PHYLOGEOGRAPHY OF RHINICHTHYS CATARACTAE (TELEOSTEI: CYPRINIDAE): PRE-GLACIAL COLONIZATION ACROSS THE CONTINENTAL DIVIDE AND PLEISTOCENE DIVERSIFICATION WITHIN THE RIO GRANDE DRAINAGE

A Thesis

by

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MASTER OF SCIENCE

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ABSTRACT

The longnose dace, *Rhinichthys cataractae*, is a primary freshwater fish inhibiting riffle habitats in small headwater rivers and streams across the North American continent, including drainages east and west of the Continental Divide. Phylogenetic analyses of 1140 base pairs (bp) of the mitochondrially encoded cytochrome *b* gene and 2298-2346 bp of the nuclear-encoded genes S7 and RAG1 were obtained from 87 individuals of *R. cataractae* (collected throughout its range) and from several close relatives recovered a monophyletic *R. cataractae* species-group that contained *R. evermanni*, *R.* sp "Millicoma dace" and *R. cataractae*; a monophyletic *R. cataractae* was not recovered. Within the *R. cataractae* species-group, two well-supported clades were identified, including a western clade (containing *R. evermanni*, *R.* sp. "Millicoma dace" and individuals of *R. cataractae* from Pacific slope drainages) and an eastern clade (containing individuals of *R. cataractae* from Arctic, Atlantic, and Gulf slope drainages). Within the eastern clade of *R. cataractae*, two well-supported groups were recovered: a southeastern group, containing individuals from the Atlantic slope, southern tributaries of the Mississippi River drainage, and the Rio Grande drainage; and a northeastern group, containing individuals from the Arctic slope and northern tributaries to the Mississippi River. Estimates of the timing of divergence within the *R. cataractae* species-group, combined with ancestral area-reconstruction methods, indicate a separation between the eastern and western clades during the Pliocene to early-Pleistocene, with a direction of colonization from the west of the Continental Divide eastward. Within the southern portion of its range, *Rhinichthys*

cataractae likely entered the Rio Grande drainage during the Pleistocene via stream capture events between the Arkansas River (Mississippi River drainage) and headwaters of the Rio Grande. A close relationship between populations of *R. cataractae* in the Rio Grande drainage and the adjacent Canadian River (Mississippi River drainage) is consistent with hypothesized stream capture events between the Pecos and Canadian rivers during the late-Pleistocene. The population of *R. cataractae* in the lower Rio Grande appears to have separated from other populations in the Rio Grande drainage (upper Rio Grande and Pecos River) and Canadian River (Mississippi River drainage) during the late-Pleistocene, well before initiation of recent and significant anthropogenic disturbance within the Rio Grande drainage.

DEDICATION

I dedicate this thesis to my parents, sister, and the cutest baby, my niece Hamie, for their support and love.

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1. INTRODUCTION

Rhinichthys cataractae, commonly referred to as the longnose dace, is a primary freshwater fish that inhabits swift flowing, steep-gradient, headwater streams across much of North America (Kuehn, 1949; Lee et al., 1980). The species is noteworthy because it has the widest geographic distribution of any member in the family Cyprinidae (carps, minnows, and their relatives) found in North America (Jenkins and Burkhead, 1994), ranging from the Atlantic coast in the east to the Pacific coast in the west, and from the Rio Grande drainage in northern Mexico to the Mackenzie River drainage in northern Canada (Lee et al., 1980) (Fig. 1).

Relatively few species of North American freshwater fishes transverse the Continental Divide, situated along the Rocky Mountains (Page and Burr, 2011). This trans-continental distribution pattern of North American freshwater fishes is hypothesized to be the result of westward, post-glacial dispersal by members of eastern lineages via drainage connections between rivers and lakes in central or north-central Canada subsequent to Wisconsin glaciation (Bernatchez and Wilson, 1998; Rempel and Smith, 1998). Results of several population-genetic studies on fishes with a trans-continental distribution (e.g., *Catostomus catostomus*; McPhail and Taylor, 1999; *Salvelinus confluentus*; Taylor et al., 1999; *Lota lota*; Van Houdt et al., 2005) have provided evidence for post-glacial or late-Pleistocene dispersal across the Continental Divide. However, Wilson and Hebert (1998) proposed that divergence between ancestral populations of *Salvelinus namaycush* in glacial refugia east and west of the Continental

Divide may have occurred in the mid-Pleistocene, much earlier than the post-glacial period of the late-Pleistocene. The trans-continental distribution of *R. cataractae* has not been studied within a modern phylogenetic framework and the timing (pre- or post-glacial) of evolutionary events that contributed to the present-day distribution of *R. cataractae* are unknown.

