.; Kosuch *et al.* 2001), salamanders (Spolsky *et al.* 1992), lizards (Macey *et al.* 1998b; Gubitz *et al.* 2000) and snakes (Zamudio & Greene 1997). We have confidence in the accuracy of this clock because it yields dates of

separation among Pacific Northwestern ranid species (Monsen and Blouin, unpubl. data; Macey *et al.* 2001) that are very similar to dates based on allozyme distances (Green 1986). For completeness we also calculated a range of estimates using two other independently calibrated anuran mtDNA molecular clocks of 0.66% sequence divergence per million years (Mulcahy & Mendelson 2000) and 1.0% sequence divergence per million years (Kosuch *et al.* 2001).

#### Estimating gene flow for comparative purposes

We estimated  $N_e m$  among the six Oregon populations under the assumption of approximate drift-migration equilibrium in an island model from  $F_{ST} = 1/(4N_e m + 1)$  (Wright 1951, 1965), and via the private allele method (Slatkin 1985) using GENEPOP version 3.2a. Many assumptions are involved in estimating number of migrants per generation ( $N_e m$ ) from  $F_{ST}$  or private alleles (equal effective sizes, demographic equilibrium, and so on; Whitlock & McCauley 1999), so the nominal values that we estimate should be taken with a healthy dose of skepticism. Nevertheless, the values are still useful for certain comparative purposes, such as testing for evidence of sex-biased gene flow by comparing mitochondrial vs. nuclear estimates, or for comparing estimates produced by different methods (i.e. estimated from  $F_{ST}$  vs. from private alleles).

## Results

### mtDNA

There were 12 mtDNA haplotypes observed throughout the range of *R. cascadae* (Figs 1 and 2). The statistical parsimony network of mtDNA haplotypes showed three distinct groups: the Olympic Peninsula, the Cascades of Oregon and Washington and California. It was not possible to connect these three groups with 95% confidence in tcs, and 14 steps were needed to force connections between them (Fig. 2). When forced, tcs originally joined Olympic allele 3 to Waldo Lake allele 7 (a rare private allele) based on a single ambiguous reversal. We changed this connection in Fig. 2 based on the geographical separation of these two populations and the other basic assumptions described by Crandall & Templeton (1993).

Of the 11 populations sampled, seven contained private alleles (Fig. 1). Three of these populations are fixed for their private allele. Three of the remaining four populations without private alleles are fixed for the most common central Cascades allele 9. The final population lacking a private allele, Screwdriver Creek, shared its two alleles (10 and 11) with the neighbouring Colby Creek, CA population. Although there are multiple alleles within the Cascades and California groups, there are few genetic differences

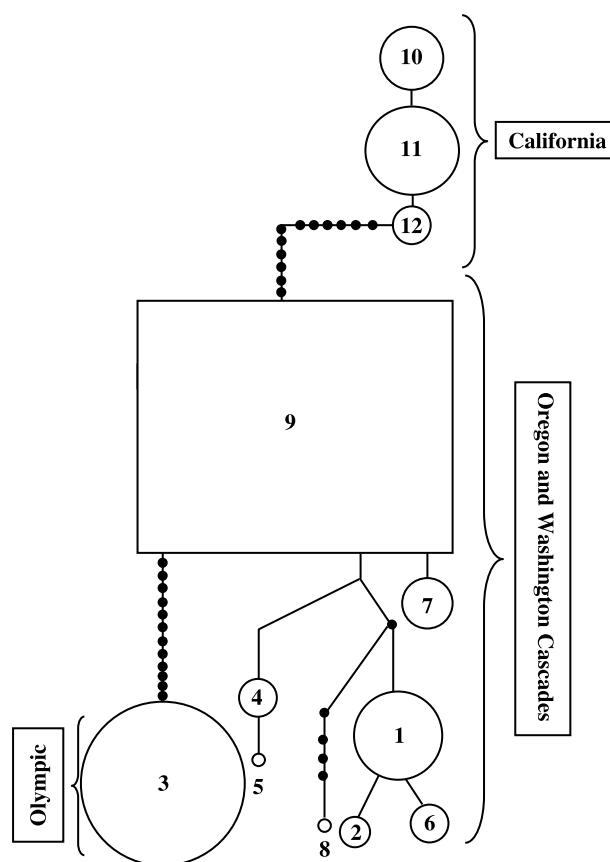


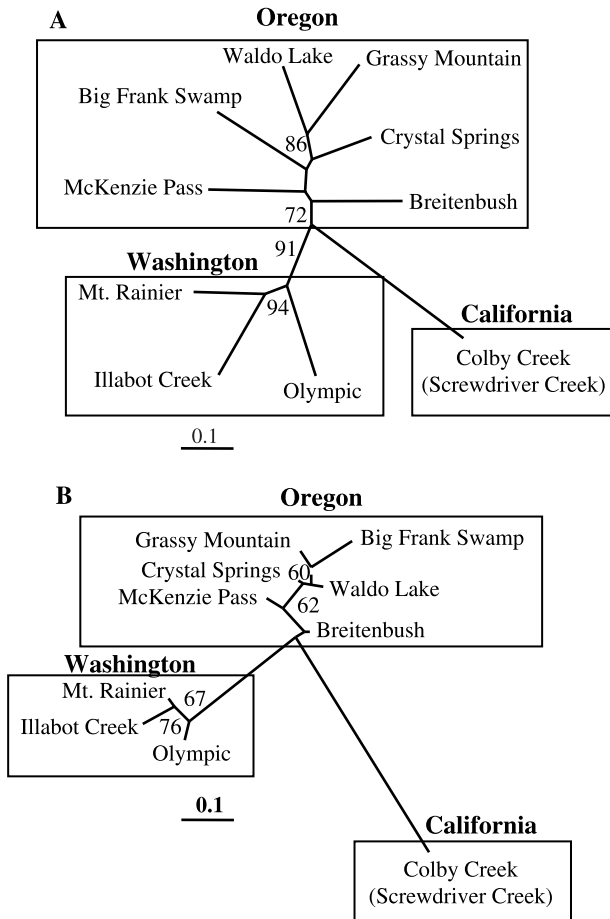
Fig. 2 Statistical parsimony network of 12 *R. cascadae* D-loop/ND1 mtDNA haplotypes. See text for details of network methods. Black dots represent mutational steps between haplotypes.

