FISHES OF THE MIO-PLIOCENE WESTERN SNAKE RIVER PLAIN AND VICINITY

II. EVOLUTION OF THE *RHINICHTHYS OSCULUS* COMPLEX (TELEOSTEI: CYPRINIDAE) IN WESTERN NORTH AMERICA

by

GERALD R. SMITH, JEFFREY CHOW, PETER J. UNMACK, DOUGLAS F. MARKLE, AND THOMAS E. DOWLING

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RECENT *MISCELLANEOUS PUBLICATIONS*

Smith, G. R., J. Chow, P. J. Unmack, D. F. Markle, and T. E. Dowling, 2017. Evolution of the *Rhinichthys Osculus* Complex (Teleostei: Cyprinidae) in Western North America. pp. 45-84, 17 figs., 5 tables, 5 maps and supplementary material.. *In*: Fishes of the Mio-Pliocene Western Snake River Plain and Vicinity. *Misc. Publ. Mus. Zool., Univ. Michigan*, No. 204 no.2.

Ruedas, L. A., S. M. Silva, J. H. French, R. N. Platt II, J. Salazar–Bravo, J. M. Mora, and C. W. Thompson. 2017. A Prolegomenon to the Systematics of South American Cottontail Rabbits (Mammalia, Lagomorpha, Leporidae: Sylvilagus): Designation of a Neotype for S. brasiliensis (Linnaeus, 1758), and Restoration of S. andinus (Thomas, 1897) and S. tapetillus Thomas, 1913. Misc. Publ. Mus. Zool., Univ. Michigan, No. 205. pp. i-iv, 1-67, 33 figs., 5 tables, 2 appendices, and supplementary material.

Stearley, R. F. and G. R. Smith, 2016. Salmonid fishes from Mio-Pliocene lake sediments in the Western Snake River Plain and the Great Basin. pp. 1-43, 17 figs., 4 tables, 3 maps. *In*: Fishes of the Mio-Pliocene Western Snake River Plain and Vicinity. *Misc. Publ. Mus. Zool., Univ. Michigan*, No. 204 no.1.

Cohn, T. J., D. R. Swanson, P. Fontana. 2013. Dichopetala and New Related North American Genera: A Study in Genitalic Similarity in sympatry and Genitalic Differences in Allopatry (Tettigoniidae: Phaneropterinae: Odonturini). *Misc. Publ. Mus. Zool., Univ. Michigan*, No. 203, pp. *i-vi*, 1-175, 11 maps, 5 tables, 5 appendices.

RECENT *OCCASIONAL PAPERS*

Kraus, Fred. 2015. A new species of the miniaturized frog genus *Paedophryne* (Anura: Microhylidae) from Papua New Guinea. *Occ. Pap. Mus. Zool., Univ. Michigan,* No. 745, pp. 1-11, 2 figs., 1 table, 1 map.

Wilkinson, M., A. O'Connor, R.A. Nussbaum. 2013. Taxonomic status of the neotropical Caeciliam genera *Brasilotyphlus* Taylor, 1968, *Microcaeilia* Taylor, 1968 and *Parvicaecilia* Taylor, 1968 (Amphibia: Gymnophiona: Siphonopidae) *Occ. Pap. Mus. Zool., Univ. Michigan,* No. 744, pp. 1-10, 2 figs., 1 table.

Smith, G.R., J.D. Stewart & N.E. Carpenter. 2013. Fossil and Recent mountain suckers, *Pantosteus*, and significance of introgression in catostomin fishes of the western United States. *Occ. Pap. Mus. Zool., Univ. Michigan,* No. 743, pp. 1-59, 12 figs., 2 appendices, supplementary material.

Lindsay, A.R. & S.C.G. Haas. 2013. DNA from feces and museum specimens confirms a first state record bird. *Occ. Pap. Mus. Zool., Univ. Michigan,* No. 742, pp. 1-10, 4 figs., 1 table.

Oldfield, R.G. 2009. Captive breeding observations support the validity of a recently described cichlid species in Lake Apoyo, Nicaragua. *Occ. Pap. Mus. Zool., Univ. Michigan,* No. 741, pp. 1-14, 6 figs., 3 tables.

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COVER PHOTOGRAPH— *Rhinichthys osculus nubilus* from the Coquille River, Oregon, male, 46 mm standard length, photograph by Douglas Markle.

MISCELLANEOUS PUBLICATIONS
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By

Gerald R. Smith[1], Jeffrey Chow[2,5], Peter J. Unmack[3], Douglas F. Markle[4], and Thomas E. Dowling[2,6]

ABSTRACT

We studied *Rhinicthys osculus* and its close relatives to discover evolutionary processes that operated to produce this widespread, polytypic fish group in an intermountain landscape of hundreds of small, isolated drainages. This group has attracted study because of its many ambiguously distinctive populations, in which homoplastic traits are shared across local geographic barriers. The observed morphological ambiguity is clarified by phylogenetic analyses of mtDNA data from 73 locations, which show deep divergences separating several dozen hypothetically monophyletic groups. Calibration of genetic distances with fossil age estimates permits identification of three groups that originated in the late Miocene —centered in the Columbia-Snake, Colorado, and Lahontan drainage basins. These mtDNA groups contain Late Pliocene lineages within the Columbia and Snake rivers, upper Green River, Lower Colorado River, Humboldt River, Death Valley, and ancient connectives. Each of these clades accumulated local, differentiated populations by subdivision in Pleistocene internal drainages, but each also shows many populations transferred to neighboring basins by stream diversions, headwater captures, and pluvial lake overflows, which are consistent with geological evidence. Population groups defined by molecular analysis correspond only approximately to named or morphologically recognized taxa. Morphological analysis of 88 samples found no unique phenotypes. Three potential explanations (independently or in concert) may explain the evolution of the *Rhinichthys osculus* complex in the west: (1) Scores of drainage changes have alternated aquatic dispersal routes with long periods of isolation resulting in vicariant divergence. Drainage and fish transfers following long periods of isolation include, for example, the connection of the Upper to Lower Colorado River Basins (~5 Ma), the connection of the Snake River to the Columbia River (~3 Ma), many lake overflows among Pleistocene Lahontan basins documented by Marith Reheis and her colleagues, and the Lake Bonneville spillover to the Snake River and the Pacific (~15 Ka). Prior to such large connection events, basins and drainages were arranged differently and sometimes isolated for periods of 10^4 - 10^6 years or more, permitting accumulation of differences. (2) Alternating pluvial and arid stages associated with more than 20 glacial cycles and over 100 subcycles over the past 3 million years subjected populations to periodically intense selection for expansion in well-watered habitats, alternating with isolation in small populations in desert habitats. (3) Pluvial overflows created secondary contact and frequent genetic mixing, leaving only one form in the basin or stream; only two instances of sympatric species persist, both in large waters of the climatically more stable Columbia and Snake River drainages. Some traits that distinguish large-river species in the north also exist in southern desert populations, but with inconsistently mixed or convergent characteristics.

KEYWORDS: Speckled Dace, cyt*b*, ND4L, morphology, speciation, biogeography, fossils, molecular clock, introgression, stream capture, lake overflow, vicariance, and dispersal.

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INTRODUCTION

The effects of timing of barriers on speciation are still incompletely understood. Geologic complexity in western United States make it well-suited to study the effects of isolation, vicariance, and dispersal on species evolution (Minckley et al.., 1986; Hershler and Liu, 2008; Riddle et al., 2014), and the *Rhinichthys osculus* (speckled dace) species group is an appropriate organism for such a study (Hubbs and Miller, 1948). This polytypic minnow (Cyprinidae) occurs in streams of eight major drainages and many small rivers of western North America (Fig. 1) from southwestern Canada to northern Mexico (Hubbs and Miller, 1948; LaRivers, 1962; Hubbs et al., 1974; Williams, 1978; Wydoski and Whitney, 2003; Wallace and Zaroban, 2013). It attained its widespread distribution by dispersal through aquatic connections including stream captures (Miller, 1958). Here, we estimate the ages of barriers and aquatic connections using fossil-calibrated mitochondrial DNA (mtDNA) phylogenies to establish a temporal and geological framework for studying genetic, morphological, and ecological divergence. *Rhinichthys* exhibits extensive morphological diversity, presumably reflecting adaptation to a broad array of habitats, from small rivulets a few centimeters deep, to warm or cold desert springs, to the largest western rivers. These features, as well as its small but important fossil record, make *Rhinichthys osculus* a useful group for study of evolutionary responses to timing of local environmental changes, drainage histories, and vicariance events.

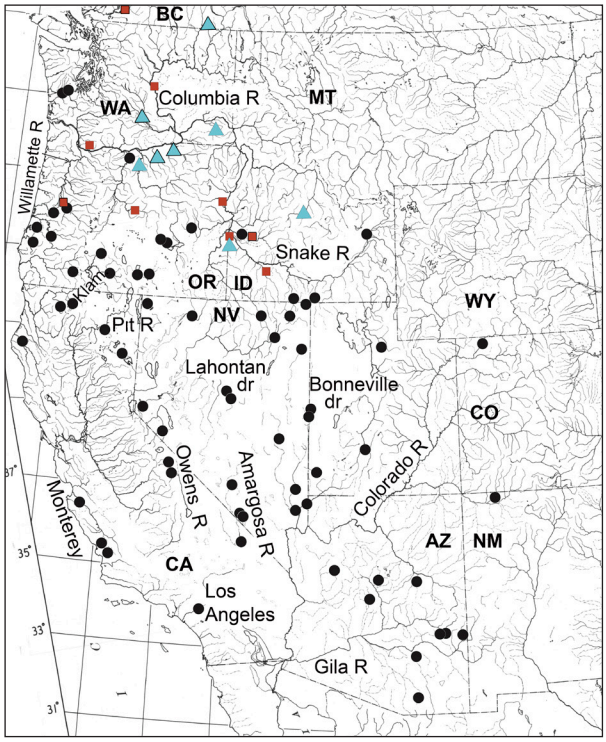


Figure 1.— Geographical context and sample localities for the *Rhinichthys osculus* group. Black circles represent *R. osculus*, blue triangles represent *R. umatilla*, red squares represent *R. falcatus*.

Rhinichthys in the Snake and Columbia river basins exists as four nominal species, the three species of the *R. osculus* complex (*R. falcatus*, *R. osculus*, and *R. umatilla*) and *R. cataractae*. *Rhinichthys cataractae* and *R. falcatus* are swift-water morphotypes, adapted by their fusiform bodies and large falcate fins to high-discharge fluvial waters with swift currents, high turbulence, and strong shear stress. Most *R. osculus* are generalized slow-riffle inhabitants, in habitats widespread throughout the west; *R. umatilla* is intermediate between extreme swift-water and generalist morphotypes. *Rhinichthys cataractae* is distributed widely across northern North America; *R. falcatus* and *R. umatilla* are restricted to the Snake and Columbia basins and some peripheral former connectives-their distribution suggests that separate Late Miocene-Pliocene histories of the Snake and Columbia basins might have contributed to the early evolution of *R. osculus* and the divergence of *R. falcatus* and *R. umatilla.*

East-west crustal extension in intermountain Western North America created over 170 linear, north-south basins and ranges over the past ~16 million years (Dickinson, 2002; Colgan et al., 2006). Mountain streams draining from these high mountain ranges into long, narrow basins in the Basin and Range Province provided habitats for diversification of numerous named subspecies of *Rhinichthys* (Hubbs and Miller, 1948; Hubbs et al., 1974; Williams, 1978; Miller, 1984). Whether reproductive isolation has occurred in addition to differentiation in currently isolated populations is a continuing question.

At least 16 million years of extension changed the courses and connections among many streams. In addition, periodic wet, pluvial climates formed composite rivers from drainages that are separated in arid times such as the present (Reheis et al., 2002; Reheis et al., 2008; Smith et al., 2002; Spencer et al., 2008). As a result, evolution in allopatry has been cyclically interrupted by gene flow among many Great Basin habitats, leaving one mixed species; but more permanent Columbia and Snake river habitats enabled evolution of sympatric, co-existing, different, and diagnosable morphologies. Millennial-scale changes in temperature, moisture, habitat size, and drainage isolation are also assumed to have influenced local divergence, especially of small populations (Hubbs and Miller, 1948). High barriers and changing climates in western North America (Chamberlain et al., 2012) also

created many diverse and isolated habitats that preserved relict lineages in a distinctive fish fauna (Miller, 1958). Ultimately, however, only about a tenth of the rapidly diverging lineages survive (Smith et al., 2010). The relict taxa are scattered in widespread drainages, including only a few localities where sympatric congeners co-occur, in contrast to species-rich aquatic habitats in the eastern U.S. (Smith et al., 2010).

Many small, isolated populations of *Rhinichthys* in the Great Basin were recognized as subspecies by Hubbs and Miller (1948), and other authors have speculated that the morphological diversity represents multiple species. Molecular trees support some differentiation hypotheses; they document many deep divisions among more than a dozen monophyletic groups (Oakey et al., 2004; Pfrender et al., 2004; Smith and Dowling, 2008; Ardren et al., 2010; Billman et al., 2010; Hoekzema and Sidlauskas, 2014; see below); however, these distinct lineages have been difficult to diagnose morphologically, except in strictly local contexts. This paper attempts to study geologic-biological interactions that caused predominance of so many single species communities (Smith, 1978), ambiguity of the molecular vs. morphological diversity, and the species level interactions of *R. falcatus* and *umatilla* in the Columbia basin while similar morphotypes in the Colorado drainage (Smith and Dowling, 2008) are isolated polymorphisms.

Observed morphological diversity in the *Rhinichthys osculus* complex (Fig. 2) is currently recognized as the species outlined above and the extinct *R. deaconi* Miller 1984, which lived in pre-metropolized Las Vegas. Differentiated populations of *R. osculus* include 14 named subspecies (most were originally in the genus *Apocope,* Hubbs and Miller, 1948; Hubbs et al., 1974): *R. o. nubila* (Girard, 1858) of coastal Oregon, Columbia, and Puget Sound drainages; *R. o. carringtoni* (Cope, 1872) of the Snake River, Northern Bonneville drainage, Harney-Malheur Basin, and northern California; *R. o. adobe* (Jordan and Evermann, in Jordan, 1891) of the Sevier River in the southern Bonneville Basin; *R. o. klamathensis* (Evermann and Meek, 1898) of the coastal Klamath and Klamath Basin (see also Pfrender et al., 2004); *R. o. robustus* (Rutter, 1903) of the Lahontan system and Sacramento drainage; *R. o. osculus* (Girard, 1856) in the Gila River basin (Minckley, 1973); *R. o. velifer* (Gilbert, 1893) of the White River system and Meadow Valley Wash, Nevada; *R. o. yarrowi* (Jordan and Evermann, in Jordan, 1891) of the Green, upper Colorado, and Virgin rivers; and *R. o. nevadensis* (Gilbert, 1893) of Ash Meadows and the Amargosa drainage, Nye Co., Nevada. Many isolated springs and intermountain valleys also contain unique forms, for example, *R. o. thermalis* Hubbs and Kuhne 1937, from Kendall Warm Springs, Sublette Co., Wyoming; *R. o. moapae* Williams 1978 of the Muddy River, Clark County, Nevada; *R. o. oligoporus* Hubbs et al., 1974 of Clover Valley, Elko Co., Nevada; *R. o. lethoporus* Hubbs et al., 1974 of Independence Valley, Elko County, Nevada; *R. o. reliquus* Hubbs et al., 1974, formerly of Grass Valley, Lander Co., Nevada; and *R. o. lariversi* Lugaski 1972 of Big Smoky Valley, Lander and Nye counties, Nevada. Many other geographically restricted and morphologically distinct speckled dace populations in the Great Basin were identified but not formally named (Hubbs and Miller, 1948; LaRivers, 1962; Hubbs et al., 1974; Miller, 1984).

Studies of geographic variation in mtDNA provided a deep perspective of phylogeography of the *R. osculus* group (Oakey et al., 2004; Pfrender et al., 2004; Smith and Dowling, 2008; Ardren et al., 2010; Billman et al., 2010; Hoekzma and Sidlauskas, 2014). Pfrender et al., (2004) reported three distinct mtDNA lineages in the Klamath drainage. Three haplotypes with different relationships are found in the Malheur/Stinking Lake/Oregon Lakes area (Ardren et al., 2010; Hoekzema and Sidlauskas, 2014). Smith and Dowling (2008) and Billman et al., (2010) found several locally distinctive lineages of mtDNA sequence variation in the Colorado River drainage, Great Basin, and Los Angeles Basin.

Here we compare morphological patterns of variation with mtDNA sequences and relate them to paleohydrographic, fossil, and molecular clock information to examine effects of timing of gene flow on evolution among populations and its influence of levels of morphological variation within this complex group of fishes. This approach provides data for examining roles of long-term fluctuating isolation, varying hydrological regimes, and climate changes that alternatively generated and suppressed diversity among populations within the *R. osculus* complex. Our hypothesis is that metapopulations (de Queiroz, 2007) within *Rhinichthys* have not diverged to species levels, because they have been interrupted by reticulate evolution (McKay and Zink, 2014).

MATERIALS AND METHODS

Samples.— Locality information for specimens used in molecular and morphological analyses are provided in Tables 1 and 2, respectively. Molecular samples of *R. osculus*, *R. falcatus,* and *R. umatilla* were obtained from 73 locations, covering most of their range and representing most of the previously described forms (Table 1, Figs. 2, 3). All molecular samples (either whole specimens or fin clips) were preserved in 95% ethanol and stored at room temperature or were frozen on dry ice and maintained at –20 C until use. Eighty-eight

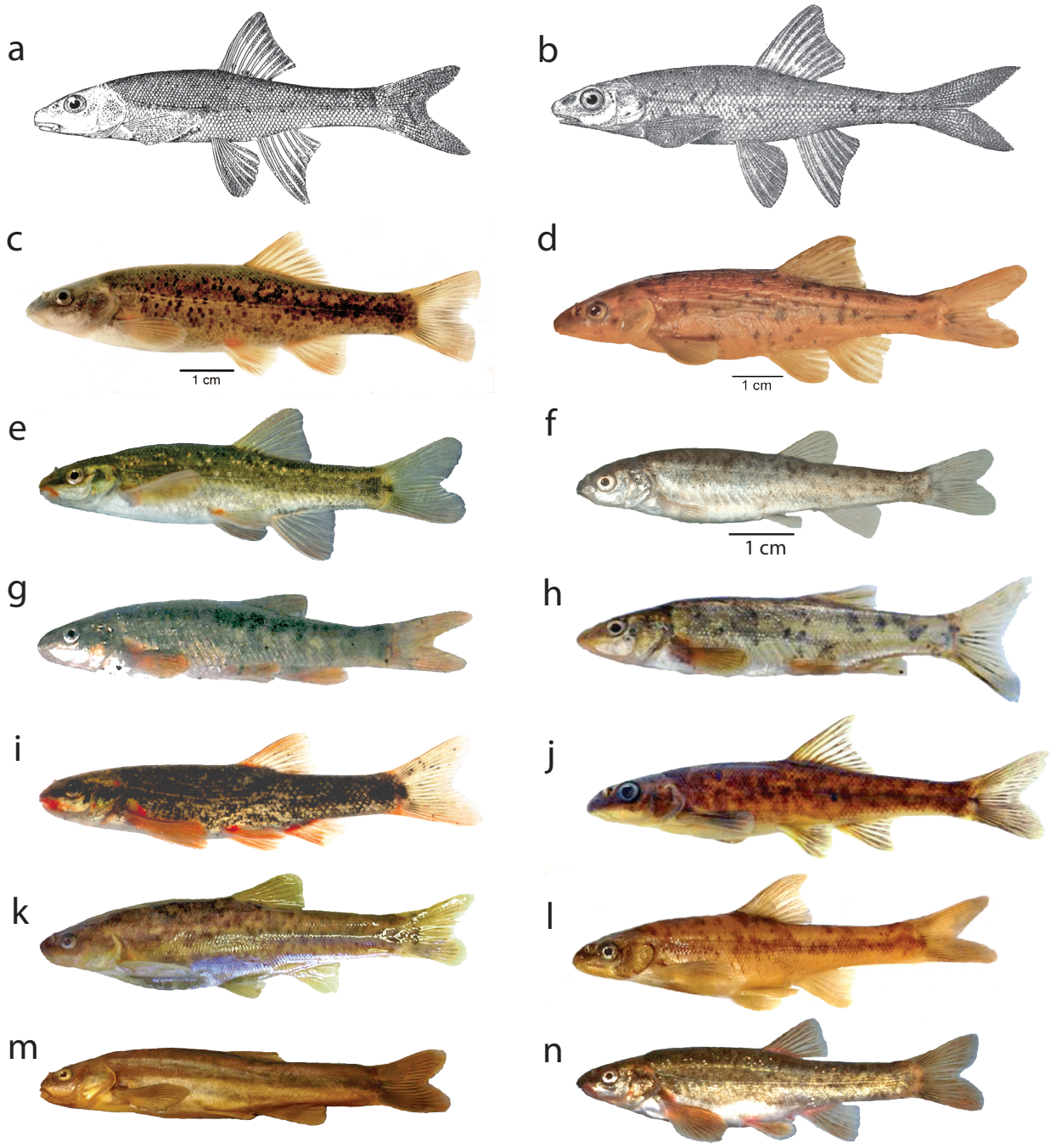


Figure 2.— Variety of morphotypes of western *Rhinichthys osculus* group: **a**, holotype illustration as published for *Rhinichthys umatilla* Gilbert and Evermann 1893, Oregon, Umatilla River near mouth; **b**, holotype of *Agosia falcata* Eigenmann 1894, Idaho, Boise River; **c**, *R. umatilla*, Umatilla R., OR; **d**, *R. falcatus*, Boise R., ID; **e**, *R. osculus*, Willamette R., OR; **f**, *R. osculus* sbsp., Harney Basin, OR; **g**, *R. osculus* (morphology) x *R. falcatus* (DNA), Boise R., ID; **h**, *R. falcatus* (morphology) x *R. osculus* (DNA), Boise R., ID; **i**, *R. osculus nubila*, OR; **j**, *R. falcatus,* Willamette R., OR; **k**, *R. osculus* sbsp., Los Angeles basin, CA; **l**, *R. osculus moapae*, Muddy R., NV; **m**, *R. osculus* sbsp, Thousand Springs, NV; **n**, *R. osculus robustus*, Barnes Valley Cr., Klamath drainage, OR.