To the east of the Continental Divide, *R. cataractae* is widespread and found in all major river systems draining the Atlantic slope (i.e., the St. Lawrence, Hudson, Potomac, and James river drainages), the Arctic slope (i.e., the Mackenzie, Churchill, and Saskatchewan river drainages), and the central plains region (i.e., the Mississippi and Rio Grande river drainages). Interestingly, *R. cataractae* is absent from the short, independent river drainages that enter the Gulf of Mexico between the Mississippi and the Rio Grande river deltas, including (from east to west) the Sabine, Neches, Trinity, Brazos, Colorado, Guadalupe, San Antonio, and Nueces river drainages. This distribution contrasts sharply with that of other geographically widespread species of primary North American freshwater fishes that also inhabit the Mississippi and Rio Grande river drainages (i.e., *Cyprinella lutrensis*, *Notropis buchanani*, and *Ictalurus punctatus*) and exhibit a continuous distribution from the lower Mississippi River across the Gulf coastal plain of Texas to the Rio Grande (Page and Burr, 2011). Furthermore, populations of *R. cataractae* in the Rio Grande drainage are highly disjunct geographically from each other. The present distribution of *R. cataractae* within the Rio Grande drainage is restricted to the upper Rio Grande upstream from Bosque, New Mexico (Sublette et al., 1990; Platania, 1991), the upper Pecos River upstream from

Carlsbad, New Mexico (Sublette et al., 1990), the lower Rio Grande in Texas between Presidio and Laredo (Hubbs, 1957; Hubbs et al., 1977), and the Rio Conchos in Mexico (Edwards et al., 2002) (Fig. 1).

Hubbs et al. (1977) and Edwards et al. (2002) hypothesized that anthropogenic disturbance transformed the middle Rio Grande and the lower Pecos River into the saline and frequently dry conditions that presently characterize these stretches of both rivers. The ichthyofauna now found in these stretches is denuded and comprised only of species with high tolerance for rapidly fluctuating temperatures, high salinity, and low water quality (Hubbs et al., 1977). Lee et al. (1980) suggested that the absence of two other cyprinid species, *Notropis jemezanus* and *N. braytoni*, from the middle Rio Grande and the lower Pecos River (upstream of the confluence with Independence Creek, Terrell County, TX) is the result of local irrigation practices. However, other freshwater fishes that require similar habitat conditions to, and are sympatric with, *R. cataractae* in the lower Rio Grande (i.e., *Cycleptus* sp., *Ictalurus lupus*, *Percina macrolepida*) are not present in the upper and middle Rio Grande or the lower Pecos River, rendering the recent, anthropogenic-disturbance hypothesis less appealing as an explanation for the absence of *R. cataractae* from these regions in both rivers.

The isolated population of *R. cataractae* in the upper reaches of the Pecos River also is of interest biogeographically. Thomas (1972) argued that the upper Canadian River (Mississippi River drainage) was captured by the upper Pecos River during the late-Pleistocene (0.13–0.01 MYA), followed by the loss of portions of the upper headwaters of the Pecos River back to the Canadian River. Based on ichthyofaunal

similarities between both rivers, Conner and Suttkus (1986) provided some support for this hypothesis, suggesting that the Pecos River may have acquired its present route by capturing the headwaters of other rivers, including the Canadian River and possibly also the Red River. Thus, the presence of *R. cataractae* in the upper Pecos River may be the result of stream capture events between the Pecos and Canadian rivers or vice versa.