between alleles within each group (Fig. 2). There was, however, substantial divergence between the three groups. The sequence divergence at the 1204 bp mtDNA ND1/tRNA fragment between the Cascades and California is 3.4%, between Cascades and Olympic is 3.2%, and between California and Olympic is 4%. The anuran ND1 clock after Macey *et al.* (1998a) dates the split of these three groups at 2.3–2.9 Mya. The full range of estimated dates based on the anuran mtDNA clocks including Mulcahy & Mendelson (2000) and Kosuch *et al.* (2001) is 2.3 Mya–6.1 Mya.

The six populations of *R. cascadae* in Oregon showed strong mitochondrial differentiation on a small geographical scale, with  $F_{ST} = 0.78$ , and  $\phi_{ST} = 0.93$ . Also, four of the six populations were either fixed for, or had private alleles in high frequency (Fig. 1).

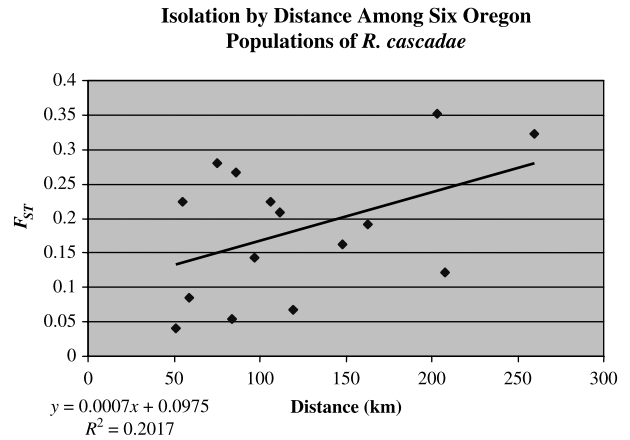
### Nuclear loci

The total number of alleles per microsatellite locus ranged from three to 20 and average expected heterozygosities within populations ranged from 0.25 to 0.87. Both nuclear gene trees (Fig. 3) suggest the presence of three groups of



**Fig. 3** Neighbour-joining trees of Cavalli-Sforza & Edward's (1967) chord distance  $D_C$  (A) and Nei's (1972) genetic distance  $D_N$  (B) for five microsatellite loci and two size variant nuclear loci among 10 *R. cascadae* populations. There are no available data for size variant locus RP123 for Screwdriver Creek, therefore this population was eliminated from this analysis. Allele frequencies and identities at all other loci in Screwdriver Creek are similar to those in the Colby Creek population. Numbers represent bootstrap values after 1000 replications. Only bootstrap values greater than 50 are reported.

populations in *R. cascadae*: an Oregon group, a Washington group, and a California group, with California being the most distinct (Fig. 3). Although these data suggest that the two Washington Cascades populations are more similar to each other than to the Olympic Peninsula population, the nuclear genetic distances among the three Washington populations are not consistent with mtDNA data, suggesting that the Olympic population is as distinct from the other Washington populations as they are from the California populations (Figs 2 and 3). On the other hand, nuclear and mitochondrial data sets both identified the California populations as being very distinct with strong bootstrap support and the largest nuclear genetic distances (Figs 2 and 3). The distinctness of California populations at the



**Fig. 4** Isolation by distance among six OR *R. cascadae* populations for seven microsatellite loci. The six populations in Oregon were used in the analysis of isolation by distance and to compare different estimates of  $N_e m$ .

nuclear loci results from unique alleles and from very different frequencies of shared alleles (data available from authors by request).

Overall microsatellite  $F_{ST}$  among the six Oregon populations was 0.16, a result consistent with that of the mtDNA in suggesting restricted gene flow over a scale of approximately 50 km. There is a positive although statistically nonsignificant correlation between genetic and geographical distance among the six populations (Fig. 4). A pattern of isolation by distance over such a small geographical scale would also suggest that movement by these frogs is restricted to very short distances.

$N_e m$  estimated among the six populations via the  $F_{ST}$  method was 4.7 times lower when using mtDNA than when using nuclear loci. For a typical outcrossing vertebrate such as frogs we expect an approximately fourfold difference (Hoelzer 1997), so this result does not support sex-biased gene flow. In contrast, the private allele method gave an mtDNA estimate that was 22 times lower than the microsatellite estimate, a result consistent with higher gene flow by males. Although at this point we consider the evidence for sex-biased gene flow in *R. cascadae* to be equivocal, the possibility is intriguing and worth further study.

