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Biogeographic patterns and current distribution of molecular-genetic variation among populations of speckled dace, *Rhinichthys osculus* (Girard)

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Abstract

We examined genetic diversity within- and among-populations of speckled dace (*Rhinichthys osculus*) in five major drainage systems in the state of Oregon in western North America. Analysis of sequence variation in a 670-bp segment of the mitochondrial cytochrome *b* gene revealed deep divergence among basins and high genetic diversity within basins. Application of a molecular clock indicated that the divergence time among basins reflects vicariant events during the late Miocene to early Pliocene. The high levels of genetic diversity observed within basins is likely due to large historic population sizes, in particular, within the Klamath Basin. Two highly divergent mtDNA lineages were found to co-occur in populations in the Klamath Basin. This result may be indicative of a complex history of isolation and reconnection in this basin and/or multiple colonization events. Based on the observed level of mtDNA divergence these lineages may represent two reproductively isolated sympatric taxa. We recommend that major basins be regarded as distinct ESUs based on high levels of subdivision, deep divergences, and reciprocal monophyly among basins.

Keywords: Biogeography; Cyprinids; Genetic diversity; mtDNA; Population genetics; Population subdivision

> Uncounted local variants occupy an almost endless number of habitats, ranging from torrential creeks in the major river systems to the tiniest and most isolated spring holes in the desiccated valleys that comprise the Great Basin—Hubbs et al., 1974.

1. Introduction

The distribution of extant populations and species is a direct consequence of the combined influence of migration and the geological history of the region. Deciphering the influence of these two factors on the current distribution of populations and species has been a major focus of evolutionary biologists for more than a century. Modern molecular techniques, and the emergence of the field of phylogeography (Avise, 2000), provide the tools and the conceptual framework to place current patterns of distribution in a historical perspective. The combination of a population–genetic analysis of genetic variation within- and among-populations and phylogenetic reconstruction of genetic lineages can reveal much about the evolutionary mechanisms driving the biogeographic history of populations and species.

The biogeographic history of the Pacific Northwest region of North America has been particularly difficult to decipher. Taxa that persist in this region have dealt with far more spectacular geologic and climatic events than their counterparts in other regions of the continent (Minckley et al., 1986). A complex history of geologic and climatic environmental events has shaped the biogeographic landscape in this region. The forces of plate tectonics (Atwater, 1970; Ernst, 1981), inland mountain building and volcanism (Armentrout et al., 1979; Montgomery, 2000), in concert with trends toward ever-increasing aridity and pronounced climatic oscillations during glaciations in the Pleistocene (Axelrod, 1979;

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Hoover et al., 1982) have had a profound effect on the distribution of taxa.

Recent molecular investigations are rapidly advancing our understanding of the complex biogeographic history of this region. Phylogeographic studies in a diverse array of terrestrial vertebrates including amphibian (Nielson et al., 2001; Tan and Wake, 1995; Wake, 1997) and reptile (Rodriguez-Robles et al., 1999; Zamudio et al., 1997) species have revealed a pattern of relatively deep divergences among populations with origins ranging from the Miocene to the Pleistocene.

Primary freshwater fish, in particular, are ideal model organisms for studying biogeographic patterns. They are restricted to lake and river drainage systems and typically exhibit low vagility. This combination links the evolutionary history of populations and species to the geological history of the landscape they occupy (Bernatchez and Wilson, 1998). One of the most specious and successful groups of primary freshwater fish is the family Cyprinidae. In the western United States cyprinids are found in virtually every primary drainage and thus comprise particularly useful biogeographic study organisms. Morphological and paleontological studies of cyprinids have long been used to reconstruct the biogeographic history of regions in western North America (Cope, 1883; Hubbs and Miller, 1948; elegantly reviewed by Minckley et al., 1986). Much of our understanding of the geological history of this region is based on the current distribution of species in this family and an extensive fossil record documenting historical distributions. Somewhat surprisingly, few molecular genetic investigations have been undertaken in this group in North America (but see Schmidt et al., 1998). In contrast, there have been extensive recent molecular studies in European cyprinids focusing on both closely related species and populations in species with large geographical ranges (Briolay et al., 1998; Gilles et al., 1998; Kotlik and Berrebi, 2001; Mesquita et al., 2001; Tsigenopoulos and Berrebi, 2000; Zardoya and Doadrio, 1998, 1999). North American studies have often focused on isolated populations (Johnson and Jordan, 2000) or endangered species (Parker et al., 1999), and are necessarily focused at a scale other than that of regional biogeography. A more appropriate scale of investigation to gain insights into regional biogeography is a phylogenetic perspective based on widespread taxa or closely related assemblages of species.

Species in the genus *Rhinichthys* have a widespread distribution in western North America. In particular, speckled dace (*Rhinichthys osculus*) have a large geographic range (Fig. 1). They are native to all major western drainages from the Columbia Basin, in Canada, south to Sonora, Mexico (Lee et al., 1980). Populations show high degrees of endemism and exhibit large differences in morphological traits (Hubbs and Miller, 1948; Woodman, 1992; Scott and Crossman, 1998). The combination of morphological differentiation and local endemism in this species has generated a lengthy and confusing taxonomic history. There have been as many as 12 separate species described in this complex (Jordan and Evermann, 1896). More recent authors have considered the complex to be a single widely distributed and highly variable species (Hubbs et al., 1974; La Rivers, 1962). Our current understanding of the relationships among populations in this complex is limited. In fact, there is no clear consensus regarding the number of distinct evolutionary lineages within *R. osculus*. This apparent gap in our knowledge is reflected in recent taxonomic treatments. For example, Deacon and Williams (1984) list 10 distinct subspecies and five undescribed taxa from the state of Nevada.

In the state of Oregon, *R. osculus* is the most frequently occurring freshwater fish (Bond et al., 1988). Speckled dace are habitat generalists (Bond et al., 1988) with high ecological versatility. As a result, they occupy a wide range of habitats. In addition to ecological flexibility, this species has a high potential-reproductive rate. Females mature in as little as two years and produce 450–2000 eggs in a season (Peden and Hughes, 1981). The combination of these two factors may explain this species' apparent historical resistance to local extinction leading to their current widespread distribution. A ubiquitous distribution and resistance to local extinction make *R. osculus* a good candidate for investigating the biogeographic history of the region.