In this study, I utilized DNA sequence data from one mitochondrial (cytochrome *b*) and two nuclear (S7 and RAG1) genes to assess whether the geographically widespread *R. cataractae* represents a monophyletic group. I then utilized molecular-clock data to test whether the trans-continental distribution of *R. cataractae* is consistent with the post-glacial dispersal hypothesis (Bernatchez and Wilson, 1998; Rempel and Smith, 1998). Finally, I tested whether the absence of *R. cataractae* in the middle Rio Grande and the lower Pecos River is consistent with a scenario of recent, anthropogenic disturbance and assess whether the isolated populations in the upper Pecos and Canadian rivers can be attributed to hypothesized late-Pleistocene stream capture events between the two rivers.

2. METHODS

A total of 87 individuals of *Rhinichthys cataractae* were sampled from 17 sites covering all major drainages (James, St. Lawrence, Mississippi, Churchill, Rio Grande, Columbia and Fraser rivers) within the range of the species (Table 1; Fig. 1). Fishes collected in the field (St. Croix, South Platte, Arkansas, and Canadian rivers and upper and lower Rio Grande) were euthanized upon capture by an overdose of MS222, preserved in 95% ethanol, and subsequently maintained at 4^{o}C at the Biodiversity Research and Teaching Collections, Texas A&M University (BRTC). Muscle-tissue samples were taken from individuals collected in the field or obtained from museum collections (Oregon State University Ichthyology Collection, OR, USA; University of British Columbia Beaty Biodiversity Museum, BC, Canada; University of Kansas Biodiversity Institute, KS, USA; Virginia Institute of Marine Science Ichthyological Collection, VA, USA).