## Discussion

### *Differentiation among populations on a local scale*

We found substantial differentiation at the smallest geographical scale (among the six populations in Oregon) for both mtDNA and microsatellites. Remarkably, four of the six Oregon populations contain private mtDNA alleles, with two of these populations fixed for a private allele (Fig. 1). The widespread presence and fixation of mtDNA private alleles has not been observed in most other anurans

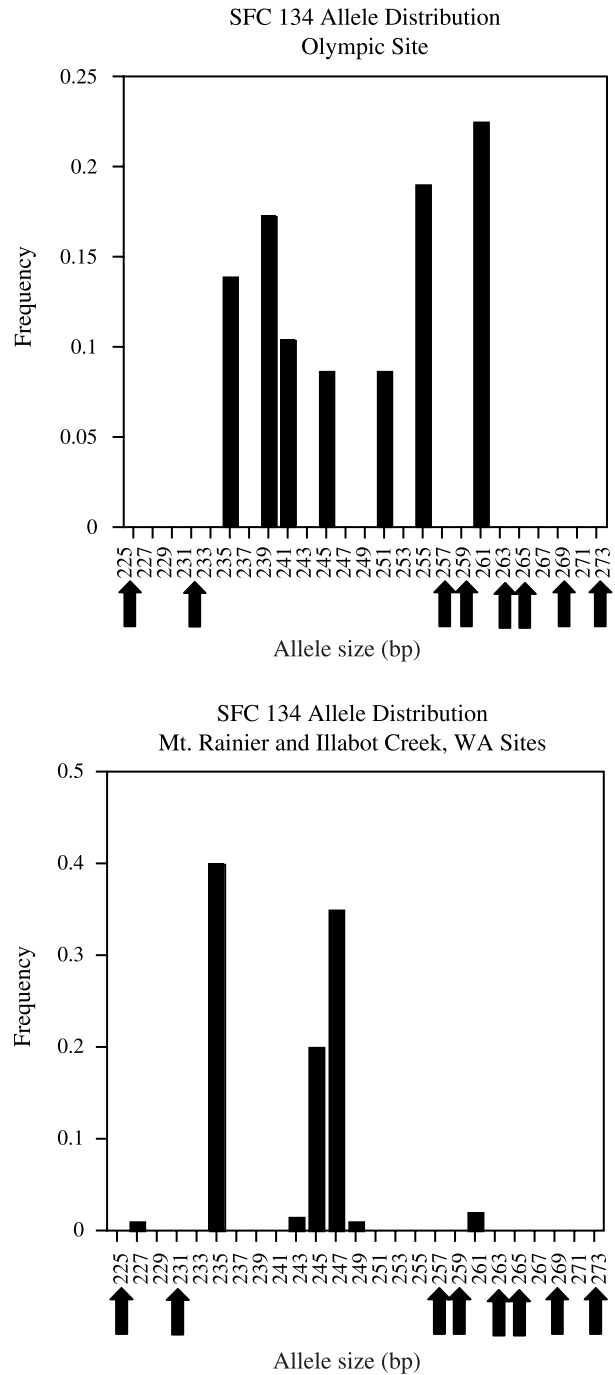
**Table 4** Genetic differentiation among six Oregon populations of *R. cascadae* for mtDNA and microsatellites, and a comparison of  $N_e m$  estimated from  $F_{ST}$  and the private alleles methods for both types of marker

|                 | $F_{ST}$ | $\phi_{ST}$ | $N_e m (F_{ST})$ | $N_e m$<br>(private alleles) |
|-----------------|----------|-------------|------------------|------------------------------|
| mtDNA           | 0.78     | 0.93        | 0.28             | 0.024                        |
| Microsatellites | 0.16     |             | 1.31             | 0.54                         |

(Yang *et al.* 1994; Wilkinson *et al.* 1996; Evans *et al.* 1997; Shaffer *et al.* 2000), but has been observed in populations of another Pacific Northwestern ranid on a similar geographical scale (*R. pretiosa*; Blouin, unpubl. data). *Rana cascadae* requires aquatic habitats that are distributed regularly but discontinuously in its Cascades mountain habitat, so a hypothesis of stepping-stone gene flow is consistent with the biology of the species. Two other lines of evidence also support this hypothesis. First, the private alleles method is expected to underestimate  $N_e m$  relative to the  $F_{ST}$  method when migration is stepping-stone (Slatkin & Barton 1989); so it is interesting that the private alleles estimate of  $N_e m$  was smaller than the  $F_{ST}$  estimate for both mtDNA and nuclear loci (Table 4). Second, that these six populations show a positive (albeit nonsignificant) correlation between geographical and genetic distance (Fig. 4) suggests that there may be isolation by distance over that geographical scale (Hutchison & Templeton 1999). Thus, several lines of evidence are consistent with the view that gene flow in *R. cascadae* is restricted to short distances and follows a stepping-stone pattern.

*Olympic vs. Washington Cascades populations*

Although mtDNA data suggest that the Olympic and Cascades populations have been separated for 2–3 million years, the microsatellite data do not support this conclusion. *R. cascadae* has a generation time of approximately 3 years (Briggs & Storm 1970), so given typical microsatellite mutation rates, after 2 million years of isolation (over 600 000 generations) there should have developed substantial differentiation between the two groups owing to mutation (Estoup & Angers 1998). If there were a large mutational component to the differentiation between the two groups we would expect  $R_{ST}$  between them to be much higher than  $F_{ST}$  (Slatkin 1995). The values are similar, with pairwise  $R_{ST}$  between the Olympic and the two central Washington sites of 0.13 and 0.28, and pairwise  $F_{ST}$  of 0.17 and 0.25. One might argue that mutational constraints on allele size have put a cap on the total microsatellite differentiation possible between the two groups, as appears to have been the case with other vertebrates separated for millions of years (Ostrander *et al.* 1993; Bowcock



**Fig. 5** Allele distributions for SFC134 in *R. cascadae* Washington populations. Alleles marked with an arrow are present in Oregon or California populations but not Washington. If there were a constraint on allele size at these loci, one would not expect to see other alleles present in other populations.