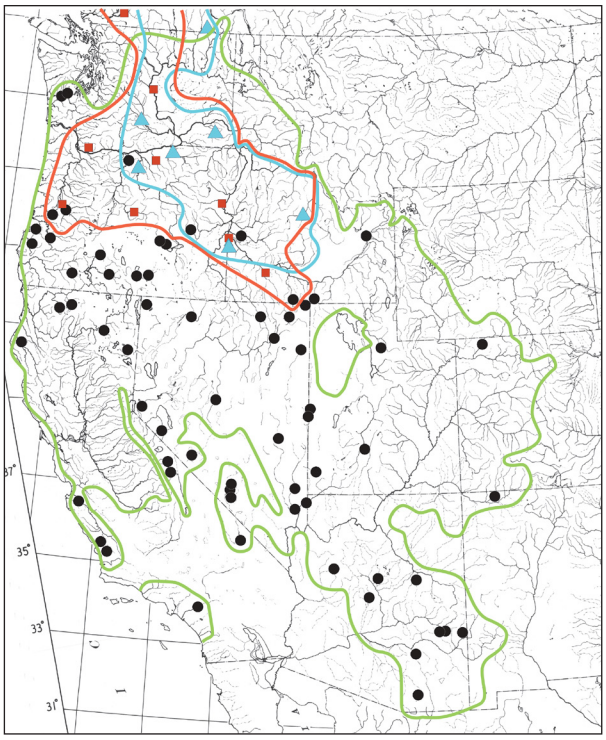


Figure 3.— Geographic distribution limits of *Rhinichthys osculus* (green lines), *R. falcatus* (red lines), and *R. umatilla* (blue lines), with molecular samples of *R. osculus* (black spots), *R. falcatus* (red squares), and *R. umatilla* (blue triangles); several morphology-only sites for *R. falcatus* and *R. umatilla* are also plotted. *Rhinichthys falcatus* and *R. umatilla* have population ranges extending north off the map into Canada (Scott and Crossman 1973, Peden and Hughes 1988).

locations (typically represented by three specimens) for morphological analysis were selected from museum collections at UMMZ, ASU, or OSU, to match as closely as practical, the localities of the molecular samples.

Molecular analyses.— DNA was obtained from muscle tissue or fin clips as described in Tibbets and Dowling (1996). For many populations, sample size was large and variation was prescreened by analysis of single-stranded conformational polymorphisms (SSCPs – Dowling et al., 1996; Sunnucks et al., 2000; Gerber et al., 2001) and direct sequencing of a 329 bp fragment from the middle of the cytochrome *b* (cyt*b*) gene (Chow unpublished data). Low levels of variation (1–2 base substitutions among individuals) were found within populations; therefore, representative samples (2–4 individuals) were used to characterize focal locations. *Rhinichthys cataractae* and *R. obtusus* were used as immediate out-groups to test for monophyly of the speckled dace complex. *Agosia chrysogaster*, *Campostoma anomalum*, *Dionda episcopa*, *Exoglossum laurae*, *Oregonichthys crameri*, *O. kalawatseti*, and *Tiaroga cobitis* were included in the first stage of analyses to provide broader taxonomic perspective.

Sequences of cytochrome *b* (cyt*b*) and NADH dehydrogenase subunit 4L (ND4L) were used to estimate phylogenetic relationships. PCR products for sequencing were typically obtained using conditions with the following amplification profile: 20-30 cycles of denaturation at 94 C for 1 min, annealing at 48 C for 1 min, and extension at 72 C for 2 min. Target sequences for the entire cyt*b* gene (1140 bp) were generated using the primers LA and HA as described by Dowling and Naylor (1997), and sequences of the ND4L gene (297 bp) generated using the primers argb-L and NAP (Bielawski and Gold, 1996) or ND4Lrhino-h (5'-GAGGCCAGTCAAATCGTTGG-3'). Sequences were obtained using Applied Biosystems automated sequencers, model 3130 at Arizona State University and model 3730 XL sequencer at the Brigham Young University DNA Sequencing Center. All sequences obtained in this study were deposited in GenBank, accession numbers, see Table 1.

Sequences were edited using Chromas Lite 2.0 (Technelysium, Tewantin, Queensland, Australia) and imported into BioEdit 7.0.5.2 (Hall, 1999). Sequences coding for amino acids were aligned by eye and checked via amino acid coding in MEGA 6.06 (Tamura et al., 2013) to test for unexpected frame shift errors or stop codons. Maximum likelihood (ML) analyses were performed using RAxML 7.2.8 (Stamatakis, 2006; Stamatakis et al., 2008) by bootstrapping with 1000 replicates with the final best ML tree being calculated using the GTRGAMMA model on the CIPRES cluster at the San Diego Supercomputer Center (Miller et al., 2010).

Morphological analyses.— Head, eye, and body measurements, meristic counts, fin shape and position, caudal peduncle length and depth, mouth, barbels, and color pattern, were selected to represent possible adaptive traits and recorded prior to clearing and staining (Appendix 1). Specimens were then cleared in trypsin, potassium hydroxide, and glycerol, and stained with Alizarin Red S for osteological study. Fifteen skeletal features were chosen, e.g., shapes of the anterior biting part of the lower jaw, condition of ventral pores of the dentary, shapes of the anterior, posterior rim, and curvature of the pharyngeal arch, shape and number of teeth, complexity of processes on the symplectic, robustness and shape of the cleithrum, dorsal extensions of the metapterygoid, and number of vertebrae, were chosen to be traits that might be adaptive, and also include characters diagnostic for identification of fossils (Appendix 1, Tables 2, 3).

Examination of morphological traits provided 31 formal characters for 88 samples of *Rhinichthys osculus,*

Table 1.— Localities, taxonomy, and sample size (N) for mtDNA samples. "NA" for ND4L were not sequenced.

Species	Drainage	Locality	N	cyt*b* acc #	ND4L acc #
Agosia chrysogaster	Gila R.	Blue R. at FSR 567, Greenlee Co., AZ	1	KY398931	KY398846
Dionda episcopa	Pecos R.	El Rito Cr., NM 91, Santa Rosa, Guadalupe Co., NM	1	KY398932	KY398847
Exoglossum laurae	Roanake R.	John's Cr., Craig Co., VA	1	KY398933	KY398848
Campostoma anomalum	Illinois R.	Mill Cr., LaPorte Co., IN	1	KY398934	KY398849
Oregonichthys crameri	Willamette R.	Lookout Point Res., Lane Co., OR	1	KY398935	KY398850
Oregonichthys kalawatseti	Umpqua R.	Calapooia R., Douglas Co., OR	1	KY398936	KY398851
Tiaroga cobitis	Gila R.	Aravaipa Cr. at Ranch House, Pinal Co., AZ	1	KY398937	KY398852
R. atratulus	Potapsco R.	So. Branch Potapsco R., Carroll/Howard Co., MD	1	KY398975	KY398890
R. cataractae	Pine R.	Bear Cr. at USFS 3113, 7.4 km (4.5 mi) SW Rudyard, Chippewa Co., MI	1	DQ990251	DQ990150
R. obtusus	Great Lakes	Rouge R., Oakland Co., MI	1	DQ990250	DQ990149
R. falcatus	Columbia R.	Willamette R. at Eugene, Willamette R. Dr., Lane Co., OR	2	KY398976, DQ990284	KY398891, DQ990183
R. falcatus	Fraser R.	Big Island Bar near Agassiz, BC	1	FJ769176	NA
R umatilla	Columbia R.	Umatilla R. at Umatilla, Umatilla Co., OR	2	KY399007, KY399008	KY398922, KY398923
R umatilla	Columbia R.	Willow Cr. at Ely Canyon Rd, Morrow Co., OR	1	KY399016, KY399017	NA
R. osculus	Amargosa R.	Oasis Valley, Nye Co., NV	2	DQ990274, DQ990275	DQ990173, DQ990174
R. osculus	Amargosa R.	Amargosa R., Tecopa, Nye Co., NV	2	DQ990270, KY398938	DQ990169, KY398853
R. osculus	Amargosa R.	Bradford Springs, Nye Co., NV	2	DQ990272, KY398939	DQ990171, KY398854
R. osculus	Amargosa R.	Jackrabbit Springs, Nye Co., NV	2	DQ990271, DQ990273	DQ990170, DQ990172
R. osculus	Bonneville	Salina Canyon Cr., near Salina, Sevier Co., UT	1	DQ990298	DQ990197
R. osculus	S. Bonneville	Park Canyon Cr., Washington Co., UT	2	DQ990312, DQ990297	DQ990211, DQ990196
R. osculus	N. Bonneville	East Canyon Cr., tributary to Weber R., Morgan Co., UT	2	DQ990283, KY399015	DQ990182, KY398930
R. osculus	N. Bonneville	Rock Springs, Elko Co., NV	2	DQ990278, DQ990281	DQ990177, DQ990180
R. osculus	Snake V.	Tributary to Fish Springs, Snake Valley, Gandy, Millard Co., UT	2	KY399001, DQ990252	KY398916, DQ990151
R. osculus	Snake V.	Lake Cr., White Pine Co., NV	2	DQ990253, DQ990300	DQ990152, DQ990199
R. osculus	Coastal CA	Alamo Cr., Santa Maria R. Dr, San Luis Obispo Co., CA	2	KY398996, KY398997	KY398911, KY398912
R. osculus	Coastal CA	Butte Cr. at Hwy 36 between Bridgeville & Dinsmore, Humboldt Co., CA	2	KY398951, KY398952	KY398866, KY398867
R. osculus	Coastal CA	San Luis Obispo Cr., San Luis Obispo Co., CA	2	KY398994, KY398995	KY398909, KY398910
R. osculus	Coastal CA	Carp Cr., Monterey Co., CA	2	KY398969, KY398970	KY398884, KY398885
R. osculus	Coastal OR	Coos R., Millicoma R., 4.0 mi upstream of Allegany, Coos Co., OR	2	KY398945, KY398946	KY398860, KY398861

Table 1.— Localities, taxonomy, and sample size (N) for mtDNA samples. "NA" for ND4L were not sequenced, cont.

Species	Drainage	Locality	N	cyt*b* acc #	ND4L acc #
R. osculus	Coastal OR	Dead Indian Cr. on Dead Indian Rd., Rogue R. drainage, Jackson Co., OR	2	KY398989, KY398990	KY398904, KY398905
R. osculus	Coastal OR	Siuslaw R. at Austa Boat Ramp, Lane Co., OR	2	KY398998, KY398999	KY398913, KY398914
R. osculus	Coastal OR	South Fk. Coquille R., Coos Co., OR	2	KY398947, KY398948	KY398862, KY398863
R. osculus	Coastal OR	Umpqua R. at mouth of N. Fk. Umpqua R, River Forks Park, Douglas Co., OR	2	KY399009, KY399010	KY398924, KY398925
R. osculus	Coastal WA	Chehalis R., Grays Harbor Co., WA	2	KY398977, KY398978	KY398892, KY398893
R. osculus	Coastal WA	Satsop R., Bridge on Hwy 12 east of Montesano, Grays Harbor Co., WA	2	KY398979, KY398980	KY398894, KY398895
R. osculus	Little Colorado	Chevelon Cr. at Mormon Crossing, Little Colorado R., Coconino Co., AZ	2	DQ990289, DQ990290	DQ990188, DQ990189
R. osculus	Colorado R.	Trout Cr., Bill Williams R. Drainage, Mohave Co., AZ	2	DQ990227, DQ990228	DQ990244, DQ990245
R. osculus	Colorado R.	Animas R. at Aztec at SR 516, San Juan Co., NM	1	DQ990221	DQ990238
R. osculus	Colorado R.	Little Snake R. at Rd 26, Moffat Co., CO	1	DQ990217	DQ990234
R. osculus	Columbia R.	Yakima R., Yakima Co., WA	2	KY398983, KY398984	KY398898, KY398899
R. osculus	Columbia R.	Deschutes R. at Deschutes R. State Rec Area, Sherman Co., OR	2	KY398949, KY398950	KY398864, KY398865
R. osculus	Columbia R.	Willamette R. at Eugene, Willamette R. drainage, Lane Co., OR	2	DQ990285, DQ990286	DQ990184, DQ990185
R. osculus	Columbia R.	Clear Cr., Stinkingwater Cr., Malheur R, Harney Co., OR	2	KY398967, KY398968	KY398882, KY398883
R. osculus	Columbia R.	Kettle R. at Midway, BC	1	FJ69177	NA
R. osculus	Gila R.	Aravaipa Cr., Pinal Co., AZ	1	DQ990231	DQ990248
R. osculus	Gila R.	Beaver Cr. on FR 26 ca 3 mi NW of US 191, Greenlee Co., AZ	1	DQ990216	DQ990233
R. osculus	Gila R.	Fossil Cr., Gila Co., AZ	2	DQ990226	DQ990243
R. osculus	Gila R.	Little Blue Cr., Gila R. drainage, Greenlee Co., AZ	1	DQ990287	DQ990186
R. osculus	Gila R.	Sonoita Cr. at Circle Z Ranch about 0.3 mi E. off AZ82, Santa Cruz Co., AZ	2	DQ990229	DQ990246
R. osculus	Gila R.	Stinky Cr., Apache-Sitgreaves Nat. Forest, Apache Co., AZ	1	KY398993	KY398908
R. osculus	Gila R.	Sycamore Cr. FR 677, Agua Fria drainage, Yavapai Co., AZ	2	DQ990223	DQ990240
R. osculus	Harney	Hibbard Springs, Harney Co., OR	2	KY398955, KY398954	KY398870, KY398869
R. osculus	Harney	Stinking Lake at the spring, Harney Co., OR	2	KY398956, KY398957	KY398871, KY398872
R. osculus	Klamath R.	Jack Cr., Klamath Co., OR	3	KY398960, KY398961	KY398875, KY398876
R. osculus	Klamath R.	Jenny Cr. at Hwy 66, Trib. to Klamath R., Jackson Co., OR	2	KY398962, KY398963	KY398877, KY398878
R. osculus	Klamath R.	S Fork Sprague R. at Sprague R. Park 2 mi. E. of Bly, Klamath Co., OR	2	KY398964, KY398965	KY398879, KY398880
R. osculus	Klamath R.	Scott R. at Jones Beach Campground below Fort Jones, Siskiyou Co., CA	2	KY398958, KY398959	KY398873, KY398874
R. osculus	Klamath R.	Yreka Cr. in Yreka, Trib. to Shasta R., Siskiyou Co., CA	2	KY398966	KY398881
R. osculus	LA Basin	East Fk. of San Gabriel R. at Los Angeles, Los Angeles Co., CA	2	DQ990291, DQ990293	DQ990190, DQ990192
R. osculus	Lahontan	Clover Valley, Elko Co., NV	2	DQ990257, DQ990258	DQ990156, DQ990157

Table 1.— Localities, taxonomy, and sample size (N) for mtDNA samples. "NA" for ND4L were not sequenced, cont.

Species	Drainage	Locality	N	cyt*b* acc #	ND4L acc #
R. osculus	Lahontan	Big Smoky Valley, Nye Co., NV	2	DQ990267, KY398940	DQ990166, KY398855
R. osculus	Lahontan	Crowley Cr., Quinn R. drainage, Humboldt Co., NV	2	DQ990264, KY398974	DQ990163, KY398889
R. osculus	Lahontan	Humboldt R. at Elko, Elko Co., NV	3	DQ990265, DQ990266, DQ990268	DQ990164, DQ990165, DQ990167
R. osculus	Lahontan	Monitor Valley, Nye Co., NV	2	DQ990261, DQ990259	DQ990160, DQ990158
R. osculus	Lahontan	Truckee Basin, Dry Ck. Diversion, Washoe Co., NV	2	KY399005, KY399006	KY398920, KY398921
R. osculus	Lahontan	Secret Cr., N. Trib. to Susan R., Lassen Co., CA	3	KY399002, KY399003, KY399004	KY398917, KY398918, KY398919
R. osculus	Lahontan	East Walker R., Lyon Co., NV	2	DQ990254, DQ990255, KY398953	DQ990153, DQ990154, KY398868
R. osculus	Oregon Lakes	Chewaucan R., Crooked Cr. at Chandler State Park, Lake Co., OR	2	KY398943, KY398944	KY398858, KY398859
R. osculus	Oregon Lakes	Wall Canyon, Washoe Co., NV	2	KY399011, KY399012	KY398926, KY398927
R. osculus	Oregon Lakes	Warner basin, 20 Mile Cr., Lake Co., OR	2	KY399013, KY399014	KY398928, KY398929
R. osculus	Owens R.	C2 drain, 9 mi WNW of Bishop, Inyo Co., CA	2	DQ990276, DQ990277	DQ990175, DQ990176
R. osculus	Owens R.	Whitmore Hot Spr. Marsh, 9 mi E. of Town of Mammoth Lakes, Inyo Co., CA	2	DQ990256, KY398971	DQ990155, KY398886
R. osculus	Sacramento R.	Canyon Cr., Trib. to Pit R. at Canyon Cr. Ranch, ca. 10 mi S.W. of Alturas, Modoc Co., CA	2	KY398972, KY398973	KY398887, KY398888
R. osculus	Snake R.	Boise R. near Middleton, Canyon Co., ID	2	KY398941, KY398942	KY398856, KY398857
R. osculus	Snake R.	Hendricks Cr., North Fk. of the Owyhee R., Elko Co., NV	2	DQ990262, DQ990263	DQ990161, DQ990162
R. osculus	Snake R.	Little Goose Cr., Goose Cr. drainage, Elko Co., NV	2	DQ990260, KY399000	DQ990159, KY398915
R. osculus	Snake R.	Salmon R., Challis boat ramp, Hwy 93 near Challis, Custer Co., ID	2	KY398981, KY398982	KY398896, KY398897
R. osculus	Snake R.	Snake R. at Pingree, Bingham Co., ID	2	KY398985, KY398986	KY398900, KY398901
R. osculus	Snake R.	Goose Cr., just N. of UT-ID border, Cassia Co., ID	2	KY398987, KY398988	KY398902, KY398903
R. osculus	Snake R.	Salmon Falls Cr., just N. of NV-ID border, Twin Falls Co., ID	2	KY398991, KY398992	KY398906, KY398907
R. osculus	Virgin R.	Muddy R., Moapa, Clark Co., NV	1	DQ990309	DQ990208
R. osculus	Virgin R.	Virgin R. at Mesquite, Clark Co., NV	2	DQ990316, DQ990295	DQ990215, DQ990194
R. osculus	Virgin R.	Meadow Valley Wash, Clark Co., NV	1	DQ990314	DQ990213
R. osculus	Virgin R.	Flag Springs, Pahranagat Valley, Lincoln Co., NV	1	DQ990305	DQ990204

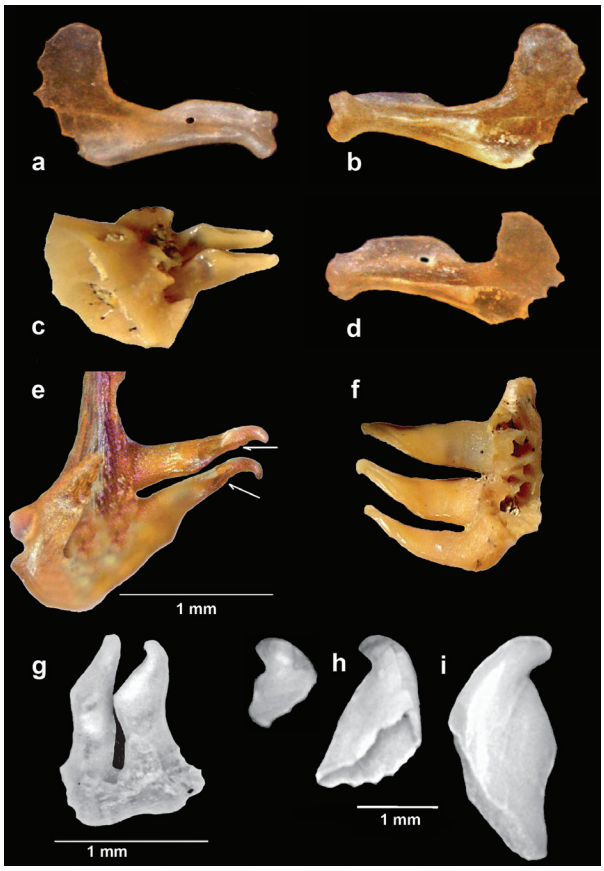


Figure 4.— Fossil *Rhinichthys* dentaries and pharyngeal teeth: **a**, lateral view of right dentary showing absence of sensory pores on ventral surface; **b**, mesial view of right dentary; **c**, left pharyngeal arch showing attachment sockets for minor teeth and major-row teeth 2 and 3 (tooth formula is 2,4-4,2); **d**, dorso-mesial view of right dentary showing diagnostic lateral flare of biting edge; **e**, left pharyngeal arch of recent *R. osculus* from the Klamath Basin showing cutting edges on posterior sides of teeth 2 and 3 (minor row of teeth absent) for comparison with f; **f**, pharyngeal arch showing cutting edge on posterior sides of teeth 2, 3, and 4; **g**, pharyngeal teeth of possible *R. osculus*; **h** and **i**, individual teeth showing diagnostic convex cutting edge below terminal hook. a-d, and f, from the Ellensburg Formation Washington (10.5 Ma, Smith *et al*., this volume); g, from Mono Lake beds, California; h, i, from White Narrows beds, Nevada (4.7 Ma, Smith *et al*., 2013).