This study was undertaken with the goal of characterizing the distribution of molecular genetic variation within- and among-populations of *R. osculus*. We focused primarily on two levels of analysis. First, an extensive sampling scheme covering the Klamath River Basin was implemented to examine the within-drainage patterns of genetic variation. Second, broader sampling that included populations from multiple major drainages provided a framework to interpret the results of the Klamath Basin survey.

2. Methods

2.1. Sample and data collection

We examined 90 individuals of *R. osculus* from 13 localities in five major river drainages throughout the state of Oregon (Fig. 1 and Table 1). Sampling within the Klamath Basin comprised six localities representing four major sub-drainages. The remaining seven localities represented the Columbia, Willamette, Snake, and Coastal drainages. We sampled two sub-drainages in the Columbia Basin: the Umatilla and John Day rivers. We sampled Johnson Creek, a tributary of the Willamette River. Three populations were sampled from coastal rivers: Millicoma Creek, a tributary of the Coos River;

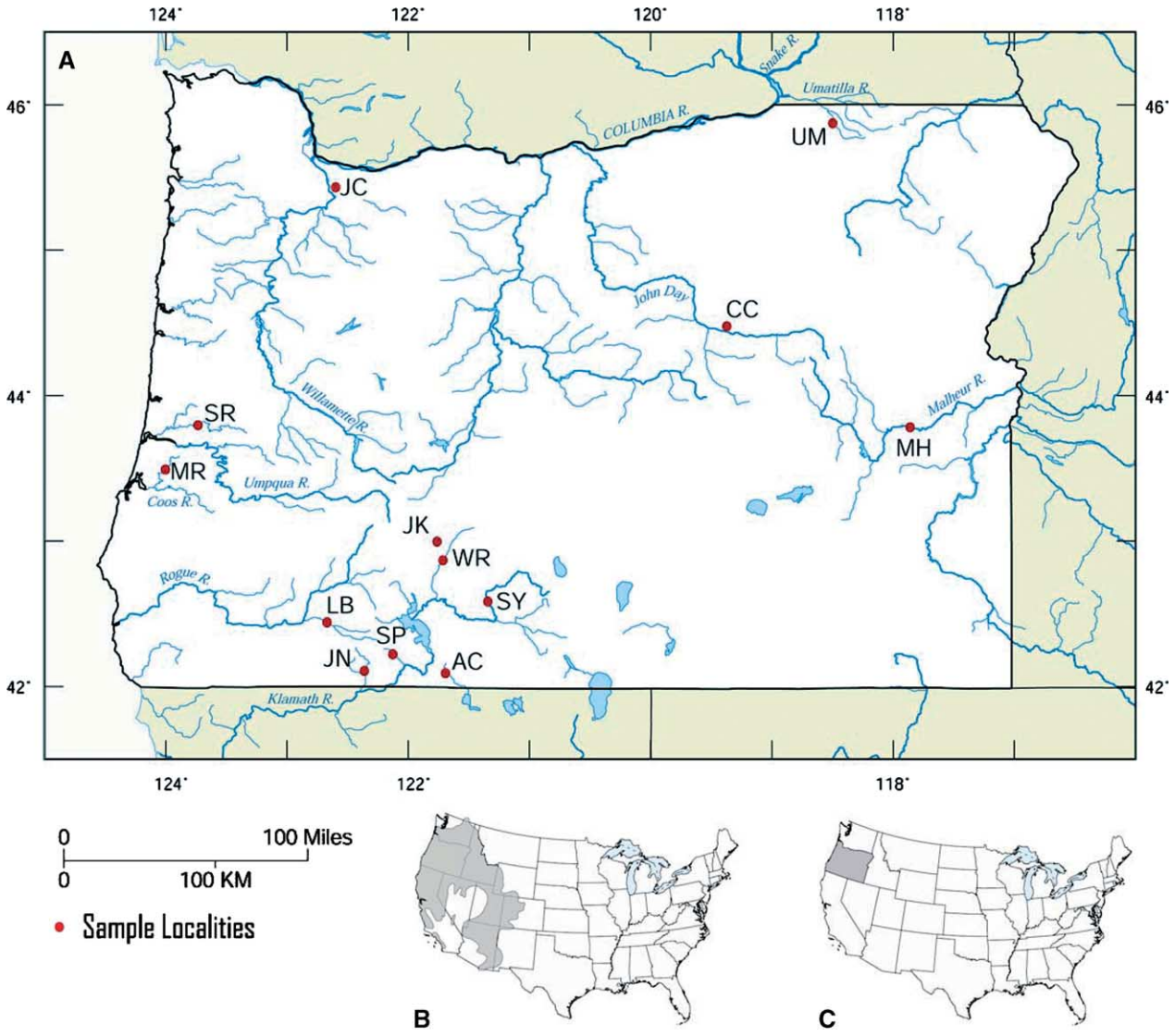

Fig. 1. (A) Map of Oregon, USA, showing the 13 collection localities used for this study. Populations are designated by two letter codes described in Table 1. (B) Distribution of speckled dace (*R. osculus*) in western North America (modified from Lee et al., 1980). (C) Shaded inset showing the location of Oregon.

the Smith River, a tributary of the Umpqua River; and Little Butte Creek, a tributary of the Rogue River.