*et al.* 1994; Garza *et al.* 1995; Paetkau *et al.* 1997). However, the Olympic and central Washington populations share the same small and size-restricted subset of the total alleles observed in this species, and they have those alleles in similar frequencies (Fig. 5). Thus, it appears

implausible that the two groups have been isolated completely for the long periods of time suggested by the mtDNA data.

Sex-biased dispersal can produce discordant patterns between mitochondrial and nuclear DNA. However, our comparison of nuclear and mitochondrial estimates of gene flow among the six Oregon populations gave equivocal evidence for lower female gene flow, and we are not aware of any other data showing sex-biased gene flow in frogs (unlike in mammals and birds, for example, where sex-biased dispersal is common; Greenwood 1980). Therefore, although we cannot reject male-biased gene flow as an explanation for the nuclear–mitochondrial discrepancy across the Olympics–Cascades divide, that hypothesis does not seem compelling. However, it will be necessary to investigate sex bias in dispersal in *R. cascadae* to dismiss it conclusively as an explanation for the discrepancy between mtDNA and nuclear data.

How, then, did the Olympic population come to be fixed for such a divergent mtDNA allele if the nuclear data show the Olympic population could not have been isolated nearly as long as the age of that allele? The Olympic Peninsula was isolated from the Cascades during the last glacial maximum that ended approximately 13 600 years ago (Waitt & Thorson 1983). One plausible scenario is that a population of *R. cascadae* was isolated on the peninsula for several million years, during which time the distinct Olympic and Cascades mtDNA lineages evolved in isolation. Following secondary contact, there would have been a mixing of Olympic and Cascades alleles as animals colonized from the East. An Olympic allele may have drifted by chance to high frequency in the population we sampled, even though the nuclear genomes were homogenized. This scenario is plausible, given the small and fluctuating effective sizes that probably typify anuran populations (Waldman & McKinnon 1993; references within), and the expected fourfold smaller  $N_e$  of mtDNA relative to nuclear loci. Furthermore, we sampled only one site on the peninsula. Even though that site appears fixed for the Olympic allele ( $n = 72$ ), if the above scenario is correct then there may be Cascades-lineage alleles still present elsewhere on the Olympic Peninsula.

We sampled a few populations throughout the species range as is typically carried out for conservation studies. This sampling scheme often means a single population is sampled from a unique genetic group when strong genetic subdivision is observed in a species. For example, we have only one population from the Olympic Peninsula, and this population appears fixed for the Olympic mtDNA allele. Other populations occur on the Olympic Peninsula. If we sampled from those additional populations, we may observe other mtDNA alleles related more closely to Cascades mtDNA alleles, yielding results more concordant with the nuclear data. Therefore, we suggest that a better

approach to these types of conservation genetics studies is to sample in two stages. First, one should sample a few populations throughout a species range and identify a general pattern of subdivision. Then, one should sample multiple populations within each region of genetic subdivision to verify the pattern of subdivision and describe the boundaries of genetic differentiation. This sampling strategy has been proposed by other authors (Baverstock & Moritz 1996).

If we had based this study on the mtDNA data alone, we would have suggested there were three DPSs in the species range of *R. cascadae*. Indeed, the three *R. cascadae* mtDNA groups are as divergent at the ND1/tRNA fragment as the sister species *R. pretiosa* and *R. luteiventris* (Monsen and Blouin, unpubl. data). One might have even suggested that the Olympic *R. cascadae* are approaching the status of subspecies or even cryptic species based on the mtDNA divergence. Clearly, the microsatellite data do not support such a division between the Olympic and other populations. This problem of discordance between molecular markers may be especially pronounced in organisms with demographically unstable populations such as frogs (Hoelzer 1997). Owing to fluctuations in population size and large variance in reproductive success, amphibians are very likely to have unstable demographic structure (Waldman & McKinnon 1993; references within). Indeed, studies of skeletochronology used to assess age structure in frog populations often find dominance of a single age class, suggesting large fluctuations in cohort survival (Friedl & Klump 1997; Driscoll 1999; Reaser 2000; Measey 2001).

#### *Washington Cascades vs. Oregon Cascades*

The nuclear data suggest a break across the Columbia River between populations in the Oregon Cascades and in the Washington Cascades (Fig. 3). Substantial genetic differentiation across the Columbia River has been observed in other taxa (Streng 1994; Soltis *et al.* 1997; Arbogast *et al.* 2001; Spruell *et al.* 2003), so the river may be a historical barrier to gene flow for many species. Apparently not enough time has passed for reciprocal monophyly to develop at the mtDNA between the two groups in *R. cascadae*, even if notable allele-frequency differences have evolved at the nuclear loci (Figs 1 and 2). However, this result may not be surprising given the highly subdivided small-scale genetic structure we see in the species at the mtDNA. Subdivision into many small populations is expected to increase the overall  $N_e$  of a group of populations (Kimura & Crow 1963; Robertson 1964; Nei & Takahata 1993; Lande 1995). Thus,  $N_e$  may be large within each group on either side of the Columbia River, which would extend the time necessary for mtDNA alleles to achieve reciprocal monophyly (Neigel & Avise 1986).