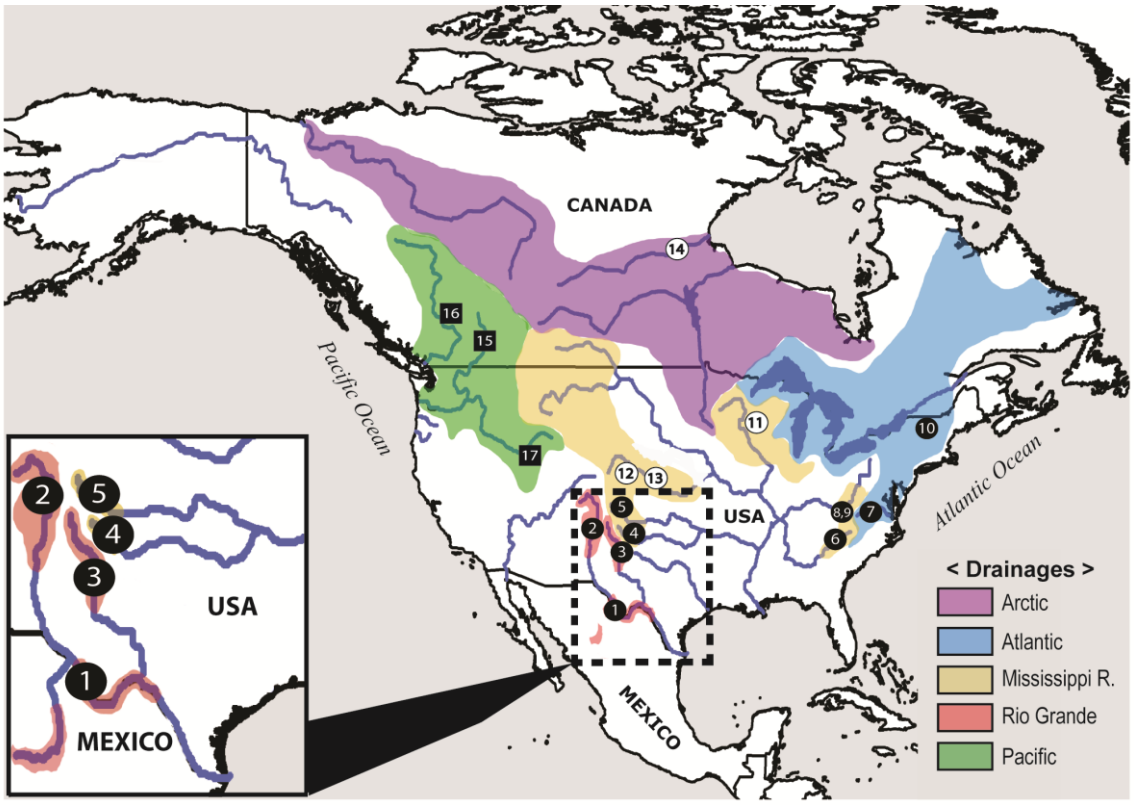


Fig. 1 Map of North America and neighboring regions showing sampling localities and distribution of the three major cyt *b* clades of *Rhinichthys cataractae* (Tables 1 and Fig. 2). Closed circles with numbers indicate sampling localities for the southeastern group, open circles with numbers indicate sampling localities for the northeastern group, and squares with numbers indicate sampling localities for the western clade. Five major drainages are highlighted in different colors: Arctic (purple), Atlantic (blue), Mississippi River (ivory), Rio Grande (red), and Pacific (green). Population names include the following: 1) lower Rio Grande 2) upper Rio Grande, 3) Pecos River, 4) Canadian River, 5) Arkansas River, 6) Tennessee River, 7) James River, 8) New River, 9) New River, 10) St. Lawrence River, 11) St. Croix River, 12) South Platte River 13) Platte River, 14) Churchill River, 15) Columbia River, 16) Fraser River, and 17) Goose Creek (see Table 1).

Genomic DNA was extracted from muscle tissue, using a DNeasy Tissue Extraction Kit (Qiagen, Inc., Valencia, CA, USA) and following manufacturer's protocols. The nearly complete mitochondrial cytochrome *b* (cyt *b*) gene was amplified using polymerase chain reaction (PCR) and primers LA-danio and HA-danio (Mayden et al., 2007). Two, single-copy nuclear loci, including S7 ribosomal protein gene intron 1 (S7) and recombination activating protein 1 (RAG1), were amplified and sequenced using primers S7RPEX1F and S7RPEX2R (Chow and Hazama, 1998) and R1 2533F and R1 4078R (Lopez et al., 2004), respectively. All PCR reactions were performed in 25.0 µl, containing 12.5 µl of GoTaq Green Master Mix (Promega, Madison, WI, USA), 5.5 µl of nuclease-free water, 300.0 ng of template DNA, and 2.0 µM of each primer (forward and reverse). PCR conditions for cyt *b* amplifications consisted of an initial denaturation at 95^{o}C for 2 minutes, followed by 35 cycles at 94^{o}C for 60 seconds each, 48^{o}C for 60 seconds, and 72^{o}C for 2 minutes, and a final extension at 72^{o}C for 5 minutes. PCR conditions for RAG1 consisted of an initial denaturation at 95^{o}C for 4 minutes, followed by 35-40 cycles with denaturation at 94^{o}C for 40 seconds, annealing at 53^{o}C for 40 seconds, and extension at 72^{o}C for 92 seconds, and a final extension step at 72 ^{o}C for 7 minutes. PCR conditions for S7 followed Bufalino and Mayden (2010). Amplified PCR products were sequenced using the HighThroughput sequencing facilities at Beckman Coulter Genomics (MA, USA). Sequences were checked for accuracy of base determination using FinchTV v.1.4.0 (Geospiza, Inc.; http://www.geospiza.com/Products/finchtv.shtml) and assembled manually.

Sequence alignment was performed with MAFFT v.6.903 (Katoh and Toh, 2010) and checked manually for accuracy. Sequences of the protein-coding genes, cyt *b* and RAG1, were translated into amino acids, using MEGA4.0 (Tamura et al., 2007), to verify gene regions and check for premature stop codons. For each gene, numbers of variable sites and parsimony-informative sites were calculated with MEGA. All sequences have been deposited on GenBank (Table 1).