Whole individuals or fin clips were preserved in 95% ethanol. Genomic DNA was obtained from muscle tissue by a standard pro-K digestion followed by phenol/chloroform/isoamyl alcohol extraction. The polymerase chain reaction was used to amplify a 670-bp mitochondrial DNA segment containing a portion of the mtDNA cytochrome *b* (cyt *b*) gene using primers L15162 (5′-TTCTTCCATGAGGACAAATAT-3′) and H15915A (5′-CCTCCGTCTTCCGGATTACAAGAC-3′). Cyt *b* has frequently been used to trace the evolutionary history of cyprinids (Briolay et al., 1998; Johnson and Jordan, 2000; Kotlik and Berrebi, 2001; Schmidt et al., 1998; Zardoya and Doadrio, 1998, 1999). In addition, numerous studies of European lineages with documented patterns of historical isolation made cyt *b* a good candidate for dating the divergence times between lineages (Zardoya and Doadrio, 1998, 1999). Amplifications were performed in 50 μl reactions using 2 μl genomic DNA extraction, 5 μl 10× PCR buffer, 5 μl of 8 mM dNTP mix, 5 μl of each 2 μM primer, 0.2 μl Taq DNA polymerase (Perkin–Elmer Cetus, Emeryville, CA) and water to final volume. Reactions consisted of an initial denaturation step at 94 °C for 5 min, followed by 35 cycles of 92 °C for 45 s, 48 °C for 60 s, and 72 °C for 90 s. Negative controls were performed with all reactions. PCR products were purified using QIAGEN spin columns (Qiagen, Valencia, CA). The DNA sequences of purified PCR products were obtained using an Applied Biosystems Inc. ABI 377 automated DNA sequencer using Dye Terminator Cycle Sequencing.

Table 1
Sampling localities listed by major basin, sub-basin, and specific locality

Basin	Sub-basin	Population	Code	*N*	Haplotypes	π
Klamath R.	Lost R.	Antelope Cr.	AC	10	5	0.0129
	Williamson R.	Upper Williamson R.	WR	9	5	0.0016
		Jack Cr.	JK	7	5	0.0152
	Sprague R.	Sycan R.	SY	11	5	0.0151
	Lower Klamath R.	Jenny Cr.	JN	8	6	0.0347
		Spencer Cr.	SP	10	7	0.0240
			Total	55	25	0.0227
Columbia R.	Umatilla R.	Umatilla R.	UM	5	2	0.0006
	John Day R.	Cottonwood Cr.	CC	7	3	0.0021
			Total	12	5	0.0086
Coastal	Umpqua R.	Smith R.	SR	5	1	0.0000
	Coos R.	Millicoma R.	MR	5	2	0.0009
	Rogue R.	Little Butte Cr.	LB	4	1	0.0000
			Total	14	4	0.0115
Willamette R.	Johnson Cr.	Johnson Cr.	JC	4	4	0.0030
Snake R.	Malheur R.	Malheur R.	MH	5	5	0.0066
All Populations				90	43	0.0434

Localities are followed by population code, number of individuals surveyed (*N*), number of unique haplotypes, and the nucleotide diversity (π). Summaries for basins with multiple samples are shown at the bottom.

PCR products were sequenced from both the forward and reverse directions. We obtained partial cyt *b* sequence from 90 *R. osculus* individuals representing 13 populations and one outgroup taxon (long-nosed dace; *Rhinichthys cataractae*).

2.2. Phylogenetic analysis and population subdivision

Protein coding sequences from forward and reverse primers were assembled into contigs with the program BioEdit vers. 5.0.9 (Hall, 1999) and aligned by eye. We inferred relationships among unique haplotypes by constructing phylogenetic trees using both maximum-parsimony (MP; Swofford et al., 1996) and maximum-likelihood (ML; Felsenstein, 1981) methods. All analyses were performed using the program PAUP* vers. 4.08d (Swofford, 2000). For the MP analyses, we used an unweighted character matrix and conducted heuristic searches with 100 random-addition sequences to minimize the effects of input order on the recovered topology. We used the options, accelerated character transformation (ACCTRAN), tree bisection–reconnection (TBR) branch swapping, and save all minimal trees (MULPARS). We used nonparametric bootstrapping (Felsenstein, 1985) with 1000 pseudoreplicates and 10 random input sequences to evaluate relative support for internal branching patterns.

To conduct ML analyses we estimated the best-fit model of evolution by using the program Modeltest vers. 3.06 (Posada and Crandall, 1998) and used the Log-likelihood Ratio Test (LRT) for model selection. Heuristic searches were conducted with 10 random-addition sequences to reduce the impact of addition order. Starting branch lengths were obtained using the least-squares method with Jukes–Cantor distances. For the ML analyses, we used nonparametric bootstrapping (Felsenstein, 1985) with 100 pseudoreplicates and random addition for each pseudoreplicate.

Historical relationships among populations that have reached reciprocal monophyly can be assessed by standard phylogenetic reconstruction. However, when populations share haplotypic constitutions and do not exhibit reciprocal monophyly, phylogenetic reconstruction can only describe the relationships among haplotypes not among populations. We assessed relationships among populations within the Klamath River Basin using a phenetic approach by constructing a tree of relationships using the UPGMA algorithm as implemented in MEGA 2.1 (Kumar et al., 2001) and a distance matrix based on the excess between-population nucleotide diversity (π) (Nei, 1987). To construct this matrix, we first estimated the nucleotide diversity within- and between-pairs of populations using the program DNAsp vers. 3.0 (Rozas and Rozas, 1999), and then removed the average within-population nucleotide diversity from the total diversity between populations.

We compared the relative levels of genetic variation by estimating nucleotide diversity (π) both within populations and within drainages (Nei, 1987). We estimated population subdivision of DNA sequence variation by calculating N_{st} (Lynch and Crease, 1990) at multiple levels including among populations, within drainages, and among drainages.

To estimate divergence times between major drainage basins, we calculated sequence-divergence estimates with PAUP* vers. 4.08d (Swofford, 2000) based on the molecular-clock constrained ML distance using the best-fit model predicted by Modeltest vers. 3.06 (Posada and Crandall, 1998). We used a molecular-clock calibration for the cyt *b* gene in cyprinid fish of 0.76%/Myr based on the results of studies in European taxa with well-established vicariant isolation dates (Zardoya and Doadrio, 1998, 1999). This rate is consistent with the value of 0.70 estimated for other ectothermic vertebrate taxa (Johns and Avise, 1998; Martins and Palumbi, 1993).

3. Results

3.1. Phylogeographic patterns among major basins

From 90 specimens of *R. osculus* examined in this study, we identified 43 unique haplotypes. Nucleotide sequences for unique haplotypes were logged in the NCBI GenBank under the accession numbers AY366257–AY366299. The number of haplotypes within drainages ranged from 1 to 25 (Table 1). The Klamath River Basin contained the highest number of unique haplotypes ($N = 25$). In two coastal populations, Smith River and Little Butte Creek, all individuals surveyed shared a single haplotype. The levels of sequence divergence between *R. osculus* haplotypes ranged from 0.16% to 16.38%. The average sequence divergence between *R. osculus* haplotypes and the outgroup, *R. cataractae*, was 20.54 (0.374) percent (throughout, values are presented as means followed by the SE in parentheses).