### Conservation implications for *R. cascadae*

Based on the mitochondrial and microsatellite data presented here, we suggest that there are at least two DPSs within the species range of *R. cascadae*: the California populations and the Oregon/Washington populations. These two groups differ by 3.2% at the mtDNA loci examined in this study, and show significant divergence of allele frequencies at nuclear loci. The fact that two of seven microsatellite loci would not amplify in the CA populations (owing presumably to changes in the primer sites) also suggests substantial divergence in the nuclear genome between CA and OR-WA populations. The two groups of populations therefore meet the definition of 'discrete and significant'. Additionally, these groups are physically separated by a known faunal break across southern Oregon and northern California (Bury & Pearl 1999). This biogeographical pattern across the Oregon/California border has been observed in numerous taxa (Steinhoff *et al.* 1983; Brown *et al.* 1997; Demboski & Cook 2001; Janzen *et al.* 2002), including several amphibians (Daugherty *et al.* 1983; Good 1989; Good & Wake 1992; Howard *et al.* 1993; Nielson *et al.* 2001). Based on the mtDNA data, California populations were most probably separated at the beginning of the last glacial maximum (approximately 2 Mya), but never experienced secondary contact after glacial retreat. This glacial history has been invoked to explain species breaks in other herpetofauna (Bury & Pearl 1999) and plants (Soltis *et al.* 1997) in the same geographical region.

The status of the Olympic population as a DPS based on molecular marker data is debatable. Many authors have criticized the designation of populations as unique based solely on molecular genetic data, citing other characteristics such as unique habitat use or physical isolation as good indicators of distinctness even when genetic data do not show a difference between populations (Pennock & Dimmick 1997; Paetkau 1999; Taylor & Dizon 1999). This opinion is supported by the observation that molecular genetic differentiation among populations usually underestimates additive genetic differentiation for quantitative traits (McKay & Latta 2002). Because of habitat restrictions it is unlikely that there is current gene flow between the Olympic Peninsula and other Washington populations, even though there has clearly been nuclear gene flow in recent evolutionary time. Therefore, given the physical isolation and unique habitat of the Olympic Mountains, we suggest that the Olympic group should be managed separately. On the other hand, the case for treating the Washington Cascades and Oregon Cascades populations as separate DPSs seems less compelling, and we simply note that there may have been a history of isolation across the Columbia River.

Finally, there was substantial genetic differentiation on a small geographical scale for both mtDNA and nuclear DNA. Remarkably, four of the six Oregon populations con-

tain private mtDNA alleles, with two of these populations fixed for a private allele (Fig. 1). The widespread presence and fixation of mtDNA private alleles has not been observed in most other anurans (Yang *et al.* 1994; Wilkinson *et al.* 1996; Evans *et al.* 1997; Shaffer *et al.* 2000), but has been observed in populations of another Pacific Northwestern ranid on a similar geographical scale (*R. pretiosa*; Blouin, unpubl. data). High genetic divergence at both nuclear and mitochondrial markers suggests that metapopulation structure is weak, with low connectivity between populations. Consistent with this conclusion is the observation that recolonization of one historic *R. cascadae* site was reported to have taken 12 years despite the presence of a *R. cascadae* population within 2 km (Blaustein *et al.* 1994). Thus, conservation of this species will require further studies on the scale of gene flow, and on the habitat features that enhance or reduce connectivity between populations.

### Acknowledgements

This research was conducted as part of K. Monsen's PhD thesis. We would like to thank Gary Fellers, Marc Hayes, Robert Hoffman, William Leonard and Don Major for help in tissue collection, and Anja Bauman and Liana Granum for help with PCR optimization. We thank William Ardren, Eric Hoffman, Merrill Peterson and Michael Pfreder for their comments on earlier versions of this manuscript.

### References

- Angers B, Bernatchez L (1998) Combined use of SMM and non SMM methods to infer fine structure and evolutionary history of closely related brook char (*Salvelinus fontinalis*, Salmonidae) populations from microsatellites. *Molecular Biology and Evolution*, **15**, 143–159.
- Arbogast BS, Browne RA, Weigl PD (2001) Evolutionary genetics and Pleistocene biogeography of North American tree squirrels (*Tamiasciurus*). *Journal of Mammalogy*, **82**, 302–319.
- Avise J, Arnold J, Ball R *et al.* (1987) Intraspecific phylogeography: the mitochondrial DNA bridge between population genetics and systematics. *Annual Review of Ecology and Systematics*, **18**, 489–522.
- Avise J, Neigel J, Arnold J (1984) Demographic influences on mitochondrial DNA lineage survivorship in animal populations. *Journal of Molecular Evolution*, **20**, 99–105.
- Baverstock P, Moritz C (1996) Project design. In: *Molecular Systematics* (eds Hillis DM, Moritz C, Mable BK), pp. 17–27. Sinauer Associates Inc., Publishers, Sunderland, MA, USA.
- Birky C, Maruyama T, Fuerst P (1983) An approach to population and evolutionary genetic theory for genes in mitochondria and chloroplasts, and some results. *Genetics*, **103**, 513–527.
- Blaustein A, Wake D, Sousa W (1994) Amphibian declines: judging stability, persistence, and susceptibility of populations to local and global extinctions. *Conservation Biology*, **8**, 60–71.
- Bowcock A, Ruiz-Linares A, Tomfohrde J, Minch E, Kidd J (1994) High resolution of human evolutionary trees with polymorphic microsatellites. *Nature*, **368**, 455–457.