R. falcatus, R. umatilla, and the out-groups *R. cataractae* and *R. obtusus*. The characters were ordinated by Principal Component Analysis of four matrices, (1) all samples, (2) Northwest clade, (3) Lahontan clade, and (4) Colorado Basin clade, to discover correlated groups of traits and relative distributions of cases based on their PC scores. Trait and case ordinations were repeated with different identifications of samples to compare whether traits or cases formed groupings correlated with molecular-based clusters, or habitats, or drainage geography.

The 31 morphological characters (Appendix 1, Table 2) were coded into two-to-five states each for parsimony analysis in PAUP* 4.0b10 (Swofford, 2002), with all but two characters (metapterygoid and minor tooth number) treated as ordered and rooted using *R. obtusus* from Michigan. Trees were recovered through heuristic search with 10,000 random addition sequence replicates. To assess the relative importance of habitat, geography, and past evolutionary history (as indicated by mtDNA), we obtained the most parsimonious trees constrained to reflect these factors (see Appendix 1 for constraint definitions). Evaluation of these three sets of constraint trees was performed through a series of contrasts against the unconstrained MP trees with PAUP using the Templeton test. Given the large number of trees recovered for each of the analyses, it was impossible to perform all pairwise comparisons (e.g., every MP tree with every constraint tree); therefore, we represented diversity in the MP trees by drawing one representative from each MP tree island and contrasted it with all constraint trees.

Principal Component Analyses (PCA) were conducted in EXELSTAT (Addinsoft, 2014). Principal Component Analysis finds orthogonal dimensions of greatest variance through the multivariate and multidimensional cloud of points representing (in this case) the correlation matrix of the original variables by cases data matrix. The several components of greatest variance (largest Eigenvalues) ordinate the cases according to their similarities and differences. Where an unknown number of possible clusters are being analyzed, components of lesser variance are examined to search for smaller clusters of interest. The method is used here to test hypotheses about species vs. population-level clades, where discriminant function analysis is inappropriate because it assumes prior knowledge of classes (Humphries et al., 1981).

Components were extracted from the (Pearson) correlation matrix. Meaningful components were identified by trial and error and by examination of the traits with loadings greater than 0.3 to determine morphological complexes in each component.

Fossil Rhinichthys.— Fossil *Rhinichthys* (Fig. 4) are diagnosed by a short dentary with the dorsal surface of the anterior biting process usually flat and nearly horizontal (variants approaching the typical upturned edge of cyprinid dentaries, as in *Richardsonius*, e.g., occur in some populations); sensory pores are absent from the anterior part of the dentary; those post-ventral to the mental foramen may be present, and these are sometimes detached from bone and embedded in skin.

Table 2.— Locations, taxonomy, and catalog numbers for morphology samples. Catalog numbers from UMMZ unless otherwise noted.

Taxon	Drainage basin	Sample location	Catalog Number
obtusus	Great Lakes	E. Branch Muskegon R., Mecosta Co. & Au Sable R., Crawford Co., MI	239292/194272
cataractae	Missouri R.	Missouri Dr., Perkins Co., SD	166824
***falcatus* group**			
falcatus	Columbia R.	Crooked R. at Prineville (Deschutes Dr.), Crook Co., OR	141307
falcatus	Columbia R.	Lacamas Cr., Lewis Co., WA	94135
falcatus	Columbia R.	Wenatchee R., Chelan Co., WA	98645
falcatus	Snake R.	Bruneau R. 7 mi above Bruneau, Owyhee Co., ID	188951, 158881
falcatus	Snake R.	Burnt R., Baker Co., OR	179505
falcatus	Snake R.	Snake R. at Homedale, Owyhee Co., ID	136193, 136192
falcatus or uma.	Columbia R.	Willow Cr., Morrow Co., OR	181722
***umatilla* group**			
umatilla	Snake R.	Sucker Cr. near Homedale, Owyhee Co., ID	130476
umatilla	Columbia R.	John Day R., Grant Co., OR	180417
umatilla	Columbia R.	Touchet R. at Dayton, Columbia Co., WA	179465
umatilla	Columbia R.	Umatilla R., Umatilla Co., OR	98783
umatilla	Columbia R.	Yakima R., Kittitas Co., WA	94127, 92226
umatilla	Snake R.	Salmon R. at Challis, Custer Co., ID	161851
umatilla	Columbia R.	E. Rock Cr., Kettle R. Dr., BC, Canada	179430
***o.nubila* group**			
o. nubila	Oregon Coast	Beaver Cr., Tillamook Dr., Tillamook Co., OR	94137
o. nubila	Oregon Coast	Millicoma R., Coos Co., OR	OSU 13860
o. nubila	Oregon Coast	Siuslaw R. at Lake Cr. outlet, Lane Co., OR	OSU 9949
o. nubila	Oregon Coast	Smith R., Umpqua Dr., Douglas Co., OR	138642
o. nubila	Oregon Coast	Yaquina R., Lincoln Co., OR	65305
o. nubila	Coastal WA	Lake Quinalt, Grays Harbor Co., WA	94133
o. nubila	Coastal OR	Coquille R., Myrtle Pt., Coos Co., OR	65306
o. nubila	Columbia R.	Willamette R., Benton Co., OR	ASU FT.0351, 180404
o. nubila	Oregon Coast	Dead Indian Cr., Rogue R., Jackson Co., OR	OSU 7918
Columbia-Snake, Klamath group			
o. klamathensis?	Klamath R.	Spencer Cr. near mouth to Klamath R., Klamath Co., OR	ASU FT.0336
o. klamathensis?	Klamath R.	Sycan R., Sprague Dr., Klamath Co., OR	136685
o. klamathensis?	Klamath R.	Lost R. below dams, near OR/CA border	146509, 146529
osculus	Coastal CA	Salinas River, Monterey Co., CA	133200, 137620
osculus	Coastal CA	San Antonio R., Salinas R. Dr., Monterey Co., CA	137620
osculus	Coastal CA	San Luis Obispo Cr., San Luis Obispo Co., CA	133192
o. pitensis	Sacramento R.	Cedar Cr., Pit R Dr., Lassen Co., CA	133730
osculus	Oregon Lakes	Warner Dr., Adel, Lake Co., Or	130515
osculus	Oregon Lakes	Chewaucan R., Paisley, Lake Co., OR	65302
osculus	Oregon Lakes	Wall Canyon, Washoe Co., NV	130545
osculus	Harney	Malheur R., Drewsey, Malheur Co., OR	106360
osculus	Harney	Harney Dr., OO Ranch, Harney Co., OR	136694
osculus sbsp	Harney	Unnamed spring, Stinking L., Harney Co., OR	OS 6291
o. carringtoni	Snake R.	Snake R., Bonneville Co., ID	158917
o. carringtoni	N.E. Bonneville	Bear L., Bear Lake Co., UT	141838
o. carringtoni	N.E. Bonneville	Ogden R., Ogden, Weber Co., UT	86644, 86625
o. robustus	Snake R. Dr.	Hendricks Cr., Elko Co., NV	ASU FT.9397
o. robustus	Snake R. Dr.	Little Goose Cr., Goose Cr Dr., Elko Co., NV	ASU FT.0304
o. robustus	N.W. Lahontan	Hornes (Painters) Cr., Madeleine Plains, Lassen Co., CA	133722
o. robustus	N.W. Lahontan	Spring stream N.E. Eagle L., Lassen Co., CA	133724
osculus sbsp	N.W. Bonneville	Thousand Springs, Elko Co., NV	132194
o. reliquus	East Lahontan	Grass Valley Ranch, Lander Co., NV	124907
o. robustus	N.E. Lahontan	Bishop Cr., Elko Co., NV	141522
o. robustus	N.W. Lahontan	Susan R., 10 mi N. Susanville, Lassen Co., CA	141559

Table 2.— Locations, taxonomy, and catalog numbers for morphology samples. Catalog numbers from UMMZ unless otherwise noted, cont.

Taxon	Drainage basin	Sample location	Catalog Number
Lahontan group			
o. robustus	S.W. Lahontan	E. Walker R., Lyon Co., CA	140319
osculus	Owens Valley	Bishop Cr., Inyo Co., CA	132159
osculus	Owens Valley	Hot Cr. Trib. to Owens R, Mono Co., CA	133097
osculus	Owens Valley	Whitmore Hot Spr., Long Valley, Mono Co., CA	124836
o. oligoporus	East Lahontan	Clover Valley, Elko Co., NV	186521, 186903
o. lethoporus	East Lahontan	Independence Valley, Elko Co., NV	186519
osculus sbsp	Snake Valley	Callao, Willow Springs, Tooele Co., UT	141421
osculus sbsp	Snake Valley	Foote Ranch, Millard Co., UT	141432, 124788
osculus sbsp	Snake Valley	Warm Spr., Gandy, Millard Co., UT	85937
osculus sbsp	Snake Valley	Warm Cr., Millard Co., UT	124788
o. nevadensis	Amargosa R.	Ash Meadows, Nye Co., NV	102106
o. nevadensis	Amargosa R.	Amargosa R. 8.8 mi N Beatty, Nye Co., NV	188858
o. nevadensis	Amargosa R.	Amargosa R. W. of Tecopa, Inyo Co., CA	139012
Colorado drainage group			
o. deaconi	Colorado R.	Las Vegas Wash, Clark Co., NV	125008
yarrowi	Colorado R.	San Juan R. at Ship Rock, San Juan Co., NM	142542
osculus	Colorado R.	Trout Cr., Bill Williams Dr., Mohave Co., AZ	ASU FT.10503
yarrowi	Gila R.	E. Fk. White R. just below Fort Apache, Navajo Co., AZ	162767
o. osculus	Gila R.	Eagle Cr., 3.5 mi S. Eagle Ranger Sta., Greenlee Co., AZ	162747
o. osculus	Gila R.	Fossil Cr., Gila Co., AZ	ASU FT.0311
o.osculus	Gila R.	Sonoita Cr., Circle Z Ranch, Salera Rd, Mohave Co., AZ	ASU FT.6505
o. osculus	Gila R.	Sycamore Cr., 2 mi S.E. Dugas, Yavapai Co., AZ	162838
o. yarrowi	Green R.	Beaver Cr., Trib. to Price R., Utah Co., UT	176921
o. yarrowi	Green R.	Confluence of Green & Yampa R., UT-CO	181753, 188971
o. thermalis	Green R.	Kendall Warm Springs, Sublette Co., WY	111287
o. osculus	Los Angeles	Santa Ana R. near Prado in the canyon, Riverside Co., CA	132986, 133164
osculus sbsp	Los Angeles	Trib. Rio Hondo, LA Co., CA	133164
o. osculus	Little Colorado	Clear Cr. W. Chevelon Ranger Station Rd, Coconino Co., AZ	178692
o. osculus	Little Colorado	E. Clear Cr. at Hwy 96, Coconino Co., AZ	188961
o. osculus	So. Bonneville	E. Branch Shoal Cr., Washington Co., UT	124778
o. adobe	So. Bonneville	Sevier R., Sevier Co., UT	117857
o. osculus	Virgin R.	Beaver Dam Wash, Lincoln Co., NV	177460
o. osculus	Virgin R.	Meadow V. Wash, Rainbow Canyon, Lincoln Co., NV	124801, 124826
o. moapae	Virgin R.	Muddy (Moapa) River headwaters, Clark Co., NV	203328
o. osculus	Virgin R.	Spring feeder to Maynard Lake, Lincoln Co., NV	136096
o. yarrowi	Virgin R.	Virgin R. 3 mi W. Rockville, Washington Co., UT	217114
o. velifer	Virgin R.	White River near Preston, Lincoln Co., NV	132181, 188956

Major teeth (except the most anterior one and sometimes the posterior one) usually have a convex, antero-dorsal blade adjacent to a slender, flat surface (similar to a grinding surface) and proximal to the terminal 'hook' (sometimes a secondary edge is posterior to the elongate grinding surface). Fossil teeth and (or) dentaries are known from the Juntura Formation, OR (10.4 Ma), Ellensburg Formation, WA (10.3 Ma), Drewsey Formation, OR (8.4 Ma), four localities in the Glenns Ferry Formation, ID (ages 4, 3, 2, and 2 Ma), Glendale NV (30 ka), Mono Basin (Pleistocene), CA, and Owens Valley (46 ka), CA, (Smith et al., 2013, and Table 3). We count three Pleistocene fossil localities, from Owens Valley, CA, Mono Lake, CA, and Glendale, NV, as one fossil horizon for analysis of density of the fossil record through time. Three Pliocene localities and three Miocene localities are counted as six horizons, because they occur in six 1-million-year time intervals.

Rhinichthys are among the smallest North American fish fossils. They were usually found in screen-washing operations intended to discover fossil mammal teeth. Radiogenic dates on nearby basalts and tephras and biostratigraphic correlations with associated mammalian fossils provided age estimates (Smith et al., 2013).

Table 3.— Summary of fossil ages of *Rhinichthys*. Horizons of 1 m.y. as recommended by Marshall (1990) method, total Horizons = 7.

Glendale, Clark Co., NV. Age 30 Ka., *Rhinichthys osculus =H1*. Pharyngeals, dermethmoid, basioccipital. Museum of Northern Arizona specimens.

Owens Valley Inyo Co., CA. Age 46 Ka., *Rhinichthys osculus =H1*. A. Jayko, collector, UMMP 42389.

Mono Lake beds, Mono Co., CA. Age Plio-Pleistocene, no *H*. A. Jayko, collector, UMMP 42390.

White Narrows beds, Clark Co., NV. Age 4.7 Ma. *=H4*. San Bernardino Museum.

Near Base of *R. osculus* node:

Glenns Ferry Formation, Twin Falls Co., ID. Hagerman 25' from top. Age 2 Ma. *Rhinichthys osculus =H2*. Some teeth with single edge; some with narrow double edge. UMMP 42391.

Glenns Ferry Formation, Twin Falls Co., ID. Hagerman 540. Age 2 Ma. *Rhinichthys osculus*? *=H2*. Large 2, 4, tooth with bladelike grinding edge. UMMP 42392.

Glenns Ferry Formation, Elmore Co., ID. RR grade locality. Age 3 Ma. *Rhinichthys osculus =H3*. One dentary with horizontal flared biting edge. UMMP 42393.

Glenns Ferry Formation, Elmore Co., ID. Highway Cut. Age 4 Ma. *Rhinichthys osculus =H4*. Many teeth and fragments. UMMP 42394.

Near *Rhinichthys* node:

Juntura Formation, Malheur Co., OR. University of Oregon 2337. Age 10.4 Ma. *Rhinichthys* sp. = *H5*. UMMP 42395; University of Oregon Museum.

Ellensburg Formation, Yakima Co., WA. Granger Clay Pit. Age 10.5 Ma. Possibly pre *Rhinichthys =H6*. UMMP 42396.

Trapa Beds, Drewsey Formation, Malheur Co., OR. Age 8.4 Ma. *Rhinichthys* sp. *=H7*. UMMP 423997.

These ages were used to calibrate ages of nodes in the maximum likelihood analysis of molecular data. Ages for crucial nodes were corrected to a best estimate by using Marshall's (1990) method to calculate an additive factor based on the number of 1 m.y. horizons (*h*) in which fossil *Rhinichthys* are known. An alpha value based on the 50% confidence interval estimates the mean age, with the 95% interval used for confidence estimates.

Divergence Time Estimates.— We combined stratigraphy and morphology to estimate ages of apomorphies shared by fossils, nodes, and branches in the phylogeny. The time-calibrated branching network provides a temporal framework for the evolution of the *R. osculus* group (as described by Unmack et al., 2014). The package of programs from BEAST 2.1.3 (Bouckaert et al., 2014) was used to estimate molecular divergence times of lineages, reported as estimates of mean divergence time and their 95% HPDs. Input files were generated using BEAUti 2.1.3. The dataset was trimmed to single representatives per/lineage (selected to avoid extremes of divergence within lineages wherever possible) because presence of a mix of within- and between-species data complicates dating owing to different processes for estimating within- versus between-species rates (Ho et al., 2008). The analysis used an uncorrelated lognormal relaxed molecular clock with rate variation following a tree prior using the calibrated Yule model and a random starting tree. We used the BEAST addon RB, which is a reversible-jump based alternative to choosing the appropriate substitution model as part of the analysis. BEAST analyses were run for 50 million generations, with parameters logged every 10,000 generations. Multiple runs were conducted to check for stationarity and that independent runs were converging on a similar result. Log and tree files from four runs were combined using LogCombiner 2.1.3 with a 10% burn-in. Outputs from LogCombiner were examined in Tracer 1.5, while mean age estimates and topology were based on the maximum clade credibility from our combined trees using TreeAnnotator 2.1.3. Analyses were also conducted excluding the sequence data to check that posterior distributions were not heavily driven solely by our fossil priors rather than the sequence data. Two calibration points based on fossils were included, one with a value of 13.7 million years at the node uniting all *Rhinichthys* species and one with a value of 6.5 at the node uniting *R. cataractae* and *R. osculus*. Each calibration point was set to be monophyletic with a log normal prior with a mean of one, a standard deviation of 1.5, with the mean offset in real space by ages of 6.5 and 13.7 million years as estimated from fossil ages. These age estimates had been corrected with the method of Marshall (1990, see above) to utilize the density of the fossil record to overcome error inherent in assuming that the earliest known fossil was actually the first of its branch or lineage. That estimate can be an underestimate if there has been reticulate evolution, as we suspect in *Rhinichthys*. We encountered no evidence that our fossils were morphologically unchanging ancestors of budding events that produced our taxa of interest (which would render an age estimate unconstrained).

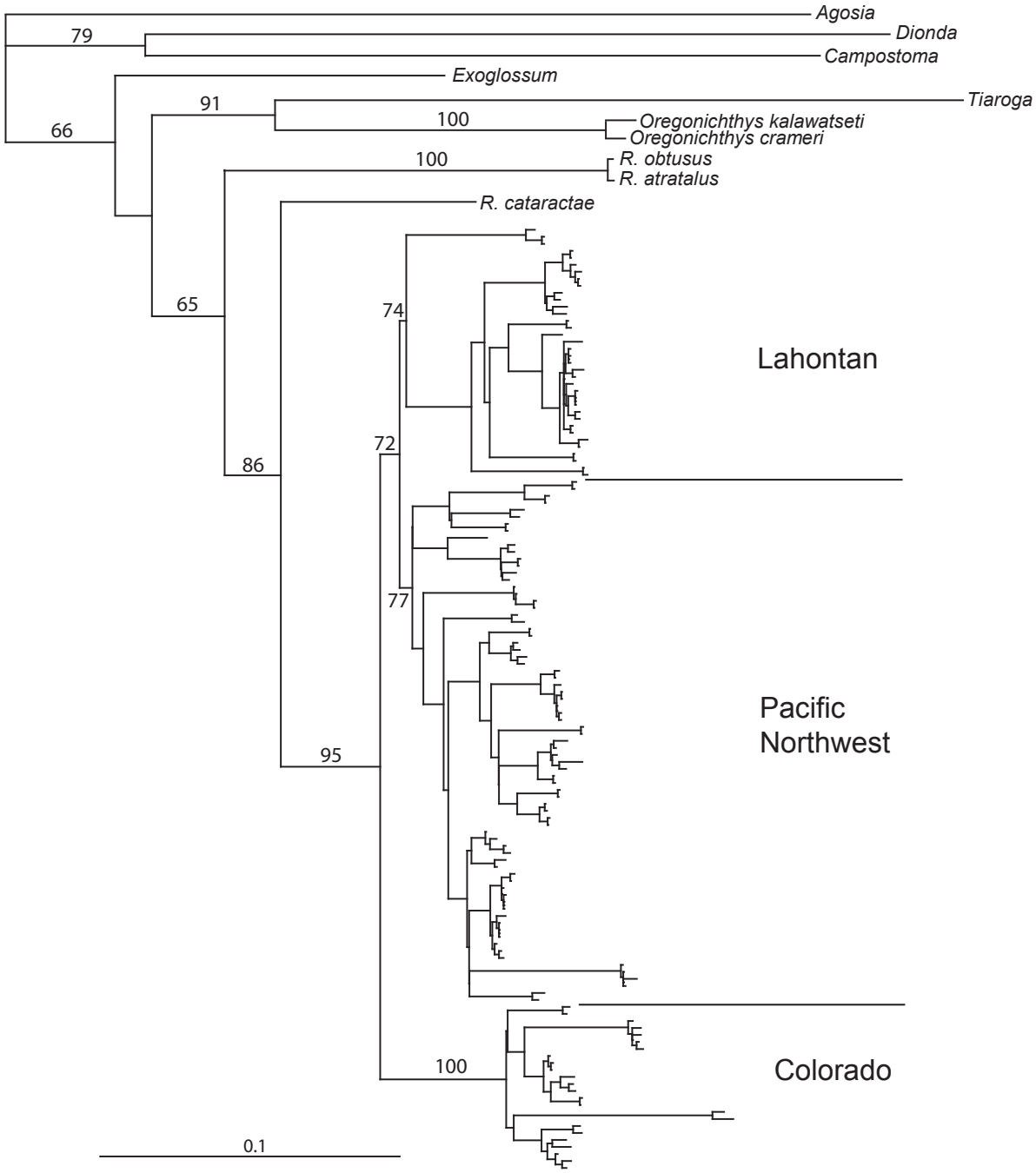


Figure 5.— Maximum likelihood tree of mtDNA relationships of *Rhinichthys osculus, R. falcatus, R. umatilla*, and out-group taxa. For more detail, see individual clades, Figs. 6, 8, 10, and maps, Figs. 7, 9, 11.