Phylogenetic analysis of 670 bp of the mtDNA cyt *b* gene revealed 138 variable characters of which 107 were parsimony informative. Based on the results of the LRT as implemented in Modeltest vers. 3.06 (Posada and Crandall, 1998), for maximum–likelihood analyses we used a substitution model with equal base frequencies, a six-step rate matrix ([A-C] = 1.0, [A-G] = 24.13, [A-T] = 1.0, [C-G] = 1.0, [C-T] = 9.391, [G-T] = 1.0), no invariant sites, and a gamma distribution shape parameter = 0.1473. These parameters correspond to a general-time-reversible model with a gamma distribution describing the rate variation among sites (GTR + Γ). The likelihood score for the resulting topology was 2227.19 (Fig. 2). Unweighted maximum-parsimony analyses resulted in a single most parsimonious tree of 243 steps, a consistency index (CI) of 0.646, and a retention index (RI) of 0.885 (Fig. 3).

The topologies obtained by MP and ML analyses showed highly structured phylogenies that were largely congruent, with the exception of the position of a basal branch comprising three haplotypes from the Klamath Basin (Klamath B in Figs. 2 and 3). Monophyletic clades of haplotypes within populations were recovered for all populations examined with the exception of those within the Klamath Basin. While only unique haplotypes were observed in the Klamath Basin, these haplotypes formed two clades that did not comprise a monophyletic grouping. No haplotype was found to occur in more than one major drainage system. The three coastal rivers formed a highly supported monophyletic clade. The two Columbia River drainages also formed a highly supported monophyletic group.

We found well-supported clades of haplotypes within populations and well-supported clades of some populations within basins. However, the deeper relationships among major drainages were not well supported by our data. Both the MP and ML analyses recovered a clade of three haplotypes found in a subset of the Klamath Basin populations (clade Klamath B, Figs. 2 and 3). The ML analysis suggested a basal position of this clade while the position of this group in the MP analysis was the sister to a clade containing Willamette + Snake + remaining Klamath haplotypes. The position of the Klamath B clade is not well supported by bootstrapping in either analysis. Our ML analyses indicated a grouping of haplotypes from coastal populations with those from the Columbia Basin and a grouping of haplotypes from the Willamette River Basin with those found in the Snake River Basin. The clade Willamette + Snake was the sister to a monophyletic clade containing the remaining 22 Klamath Basin haplotypes. These results were largely corroborated by the MP analyses with the exception the there was no grouping of coastal and Columbia populations in the single most-parsimonious tree recovered.

3.2. Subdivision among basins and populations

Estimates of population subdivision among major drainages based on N_{st} (Lynch and Crease, 1990) revealed high levels of genetic differentiation (0.823 (0.027)). Similarly, subdivision among subdrainages within major basins was substantial, ranging from 0.226 (0.045) among subdrainages in the Klamath River Basin to 0.909 between the two tributaries in the Columbia River Basin. This result was not surprising because, with the exception of subdrainages within the Klamath River Basin, there were no shared haplotypes found among any of the populations we sampled. The relatively low N_{st} among Klamath subdrainages contrasts with high levels of nucleotide diversity (π) found in this basin. This result was due to subdrainages in this basin sharing highly divergent haplotypes (see below).

Nucleotide diversity (π) within subdrainages ranged from 0.0 to 0.0347 (Table 1). Comparisons of π between basins showed a wide range of values. The Klamath Basin contained relatively high nucleotide diversity (0.023) having a level of twice that found in any other basin.

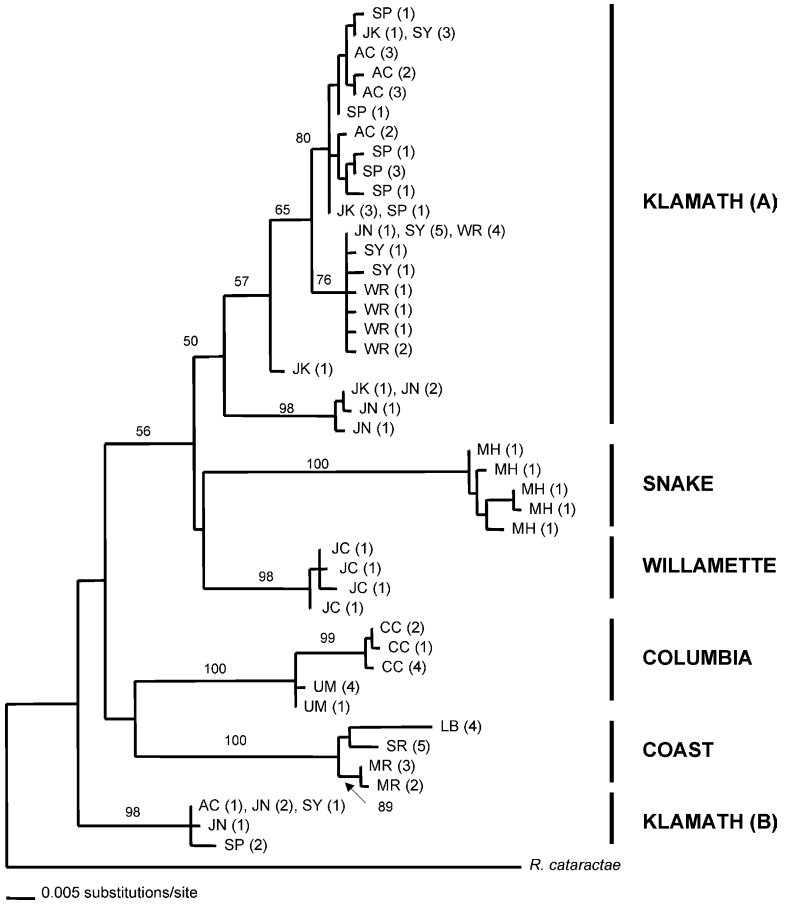

Fig. 2. Maximum-likelihood tree of unique haplotypes based on a GTR + Γ model. Branch lengths are drawn proportional to divergence. Major river basins are shown to the right of the tree. Localities (see Table 1 for locality codes) and number of occurrences for each haplotype are shown at the terminal branches. Bootstrap proportions from 100 pseudoreplications of the data are shown above branches where the values exceeded 50%.