- Bowen B (1998) What is wrong with ESUs?: the gap between evolutionary theory and conservation principles. *Journal of Shellfish Research*, **17**, 1355–1358.
- Briggs J, Storm R (1970) Growth and population structure of the cascades frog, *Rana cascadae* Slater. *Herpetologica*, **26**, 283–300.
- Brown JM, LeebensMack JH, Thompson JN, Pellmyr O, Harrison RG (1997) Phylogeography and host association in a pollinating seed parasite *Greya politella* (Lepidoptera: Prodoxidae). *Molecular Ecology*, **6**, 215–224.
- Buonaccorsi V, Reece K, Morgan L, Graves J (1999) Geographic distribution of molecular variance within the blue marlin (*Makaira nigricans*): a hierarchical analysis of allozyme, single-copy nuclear, and mitochondrial DNA markers. *Evolution*, **53**, 568–579.
- Bury R, Pearl C (1999) Klamath–Siskiyou herpetofauna: biogeographic patterns and conservation strategies. *Natural Areas Journal*, **19**, 341–350.
- Cavalli-Sforza LL, Edwards AWF (1967) Phylogenetic analysis: models and estimation procedures. *American Journal of Human Genetics*, **19**, 233–257.
- Clement M, Posada D, Crandall K (2000) rcs: a computer program to estimate gene genealogies. *Molecular Ecology*, **9**, 1657–1659.
- Crandall K, Templeton A (1993) Empirical tests of some predictions from coalescent theory with applications to intraspecific phylogeny reconstruction. *Genetics*, **134**, 959–969.
- Daugherty CH, Allendorf FW, Dunlap WW, Knudsen KL (1983) Systematic implications of geographic patterns of genetic variation in the genus *Dicamptodon*. *Copeia*, **1983**, 679–691.
- Demboski JR, Cook JA (2001) Phylogeography of the dusky shrew *Sorex monticolus* (Insectivora, Soricidae): insight into deep and shallow history in northwestern North America. *Molecular Ecology*, **10**, 1227–1240.
- Driscoll D (1999) Skeletochronological assessment of age structure and population stability for two threatened frog species. *Australian Journal of Ecology*, **24**, 182–189.
- Estoup A, Angers B (1998) Microsatellites and minisatellites for molecular ecology: theoretical and empirical considerations. In: *Advances in Molecular Ecology* (ed. G Carvalho), p. 55. IOS Press, Amsterdam, The Netherlands.
- Evans B, Morales J, Picker M, Kelley D, Melnick J (1997) Comparative molecular phylogeography of two *Xenopus* species, *X. gilli* and *X. laevis*, in the south-western Cape Province, South Africa. *Molecular Ecology*, **6**, 333–343.
- Excoffier L, Smouse P, Quattro J (1992) Analysis of molecular variance inferred from metric distances among DNA haplotypes: application to human mitochondrial DNA restriction data. *Genetics*, **131**, 479–491.
- Fellers G, Drost C (1993) Disappearance of the cascades frog *Rana-cascadae* at the southern end of its range, California USA. *Biological Conservation*, **65**, 177–181.
- Franck P, Garnery L, Loiseau A *et al.* (2001) Genetic diversity of the honeybee in Africa: microsatellite and mitochondrial data. *Heredity*, **86**, 420–430.
- Friedl T, Klump G (1997) Some aspects of population biology in the European tree frog, *Hyla arborea*. *Herpetologica*, **31**, 52–59.
- Garza J, Slatkin M, Freimer N (1995) Microsatellite allele frequencies in humans and chimpanzees, with implications for constraints on allele size. *Molecular Biology and Evolution*, **12**, 594–603.
- Good DA (1989) Hybridization and cryptic species in *Dicamptodon*. *Evolution*, **43**, 728–744.
- Good DA, Wake DB (1992) Geographic variation and speciation in the torrent salamanders of the genus *Rhyacotriton* (Caudata: Rhyacotritinidae). *University of California Publications in Zoology*, **126**, 1–91.
- Goudet J (1995) FSTAT (version 1.2): a computer program to calculate F-statistics. *Journal of Heredity*, **86**, 485–486.
- Green D (1986) Systematics and evolution of western North American frogs allied to *Rana aurora* and *Rana boylei*: electrophoretic evidence. *Systematic Zoology*, **35**, 283–296.
- Greenwood PJ (1980) Mating systems, philopatry and dispersal in birds and mammals. *Animal Behavior*, **28**, 1140–1162.
- Gubitz T, Thorpe RS, Malhotra A (2000) Phylogeography and natural selection in the Tenerife gecko *Tarentola delalandii*: testing historical and adaptive hypotheses. *Molecular Ecology*, **9**, 1213–1221.
- Hare MP, Avise JC (1998) Population structure in the American oyster as inferred by nuclear gene genealogies. *Molecular Biology and Evolution*, **15**, 119–128.
- Hillis D, Mable BK, Larson A, Davis S, Zimmer E (1996) Nucleic acids IV: sequencing and cloning. In: *Molecular Systematics* (eds Hillis D, Moritz C, Mable B), pp. 342–343. Sinauer Associates Inc., Publishers, Sunderland, MA, USA.
- Hoelzer G (1997) Inferring phylogenies from mtDNA variation: mitochondrial-gene trees versus nuclear-gene trees revisited. *Evolution*, **51**, 622–626.
- Howard JH, Seeb LW, Wallace R (1993) Genetic variation and population divergence in the *Plethodon vandykei* species group. *Herpetologica*, **49**, 238–247.
- Hutchison DW, Templeton AR (1999) Correlation of pairwise genetic and geographic distance measures: inferring the relative influences of gene flow and drift on the distribution of genetic variability. *Evolution*, **53**, 1898–1914.
- Jack PL, Dimitrijevic T, Mayes S (1995) assessment of nuclear, mitochondrial and chloroplast RFLP markers in oil palm (*Elaeis guineensis jacq.*). *Theoretical and Applied Genetics*, **90**, 643–649.
- Janzen FJ, Krenz JG, Haselkorn TS, Brodie ED (2002) Molecular phylogeography of common garter snakes (*Thamnophis sirtalis*) in western North America: implications for regional historical forces. *Molecular Ecology*, **11**, 1739–1751.
- Kimura M, Crow J (1963) The measurement of effective population number. *Evolution*, **17**, 279–288.
- Kosuch J, Vences M, Dubois A, Ohler A, Bohme W (2001) Out of Asia: mitochondrial DNA evidence for an oriental origin of tiger frogs, genus *Hoplobatrachus*. *Molecular Phylogenetics and Evolution*, **21**, 398–407.
- Lande R (1995) Breeding plans for small populations based on the dynamics of quantitative genetic variance. In: *Population Management for Survival and Recovery: Analytical Methods and Strategies in Small Population Conservation* (eds Ballou J, Gilpin M, Foose T), pp. 318–340. Columbia University Press, New York.
- Macey J, Schulte J II, Larson A *et al.* (1998a) Phylogenetic relationships of toads in the *Bufo bufo* species group from the eastern escarpment of the Tibetan Plateau: a case of vicariance and dispersal. *Molecular Phylogenetics and Evolution*, **9**, 80–87.
- Macey J, Schulte J II, Ananjeva N *et al.* (1998b) Phylogenetic relationships among agamid lizards of the *Laudakia caucasia* species group: testing hypotheses of biogeographic fragmentation and an area cladogram for the Iranian Plateau. *Molecular Phylogenetics and Evolution*, **10**, 118–131.
- Macey J, Strasburg J, Brisson J, Vredenburg V, Jennings M, Larson A (2001) Molecular phylogenetics of western North American frogs of the *Rana boylei* species group. *Molecular Phylogenetics and Evolution*, **19**, 131–143.