RESULTS

Maximum Likelihood Analysis of molecular data.— We generated sequences from 145 individuals, representing 74 localities and 10 out-group taxa, yielding 889 invariant characters and 548 variable sites. Maximum Likelihood (ML) analysis recovered one tree with a likelihood of -13532.738126 (Fig. 5). In general, bootstrap analyses provide moderate support (> 70%) for many of the deeper nodes (reduced support values are provided for some nodes discussed in the text). In this tree, the *R. osculus* group is divided into three broadly distributed geographic groups, (1) a Northwest group that includes the Columbia, Snake and Klamath drainages, and coastal drainages in Washington, Oregon, and northern California, and the northern Bonneville

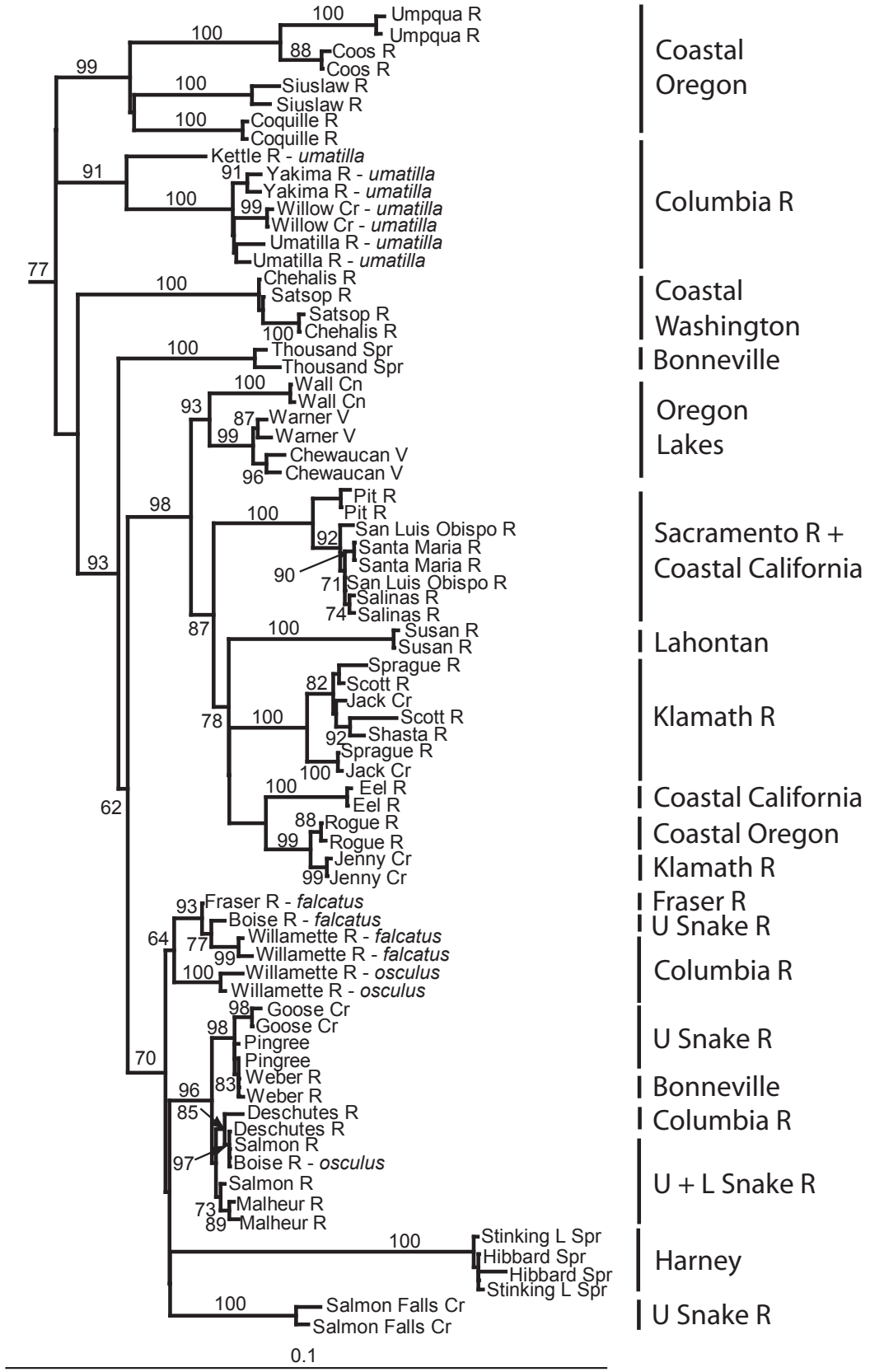


Figure 6.— Maximum likelihood tree of mtDNA relationships of samples in the Northwest clade. See map, Fig. 7.

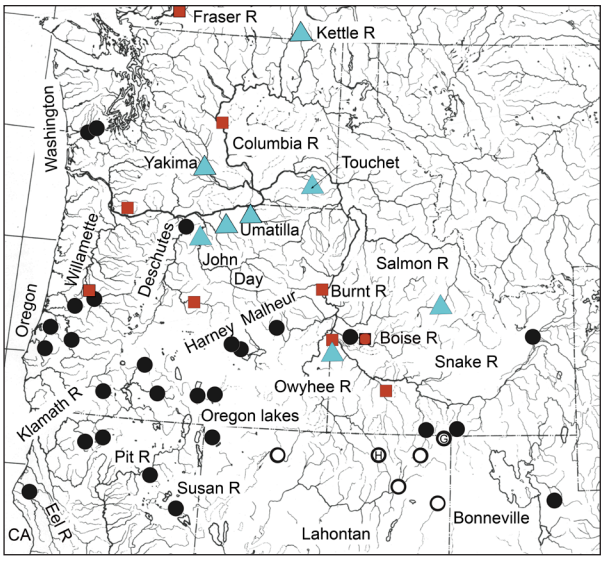


Figure. 7.— Distribution of samples of *R. osculus* (black spots), *R. falcatus* (red squares), and *R. umatilla* (blue triangles) of the Northwest clade. Coastal California samples from near Monterey and San Luis Obispo not shown (see Figs. 1, 3). Open circles represent samples in the neighboring Lahontan clade; 'H' (Hendricks Cr.) and 'G' (upper Goose Cr.) are members of the Lahontan clade captured by tributaries of the Snake River. The Susan River (of the Lahontan basin) sample is polymorphic, with haplotypes belonging to Lahontan and (probably) Pit or Klamath headwater clades. Other peripheral samples indicating stream captures are in the (now) unconnected Fraser River, Coastal Chehalis (Chehalis and Satsop drainages), Coastal Tyee (Coos, Umpqua, Siuslaw, and Coquille drainages), Coastal Klamath (Rogue and Klamath drainages), and other northern California coastal drainages. Peripheral samples that indicate severance of former connected drainages include the Bonneville Basin (Thousand Springs drainage, with ancient haplotypes sister to most of the rest of the Columbia-Snake River localities (see Figs. 8, 9), and Weber River, Harney Basin, Chewaucan drainage, and Wall Canyon drainage.

Basin as well as the included species, *R. falcatus* and *R. umatilla*, (2) a central group focused in the Lahontan Basin with its peripheral offshoots, and (3) a southeastern group comprising individuals from the Colorado River Basin, southern Bonneville Basin, and Los Angeles Basin. Groups (1) and (2) are sister lineages and (1)+(2) are sister to (3); however, these relationships are weakly-supported by bootstrap analysis, occurring in only 65% of the replicates. Samples from the Bonneville Basin are found in all three groups (Figs. 6, 8 and 10).

The Pacific Northwest mtDNA Group.— The large group from the Pacific Northwest includes 13 distinctive molecular lineages, which are included in 75% of the bootstrap replicates (Figs. 6, 7). Four lineages form a polytomy at the base of this group: (1) eight samples from the Umpqua group of four Oregon coastal drainages (coastal Tyee region of Kettretad and Markle, 2010), (2) seven samples of *R. umatilla* representing four drainages from the middle and upper Columbia River, (3) four samples from two Washington coastal drainages (coastal Chehalis), and (4) a large number of samples representing 29 locations from the central Columbia and Snake rivers and former connectives. These four groups are well-supported (>90% of bootstrap replicates); however, relationships among them are not resolved (<50% of bootstrap replicates).

Many of the clades in this large fourth group are well-supported. The Rock Springs samples in the Thousand Springs area in extreme northeast Nevada (now tributary to the Bonneville drainage) are sister to diverse lineages from central and coastal California, the Klamath drainage, Oregon Lakes basins of the Great Basin, and present or former tributaries of the Columbia and Snake Rivers, scattered in Utah, Idaho, and Oregon. The most divergent clade in this group comes from the Oregon Lakes, including Warner Valley, Chewaucan basin, and Wall Canyon. It is sister to four groups of samples found in two clusters, one that includes the Pit River and central California coastal rivers, while the other includes samples from the Susan, Klamath, Eel, and Rogue rivers. In the latter group, the Susan River is most divergent; the Klamath River is sister to an Eel River plus Rogue River clade (coastal Klamath). Samples from Jenny Creek in the Klamath River basin cluster with samples from the Rogue River, Oregon.

In the Columbia and Snake River group, samples from the Willamette River are especially important: mtDNA from the local form of *R. osculus* and the sympatric and morphologically and ecologically divergent *R. falcatus* are sisters in gene trees. *Rhinichthys falcatus* mtDNA haplotypes were also found in samples from the Boise and Fraser rivers, forming a well-supported monophyletic group; however, these were found in a few morphologically identified *R. osculus* from the Boise River sample. MtDNA haplotypes from the majority of Boise River *R. osculus* are related to samples of *R. osculus* from the upper Snake River. The sisters to these populations form a complex polytomy with populations from (1) Harney Basin, Oregon (with a long branch); (2) Salmon Falls Creek, Nevada; and (3) a broadly distributed group of 13 samples including Malheur and Deschutes rivers, Oregon; Goose Creek and Boise, Salmon, and upper Snake rivers, Idaho; and Weber River, in the northern Bonneville Basin, Utah. This large group includes some individuals identified as *R. falcatus* (Malheur River) and *R. umatilla* (Salmon River) based on morphology. The three groups above are, or were recently, connected through the Snake River.

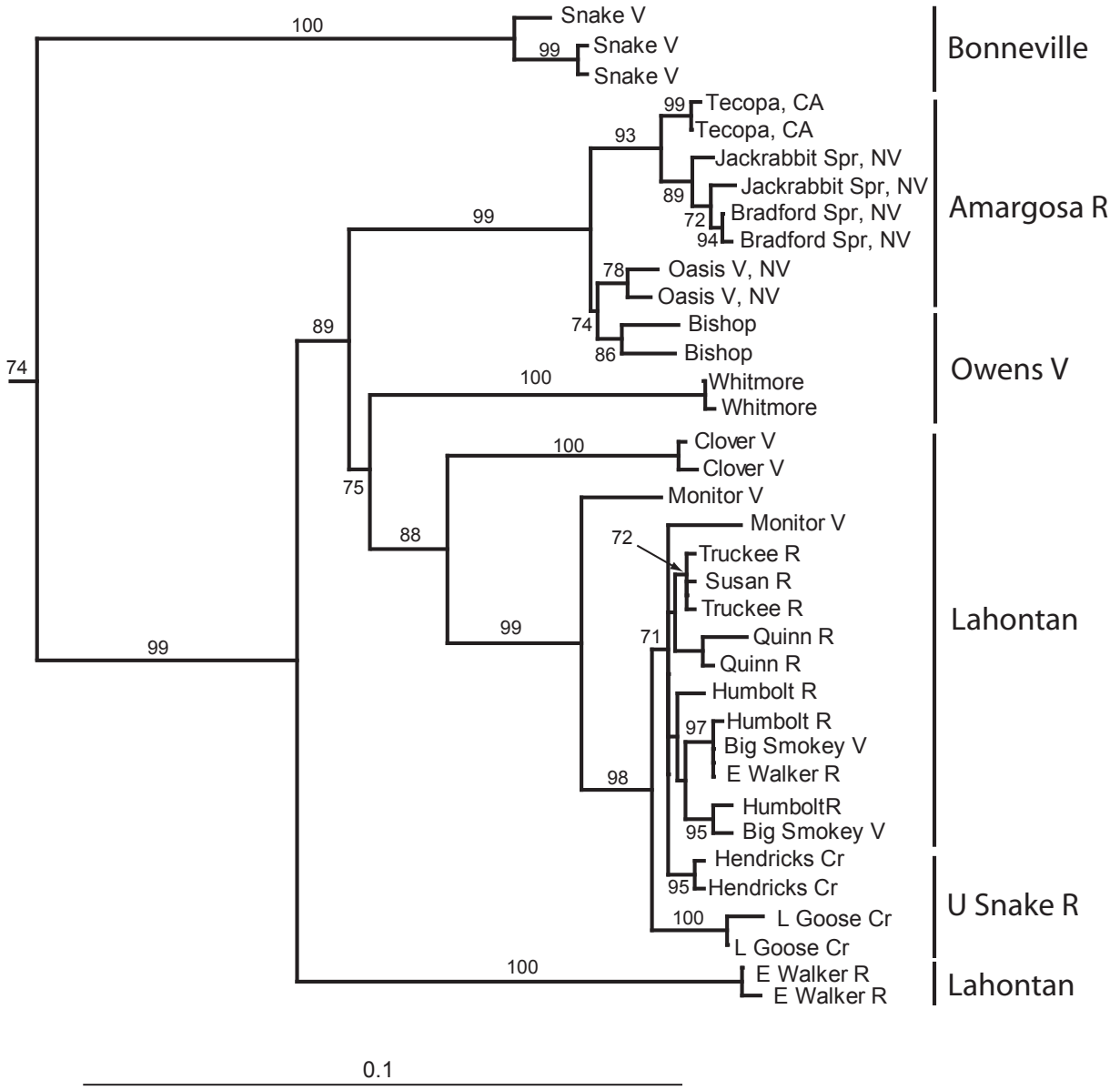


Figure 8.— Maximum likelihood tree of mtDNA relationships of samples in the Lahontan clade. See map, Fig. 9.

The Western Snake River Plain is hydrographically connected to most of the northwestern region; its Pliocene to modern geological history is central to biogeography of western fishes, including *R. osculus*, *R. falcatus*, and *R. umatilla* (see discussion and Carpenter and Smith, this volume).

Lahontan Group.— The Lahontan group comprises many distinct lineages found in currently isolated drainages in and around the edges of the basin (Figs. 8, 9). The most divergent lineages are found in Snake Valley, a long north-south desert stream near the Nevada-Utah line, now in the Bonneville Basin; it was identified as a member of the Lahontan group, but in only 75% of the bootstrap replicates. Some individuals from Snake Valley exhibit haplotypes found in the nearby Lower Colorado group (Figs. 8, 9).

The remaining 33 samples from 16 Lahontan basin locations form a strongly supported clade, with the most divergent lineage from the southwestern side of the Lahontan Basin, samples from East Walker River, with remaining samples representing the main Lahontan complex, Owens Valley, and a sister clade from the Amargosa River and Death Valley. The Amargosa clade includes a haplotype living in Bishop Creek, Owens Valley. Within the Amargosa and Death Valley group, samples from Bishop Creek, Owens Valley, and Oasis

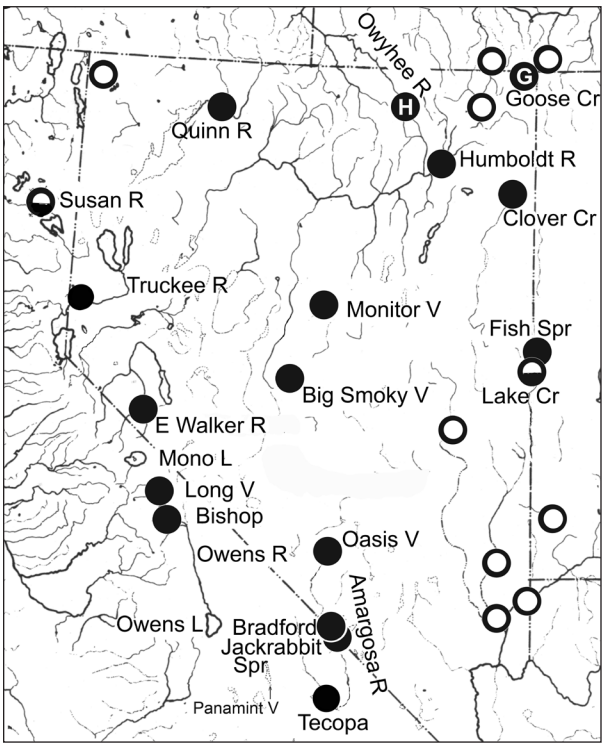


Figure 9.— Distribution of samples in the Lahontan clade (solid black symbols). Open circles are localities belonging to the Northwest clade (N) and Colorado River clade (SE). The Susan River is polymorphic for haplotypes belonging to the Lahontan as well as Northwest clade. Molecular relationships are shown in Fig. 8. The Hendricks Creek (H) and Goose Creek (G), headwaters in the Snake River drainage, contain *R. osculus* with Lahontan haplotypes. These creeks formerly flowed south, but now turn northward at the points of diversion. The Goose Creek headwater formerly flowed into the now-isolated Thousand Springs drainage, but now flows into the Snake River drainage. Goose Creek was once part of the Lahontan drainage, according to the Humboldt drainage haplotype in *R. osculus* in Goose Creek. Similarly, the Lake Creek and Fish Springs fish carry Lahontan haplotypes, despite now being in the Lake Bonneville Basin. Some Lake Creek fish carry Sevier River haplotypes derived from the Colorado River clade (See Figs. 10, 11). The diverse Amargosa River (Death Valley) populations carry Lahontan-related haplotypes. Their most direct, relatively recent, relationship is with fish sampled in C2 drain in the Owens drainage near Bishop, possibly via a headwater diversion between Lower Centennial Flat and Santa Rosa Wash north of Talc City Hills, California. Owens basin fish display two haplotypes: one connected to the Amargosa drainage at 0.45 Ma and one connected to the Lahontan at 2.3 Ma, in agreement with the southward shift in Mono basin drainage at about 1.3 Ma, as worked out by Reheis et al., (2002). Fish in Big Smoky Valley are close relatives of *R. osculus* in the East Walker and Humboldt clade, supporting the hypothesized northern connections through Lake Toiyabe and Grass Valley (Lake Gilbert) as outlined by Reheis et al., (2002; fig. 1).

Spring in Death Valley, are sister lineages, as are the Amargosa River, with Jackrabbit Spring, and Bradford Spring.

The main clade in the Lahontan complex includes 23 samples with similar haplotypes. Early branches are scattered around the edges of the basin. Whitmore Pond (near Bishop in northwestern Owens Valley, now isolated from the Lahontan Basin, Fig. 9) is sister to the following branching sequence: Clover Valley (in northeastern Nevada), then a sample from Monitor Valley (in south-central Lahontan Basin), and finally a sample from the headwaters of Little Goose Creek. The remaining 14 samples are from the Humboldt River, East Walker River, Monitor Valley, Big Smokey Valley, Truckee River, Quinn River, and Susan River. These samples form a large polytomy consistent with recent connections and likely gene exchange among these locations (including Monitor Valley, represented by a divergent haplotype mentioned earlier).

Some samples provide evidence for stream capture events. Within the Central Lahontan group, two samples from Hendricks Creek, in north-central Nevada, live in a south-flowing headwater of a fish-hook-shaped former Lahontan tributary, which was captured and turned northwards into the Owyhee River, a tributary of the Snake River drainage. These still retain their Lahontan basin mtDNA signature. Upper Goose Creek drainage is also a former Lahontan tributary that is now a captured tributary of the Snake River in northeastern Nevada; our sample from further downstream in the system is more closely related to Snake River drainage samples (Figs. 6, 7). There are no sympatric forms of the *Rhinichthys osculus* group within the Lahontan Basin.