Localities samples within the Klamath Basin showed extensive mixing of haplotypes, with no single locality containing a unique monophyletic grouping. There was significant structure among the haplotypes observed within the Klamath Basin, with four distinct clades of haplotypes. The first contained three haplotypes found in Antelope (AK), Jenny (JN), and Spencer (SP) creeks, and the Sycan River (SY) (Klamath B, Figs. 2 and 3). Haplotypes in Klamath Clade B were highly divergent from the other haplotype groups found in the Klamath Basin with an average uncorrected sequence divergence of 0.052 (0.0004). The maximum sequence divergence among the remaining 22 haplotypes (Klamath A, Figs. 2 and 3) was 0.040. These haplotypes formed three distinct and well-supported clades that did not exhibit strong geographical structure.

The preceding analytical approach, based on unique haplotypes, is particularly useful for reconstructing the evolutionary history of a particular gene, a gene genealogy. When all populations have achieved reciprocal monophyly this gene genealogy may be concordant with the population phylogeny. However, when there is substantial mixing of haplotypes among subdrainages, as is the case in the Klamath Basin, a gene genealogy provides little information on the relationships among subdrainages. To assess the pattern of relationship in this basin, we constructed a UPGMA phenogram based on excess between-population nucleotide diversity. This analysis used all the individuals we examined, not simply the unique haplotypes, and thus took into account not only the divergence among-haplotypes but the frequency of haplotypes as well. The resulting topology showed a number of interesting patterns (Fig. 4). Within the Klamath there were three groupings of localities: (1) two northeastern populations, Sycan (SY) and Upper Williamson (WR) rivers, showed a close association; (2) three localities, Antelope (AK), Jack (JK), and Spencer (SP) creeks, formed a group closely allied to the northeastern populations; and (3) the furthest downstream subdrainage, Jenny Creek (JN), occupied a basal position and was the most divergent.

The patterns of relationships among major basins, based on nucleotide diversity, showed a grouping of the Klamath and Willamette populations. This grouping was closely associated with the Snake River Basin, and the complex of Klamath–Willamette–Snake populations

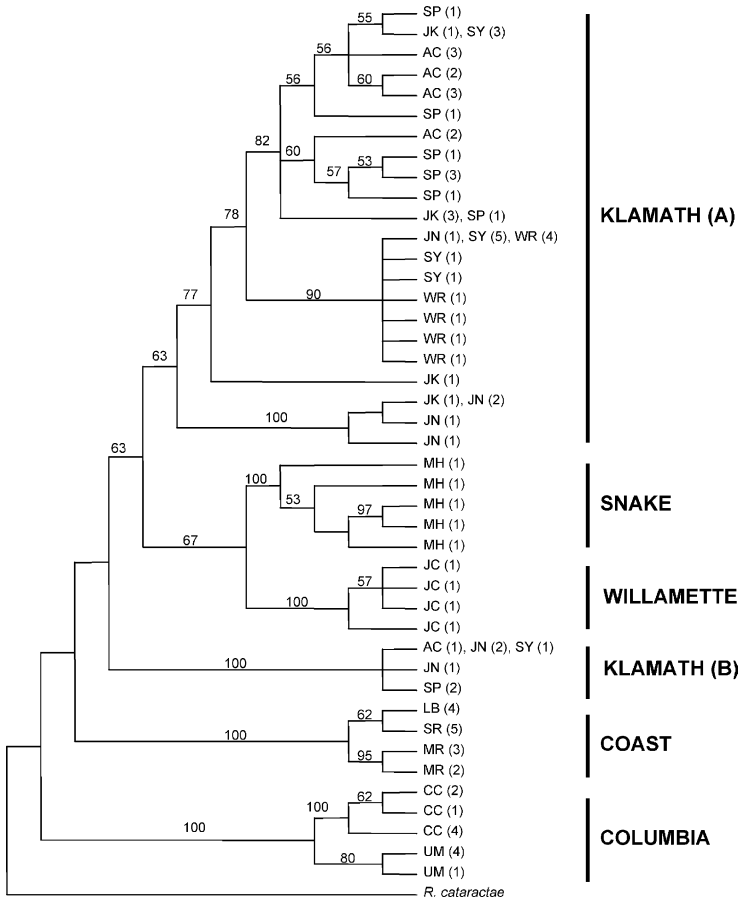

Fig. 3. Unweighted maximum-parsimony tree of unique haplotypes. Maximum-parsimony analyses resulted in a single most parsimonious tree of 243 steps, a consistency index (CI) of 0.646, and a retention index (RI) of 0.885. Localities (see Table 1 for locality codes) and number of occurrences for each haplotype are shown at the terminal branches. Bootstrap proportions from 1000 pseudoreplications of the data are shown above branches where the values exceeded 50%.

was associated with the two populations from the Columbia River Basin. The most distantly related grouping comprised populations found in the three coastal drainages.

3.3. Divergence times among lineages

Estimates of the average sequence divergence between major basins based on maximum-likelihood corrected distance ranged from 5.92 (0.09) percent for the comparison of the Klamath and Willamette basin, to 14.61 (0.24) percent between the Snake River and coastal basins (Table 2). Assuming a molecular clock calibration of 0.76% per Myr the range of divergence times among basins was 3.89–9.61 Myrs. On average haplotypes in major river basins in Oregon shared a most recent common ancestor 6.81 MYA.

The average sequence divergences between basins were much larger than the divergences between haplotypes within basins. With the exception of haplotypes within the Klamath Basin, the average sequence divergence within basins was less than 2% (Table 3). These results indicated that that the maximum coalescent time within basins is on the order of 1.7 Myrs for all basins except the Klamath. The presence of two highly divergent clades of haplotypes in the Klamath Basin (Klamath A and B, Figs. 2 and 3) elevated the average divergence in this basin to 3.66%. Considering the maximum divergence between haplotypes, there may have been as long a coalescent time as 7.1 Myrs in this basin.