Outgroup taxa used during the gene-tree analyses included the congeners *R. atratulus*, *R. evermanni*, *R. falcatus*, *R. osculus* and *R.* sp. "Millicoma dace" (*sensu* McPhail and Taylor, 2008) and selected representatives from other closely related genera (*Mylocheilus*, *Oregonichthys*, and *Tiaroga*) within the cyprinid subfamily Leuciscinae (following Simons and Mayden, 1999; Bufalino and Mayden, 2010; Schönhuth et al., 2012) (Table 1). Sequence data for samples for which I was unable to obtain in the field or from museum collections were downloaded from GenBank (Table 1). *Mylocheilus caurinus* was used to root trees for gene-tree analyses, based on my preliminary analyses. For gene-tree analyses, sequence data were pruned to include only unique mitochondrial haplotypes or nuclear alleles. Best-fit models of sequence evolution, according to codon positions, for cyt *b* sequences and an unpartitioned data set of nuclear sequences were chosen using MrModeltest v.2.3 (Nylander, 2004), based on the Akaike Information Criterion (AIC; Posada and Buckley, 2004; Fig. 2). Gene-trees were inferred in a Bayesian framework (MrBayes 3.2.1; Ronquist et al., 2012).

Table 1 List of specimens, locality information, the number of specimens (*N*), catalog numbers or references, and GenBank accession numbers for loci examined (cyt *b*, S7, RAG1) for each species used in this study (except for *Rhinichthys osculus*). Numbers next to the drainage name are locality numbers for specimens of *R. cataractae*, corresponding to those in Fig. 1. Superscript letters next to the taxon name indicate alcohol-preserved muscle tissue samples obtained from the following museums: [a], Oregon State University Ichthyology Collection (OR, USA); [b], University of British Columbia Beaty Biodiversity Museum (BC, Canada); [c], University of Kansas Biodiversity Institute (KS, USA); [d], Virginia Institute of Marine Science Ichthyological Collection (VA, USA). TCWC catalog numbers are given to specimens collected in the field that are deposited at the Biodiversity Research and Teaching Collections, Texas A&M University (BRTC).

Taxon	Locality (drainage, locality number)	Lat/Long	*N*	Catalog No. or Reference	Locus data collected		
					cyt *b*	S7	RAG1
Rhinichthys cataractae	Lower Rio Grande (Rio Grande, 1)	29.338/-104.056	14	TCWC _____	KF640103 – KF640108	KF640180 – KF640184	KF640159 – KF640160
Rhinichthys cataractae	Upper Rio Grande (Rio Grande, 2)	36.208/-105.959	10	TCWC 15785.02	KF640109 – KF640115	KF640199	KF640170 – KF640172
Rhinichthys cataractae	Pecos River (Rio Grande, 3)	35.417/-105.494	10	TCWC 15784.06	KF640121 – KF640128	KF640191	KF640164 – KF640166
Rhinichthys cataractae	Cimarron River (Canadian – Arkansas – Mississippi River, 4)	36.507/-104.920	10	TCWC 15786.04	KF640129 – KF640137	KF640191	KF640161 – KF640163
Rhinichthys cataractae	Arkansas River (Mississippi River, 5)	38.438/-105.206	10	TCWC 15787.04	KF640138 – KF640146	KF640186 – KF640190	KF640169
Rhinichthys cataractae	Swannanoa River (Tennessee River – Mississippi River 6)	–	1	Nagle and Simons (2012)	JQ712320	–	–
Rhinichthys cataractae[d]	Craig Creek (James River, 7)	–	1	VIMS 12337	KF640149	KF640202	–
Rhinichthys cataractae[d]	Big Stony Creek (New – Ohio – Mississippi River, 8)	–	1	VIMS 12610	KF640148	KF640203	KF640173
Rhinichthys cataractae[d]	Big Stony Creek (New – Ohio – Mississippi River, 8)	–	1	VIMS 12609	KF640147	KF640200	–
Rhinichthys cataractae[d]	Little River (New – Ohio – Mississippi River, 9)	–	1	VIMS 12748	KF640151	KF640201	–
Rhinichthys cataractae[d]	Little River (New – Ohio – Mississippi River, 9)	–	1	VIMS 12751	KF640150	–	–
Rhinichthys cataractae[c]	Lewis Creek (Rivière Richelieu - St. Lawrence River, 10)	44.277/-73.184	1	MCZ 163564	KF640096	KF640185	KF640178
Rhinichthys cataractae	Rock Creek (St. Croix – Mississippi River, 11)	45.716/-92.886	8	TCWC _____	KF640097 – KF640102	KF640192 – KF640194	KF640168
Rhinichthys cataractae	South Platte River (Platte – Mississippi River, 12)	39.008/-105.367	10	TCWC 15788.04	KF640116 – KF640120	KF640195 – KF640198	KF640167
Rhinichthys cataractae	Platte River (Mississippi River, 13)	–	1	Schönhuth et al. (2012)	JX442982	JX443213	JX443315
Rhinichthys cataractae[b]	Churchill River (Churchill River, 14)	–	3	–	KF640153 – KF640154	KF640205 – KF640206	KF640168