The Colorado River Clade.— The Colorado River group is a well-supported monophyletic group of five major lineages: (1) Gila River drainage, (2) Los Angeles Basin, (3) Little Colorado River, (4) Green River, upper Colorado River, Bill Williams River, and southern Bonneville Basin complex, and (5) Pluvial White River, Moapa, Meadow Valley Wash, Virgin rivers, and southern Bonneville complex (Fig. 10, 11). Samples from the Los Angeles Basin and those from Gila River drainage, Arizona and New Mexico, form a weakly-supported group (67% of bootstrap replicates) that is found in a trichotomy with samples from the Little Colorado River and all remaining samples from lower and upper Colorado River as well as the Bonneville basin.

Populations from the Bill Williams River, a tributary to the Lower Colorado River in western Arizona, southern Bonneville Basin, and upper Colorado River are sister to the Virgin and Pluvial White river clade. Within the former group, samples from the Bill Williams River are sister to populations in the southern Bonneville Basin, Utah.

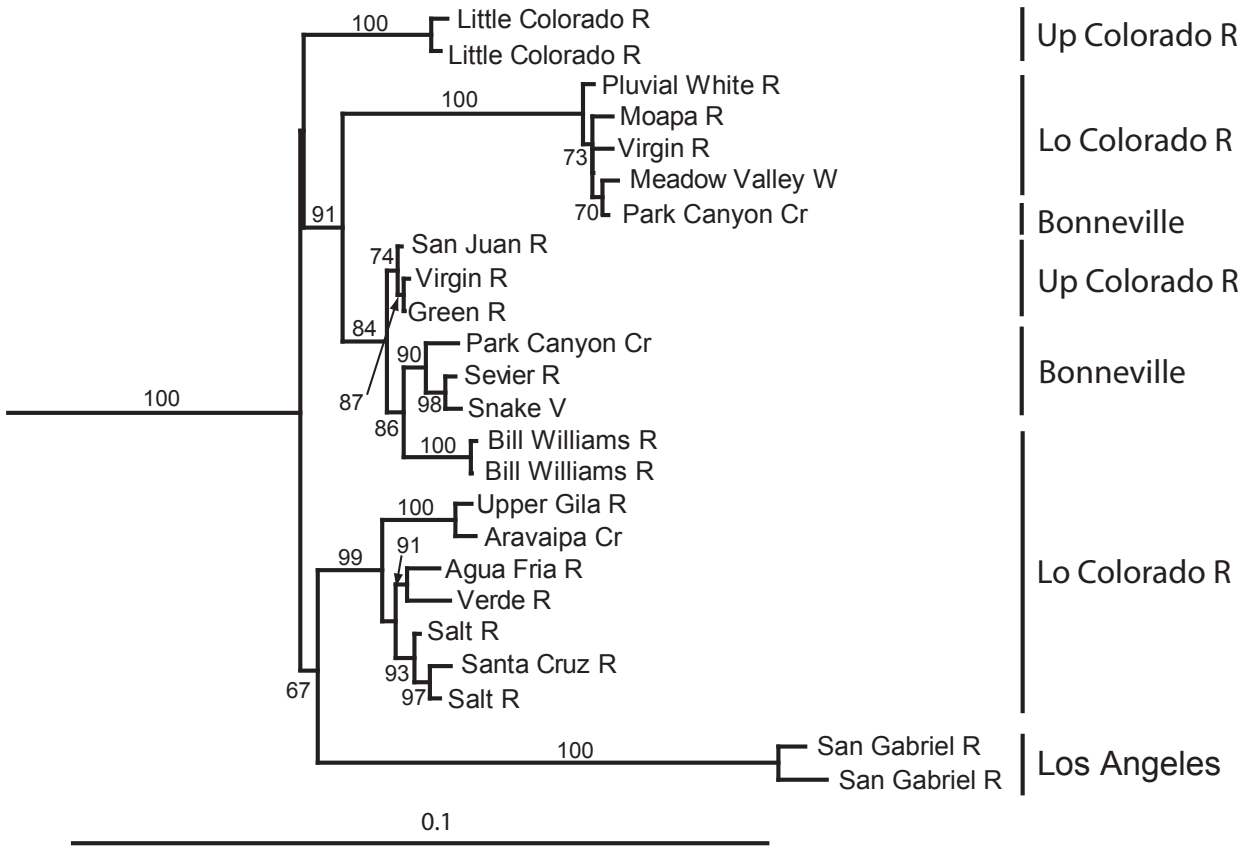


Figure 10.— Maximum likelihood tree of mtDNA relationships of samples in the Colorado River clade. See map, Fig. 11.

Some collections exhibit membership in multiple well-resolved lineages. Examples are provided by different haplotypes from the Virgin River which are related to sister populations in the Upper Green River, Wyoming, and San Juan River, Colorado, as well as to clades in the Pluvial White River, Nevada, and Shoal Creek (Park Canyon Creek), Utah. Different haplotypes from Shoal Creek are related to clades in the Sevier River, Utah, as well as Meadow Valley Wash, Nevada. There are no sympatric forms of the *Rhinichthys osculus* group within the Colorado River drainage and southern Bonneville Basin.

Results of morphological analyses.— The morphological variation in *Rhinichthys osculus* is illustrated in Fig. 2. Parsimony analysis yielded 156 most parsimonious trees of 842 steps, with large clusters of geographical mixtures (Fig. 12). Samples of *Rhinichthys falcatus* and *R. umatilla* clustered approximately by species; *R. osculus* was scattered through the morphological tree and samples were distributed in a manner that usually failed to represent any drainage or biogeographical entities. None of the suspected species or subspecies relationships emerged consistently in the morphological tree; homoplasy dominated the matrix (Table 4).

Principal Component Analyses of a correlation matrix of 31 morphological characters identified 10 components with Eigenvalues greater than 1.0, with the first three accounting for 42% of the variability. Component I (accounting for 23% of the variance) is positively correlated with all measures of size. Component II (11% of the variance) has high loadings (>0.3) on the following characters (in order of magnitude of the loading): caudal peduncle depth, head length, anal fin position, dorsal fin rays, suborbital bones, fin shape, snout length, pigment pattern, barbels, caudal peduncle length, eye size, anal fin rays, ventral flange on the dentary, dentary shape, vertebral number, dentary pores, absence of lateral stripe, and cleithrum shape. These are traits that characterize *R. falcatus, R. umatilla,* and many *R. osculus* (not shown). Component III (7% of the variance) has high loadings for the presence/absence of the supraorbital bone, number of minor teeth, tooth shape, shape of the pharyngeal arch, number of lateral line scales, anal fin origin, and frenum. These traits identify *R. cataractae* and some similar *R. osculus,* and the unique absence of supraorbital bones in the

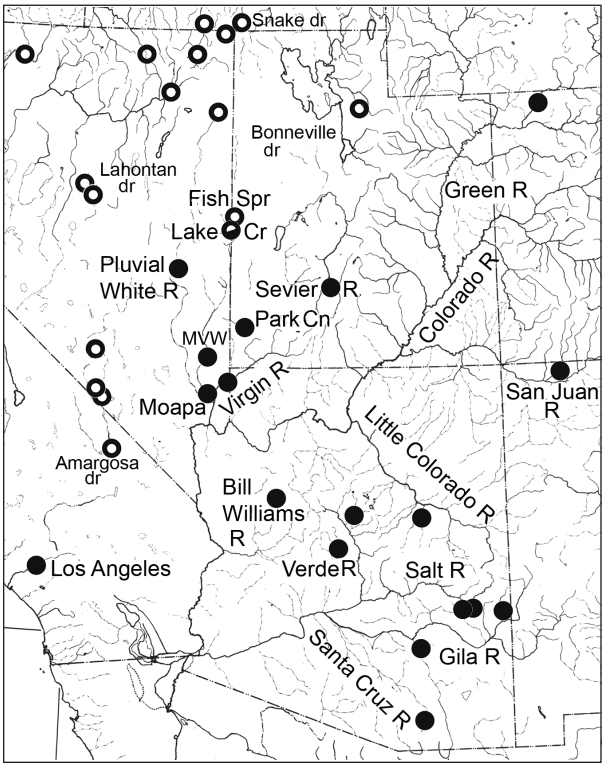


Figure 11.— Distribution of samples in the Colorado River clade (solid black symbols). Open circles are localities of *Rhinichthys* in the Snake River and Lahontan clades. One Lake Creek population is polymorphic with haplotypes from the Colorado River (via the Sevier River) and the Lahontan clade. Three clades represent Colorado River dace (Fig. 10): (1) The upper Colorado River, including the Green River, San Juan River, Virgin River, Sevier River, part of Lake Creek, Pluvial White River, Meadow Valley Wash, Bill Williams River, and Park Canyon Creek; (2) the Little Colorado River; and (3) the Lower Colorado River, including Gila River tributaries and the San Gabriel River in Los Angeles. Two haplotypes are in the Virgin River, one from the upper Colorado and one from the lower Colorado drainage. Stream captures are indicated between Lake Creek in Snake Valley and the Sevier drainage (southern Bonneville drainage), relatively recently, and the Lahontan drainage, 4.32 Ma; and between Park Canyon Creek and Meadow Valley Wash, relatively recently. Connections between the Bill Williams River and the Sevier drainage are unexplained. A connection between the Los Angeles Plain and the Gila drainage was postulated by Howard (1996, 2000) but was questioned by Spencer et al., (2008). See Smith and Dowling (2008) for more discussion and more complete map.

Los Angeles populations. Component IV (6% of the variance) has high loadings on number of anal fin rays, dentary length, symplectic shape, metapterygoid shape, flange on the postmesial edge of the dentary, and barbels, traits that vary widely among *R. osculus*. Component V (6% of the variance) has high loadings on number of lateral line scales, metapterygoid shape, frenum, head shape, lateral stripe, dentary length, pigment pattern, and vertebral number, which vary among Colorado River populations. Component VI (5% of the variance) has high loadings with head shape, dentary flange, barbels, cleithrum shape, number of anal fin rays, lateral scales, and dentary shape. Remaining components have high loadings on mouth bones, mouth soft anatomy, and color traits that appear to be habitat related homoplasies.

Examination of the clusters of correlated traits suggests extensive homoplasy throughout the matrix (Figs. 13-15). The ordination of the full sample of cases showed broad overlap, with no unambiguous clusters representing taxa or geographical sample groups. Analysis of the three major clades separately showed useful patterns, reflecting some biogeography and differentiation.

Principal Component Analyses of the Northwest Clade showed a tendency for cases of *R. falcatus* and *R. umatilla* to cluster by species, especially on component II, but with definite overlap along all components (Fig. 13). Samples of *R. osculus* showed some separation from *R. falcatus* and *R. umatilla* on component II; the northwest coastal group, and the Lahontan samples also showed some differentiation, but not without overlap with each other and other *R. osculus*.

The Lahontan clade shows morphological separation of Snake Valley vs. Amargosa River vs. Lahontan plus Owens samples on component I (Fig. 14). Geographic or habitat differentiation is not reflected in components II, III, or IV (not shown).

Principal Component Analysis of samples in the Colorado drainage clade (Fig. 15) shows no noteworthy differentiation by drainage. On combined components II x III, however, the samples from the upper Colorado Basin are separate from the Lower Colorado Basin samples (Fig. 15, II x III).

Because of the limited phylogenetic resolution with morphological characters, we explored the potential contribution of various factors (e.g., current geographic connections, habitat, and past geographic connections [as indicated by mtDNA]) to morphological variation in this group. To achieve this we re-analyzed the morphological data, applying phylogenetic constraints to maintain groups (identified in Appendix 1) expected from current drainage connections, similarity of habitat (e.g., standing water, creeks, and large rivers) or the three major mtDNA groups (Fig. 5). This yielded 48 trees that

Table 4.— Consistency and retention indices (CI and RI, respectively) for each of the 31 morphological characters examined in this study (character abbreviations provided in Appendix 1) from the 156 MP trees (length = 842). Rows provided scores for trees representative of each tree island identified in the MP analysis, with the last two rows identifying the best (highest) and worst (lowest) score for each character.

Tree	1 (size)		2 (hdshape)		3 (hdln stln)		4 (eye hdln)		5 (snoutln hdln)		6 (finshape)		7 (Drays)	
	CI	RI	CI	RI	CI	RI	CI	RI	CI	RI	CI	RI	CI	RI
5	0.100	0.514	0.080	0.603	0.091	0.302	0.121	0.370	0.094	0.517	0.130	0.692	0.121	0.453
10	0.100	0.514	0.080	0.603	0.091	0.302	0.125	0.391	0.094	0.517	0.130	0.692	0.121	0.453
18	0.100	0.514	0.080	0.603	0.091	0.302	0.121	0.370	0.094	0.517	0.130	0.692	0.121	0.453
26	0.105	0.541	0.077	0.586	0.091	0.302	0.125	0.391	0.094	0.517	0.130	0.692	0.121	0.453
31	0.100	0.514	0.080	0.603	0.091	0.302	0.125	0.391	0.094	0.517	0.130	0.692	0.121	0.453
62	0.105	0.541	0.080	0.603	0.094	0.326	0.129	0.413	0.091	0.500	0.130	0.692	0.125	0.472
78	0.105	0.541	0.080	0.603	0.094	0.326	0.129	0.413	0.091	0.500	0.130	0.692	0.125	0.472
148	0.105	0.541	0.087	0.638	0.094	0.326	0.129	0.413	0.094	0.517	0.130	0.692	0.121	0.453
149	0.105	0.541	0.087	0.638	0.094	0.326	0.125	0.391	0.094	0.517	0.130	0.692	0.121	0.453
152	0.105	0.541	0.087	0.638	0.094	0.326	0.125	0.391	0.094	0.517	0.130	0.692	0.121	0.453
Best	0.105	0.541	0.087	0.638	0.094	0.326	0.129	0.413	0.094	0.517	0.130	0.692	0.125	0.472
Worst	0.100	0.514	0.077	0.586	0.091	0.302	0.121	0.370	0.091	0.500	0.130	0.692	0.121	0.453

Tree	8 (Arays)		9 (latlinscls)		10 (latlinecompl)		11 (barbls)		12 (frenum)		13 (Afinorig)		14 (cpdep cpln)	
	CI	RI	CI	RI	CI	RI	CI	RI	CI	RI	CI	RI	CI	RI
5	0.250	0.368	0.121	0.637	0.143	0.760	0.091	0.487	0.167	0.091	0.167	0.583	0.109	0.529
10	0.250	0.368	0.121	0.637	0.143	0.760	0.091	0.487	0.167	0.091	0.167	0.583	0.109	0.529
18	0.250	0.368	0.121	0.637	0.143	0.760	0.091	0.487	0.167	0.091	0.167	0.583	0.111	0.540
26	0.250	0.368	0.121	0.637	0.143	0.760	0.087	0.462	0.167	0.091	0.167	0.583	0.109	0.529
31	0.250	0.368	0.121	0.637	0.143	0.760	0.091	0.487	0.167	0.091	0.167	0.583	0.109	0.529
62	0.267	0.421	0.121	0.637	0.111	0.680	0.095	0.513	0.167	0.091	0.200	0.667	0.106	0.517
78	0.267	0.421	0.121	0.637	0.111	0.680	0.095	0.513	0.167	0.091	0.200	0.667	0.106	0.517
148	0.267	0.421	0.121	0.637	0.125	0.720	0.100	0.538	0.167	0.091	0.200	0.667	0.106	0.517
149	0.267	0.421	0.121	0.637	0.125	0.720	0.100	0.538	0.167	0.091	0.200	0.667	0.106	0.517
152	0.267	0.421	0.121	0.637	0.125	0.720	0.100	0.538	0.167	0.091	0.200	0.667	0.106	0.517
Best	0.267	0.421	0.121	0.637	0.143	0.760	0.100	0.538	0.167	0.091	0.200	0.667	0.111	0.540
Worst	0.250	0.368	0.121	0.637	0.111	0.680	0.087	0.462	0.167	0.091	0.167	0.583	0.106	0.517

Tree	15 (pigmntspots)		16 (stripe)		17 (dentlngth)		18 (dentshape)		19 (dentpores)		20 (dentflang)		21 (metaptrgd)	
	CI	RI	CI	RI	CI	RI	CI	RI	CI	RI	CI	RI	CI	RI
5	0.083	0.676	0.143	0.625	0.083	0.476	0.083	0.598	0.133	0.618	0.086	0.448	0.190	0.528
10	0.083	0.676	0.143	0.625	0.080	0.452	0.083	0.598	0.133	0.618	0.086	0.448	0.190	0.528
18	0.077	0.647	0.143	0.625	0.083	0.476	0.083	0.598	0.133	0.618	0.086	0.448	0.190	0.528
26	0.083	0.676	0.143	0.625	0.080	0.452	0.083	0.598	0.133	0.618	0.086	0.448	0.190	0.528
31	0.083	0.676	0.143	0.625	0.080	0.452	0.083	0.598	0.133	0.618	0.086	0.448	0.190	0.528
62	0.083	0.676	0.111	0.500	0.080	0.452	0.107	0.695	0.154	0.676	0.071	0.328	0.200	0.556
78	0.083	0.676	0.111	0.500	0.080	0.452	0.107	0.695	0.154	0.676	0.071	0.328	0.200	0.556
148	0.083	0.676	0.118	0.531	0.077	0.429	0.107	0.695	0.133	0.618	0.073	0.345	0.190	0.528
149	0.083	0.676	0.125	0.563	0.077	0.429	0.107	0.695	0.133	0.618	0.073	0.345	0.190	0.528
152	0.083	0.676	0.125	0.563	0.077	0.429	0.107	0.695	0.133	0.618	0.071	0.328	0.190	0.528
Best	0.083	0.676	0.143	0.625	0.083	0.476	0.107	0.695	0.154	0.676	0.086	0.448	0.200	0.556
Worst	0.077	0.647	0.111	0.500	0.077	0.429	0.083	0.598	0.133	0.618	0.071	0.328	0.190	0.528

Table 4.— Continued.

Tree	22 (cleithrmsh)		23 (suprorb)		24 (suborb)		25 (symplect)		26 (toothshp)		27 (minteeth)		28 (phararchshp)	
	CI	RI	CI	RI	CI	RI	CI	RI	CI	RI	CI	RI	CI	RI
5	0.083	0.542	0.097	0.533	0.088	0.475	0.053	0.507	0.118	0.423	0.136	0.472	0.045	0.222
10	0.083	0.542	0.097	0.533	0.091	0.492	0.053	0.507	0.118	0.423	0.130	0.444	0.045	0.222
18	0.083	0.542	0.097	0.533	0.088	0.475	0.053	0.507	0.118	0.423	0.136	0.472	0.047	0.241
26	0.083	0.542	0.097	0.533	0.088	0.475	0.053	0.507	0.118	0.423	0.130	0.444	0.045	0.222
31	0.083	0.542	0.094	0.517	0.091	0.492	0.053	0.507	0.118	0.423	0.130	0.444	0.045	0.222
62	0.083	0.542	0.083	0.450	0.086	0.458	0.050	0.479	0.118	0.423	0.120	0.389	0.049	0.278
78	0.083	0.542	0.081	0.433	0.086	0.458	0.050	0.479	0.118	0.423	0.120	0.389	0.050	0.296
148	0.087	0.563	0.081	0.433	0.083	0.441	0.050	0.479	0.118	0.423	0.120	0.389	0.049	0.278
149	0.087	0.563	0.081	0.433	0.083	0.441	0.050	0.479	0.118	0.423	0.125	0.417	0.049	0.278
152	0.087	0.563	0.081	0.433	0.083	0.441	0.050	0.479	0.118	0.423	0.125	0.417	0.050	0.296
Best	0.087	0.563	0.097	0.533	0.091	0.492	0.053	0.507	0.118	0.423	0.136	0.472	0.050	0.296
Worst	0.083	0.542	0.081	0.433	0.083	0.441	0.050	0.479	0.118	0.423	0.120	0.389	0.045	0.222

Tree	29 (vntcorner)		30 (pwvert)		31 (Rw)	
	CI	RI	CI	RI	CI	RI
5	0.056	0.244	0.093	0.519	0.065	0.341
10	0.056	0.244	0.093	0.519	0.065	0.341
18	0.056	0.244	0.093	0.519	0.065	0.341
26	0.057	0.267	0.095	0.531	0.065	0.341
31	0.056	0.244	0.095	0.531	0.065	0.341
62	0.054	0.222	0.098	0.543	0.069	0.386
78	0.054	0.222	0.098	0.543	0.069	0.386
148	0.053	0.200	0.098	0.543	0.069	0.386
149	0.053	0.200	0.098	0.543	0.067	0.364
152	0.053	0.200	0.098	0.543	0.067	0.364
Best	0.057	0.267	0.098	0.543	0.069	0.386
Worst	0.053	0.200	0.093	0.519	0.065	0.341

were 907 steps long, 24 trees that were 911 steps long, and 19 that were 885 steps long, respectively (Table 5). Each one of these constraint trees was contrasted against five representatives from each tree island from the unconstrained analysis to assess the importance of these factors on patterns of morphological variation. Results of these tests (Table 5) indicate that contrasts of the MP trees with constraint trees defined by mtDNA clades, current geography, and habitat were nearly always significant, indicating that the solution provided by parsimony analysis of the morphological data does not support a role of these other factors in shaping the distribution of the data among these populations.

The PCA scatter plots of all cases provided the basis (repeated for all comparisons) for a visual contrast of constraints by habitat, geography, and clade. Plots of PCII and PCIII, with cases identified to their mtDNA clade, habitat, and drainage basin (Fig. 16 upper, middle, and lower, respectively) were consistent with analyses of phylogenetic constraints, because there is greater separation among groups identified by PCA when identified by mtDNA clade than when considering drainage basins and habitats.