4. Discussion

4.1. Phylogeographic relationships among basins

The most striking result of this study is the pattern of deep divergences among major drainage basins. Populations in major river basins form reciprocally monophyletic clades with high levels of sequence divergence. The amounts of divergence observed among *R. osculus* occupying major basins is on the order of species-level differences noted in other studies of cyprinids (McPhail

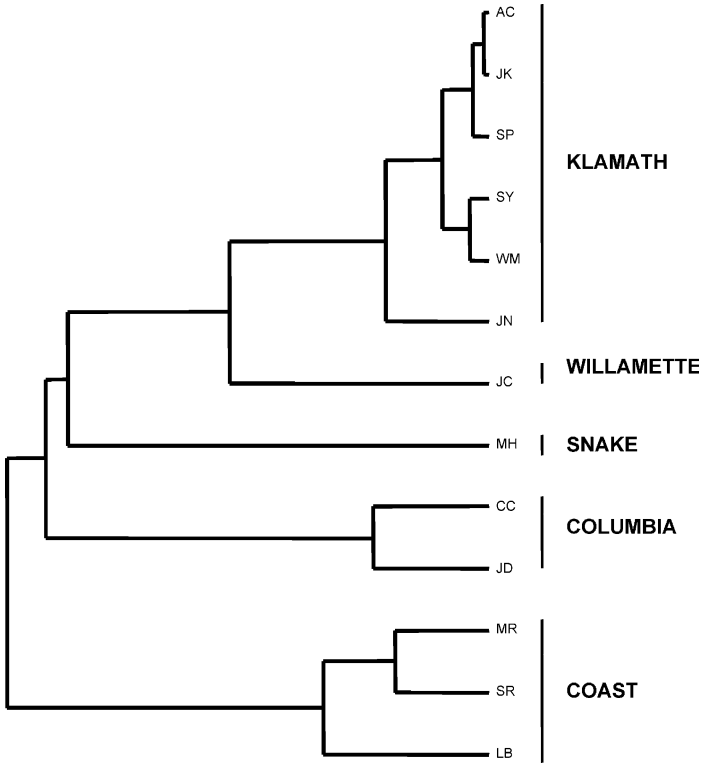

Fig. 4. UPGMA phenogram of populations based on the excess between-population nucleotide diversity (π). Major river basins are shown to the right of the phenogram. See Table 1 for locality codes.

Table 2
Sequence divergence and estimated times of divergence between major basins

Comparison	% sequence divergence			Divergence time (MYA)	
	Range	Mean	SE	Mean	SE
Klamath River vs.					
Columbia River	7.56–12.12	10.07	0.09	6.63	0.12
Coastal	8.18–13.76	10.40	0.10	6.84	0.14
Willamette River	4.24–8.89	5.92	0.09	3.89	0.12
Snake River	8.25–14.91	11.20	0.10	7.37	0.14
Columbia River vs.					
Coastal	8.66–12.34	10.24	0.23	6.74	0.31
Willamette River	7.98–10.26	9.13	0.16	6.01	0.22
Snake River	11.84–14.93	13.28	0.18	8.74	0.24
Coastal vs.					
Willamette River	9.11–11.32	10.11	0.16	6.65	0.22
Snake River	12.87–16.38	14.61	0.24	9.61	0.32
Willamette River vs.					
Snake River	7.25–9.89	8.60	0.18	5.66	0.23

Sequence divergence estimates are based on a molecular clock constrained maximum-likelihood analysis (see text). Divergence times are estimated based on a molecular clock calibration for the cyt *b* gene in cyprinid fishes of 0.76%/per MY (Zardoya and Doadrio, 1999).

and Taylor, 1999). For the same gene region, European *Barbus* species vicariantly separated by the flooding of the Strait of Gibraltar differ by an average of 7.97% (Zardoya and Doadrio, 1999) and species in the North American cyprinid genus *Lythrurus*, differ by an average of 10.3% (Schmidt et al., 1998). In these cases, major speciation events likely occurred during the Miocene. The average sequence divergence among basins in this study ranged from 5.92% to 14.61%. If we assume a molecular clock calibrated for the cyt *b* gene in cyprinid fishes, these levels of sequence divergence correspond to divergence dates ranging from 3.89 to 9.61 MYA

Table 3
Percent sequence divergence between haplotypes within major basins

Basin	Range	Mean	SE
Klamath River	0.160–10.817	3.664	0.191
Columbia River	0.161–1.990	1.167	0.345
Coastal	0.162–2.607	1.654	0.413
Willamette River	0.160–0.501	0.329	0.068
Snake River	0.160–1.018	0.735	0.116

Divergence estimates are based on a molecular clock constrained maximum-likelihood analysis (see text).

(Table 2). These dates are likely overestimates of the actual divergence time because they reflect the age to the most recent common ancestor (MRCA) and population isolation may well have occurred subsequent to the origin of haplotype clades. Additionally, population structure may contribute to the maintenance of divergent lineages and increase the coalescence time (Hoelzer et al., 1998). Even if our estimates are somewhat inflated it seems clear that the biogeographic patterning of *R. osculus* populations distributed among major river basins in this region reflects vicariant and dispersal events that occurred during the Pliocene and Miocene. The divergence levels between drainages in this system contrast to the signature of Pleistocene events observed in other taxa from this region. For example, in salmonids such as bull trout (*Salvelinus confluentus*), the most divergent mtDNA lineages differ by 0.8% (ND1 sequence) (Taylor et al., 1999). In a more closely related cyprinid taxon, the longnose sucker (*Catostomus catostomus*), McPhail and Taylor (1999) found similarly low levels of divergence between haplotypes (Cyt *b* and ND4 sequence). Both of these studies revealed within-species levels of mtDNA divergence that likely reflect dispersal from Pleistocene refugia.

The historical relationships among major basins in Oregon are far from clear. In most cases, physiographic evidence of interbasin connections is lacking (Minckley et al., 1986) and associations based on faunal assemblages are often biased by the occurrence of a few extinction-resistant species (Smith, 1978). Unfortunately, the results of this study do little to elucidate these interbasin relationships. There is lack of resolution among the basal branches of phylogenies constructed with both MP and ML methodologies. One possibility is that these data do not provide sufficient informative characters to resolve these relationships. In this case, additional DNA sequence data might be useful (de Queiroz et al., 2002). Alternatively, the lack of resolution in the deeper branches of the phylogeny might reflect a rapid radiation resulting in short internodes.