Table 1 Continued

Taxon	Locality (drainage, locality number)	Lat/Long	*N*	Catalog No. or Reference	Locus data collected		
					cyt *b*	S7	RAG1
Rhinichthys cataractae[b]	Beaver Creek (Columbia River, 15)	–	4	–	KF640155 – KF640157	KF640207 – KF640208	KF640175 – KF640177
Rhinichthys cataractae[b]	Bonaparte River (Fraser River, 16)	–	1	–	KF640158	–	–
Rhinichthys sp.[a] "Millicoma dace"	South Coos River (Coos River)	43.361/-123.930	1	OS18142-1300-100	KF640152	KF640204	–
Rhinichthys evermanni	Umpqua River (Umpqua River)	–	1	McPhail and Taylor (2008)	EU780890	–	–
Rhinichthys osculus	Kettle River (Columbia River)	–	1	McPhail and Taylor (2008)	FJ769177	–	–
Rhinichthys osculus	–	–	1	Bufalino and Mayden (2010)	–	GU134265	GU136359
Rhinichthys osculus[a]	South Coos River (Coos River)	43.361/-123.930	1	OS18142-1300-098	–	–	–
Rhinichthys osculus[a]	Silver Lake (endorheic Harney Lake, Oregon)	–	1	–	–	–	–
Rhinichthys osculus[a]	Goose Lake (endorheic Goose Lake, Oregon)	–	1	–	–	–	–
Rhinichthys osculus[a]	Warner Lakes (endorheic Warner Lakes, Oregon)	–	1	–	–	–	–
Rhinichthys osculus[a]	Malheur Lake (enderheic Malheur Lake, Oregon)	–	1	–	–	–	–
Rhinichthys falcatus	Fraser River (Fraser River)	–	1	McPhail and Taylor (2008)	FJ769176	–	–
Rhinichthys atratulus[c]	Lewis Creek (Rivière Richelieu - St. Lawrence River)	44.277/-73.184	1	MCZ 163567	KF640095	KF640179	–
Rhinichthys atratulus	South Fork Zumbro River (Zumbro – Mississippi River)	43.907/-92.703	1	TCWC _____	KF640094	–	KF640174
Oregonichthys crameri	Shady Del Pond (Willamette - Columbia River)	–	1	Schönhuth et al. (2012)	JX442990	JX443219	JX443321
Tiaroga cobitis	Aravaipa Creek (Gila - Colorado River)	–	1	Schönhuth et al. (2012)	JX442987	JX443216	JX443318
Mylocheilus caurinus	North Thompson River (Fraser River)	–	1	Schönhuth et al. (2012)	JX443008	JX443233	JX443336

Two independent runs of 106 generations with four chains were performed for each gene, sampling trees every 1000 generations. Tracer v.1.5 (Rambaut and Drummond, 2009) was used to check convergence and stationarity, to determine the number of generations discarded as burn-in, and to confirm that effective sample size (ESS) values were over 200. Tree samples were used to construct a 50% majority–rule consensus tree after discarding burn-in. Trees were visualized in FigTree v.1.3.1 (http://tree.bio.ed.ac.uk/software/figtree).

Divergence time estimation was inferred from species-tree reconstruction, using *BEAST (implemented in BEAST v.1.7.4; Drummond et al., 2012). For the purpose of this analysis, taxon sampling was reduced to include only members of *Rhinichthys*. Individuals with a large amount of missing sequence data from one or more of the three target genes were excluded from analysis. This included individuals of *R. cataractae* from the James and Fraser rivers, *R. evermanni*, and *R. falcatus*. Each of the remaining populations of *R. cataractae* was assigned as separate Operational Taxonomic Unit (OUT). All sequence data for utilized individuals, including shared haplotypes (when present), were included for analysis. Analyses were conducted under a model of uncorrelated lognormal distribution rate, unlinking substitution models across partitions, utilizing a Yule tree prior, and a calibration point derived from the fossil record of the oldest known fossil placed in *Rhinichthys*. This fossil specimen was identified as *R. osculus* and estimated to be roughly 4.5 MYA (Smith and Dowling, 2008). I treated the fossil calibration point as a minimum age at the stem of the *R. osculus* lineage, using a lognormal distribution with a mean of 1.95, a standard deviation of 1.2, and an offset of