Fossil *Rhinichthys*.— Origins of *Rhinichthys*-, *Tiaroga*-, and *Oregonichthys*-like traits in the late middle Miocene of the Northwest is suggested by the concentration of fossils and general apomorphies of that age in Oregon and Washington. Three Pleistocene fossil localities, from Owens Valley, CA, Mono Lake, CA, and Glendale, NV, three Pliocene localities from Idaho, and three Miocene localities from Oregon and Washington provide support for the hypothesis that the fossil record of *Rhinichthys* is a plausible basis for dating most of the divergences over the past 12 million years. We suspect

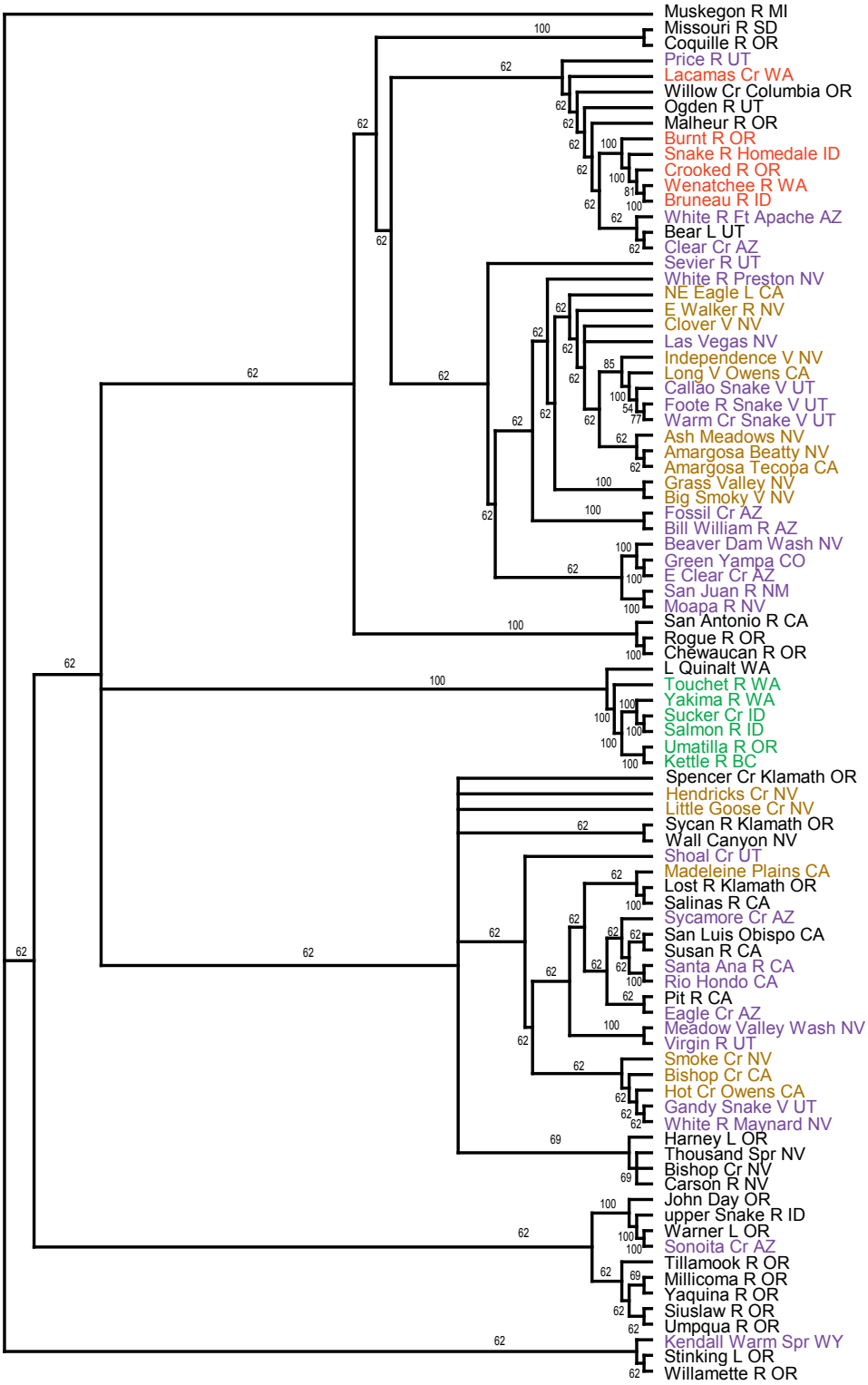


Figure 12.— Morphological tree for samples on the *R. osculus* group. Majority rule (50%) consensus representation of 156 Maximum Parsimony trees of 842 steps (from two tree islands) for 88 taxa based on 31 characters. Colors represent mtDNA maximum likelihood relationships: Brown, Lahontan Basin and peripheral captures; purple, Colorado drainage; red, *R. falcatus*; green, *R. umatilla*; black, Northwest clade. Three morphological samples of uncertain mtDNA relationships were drawn from streams with polymorphic haplotypes: Susan R., Lahontan and Northwest; Snake Valley, Lahontan and Colorado drainage; Goose Cr. Lahontan and Snake River.

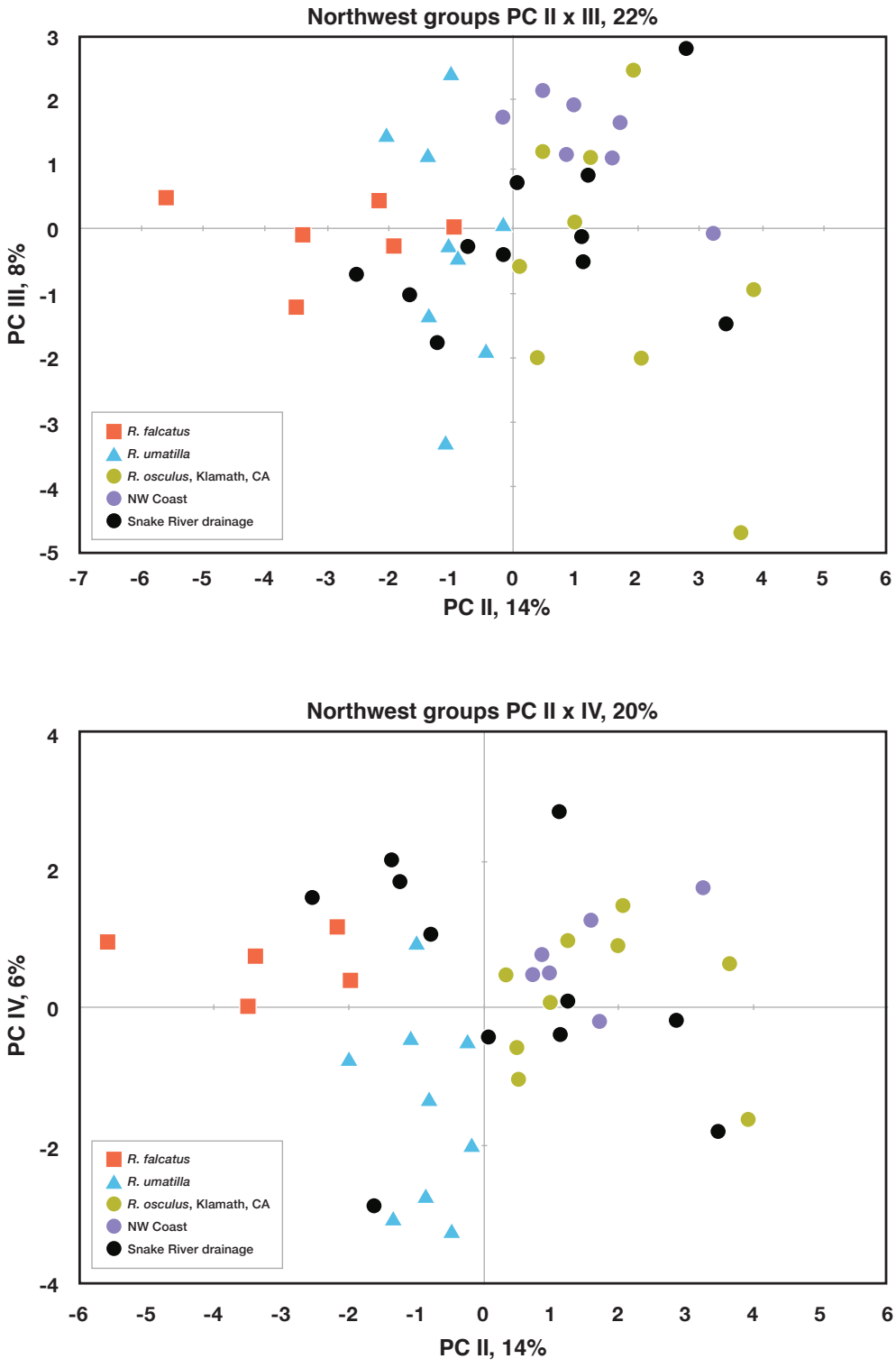


Figure 13.— Principal Component Analysis (PCA) of samples in the Northwest clade. Scatter plot of PC II X PC III demonstrates some cohesive similarities within *R. falcatus* on II and III; within *R. umatilla* on II and III; and within the northwest coastal samples on II and III. Samples from the Snake and Klamath drainages are dispersed. Components II and IV show better cohesiveness of the northwest coastal group and the Klamath group.

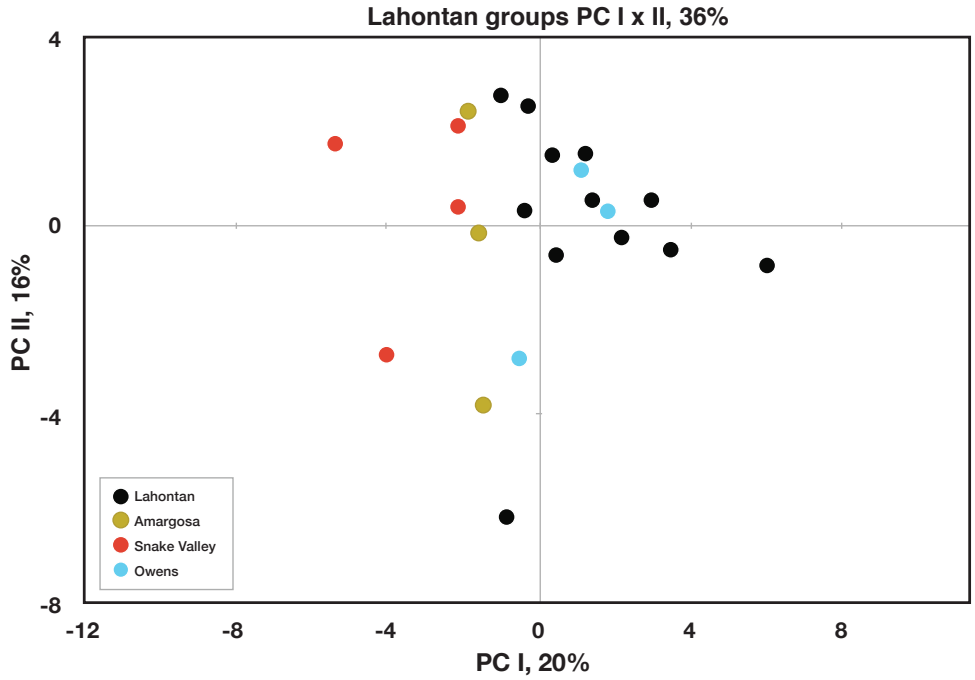


Figure 14.— PCA of samples in the Lahontan clade. Snake Valley samples are almost isolated on PC I and II, partly because these are small fishes, as are the Amargosa fish. There is no tendency for discrimination of any populations on components II and IV (not shown).

that the genus was absent before the middle Miocene, based on the lack of earlier fossils and results of the correction method of Marshall (1990). Based on such fossils, the *R. cataractae – R. osculus* ancestral lineage appeared to separate from eastern *Rhinichthys* at13.7 Ma while the *R. osculus* group appears to be identifiable as a lineage separate from *R. cataractae* in the Columbia and Snake rivers by about 7.8 Ma (estimates corrected by the method of Marshall, 1990).

The diagnostic features that identify these taxa include the flat, horizontal biting edge of the lower jaw, the separation of anterior sensory pores from the dentary bone, the distinctive acute angular shape of the post lateral edge of the pharyngeal arch, and the development of convex and sometimes slightly serrated cutting blades on the antero-dorsal sides of the grinding surface of the middle two (or more) pharyngeal teeth in the main row.

BEAST analysis of molecular data.— Molecular clock estimates (in millions of years) obtained from the BEAST analyses are presented based on their mean values and 95% highest posterior density (HPD) (Fig. 17). All parameter estimates had effective sample sizes greater than 4188. Running the analysis with the same settings, but without data confirmed that our input settings actually produced the desired prior probability distributions on our calibrated nodes and that our data were responsible for our results rather than our priors. The overall topology was similar to that obtained via ML, especially where bootstrap support for nodes was high. In a few cases well-supported nodes in the ML topology were recovered differently in the BEAST tree (e.g., the Amargosa River lineage, Fig. 8 vs. Fig. 17). In most cases the differences between BEAST and ML topologies were at nodes with poor or no bootstrap support (e.g., the Los Angeles lineage, Fig. 7 vs. Fig. 17). We base our discussions below on the ML tree because it has more complete sampling, while obtaining the estimates of divergence time and their HPDs from the BEAST analysis. These estimates of divergence time are based on mtDNA; therefore, the molecular clock may have been reset by introgression and must be interpreted with this in mind.

DISCUSSION

Patterns of evolution in the western *Rhinichthys osculus* complex appear to be unusual; there is less consistent morphological divergence than expected given the ~7.5 million years of extensive geological and geographic isolation (Figs. 12, 13, 14, 15). We investigated the potential roles of geography, habitat, and past history (as inferred from mtDNA) on evolution of *R. osculus* to explain the lack of unambiguous morphological diversification (Table 5, Fig. 16).

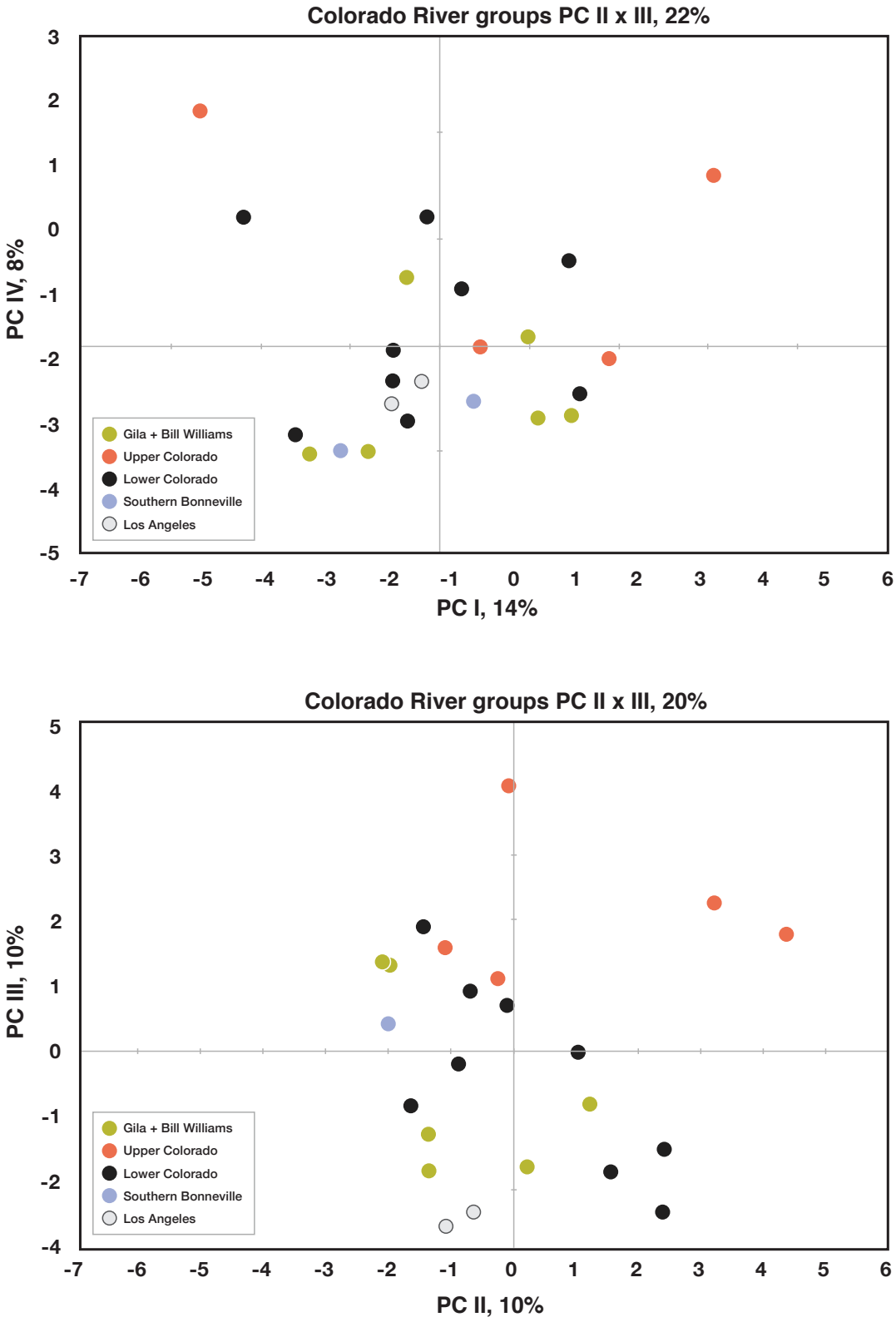


Figure 15.— PCA of samples in the Colorado R. drainage clade. There is no tendency for the samples from geographic units to form clusters, except that four samples from the Upper Colorado R. group tend toward the upper right quadrat in PC II x III. The two samples from Los Angeles are similar but not unique in either plot.

Table 5.— Statistical results from the three constraint analyses performed using PAUP (see Methods section for description of analyses). These analyses were designed to examine the relationship between habitat, current geography, and mtDNA clades on measured morphological variation. Constraint groups for each of the three analyses are provided in Appendix 1, and resulting trees tested against the MP trees. Statistics include length and number of constraint trees, number of statistical analyses for each constraint analysis, number and proportion of those tests that were significant, and the minimum, maximum, average and median significance values from all tree contrasts within each constraint analysis.

	MP	mtDNA	Geography	Habitat
length	842	885	907	911
# of trees	156	19	48	24
# tests	NA	190	480	240
# sig tests	NA	184	480	238
% sig	NA	0.968	1.000	0.992
min P	NA	0.032	0.008	0.018
max P	NA	0.041	0.017	0.027
average P	NA	0.035	0.012	0.022
median P	NA	0.035	0.012	0.022

Contrast of constraint trees representing each of these factors and the most parsimonious tree indicated that morphological data are not consistent with expectations generated from consideration of the mtDNA topology, current geographic distributions, and habitat. Results of morphological Principal Component Analyses were visually consistent with phylogenetic analyses of constraint trees (Fig. 16).

Analysis of the processes responsible for these patterns of evolution in western *Rhinichthys* begins with estimates of the places these dace populations occupied and the times of geological/hydrological barrier formation (vicariance) or breaching of geological barriers (dispersal) (Minckley et al., 1986; Hershler and Liu, 2008), with the further insight that each stream capture entails one vicariance and one dispersal event, often with initiation of at least one secondary contact. This geologic framework is helpful to understand controls on episodes of divergence and gene flow in fluvial fishes. Past climates and habitats, when known, can add more information about ecological divergence.