The reconstructed phylogenies relating major basins contained some well-supported structural features. The Columbia and coastal drainage systems occupy basal positions relative to the other basins. Coastal valleys were eroded during Pliocene and Pleistocene uplift of the Coast Range and these drainages have thus been isolated from the Columbia and other systems at least since the Early Pliocene (Baldwin, 1959; Minckley et al., 1986). The Snake River and the Willamette River basins form a moderately supported clade in the both the ML and MP analyses suggesting a more recent association between these basins. Populations within these two basins are more closely related to the major clade of Klamath Basin haplotypes (Klamath A, Figs. 2 and 3) than to either the Columbia or coastal drainages. This finding is also supported by our UPGMA analysis of population relationships (Fig. 4). These results indicate that there may have been a connection between the Klamath Basin and the Willamette Basin. Furthermore, the presence of two highly divergent clades in the Klamath Basin may be the signature of multiple colonization events with the more recent being derived from the Willamette or Snake basins.

It has been suggested that the Umpqua River was isolated from the Willamette River by stream capture some time during the Pleistocene isolating five species of cyprinids from the Columbia Basin (Baldwin, 1959). However, the levels of sequence divergence in *R. osculus* among these major basins are more consistent with a Pliocene or Miocene sundering of gene flow between major basins. Miocene isolation of these river systems is substantially earlier than has previously been suggested. The pronounced genetic divergence has been accompanied by morphological divergence among basins. For example, distinct patterns of morphological divergence have been noted in *R. osculus* and congeneric co-occurring species. Populations of *R. osculus* in coastal streams have diverged from Willamette stocks (Zirges, 1973) and *R. cataractae* exhibits distinct morphological differences between Columbia Basin and coastal populations (Bisson and Reimers, 1977).

The cladogenic events separating sub-basins within the Columbia and coastal drainages are more recent. Populations within these basins likely diverged between 0.77 and 1.1 MYA (Table 3). The observed divergence among these populations is consistent with Pleistocene events separating these more closely related groups. Even though there has been a shorter isolation time there has been ample opportunity for morphological divergence among some of these populations. In a closely related taxon (*R. cataractae*) there is substantial morphological divergence among coastal populations (Bisson and Reimers, 1977). The monophyletic grouping of the costal drainages and the shallow divergence among haplotypes suggests a recent common origin.

4.2. Genetic diversity in major basins

High levels of genetic diversity characterized populations of speckled dace in Oregon. The levels of

nucleotide diversity (π) observed within major basins ranged from 0.0066 in the Snake River Basin to 0.0227 in the Klamath River Basin. Given the limited sampling in some basins the observation of high genetic diversity is suggestive of large population sizes. In particular, the Klamath Basin may have had a historically large genetic effective-population size. Because the coalescence time to the MRCA for mtDNA sequences is on the order of $2N_f$ (Tajima, 1990), where N_f is the effective number of females, the divergence times between haplotypes within basins can be used as a rough correlate of effective-population size. Using the divergence dates within basins as a guideline, the Klamath Basin has an almost threefold higher historic N_f than any other basin (Table 3). Among major basins there is a positive correlation between sample size and nucleotide diversity ($r = 0.97$, $p < 0.0001$), which suggests that larger sample sizes within basins and the inclusion of multiple subdrainages would likely reveal even greater diversity.

4.3. Genetic structure in the Klamath Basin

The Klamath River consists of two distinct faunal segments. The fauna of the lower river shows affinities to the Rogue River and other coastal streams (Minckley et al., 1986). The upper river supports a number of unique fish species that differ markedly from those found in other drainages. For example, there are three recognized lamprey species endemic to this basin (Lorion et al., 2000). The zoogeographic associations of the aquatic vertebrate fauna in the Klamath Basin may be the result of a complicated historical pattern of drainage connections. It has been suggested that there are affinities between Columbia and Pit River forms of *Catostomus* and those found in the Klamath (Snyder, 1908). Smith (1978) has pointed out faunal associations between the Snake River and the Klamath Basin, and Kimmel (1975) suggested a possible Miocene connection between these systems. Hubbs and Miller (1948) proposed that stream capture from the Sycan Marsh area could explain the presence of *Gila bicolor* in the Silver Lake drainage. It has also been suggested that the basin of Upper Klamath Lake was once isolated without the current outlet draining South through the Klamath River (Hubbs and Miller, 1948).

Lorion et al. (2000) speculated that the current distribution of the lamprey *Lampetra minima* is consistent with an historical connection of the upper Sycan River and the Williamson River. The close relationship between dace sampled in these two drainages provides some further support for this conjecture. These authors also observed high haplotypic diversity in Klamath populations of *Lampetra*. The repeated observation of high genetic diversity across taxa suggests that a complicated pattern of historical isolation and reconnection, not simply large effective genetic population size, has contributed to the maintenance of genetic variation in this basin. Other species with similar distributions in the Klamath Basin include bull trout (*Salvelinus confluentus*) and tui chub (*Gila bicolor*). It would be informative to examine the population-genetic structure in these other species to determine whether a pattern of high haplotypic diversity is a general feature of this basin.

We found evidence of strong genetic subdivision among subdrainages within the Klamath Basin. The observed N_{st} (Lynch and Crease, 1990) in the Klamath Basin was 0.226 (0.003). The phylogeographic structure of subdrainages did not show a striking pattern. There was some geographic structure detected as shown by a grouping of individuals in the Sycan and Williamson rivers, consistent with an historical connection between these drainages (Fig. 3).

The highest nucleotide diversities observed in any of the Klamath Basin subdrainages were found in Jenny and Spencer creeks. Of all our sampling localities, these two occupy the lowest positions in the basin. One possibility is that the high nucleotide diversity is the result of directional gene flow from populations higher in the basin to populations lower down. However, a barrier falls near the confluence of Jenny Creek and the mainstream Klamath makes recent gene flow an unlikely explanation for the high diversity in this subdrainage. High allelic diversity has also been found in populations of Redband Trout in this subdrainage (S. Reid, personal communication). Taken together these observations may indicate that the history of the Jenny Creek involves a complex pattern of headwater captures.