- McKay JK, Latta RG (2002) Adaptive population divergence: markers, QTL and traits. *Trends in Ecology and Evolution*, **17**, 285–291.
- Measey G (2001) Growth and ageing of feral *Xenopus laevis* (Daudin) in South Wales, UK. *Journal of Zoology*, **254**, 547–555.
- Melnick DJ, Hoelzer GA (1992) Differences in male and female macaque dispersal lead to contrasting distributions of nuclear and mitochondrial DNA variation. *International Journal of Primatology*, **13**, 1–25.
- Moore W (1995) Inferring phylogenies from mtDNA variation: mitochondrial-gene trees versus nuclear-gene trees. *Evolution*, **49**, 718–726.
- Moritz C (1994) Defining 'evolutionarily significant units' for conservation. *Trends in Ecology and Evolution*, **9**, 373–375.
- Mulcahy DG, Mendelson JR (2000) Phylogeography and speciation of the morphologically variable, widespread species *Bufo valliceps*, based on molecular evidence from mtDNA. *Molecular Phylogenetics and Evolution*, **17**, 173–189.
- Nagae Y (1988) The primary and secondary structures of the ribosomal RNAs of *Rana catesbeiana* mitochondrion. *Journal of the Nippon Medical School*, **55**, 417–430.
- Nei M (1972) Genetic distance between populations. *American Naturalist*, **106**, 283–292.
- Nei M, Takahata N (1993) Effective population size, genetic diversity, and coalescence time in subdivided populations. *Journal of Molecular Evolution*, **37**, 240–244.
- Neigel J, Avise J (1986) Phylogenetic relationships of mitochondrial DNA under various demographic models of speciation. In: *Evolutionary Processes and Theory* (eds Nevo E, Karlin S). Academic Press, New York.
- Nielson M, Lohman K, Sullivan J (2001) Evolution and phylogeography of the tailed frog (*Ascaphus truei*): insights on the biogeography of the Pacific Northwest. *Evolution*, **55**, 147–160.
- Nyakaana S, Arctander P (1999) Population genetic structure of the African elephant in Uganda based on variation at mitochondrial and nuclear loci: evidence for male-biased gene flow. *Molecular Ecology*, **8**, 1105–1115.
- Orita M, Iwahana H, Kanazawa H, Hayashi K, Sekiya T (1989) Detection of polymorphisms of human DNA by gel electrophoresis as single-strand conformation polymorphisms. *Proceedings of the National Academy of Sciences USA*, **86**, 2766–2770.
- Ostrander E, Sprague G, Rine J (1993) Identification and characterization of dinucleotide repeat (CA)<sub>n</sub> markers for genetic mapping in dog. *Genomics*, **16**, 207–213.
- Paetkau D (1999) Using genetics to identify intraspecific conservation units: a critique of current methods. *Conservation Biology*, **13**, 1507–1509.
- Paetkau D, Waits L, Clarkson P, Craighead L, Strobeck C (1997) An empirical evaluation of genetic distance statistics using microsatellite data from bear (Ursidae) populations. *Genetics*, **147**, 1943–1957.
- Page R (1996) TREEVIEW: an application to display phylogenetic trees on personal computers. *Computer Applications in the Biosciences*, **12**, 357–358.
- Pennock D, Dimmick W (1997) Critique of the evolutionarily significant unit as a definition for 'distinct population segments' under the US endangered species act. *Conservation Biology*, **11**, 611–619.
- Raymond M, Rousset F (1995) GENEPOP version 1.2: population genetics software for exact tests and ecumenicism. *Journal of Heredity*, **86**, 248–249.
- Reaser J (2000) Demographic analysis of the Columbia spotted frog (*Rana luteiventris*): case study in spatiotemporal variation. *Canadian Journal of Zoology*, **78**, 1158–1167.
- Robertson A (1964) The effect of non-random mating within inbred lines on the rate of inbreeding. *Genetic Research*, **5**, 164–167.
- Ryder O (1986) Species conservation and systematics: the dilemma of subspecies. *Trends in Ecology and Evolution*, **1**, 9–10.
- Schneider S, Roessli D, Excoffier L (2000) ARLEQUIN, version 2.000: a Software for Population Genetic Data Analysis. Genetics and Biometry Laboratory, University of Geneva, Switzerland.
- Shaffer HB, Fellers G, Magee A, Voss R (2000) The genetics of amphibian declines: population substructure and molecular differentiation in the Yosemite toad, *Bufo canorus* (Anura, Bufonidae) based on single-strand conformation polymorphism analysis (SSCP) and mitochondrial DNA sequence data. *Molecular Ecology*, **9**, 245–257.
- Slatkin M (1985) Rare alleles as indicators of gene flow. *Evolution*, **39**, 53–65.
- Slatkin M (1995) A measure of population subdivision based on microsatellite allele frequencies. *Genetics*, **139**, 457–462.
- Slatkin M, Barton NH (1989) A comparison of three indirect methods for estimating average levels of gene flow. *Evolution*, **43**, 1349–1368.
- Soltis D, Gitzendanner M, Strenge D, Soltis P (1997) Chloroplast DNA intraspecific phylogeography of plants from the Pacific Northwest of North America. *Plant Systematics and Evolution*, **206**, 353–373.
- Spolsky C, Phillips C, Uzzell T (1992) Antiquity of clonal salamander lineages revealed by mitochondrial DNA. *Nature*, **356**, 706–708.
- Spruell P, Hemmingsen AR, Howell PJ, Kanda N, Allendorf FW (2003) Conservation genetics of bull trout: geographic distribution of variation at microsatellite loci. *Conservation Genetics*, **4**, 17–29.
- Steinhoff RJ, Joyce DG, Fins L (1983) Isozyme variation in *Pinus monticola*. *Canadian Journal of Forest Research*, **13**, 122–132.
- Strenge D (1994) *The intraspecific phylogeography of Polystichum munitum and Alnus rubra*. MSc Thesis, Washington State University.
- Takezaki N, Nei M (1996) Genetic distance and reconstruction of phylogenetic trees from microsatellite DNA. *Genetics*, **144**, 389–399.
- Taylor B, Dizon E (1999) First policy then science: why a management unit based solely on genetic criteria cannot work. *Molecular Ecology*, **8**, S11–S16.
- Templeton A, Crandall K, Sing C (1992) A cladistic analysis of phenotypic associations with haplotypes inferred from restriction endonuclease mapping and DNA sequence data. III. Cladogram estimation. *Genetics*, **132**, 619–633.
- US Department of the Interior and US Department of Commerce (1996) Policy regarding the recognition of distinct vertebrate population segments under the endangered species act. *Federal Register*, **61**, 4722–4725.
- Waitt R, Thorson R (1983) The Cordilleran ice sheet in Washington, Idaho, and Montana. In: *Late Quaternary Environments of the United States*, vol. 1. *The Late Pleistocene* (eds Wright H Jr, Porter S), pp. 53–70. University of Minnesota Press, Minneapolis.
- Waldman B, McKinnon J (1993) Inbreeding and outbreeding in fishes, amphibians, and reptiles. In: *A Natural History of Inbreeding and Outbreeding, Theoretical and Empirical Perspectives* (ed. Wilmsen Thornhill N), pp. 250–282. University of Chicago Press, Chicago and London.