Time and place of origin of *R. osculus and R. cataractae*

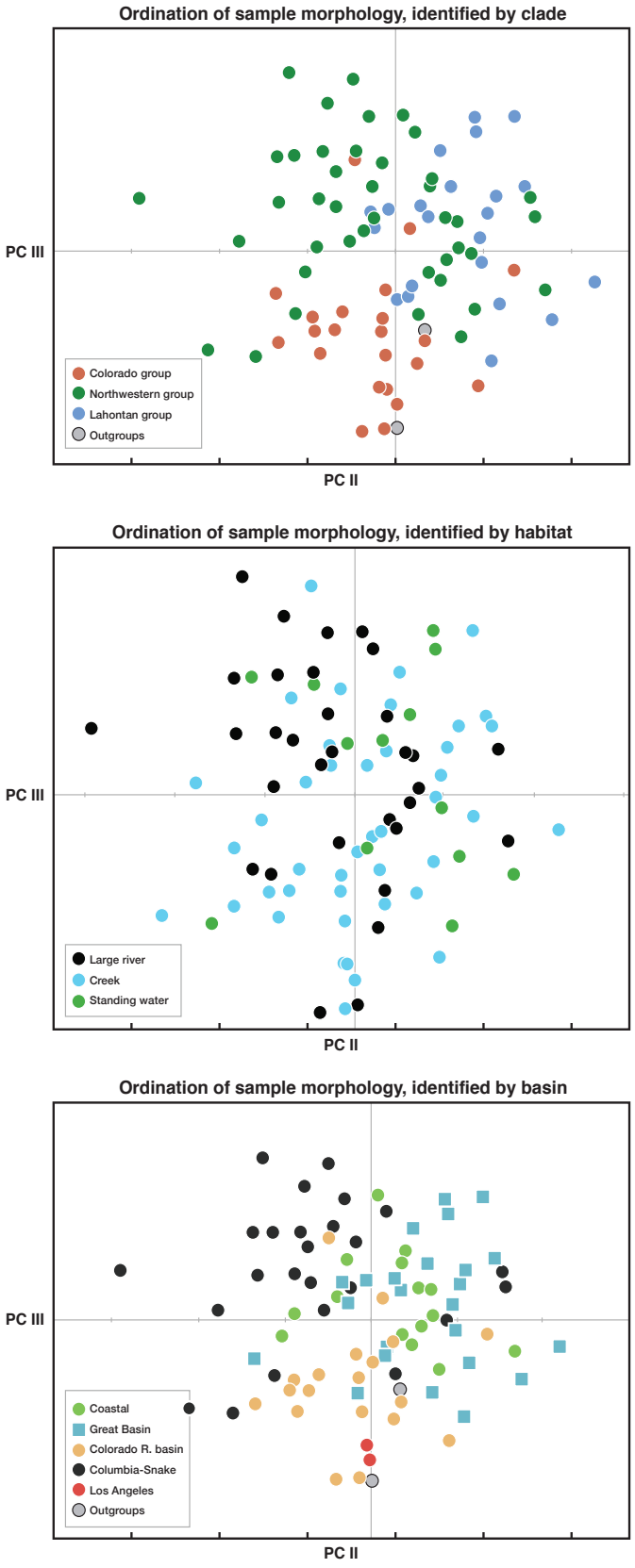
To estimate the time and place of origins of the *R. osculus* group and *R. cataractae*, we seek the best fit between the molecular estimate of calibrated age of the node of split between the two lineages, the location, age, and morphology of fossils near that node, possible barriers causing vicariance, the most plausible location of the ancestral species ~8–6 Ma, and geographic ranges of the two daughter lineages. Other indicators are the age and sister relationships of the basal nodes of the daughter lineages-the Columbia and coastal Pacific Northwest for *R. cataractae* group (including *R. evermanni*, Taylor et al., 2015) and the Columbia-Snake-Green-Colorado drainages for the *R. osculus* group (Fig. 17).

Regions that contain both the *R. osculus* and *R. cataractae* groups, parapatric or sympatric, as well as plausible barriers that could have led to their divergence, are in the northwest between the Continental Divide and the Pacific Coast. The place of origin is, therefore, estimated by locating the areas of geographic contiguity of the daughter species, plausible locations of basal nodes of the maximum likelihood tree, fossil *Rhinichthys*, sister out-groups in the east (Tipton et al., 2011), and possible ancient barriers for vicariance.

Rhinichthys osculus lives across most of the United States and southernmost Canada west of the Continental Divide. The western half of *Rhinichthys cataractae* lives in Atlantic drainages from the Rio Grande north to the Arctic, and the Snake, Columbia, and north Pacific drainages from the northern Bonneville Basin to the Arctic. Both species are found in the northern Bonneville, Snake, Columbia, and northwestern coastal drainages. Within those regions, possible barriers causing the split are the Cascades, the Blue Mountains between the Snake and Columbia rivers, and the Snake River - Green River divide south of Jackson Hole, Wyoming.

The sister relationship of *R. osculus* and *R. cataractae* groups is unambiguous, based on molecular and morphological data. The estimated mean age of their split is 7.8 Ma (95% HPD 9.9–6.5 Ma, Fig. 17; see also Smith and Dowling, 2008; Kim, 2013). The estimated time range of the *R. cataractae – R. osculus* ancestor is 13.7 to 7.8 Ma (95% HPD 15.4–6.5 Ma) based on two nodes estimated by BEAST (Fig. 17). The fossils of the earliest known *Rhinichthys* are from the Juntura Formation and Drewsey formations of eastern Oregon, and the Ellensburg Formation, central Washington (10.4–8.3 Ma). These fossils are morphologically consistent with expected apomorphies of the stem, *Rhinichthys* sp. The separation of the oldest of these fossils from *R. obtusus*, *Tiaroga,* and *Oregonichthys* is not certain (Table 3). If some of these Middle Miocene fossils actually represent related genera, the two unexpectedly long early branching lineages in Fig. 17 could be wrong.

The possible vicariance in the *R. osculus*-*R. cataractae* split possibly involved the Snake and Colorado rivers in the Jackson, Wyoming, area, the



Snake and Columbia rivers in the Blue Mountains, Oregon, area, or the Columbia or Snake and Hudson Bay drainages (Lemke et al., 1965) in western Montana or Wyoming. Other possibilities have been considered because of sympatry of *R. osculus* and *R. cataractae* groups in the Northwest. The Umpqua-Coos-Siuslaw-Coquille and Umatilla node is sister to the remainder of the Northwest clade and near the node connecting the Northwest clade to the Lahontan clade, but that node is more recent than the basal *R. osculus* node. Mean molecular clock estimates of ages of these populations are 3–2 Ma, much younger than 7.8 Ma datum.

The possibility of vicariance between the Snake River and Columbia region is consistent with the ages and localities of fossil *Rhinichthys*. The Blue Mountain (pre-Hells Canyon) barrier between the Snake and Columbia drainages in the late Miocene could have caused vicariance in the required 13.5–7.8 Ma time frame. That region is not far from the basal nodes, but is approximately equidistant from probable locations of all of them. The Bonneville Flood (~15 ka) contributed to some instances of gene exchange from the Upper Snake River to the Pacific Coast much later–a homogenization event recognized by application of the name "*carringtoni*" to populations in the northern Bonneville Basin, eastern Oregon, and California.

Figure 16.— Upper, Principal component ordination of samples of the *Rhinichthys osculus* group based on morphology, colored according to DNA clade groupings. *Rhinichthys falcatus* and *R. umatilla* are in the upper left of the northwestern clade. The clusters are consistent with the DNA cladistics, but with broad overlap of groups. Middle, Principal component ordination of *Rhinichthys osculus*-group morphology colored by habitat classification. Similarities that might relate habitat classification to general morphological groupings are largely absent, indicating dominant control by clade membership (as indicated by DNA, Fig 5) and drainage control by geology (below). See text and Table 5 (constraint analysis). Similarities to scatter plots above and below are obscure except for some large river phenotypes in the upper left (Columbia River) and lower right (Colorado River). Lower, Principal component ordination of morphological samples of the *Rhinichthys osculus* group colored by drainage basin of origin. The upper tan outlier of the Colorado drainage is from the Green River; the tan symbol near the center is *R. deaconi* from Las Vegas. *Rhinichthys falcatus* and *R. umatilla* are in the upper left, see Figs. 6, 13). Klamath and Great Basin populations are unusually diverse, unlike clusters colored according to DNA clades (upper panel).

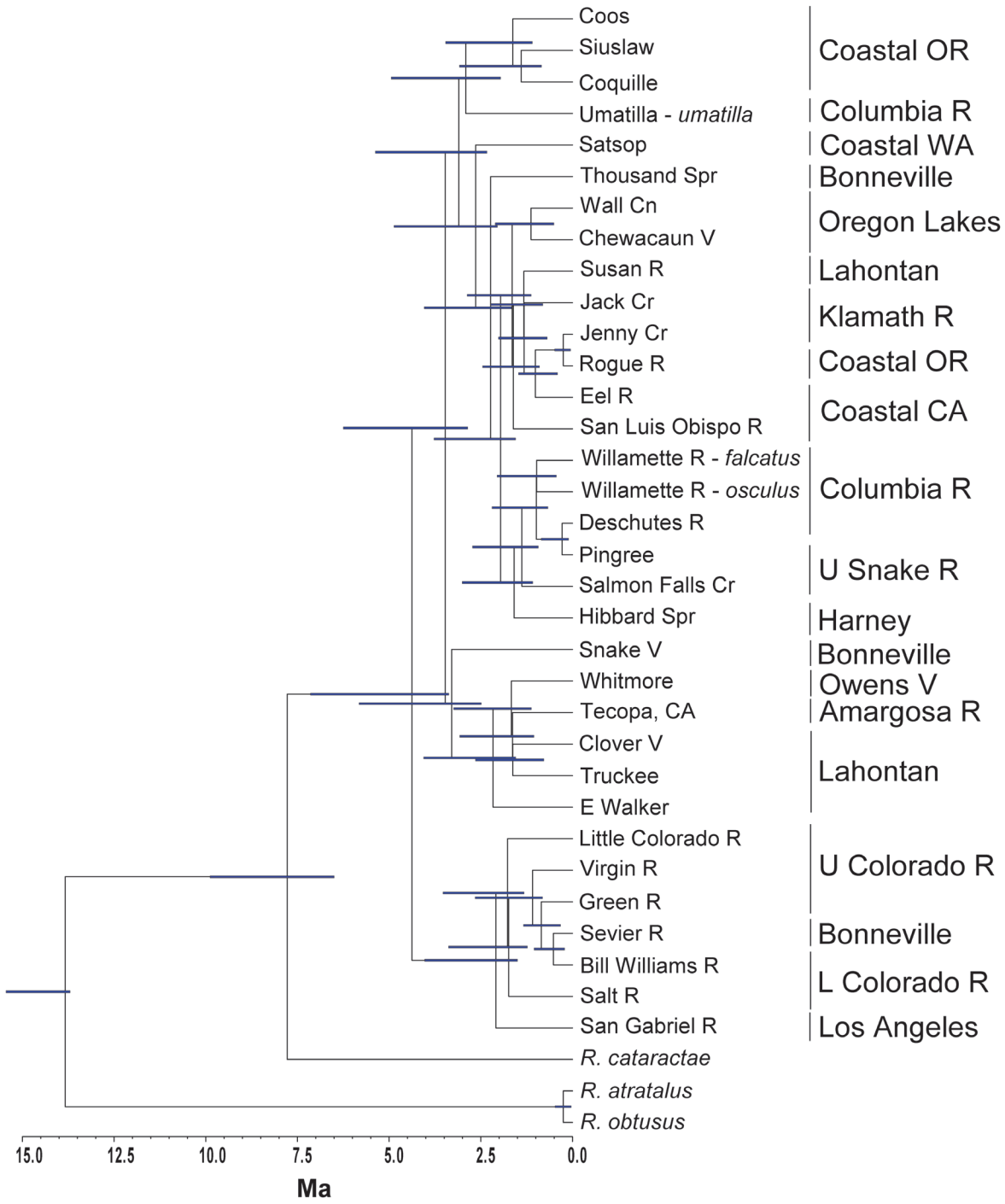


Figure 17.— Age estimates of nodes of *Rhinichthys* clades, with BEAST averaged confidence intervals.

The eastward shifting Continental Divide (Beranek et al., 2006; Spencer et al., 2008) between Hudson Bay and Columbia drainages in Washington, Idaho, and Montana is a possible place to separate populations, assuming that the *R. cataractae-R. osculus* ancestor was in the drainages being redirected by the migrating divide. The daughter species are in that region now.

The same applies to the Jackson region, Wyoming, with additional support from its earlier timing and its position near the likely site of the node connecting the Snake-Columbia-Lahontan clade to the Green-Colorado clade near their headwaters ca 6 Ma. (Beranek et al., 2006). Both species were west of the continental divide by the end of the Miocene (Kim, 2013). A barrier

between the upper Snake and Green River drainages south of Jackson, Wyoming is required to separate Snake River *R. osculus* from the Colorado basin. The estimated mean age of the basal molecular branch between those drainages is 4.4 Ma (95% HPD 7.1–3.4 Ma, Fig. 17). Support for a divide is provided by oxygen isotope evidence for high elevation near the Camp Davis Formation (Drummond et al., 1993) near Jackson, but the hypothesis that a barrier was in south-central Idaho in the Late Miocene (Beranek et al., 2006) is a possibility.

Headwater stream connections at high altitudes could have been similar to modern examples at Two-Ocean Pass in the Teton-Bridger National Forest, Wyoming (Evermann, 1895) and Isa Lake in Yellowstone National Park. The calibrated tree (Fig. 17) suggests that gene flow between *R. cataractae* and *R. osculus* ended 7.8 Ma (95% HPD 9.9–6.5 Ma). The vicariance event between the Snake River and Green River allowed divergence of the northwest (Columbia River and derived lineages in the Lahontan Basin) from the southeast (Colorado River) clades, in the Pliocene, 4.4 Ma (95% HPD 7.1–3.4 Ma). Temporal and geographical evidence for a former transfer and connection between the Green and Upper Snake drainages is provided by Beranek et al., (2006, figs. 8, 10).

Dispersal of *Rhinichthys osculus* south from the Green River through the Colorado River probably occurred during the first million years of transfer, thence to the Kaibab Plateau, and finally into the lower Colorado River basin after passage through Grand Canyon and Grand Wash ~5.5 Ma (Spencer et al., 2008). The divergence, or at least the most recent genetic mixing of lower Colorado River basin *R. osculus*, is younger than 4.4 Ma (95% HPD 7.1–3.4 Ma, Fig. 17). This estimate is close to the age of fossil *Rhinichthys* in the White Narrows beds (4.9–4.3 Ma, Smith et al., 2013). A morphological marker of the *R. cataractae – R. osculus* relationship occurs in the Colorado drainage, where fast-water *R. osculus* uniquely share *R. cataractae's* diagnostic frenum, a bridge of skin from the upper lip to the snout (Minckley and Marsh, 2009).

Divergence within the *R. osculus* group in Plio-Pleistocene Basins

Gene flow between the Snake River Plain and Washington and Oregon (Figs. 5, 6, 7) (Smith and Van Tassell, this volume) is estimated to be 3 Ma (95% HPD 6.3–2.9 Ma, Fig. 17). Evidence for initiation of a Late Pliocene fluvial connection between the Snake and Columbia rivers through Hells Canyon was presented by Wheeler and Cook (1954) and Smith et al. (2000). Isolation of the major branches of *Rhinichthys osculus* in major drainages of western United States occurred with the initial splitting of the Colorado River clade at 4.4 Ma (95% HPD 7.1–3.4 Ma), followed by the division of the Pacific Northwest and Lahontan Basin clades at 3.5 Ma (95% HPD 6.3–2.9 Ma, Fig. 17). *Rhinichthys* could have dispersed among rivers and streams tributary to the large Glenns Ferry Lake and later the Snake River on the Western Snake River Plain. Regional hydrographic diversity fostered metapopulation connections through dispersal routes and habitats aided by the long-lived lake (4.5–2.8 Ma). This is consistent with local generation of species-level taxonomic diversity in the *Rhinichthys osculus* species group (*R. umatilla* and possibly *R. falcatus*, see below).

The long-term persistence of aquatic habitat on the Snake River Plain served as a source for *Rhinichthys* in the Lahontan basin. The basal node (~3.3 Ma, 95% HPD 5.8–2.5 Ma) of the Lahontan clade is represented by one of the haplotypes in Snake Valley on the edge of the Bonneville Basin. Also on the divide between the Lahontan and Bonneville Basin, 300 km due north, the Rock Creek sample in Thousand Springs drainage is at a basal node in the Snake River - Bonneville drainage, at 2.2 Ma (95% HPD 4.0–1.7 Ma). The connections between the Lahontan and Bonneville basins, 3.1–2.5 Ma, must have been restricted to headwater transfers because they are not shared by suckers and other minnows in these basins, which were isolated earlier. The peripheral connectives on the west side of the Lahontan Basin are Death Valley and Owens Valley (Figs. 5, 8, 9) in the Pliocene, 3.5 Ma (Fig. 17). The complex geological basis for these connections was documented by Reheis et al. (2002).

Late Pliocene drainage changes in the Pacific Northwest permitted gene flow among *Rhinichthys osculus*-group ancestors and coastal streams of California, Washington, and Oregon at 3.1 Ma (95% HPD 5.4–2.3Ma, Fig. 17). The maximum morphological and DNA diversity in *Rhinichthys* occurs in the Pacific Northwest U.S., including the Snake River Plain. *Rhinichthys cataractae, R. osculus, R. falcatus,* and *R. umatilla* co-occur where the drainages were dominated by the Pliocene Glenns Ferry lake, which existed in the Western Snake River plain but not the Columbia drainage over the time period during which *R. umatilla* and *R. falcatus* diverged from *R. osculus*.

Rhinichthys falcatus* and *Rhinichthys umatilla.—Our examination of the literature and museum specimens suggests problems with identifications within the *R. osculus* group. *Rhinichthys falcatus* (from the Boise, Snake, Bruneau, Burnt, Lacamas, Wenatchee, and Willamette rivers, Figs. 2, 6, 7, Table 2) is highly apomorphic, generally characterized by long, streamlined snouts, large, falcate dorsal fins, anal fin origin usually ventral to the last dorsal ray, slender caudal peduncles, 34-37 vertebrae, robust cleithrum and radials, and large, dark "leopard" blotches on the sides consisting of irregular groups of melanophores not

restricted to individual scales (Appendix 1, Fig. 2, see also Wallace and Zaroban, 2013, p.115). The fusiform body shape and unusual fins suggest adaptation to rapid currents of rivers. The color pattern suggests cryptic avoidance of predation by piscivorous birds in clear water. The color pattern of *R. falcatus* in the Willamette River collected by DFM and TED are consistent with a swift, clear-water habitat hypothesis.

Samples of *R. umatilla* (from the Umatilla, Touchet, John Day, Salmon, Yakima rivers, and Sucker Creek, Appendix, Figs. 2, 6) usually have a smaller, less pointed snout, less streamlined bodies, smaller less falcate fins, the origin of the anal fin antero-ventral to the last dorsal fin ray, thicker caudal peduncle, 33–36 vertebrae, teeth often with grinding surfaces behind the cutting blades, less robust cleithrum and radials, and melanophores prominently confined to individual, dispersed scales on the sides. These features are consistent with the hypothesis that this species is adapted to slower parts of rivers.

Rhinichthys osculus usually has a smaller, less pointed snout, less fusiform body, smaller, less falcate fins, origin of the anal fin post-ventral to the last dorsal ray, thick caudal peduncle, 33–36 vertebrae, less robust cleithrum and radials, and minute melanophores, often forming a horizontal stripe or stripes on the sides. Much variation exists, consistent with the hypothesis that *R. osculus* is a generalized species with many locally-adapted lineages (Appendix 1, Figs. 2, 12).

Morphological, osteological, meristic, and mtDNA traits among the three species overlap at many localities, comparable to the decoupled morphological, allozyme, and mtDNA distributions among individuals of *Luxilus cornutus* and *L. chrysocephalus* (Dowling and Moore, 1984; Dowling et al., 1989, 1997). Distribution and variation of key characters of western *Rhinichthys* species suggest high levels of homoplasy, influenced by past introgression and convergence. Much taxonomic confusion resulted from illustrations of the type specimen of *R. umatilla* from the mouth of the Umatilla River (Fig. 2a) because the type illustration depicts a specimen with a rather slender caudal peduncle and falcate fins as found in *R. falcatus*, not *R. umatilla*. Carter Gilbert (pers. comm., January 26, 2016) examined the type and compared the illustration and suspects that they might not be the same individual. Based on these non-congruent traits, we suspect that the specimen figured in Gilbert and Evermann (1894) was a hybrid between *R. falcatus* and *R. umatilla*. As a consequence, many early northwest collections by Hubbs and Schultz and their followers were misidentified.

More seriously, mtDNA sequences of some individuals are inconsistent with morphologies of *R. falcatus, R. umatilla*, and *R. osculus* where they co-occur. For example in the Boise River, 9 of 10 specimens in our sample of morphological *R. falcatus* have *R. osculus* mtDNA, reflecting introgressive hybridization between these species. Additionally, *R. falcatus* and *R. umatilla* phenotypes in British Columbia are slightly inconsistent with the phenotypes of those species at the type localities and elsewhere in the Columbia and Snake River drainages (Peden and Hughes, 1988, figs. 2, 3, 4, 6; and Fig. 2 of this paper). Peden and Hughes's fig. 2 is, as they indicate, *R. osculus*, with a blunt snout, small fins, subterminal mouth, non-falcate fins, nondescript color pattern, and a deep caudal peduncle. Peden and Hughes's fig. 3, labeled *R. umatilla* from Kettle River, possibly followed from a comparison with the type specimen and differs from our *R. umatilla* in its sharp snout, ventral mouth, falcate fins and leopard blotched color pattern, which diagnose *R. falcatus* in Idaho and Oregon. Peden and Hughes's fig. 4, labeled *R. umatilla* from the Similkameen drainage would be identified as that taxon if it were found in the southern Columbia drainage. Peden and Hughes fig. 6, labeled *R. falcatus* from the Fraser River, is typical of our concept of that species, with its sharp snout, pelvic stays, blotched leopard color pattern, falcate anal fin, and slender peduncle, but the non-falcate dorsal fin is uncharacteristic of *R. falcatus*. The mtDNA sequence and position in the tree (Fig. 6) with our Fraser River sample is consistent with *R. falcatus* (McPhail and Taylor, 2009). The *R. osculus* figured in Wallace and Zaroban (2013), p. 119, appears to be a mixture of *R. osculus* and *R. falcatus*, as are most of our samples from the Boise River, because of its long snout, slightly slender peduncle, large rather falcate fins, and blotched pigment pattern. The *R. falcatus* figured in Wallace and Zaroban (2013), p. 115, is a typical representative of that species. (The fleshy pelvic stays figured there are often referred to as a key character in *R. falcatus* but are not universally present.)