The co-occurrence of two highly divergent clades of haplotypes in the Klamath Basin raises the possibility that these two lineages are actually reproductively isolated taxa. Moyle (2002) suggested the possibility of two co-occurring forms in the Klamath Basin. There has been time for reproductive isolation to develop since the level of sequence divergence between these two clades is consistent with species level differences in other cyprinids. However, Briolay et al. (1998) noted the occurrence of hybrids between cyprinid lineages whose most recent common ancestor is as old as 10–15 MY. They suggested that these intergeneric hybrids are the consequence of a high level of genetic compatibility in cyprinid fishes. Many instances of hybrid swarms have been documented in a diverse array of cyprinid fishes (Minckley et al., 1986). Interspecific hybridization has even lead to the formation of new species in the closely related genus *Gila* (DeMarais et al., 1992). In light of these observations we cannot rule out that the divergent lineages in the Klamath Basin may in fact represent freely interbreeding members of a single population. If these divergent haplotypes are derived from a reproductively cohesive population, there are a number of competing hypotheses regarding the origin and maintenance of the observed genetic diversity to be evaluated.

These alternative hypotheses include: (1) Historically large genetic effective-population size and the resulting incomplete lineage sorting (Tajima, 1990); (2) A complicated history of population isolation and reconnection within the Klamath Basin resulting in the retention of ancestral polymorphisms (Hoelzer et al., 1998); and (3) Multiple colonization events. The basal position of one haplotype clade and the derived position of the other are consistent with multiple colonization events. However, the concordant observations of high genetic diversity in other Klamath Basin taxa (Lorion et al., 2000) indicate that large population size and a pattern of isolation and reconnection may also have contributed to the current pattern.

It is possible that inclusion of two reproductively isolated lineages could have an effect on our estimate of genetic subdivision in this basin. However, N_{st} actually increases to 0.418 (0.066) when the basal haplotypes are removed from the analysis. The levels of genetic subdivision in the Klamath Basin may, in fact, be substantially higher if the basal haplotypes represent a cryptic and reproductively isolated lineage. In this case, there would be high levels of structure in the basin as a whole, indicating restricted levels of historic gene flow.

4.4. Taxonomic and conservation implications

Given the high levels of subdivision and isolation observed among basins, how should these populations be viewed from a taxonomic and conservation perspective? Clearly, the levels of sequence divergence among basins are consistent with species-level differences found in other cyprinids. Should populations occupying major river basins be considered as separate species? While there is ample evidence of not only genetic, but also morphological, divergence among basins, two factors argue against considering separate basin populations as distinct species. First, this study was based solely on nucleotide variation in a single mtDNA gene. This information provides no direct way to infer the levels of historic gene flow or assess whether these populations are reproductively isolated. The application of techniques that directly examine genetic variation at nuclear loci would be particularly helpful to resolve these issues. However, it may not be possible to further resolve the issue of reproductive incompatibility when populations in different basins are allopatric. Second, the sparse sampling scheme in this study gives us limited ability to detect intermediate haplotypes in geographically proximate basins. Our study transects the range of at least two described subspecies of *R. osculus* (*R. o. klamathensis* and *R. o. carringtoni*). However, the extent of the geographic range of these two subspecies and their evolutionary relationship to other described forms is unknown. What is well understood is the critical need to gather such information. Currently there are at least 8 subspecies of *R. osculus* listed as threatened or species of special concern and at least three local endemics have gone extinct in the past 50 years (Williams et al., 1989).

While it would be premature to recognize the populations in these drainages as distinct species, there is ample evidence to regard them as distinct ESUs (Moritz, 1994; Waples, 1991). Two criteria suggested by Moritz (1994) are: (1) populations have diverged sufficiently to achieve reciprocal monophyly for mtDNA and (2) there is divergence of nuclear loci. Additionally, Waples (1991) specifically pointed out that divergence in adaptive characters such as morphology should be considered. In *R. osculus* we have evidence for reciprocal monophyly of mtDNA genes and morphological divergence among basins.

The Klamath Basin should be viewed as a unique reservoir of genetic diversity. Subdrainages within this basin contain the highest levels of nucleotide and haplotype diversity of any examined. This observation is consistent with the presence of many endemic species from other taxonomic groups such as the Miller Lake Lamprey and Klamath Suckers. Additionally, there is the possibility of the existence of two reproductively isolated sympatric lineages of dace in this basin. Clearly, there is a need for further genetic and morphological investigation in this basin. The application of nuclear markers would be particularly revealing regarding the status of these two divergent lineages.

5. Concluding remarks

The results of our study revealed a surprising degree of isolation and genetic divergence among major river basins as well as a high level of genetic variation within basins. This study was somewhat limited in the extent of the current geographical range of *R. osculus* we examined and limited by small within-basin sampling in most basins. The discovery of two highly divergent haplotype clades in the Klamath system was the result of much more extensive sampling within this basin. Equally intense sampling of other drainages is warranted and would surely reveal more genetic diversity. Certainly, a more encompassing study across the entire range of *R. osculus* would reveal more biodiversity. Definitive taxonomic and biogeographic assessment of *R. osculus* will require this broader geographic perspective. A clear result of our study is that the divergence among major river basins is far older than can be explained by hydrofluctuations during Pleistocene cycles of glaciation. This assertion is consistent with the viewpoint of Minckley et al. (1986) who reviewed both faunal distributions and fossil evidence for the zoogeographical associations of this region. It seems likely that vicariance and dispersal events during the Miocene and Pliocene contributed to the genetic isolation underlying the cur-

rent phylogeographic patterns. Closer examination of other widespread cyprinid taxa would likely reveal much higher taxonomic diversity than has been recognized by current taxonomy.

Further investigation into phylogeographic patterns of *R. osculus*, a widespread and highly subdivided species, will form an important contribution to understanding the historical biogeography of western North America. Since the distribution of primary freshwater fishes is directly connected to paleobiogeography of the region, other freshwater animals should exhibit similar patterns.

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