- Waples R (1991) Pacific salmon, *Oncorhynchus* spp. and the definition of 'species' under the endangered species act. *US National Marine Fisheries Service Marine Fisheries Review*, **53**, 11–22.
- Waples R (1995) Evolutionarily significant units and the conservation of biological diversity under the endangered species act. *American Fisheries Society Symposium*, **17**, 8–27.
- Waples R (1998) Evolutionarily significant units, distinct population segments, and the endangered species act: reply to Pennock and Dimmick. *Conservation Biology*, **12**, 718–721.
- Weir B, Cockerham C (1984) Estimating  $F$ -statistics for the analysis of population structure. *Evolution*, **38**, 1358–1370.
- Whitlock M, McCauley D (1999) Indirect measures of gene flow and migration:  $F_{ST}$  does not equal  $1/4Nm + 1$ . *Heredity*, **82**, 117–125.
- Wilkinson J, Matsui M, Terachi T (1996) Geographic variation in a Japanese tree frog (*Rhacophorus arboreus*) revealed by PCR-aided restriction site analysis of mtDNA. *Journal of Herpetology*, **30**, 418–423.
- Wright S (1951) The genetical structure of populations. *Annals of Eugenetics*, **15**, 323–354.
- Wright S (1965) The interpretation of population structure by  $F$ -statistics with special regard to systems of mating. *Evolution*, **19**, 395–420.
- Yang Y, Yao-Sung L, Wu J, Hui C (1994) Variation in mitochondrial DNA and population structure of the Taipei tree frog *Rhacophorus taipeinus* Taiwan. *Molecular Ecology*, **3**, 219–228.
- Yoneyama Y (1987) The nucleotide sequences of the heavy and light strand replication origins of the *Rana catesbeiana* mitochondrial genome. *Nippon Ika Daigaku Zasshi*, **54**, 429–440.
- Zamudio K, Greene H (1997) Phylogeography of the bushmaster (*Lachesis muta*: Viperidae): implications for neotropical biogeography, systematics, and conservation. *Biological Journal of the Linnean Society*, **62**, 421–442.

---

Kirsten Monsen has recently completed her PhD studying the population and conservation genetics of *Rana cascadae*, and is currently investigating population genetic structure within a beetle hybrid zone. Michael Blouin's laboratory focuses on the causes and consequences of genetic structuring, and on applications of methods for pedigree reconstruction in natural populations. He works on a variety of taxa including fish, amphibians and parasites.

---