Wydoski and Whitney (2003, figs. 43–46, pages c21, c22) illustrate five *Rhinichthys* specimens with helpful color photographs. Figure 43 shows typical traits of *R. cataractae*. Figure 44 illustrates the characteristic long snout, slender caudal peduncle, falcate fins, anal origin behind the last dorsal fin ray, and large blotches on a pale background of *Rhinichthys falcatus*, the Leopard Dace, as they indicate. Figure 45, called an Umatilla dace, shows a specimen that we would identify as a hybrid between *R. falcatus* and *R. umatilla* or *R. osculus* because of the combination of long snout, large blotches, moderately long, intermediate caudal peduncle, and falcate fins (of *R. falcatus*), small eye, and anal fin origin antero-ventral to the last dorsal fin ray of *R. umatilla* or *osculus*. Figures 46a and 46b are identified as *Rhinichthys osculus* by Wydoski and Whitney (2003). In our analysis, fig. 46a, from the Yakima River, would

be called *R. umatilla* because of its robust body shape, short snout, small rounded fins, and black spots confined to random scales on a paler background. Specimen 46b, from Swamp Creek, tributary to Lake Washington, has a small, rounded snout, faint spots confined to random scales on a pale background, and anal fin origin ahead of the last dorsal fin ray, as in *R. osculus*. It has several intermediate traits–an intermediate caudal peduncle and medium large, slightly falcate fins–suggestive of *R. falcatus*.

The hypothesis that *R. umatilla* originated from hybridization between *R. osculus* and *R. falcatus* (Haas, 2001) presumes an earlier origin of *R. falcatus* than 1.0 Ma (2.1–0.4 HPD) Ma (Figs. 6, 17), but our phylogeny suggests that *R. umatilla* may be much older ~3.1 Ma (5.4–2.3 HPD) Ma than *R. falcatus*. The ages, distributions, and osteology do not support a hybrid origin for *R. umatilla*.

Willamette River populations of *R. falcatus* and *R. osculus* appear to be reproductively separate and independent lineages (Fig.6), while Boise River *R. falcatus* show introgression with *R. osculus*. *Rhinichthys falcatus* forms a monophyletic cluster imbedded within Columbia River *R. osculus*, and is sister to Willamette River *R. osculus* (Fig. 6). What we do not see in the Willamette River is *R. umatilla* or a hybrid swarm.

If this complex has experienced reticulate evolution, we do not expect algorithms designed to discover bifurcation to recover the actual pattern (Smith, 1992; Martinsen et al., 2001; Mallet, 2005), nor that the recovered dendritic patterns make geographic sense. *Rhinichthys osculus, R. falcatus,* and *R. umatilla* deserve further study of their reproductive biology and the relationship of convergence and (or) introgressive hybridization to adaptive phenotypes.

Samples of mtDNA from *R. umatilla* may be embedded within the Columbia River subclade of the Northwest clade and are distributed most widely below the Pliocene barrier between the Snake and Columbia basins, which existed until 3.5-2.8 Ma. Samples of *R. falcatus* mtDNA are embedded deeply within the Snake River subclades of the Northwest clade and their distributions are slightly weighted toward the Snake River basin, above the Blue Mountains-Seven Devils Barrier, cut by Hells Canyon in 3.5-2.8 Ma. The ages of *R. umatilla* (~3 Ma) and *R. falcatus* (~1 Ma) are consistent with the hypothesis that these two species are descendants of an ancestor, sister to *R osculus*, which was distributed in the Columbia and Snake river basins before and during their isolation from each other in the Pliocene. Each species is now distributed above and below Hells Canyon and they exhibit evidence of introgression. Alternatively, these two species may have arisen within the Pacific Northwest mtDNA lineage, making *R. osculus* paraphyletic. The latter alternative is rejected because there are many instances of morphologically-identified *R. osculus*, *R. umatilla*, and *R. falcatus* with discordant mtDNA haplotypes. In either interpretation, the *R. osculus* group members meet the criteria for a metapopulation as described by de Queiroz (2007).

Pacific Northwest Lineage Divergences.— The inconsistencies among traits in the *Rhinichthys osculus* group suggest past gene flow among taxa. Prior to the Miocene, the Willamette-Puget Sound lowland was a shallow sea as far south as Salem, OR with a broad coastal plain presumably with multiple drainages from the Western Cascades (Orr et al., 1992). The oldest event in this clade (Figure 6) involves the Coastal Oregon, Umatilla, and Columbia/Klamath drainages. After the Western Cascades drainages were colonized by ancestral dace ~3 million years ago, the north coastal Oregon drainages could have been isolated from Columbia and Willamette drainages by Cascade volcanism. The oldest fluvial deposits in the Willamette Valley are poorly dated but paleobotanical evidence suggests late Miocene to Pliocene (Yeats et al., 1996). Our estimates for the polytomy (Figure 6) range from 3.1–2.6 Ma (95% HPD 5.4–2.1 Ma, Fig 17), which are consistent with Pliocene events. The central Oregon coastal fauna has a similar position in the *Catostomus macrocheilus* clade (Kettretad and Markle, 2010) and, with the Chehalis fauna, in the *R. cataractae* clade (Kim, 2013; Taylor et al., 2015).

The primary dichotomy in the remainder of Figure 6 is the Klamath/Sacramento versus Columbia/Snake divergence. These clades diverged in the early Pleistocene, 2.0 Ma (95% HPD 3.8–1.6 Ma), and subsequently became unusually diverse and geographically widespread. A similar dichotomy is seen in *Catostomus* (Kettretad and Markle, 2010). Divergence events within these clades were probably influenced by cascade volcanism and Pleistocene climate fluctuations, which have since obscured much evidence of barriers and hydrography.

The Columbia-Snake drainage is currently the fourth largest watershed in the United States at 668,000 km^2 (258,000 mi^2); it is densely inhabited by a diversity of *Rhinichthys* (Fig. 2), its most common fishes. Pleistocene Lake Bonneville (51,000 km^2, 19,691 mi^2) augmented the area of the Columbia-Snake drainage to ~ 719,000 km^2 when it was tributary to it, about 15 ka (Malde, 1968; Link et al., 1999). MtDNA sequence divergences among pairs of fishes indicate connections between the Bonneville and Snake basins at 2.2 Ma (95% HPD 4.0–1.7 Ma, Fig. 17; Johnson and Belk, 1999; Johnson, 2002). The pre-Lahontan basin (more than 22,000 km^2, 8,500 mi^2) could also have significantly augmented the Columbia-Snake basin if

and when it was tributary to it (Reheis et al., 2002). The presence of some Bonneville Basin fish in all three major clades (Figs. 6, 8, and 10) suggests complex pre-Bonneville vicariance and dispersal. In the Columbia-Klamath clade, Bonneville fish are sister to both as well as imbedded in the Columbia-Snake clade. Pleistocene overflow of the Bonneville Basin and perhaps the pre-Lahontan basin into the Snake-Columbia River drainage would have been significant gene flow events in *Rhinichthys* history.

The isolated fauna of Harney Basin has been described by Bisson and Bond (1971) and Hoekzema and Sidlauskas (2014). Both studies suggest multiple invasions and two lineages of dace in the basin. We sampled one of the forms, Harney Spring Dace (including dace from Hibbard Spring and Barnyard Spring plus the Stinking Lake Spring Dace of Hoekzema and Sidlauskas, 2014). Dace from the adjacent Malheur River are sister to Snake River (Salmon River, Fig. 6), but the polytomy including Harney Spring Dace does not inform relationships (Fig. 6). Harney Spring Dace are related to Columbia *R. falcatus* and Columbia *R. osculus*, with an estimated mean divergence of 1.6 Ma (95% HPD 3.0–1.1 Ma, Fig. 17). Hoekzema and Sidlauskas (2014) provided a broader divergence estimate for Stinking Lake Spring Dace, 1.3 – 4.0 Ma, noting that it was not closely related to dace in the basins southwest of Harney Basin, in the Oregon Lakes. Our analyses align Oregon Lakes dace with the Klamath/Sacramento clade and the Harney Spring Dace with the Columbia/Snake clade (Figs. 6 and 17). Harney Basin is a large, closed depression with a volcanic Miocene-Pliocene history (Orr et al., 1992). The oldest fluvial sedimentary rocks in the basin are the Wrights Point Stratum (2.4 Ma) which includes fish bones (Niem, 1974) while some lacustrine sediments may be middle to late Miocene (Walker, 1979), It therefore seems likely that the Harney Spring Dace are relicts of a widespread ancestor, possibly including all Columbia/Snake *R. osculus*, exclusive of *R. umatilla* and the central Oregon coastal and Chehalis isolates (Fig. 6).

Differentiation of *R. umatilla* in the Umatilla, Yakima, and other Columbia drainages, as well as *R. osculus* in the Coos, Umpqua, Siuslaw, and Coquille coastal drainages in Oregon, occurred in Late Pliocene and early Pleistocene (Figs. 5, 6, 7), 2.9 Ma (95%HPD 4.9–2.0 Ma). At the same time, the *Rhinichthys* group in the western Snake River plain tributaries and distributaries–the Upper Snake, Bonneville, Thousand Springs, Salmon Falls, Snake Valley, Oregon Lakes, Boise, Harney, Malheur, Klamath, Sacramento, and their connectives–would have been accumulating their distinctive genetic combinations and morphological divergence (Figs. 5, 6, 13).

Lahontan Lineage Divergences.— MtDNA evidence (Figs. 8, 9) for crossing of the barrier between the Snake River drainage (including the Oregon Lakes region) and the Lahontan basin is suggested by ambiguous geological evidence. Reheis et al. (2002) noted the possibility of high pre-Lahontan lake levels in the Pliocene and suggested a possible connection across the barrier between the pre-Lahontan basin in Nevada and Alvord Valley in Oregon (Reheis et al., 2002, fig. 1). The last gene flow between *Rhinichthys* of the Snake River and the western Great Basin occurred 3.5 Ma (95% HPD 6.3–2.9 Ma) according to molecular clock estimates (Fig. 17). After colonization of the pre-Lahontan Basin in the later Pliocene and early Pleistocene, sorting of *Rhinichthys* genetic combinations around the edges of the Humboldt River drainage would have been under way. Snake Valley, at the eastern edge of the basin, and East Walker River, at the southwest corner of the basin (Figs. 8, 9), are the earliest branching lineages to retain their distinct haplotypes (3.3 Ma, 95% HPD 5.8–2.5 Ma and 2.2 Ma, 95% HPD 4.1–1.6 Ma, respectively, Fig. 17). The northern edge and Humboldt drainage should have been colonized first; their more recent divergence times (2.2–1.6 Ma; Figs. 8, 9) probably reflect gene flow during contact among populations in the Humboldt drainage (Figs. 9, 14). Panmictic genetic contact through the Humboldt drainage would have occurred later, when the Pleistocene glacial and pluvial cycles were creating humid climates, large fluvial discharge and overflow among Great Basin lakes (Reheis et al., 2002).

Colorado River Drainage lineages.— *Rhinichthys* colonized the Green-Colorado drainage on the Colorado Plateau prior to 4.4 Ma (95% HPD 7.1–3.4 Ma, Fig. 17) from the upper Snake River drainage, before they reached the Lahontan Basin and before they had access to the Lower Colorado drainage in the Great Basin (Figs. 9, 10). Possible alternations of isolation and dispersal are seen in the high frequency of post-3 million year old nodes in Fig. 10 (dates in Fig. 17), which could have occurred during 20 to 100 major Pliocene and Pleistocene climate fluctuations (Andrews, 2000; Wright, 2000) and pluvial periods. Other branches in this multiple-branched node include connectives of the Green-Colorado drainage and the southern Bonneville Basin (Smith and Dowling, 2008), with nodes dated at 2.1–0.5 Ma, and the Gila Basin, also with Pleistocene nodes (Figs. 10, 17). The most important aspect of this branching pattern and its dates is evidence that immigration from the upper Colorado River on the Colorado Plateau to the Lower Colorado River in the Great Basin (Virgin, Pluvial White, Bill Williams, Gila drainages) occurred after the connection of the Colorado River through Grand Canyon and Grand Ledge at about 5.5 Ma (Faulds et al., 2008; Dorsey, 2012). The long

branch (Fig. 10) and estimated date of the Los Angeles Basin *Rhinichthys* (10, 17) are attributed to the rapid molecular substitution rate in these fish, predicted by the low elevation and warm temperatures of the Los Angeles Basin (Estabrook et al., 2007). Colonization of the Los Angeles Basin might have occurred at about 2.1 Ma (95% HPD 4.0–1.5 Ma), according to the dates (Fig. 17) for the nodes for the lower Colorado River basin (Fig. 10).

Summary: Divergent, Convergent, and Introgressed Evolution

Deep divergences among branches of mtDNA sequences indicate that lengthy isolation has been important in the evolution of the *Rhinichthys osculus* complex. Patterns of mtDNA variation are generally hierarchical, reflecting hydrographic isolation over the last several million years (Figs. 6, 8, 10, 17). Despite the history of isolation indicated by mtDNA, morphological divergence has been incomplete and mixed. This may be because divergence in small, fluvial fishes like western *Rhinichthys* is allopatry-based, requiring isolation of drainages by distance or by geological barriers that remained in place long enough to permit accumulation of adaptive genetic variation.

Figures 5 and 17 show long early branches with no nodes between 13.8–7.8 and 7.8–4.4 Ma. We assume that *R. osculus* was widespread through at least the Snake and Columbia river drainages during this long period and that population differentiation occurred in the many separate drainages in the Northwest. If so, the absence of ancient branches from this time implies a large metapopulation, somewhat like the modern one, with lineages subject to random local extinction and replacement or occasional introgression, with mtDNA replacement.

During the entire ~10 million year history of *Rhinichthys*, fish are assumed to have responded, ecophenotypically and genetically, to local water velocity, seasonal and longer-term temperature, water-abundance cycles, food sources, nutrition for growth, predators, predator-avoidance shelters (or not), light penetration, or stochastic change associated with fluctuating population sizes. We assume that barriers and habitat conditions remained relatively stable for sufficient times to accumulate and fix morphological, locomotor, and physiological differences, but our examination of mtDNA and morphological structure among populations of these fishes, suggests incomplete genetic separation of evolutionary lineages. Because molecular markers and morphological structures are not congruent, long branches of DNA markers are not sufficient, without morphological evidence, to demonstrate evolutionary independence.

The lack of unique (unshared) morphological traits for most of the populations or groups of *Rhinichthys osculus* suggests that environmental stability and isolation have not persisted sufficiently long to form irreversibly independent lineages. Neogene climate fluctuations and stream connections possibly caused frequent local extinction or allowed gene exchange that interrupted selection for diverging adaptive morphotypes, preventing assortment of unique morphological traits in lineages. We hypothesize that many diverging populations were terminated by extinction or reversed by temperature and moisture fluctuations in the rain-shadows of the California Coast Ranges, Sierra Nevada, and Cascades (Kohn et al., 2002), and pluvial cycles in the Great Basin (Reheis, 2008).

Because of the hundreds of isolated drainages in the Basin and Range province, there have been many potential opportunities for vicariance and habitat differences to contribute to divergence. The maximum likelihood tree demonstrates many diverse groups in a variety of drainages and habitats; however, most of these groups are not unambiguously diagnosed by unique, non-overlapping morphological characteristics (Figs. 13, 14, 15). The most extreme example of molecular divergence involves *Rhinichthys* from the Los Angeles Plain, with mtDNA divergence of ~7% and a suggested origin about 2 Ma; however, no external morphological traits and only one osteological difference was identified. The Los Angeles populations defied previous attempts to discover evidence of morphological uniqueness (Cornelius, 1969).

Only in the large and stable Mio-Pliocene rift lake drainages of the Snake River Plain and the high water volumes of the Columbia River drainage have climatic and hydrologic conditions persisted long enough (1–4 my) to permit accumulation of unique morphological traits. The process of speciation in these fishes included physiological and morphological responses to ecological conditions that promoted different ecological preferences. Here we find three species, *R. osculus, R. falcatus,* and *R. umatilla*, in addition to *R. cataractae*, that have evolved recognizable sets of locomotor adaptations (different snout, fin, and body shapes) to different velocities and turbulent shear stresses of rivers. Body-shape, fin-shape, and behavioral adaptations permit individuals to choose diverse foraging zones and possibly mate assortatively at different spawning times and places in many northwest drainages (Gee and Northcote, 1963; Scott and Crossman, 1973; Wydoski and Whitney, 2003; Wallace and Zaroban, 2013).

Inconsistent combinations of morphological and molecular data demonstrate that either convergence or hybridization occurred often throughout the *Rhinichthys osculus* group. The dearth of sympatric species suggests that divergence has advanced, but has been interrupted, a process described by McKay and Zink (2014) as Sisyphean evolution—divergent trends interrupted by gene flow.

Convergent adaptations to flow are suggested by terete body shapes with large falcate fins in other swift-water inhabitants in the intermountain west. *Rhinichthys cataractae* is terete and is also more completely reproductively isolated from *R. osculus, R. falcatus,* and *R. umatilla* as a consequence of longer competitive evolution with them in the more stable, high-gradient tributaries of the north (Gee and Northcote, 1963). In the south, however, large volume, high-gradient tributaries have been geologically and climatically less stable. As a consequence, some *R. osculus* in the Colorado River drainage have slender, swift-water-adapted bodies, large falcate fins, and reduced swim-bladders (Smith and Dowling, 2008, fig. 4, bottom) similar to species in the Columbia River drainages (Gee and Northcote, 1963; Peden and Hughes, 1988, figs. 2, 4). Convergent forms in the Green and Colorado rivers (Wyoming, Colorado, Utah, and Arizona), the pluvial White River (Nevada), and White River (Arizona) indicate large-volume fluvial habitats that selected for swift-water phenotypes in pluvial stages of the Pleistocene. Existence of the large-river phenotype in the incised pluvial White River canyon (Hubbs and Miller, 1948, figs. 25, 26, 27), with its anomalously small modern stream, suggest that aridity has reduced those fluvial habitats, but some rare derived phenotypes remain in place (Williams, 1978) as ghosts of selection past. In the present analysis, only *R. cataractae,* and to a lesser extent, *R. osculus, R. falcatus,* and *R. umatilla,* can be diagnosed as species with genetically separate evolutionary trajectories. The populations in the Colorado River, Moapa River, and White River, Arizona, are divergent with locally distinctive shapes, so may be genetically separate from their nearby congeners (Smith and Dowling, 2008); future population genetic analysis of more developmental genes and characters will be important.

Many molecular markers are effectively neutral, having little to no impact on fitness. This contrasts directly with many morphological features, which respond to challenges provided by the environment; therefore, molecular markers and morphological structure often describe different histories. Evidence of splitting in neutral molecular markers may indicate genetic contact that postdates initial splitting of populations although these synapomorphies and splits are usually assumed to date the initial splitting of lineages (Neigel and Avise, 1986).

Introgression is expected to be more common in populations that diverged in allopatry, but were secondarily placed together in small habitats by stream diversions. This process is mimicked by Federal and State agency introductions of fishes into rivers containing native species with no historical exposure and lacking isolating mechanisms (Halverson, 2010).

Implications for conservation.— Evidence from patterns of mtDNA and morphological variation indicates that *Rhinichthys osculus* persists as a series of metapopulations. During periods of increased aridity, subpopulations are isolated and smaller, causing them to lose genetic variation more rapidly and become divergent from other independently evolving subpopulations. As water levels rise, divergent subpopulations become reconnected, allowing for exchange of genetic material and replenishment of genetic variation in newly connected subpopulations.

This structure has significant implications for the evolution and conservation of this group. The cyclical nature of this system has been quite regular, with pluvial high water periods occurring approximately every 100 ka (Shackleton, 2000). Such periodic exchange allows for divergence of neutral markers; however, cyclical fluctuations of water levels select for maintenance of a generalist phenotype typical of most subpopulations (Smith and Dowling, 2008, fig. 4, bottom). The future is expected to be different, with fewer chances for a return to pluvial conditions. The well-watered Columbia-Snake system has been more protected from aridity in the past, and this region exhibits some concordance between molecular and morphological diversity. The future is expected to be drier also because of water withdrawal for agriculture, overgrazing destruction of vegetation, soil, and permanent water (Smith and Stearley, in press), and global climate change.

While such systems can function when the natural balance is maintained, they are sensitive to human impacts and pose especially difficult problems for conservation and management. Increased demands for water caused by urban growth and agriculture have reduced habitat volume and connectedness of populations (Smith and Stearley, in press). Additional threats posed by human activities (e.g., habitat degradation, pesticide poisoning, and introduction of nonnative fishes) make it difficult for these and other desert fishes to persist in such modified environments. The ultimate result is the loss of local subpopulations, reducing the accumulation of genetic variation in the metapopulation, and reduction of the potential for connection, preventing replenishment of genetic variation through exchange among formerly isolated subpopulations. Given these issues, the ultimate challenge for managers is to find ways to prevent extirpation of subpopulations as well as devising means for replenishing genetic variation by developing programs that foster communication among individuals in divergent subpopulations. While the former task is difficult in its own right, the latter is especially problematic because it necessitates a firm understanding of the evolutionary process and development of protocols for facilitating natural exchange of individuals among

isolated subpopulations in changing climates. Without such an approach, these metapopulations will eventually fail, leading to increased rates of extinction and human-fueled loss of biodiversity.

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