4.5. I also conducted an analysis without the fossil calibration, employing a cyt *b* substitution rate of 0.76% per lineage per million years derived from European leuciscin cyprinids (Zardoya and Doadrio, 1999). A lognormal distribution was applied for the rate prior with a mean of 0.76, a standard deviation of 0.49, and an offset of 0.0 to set a 95% confidence interval to 0.30-1.51%. Four independent *BEAST runs of 160 million generations were performed, sampling parameters every 2000 generations. Using Tracer, I checked convergence and stationarity, determined the number of generations discarded as burn-in, and confirmed that effective sample size (ESS) values were over 200. These three runs were combined with LogCombiner v.1.7.4 (Drummond et al., 2012). The resulting trees were annotated with TreeAnnotator v.1.7.4 (Drummond et al., 2012) and the final tree was visualized in FigTree.

To investigate the geographical origin of the *R. cataractae* species group (east vs. west of the Continental Divide), I conducted a Bayesian Binary MCMC (BBM) analysis, using RASP (Yu et al., 2012). The analysis was based on the post burn-in species-trees; the annotated final tree was utilized as the "condense tree" in RASP. I applied two geographic areas that represent the current distribution of the terminal taxa that were utilized during the species-tree estimation: A, drainages flowing to the east of the Continental Divide; and B, drainages flowing to the west of the Continental Divide. The number of maximum areas at each node was set to two. A null distribution was applied to the root distribution. Ten MCMC chains with F81+G model were run for 10^6 generations and the state was sampled every 100 generations.

For each gene, means of the corrected genetic distances within and between populations of the *R. cataractae* species group were calculated with MEGA4.0, using "within group means", "between groups means", and "net between groups means" options. Standard errors of the genetic distances were calculated using 1000 bootstrap replicates. I chose the closest distance metric to the best-fit model for each locus.

3. RESULTS

Phylogenetic analyses did not recover *R. cataractae* as a monophyletic group. Monophyly of *R. cataractae* was violated by *R.* sp. ("Millicoma dace") and *R. evermanni* in the cyt *b* gene-tree (Fig. 2A), by "Millicoma dace" in the S7 gene-tree (Fig. 2B) and the species-tree analyses (Fig. 2D). *Rhinichthys osculus* was recovered as the sister group to the clade comprised of *R. cataractae*, *R. evermanni*, and "Millicoma dace" (henceforth *R. cataractae* species-group), with moderate statistical support in cyt *b* and RAG1 gene-tree analyses (BPP=0.92; Fig. 2A and BPP=0.84; Fig. 2C, respectively) and strong support in the species-tree analysis (BPP>0.95; Fig. 2D). Haplotypes within the lineage comprised of *R. cataractae* and "Millicoma dace" differed from each other by an average of 3.2% (cyt *b*), 0.6% (S7), and 0.2% (RAG1) sequence divergence.

Members of the *R. cataractae* species-group were comprisedof two well-supported clades, representing eastern and western clades, in topologies resulting from gene-tree analyses for nuclear loci and species-tree analyses (Fig. 2B-D). The eastern clade included all samples of *R. cataractae* from Atlantic and Arctic drainages; the western lineage included *R. evermanni*, "Millicoma dace", and all samples of *R. cataractae* from Pacific drainages (Fig. 2B-D). Corrected mean genetic distances between the two lineages were 1.6% (S7) and 0.8% (RAG1) or 1.1% (S7) and 0.7% (RAG1), after accounting for variation within lineages. The gene-tree analysis for cyt *b* did not recover distinct eastern and western clades, instead grouping populations from east and west of the Continental Divide within a poorly resolved polytomy (Fig. 2A). Interestingly, two

Fig. 2 The 50% majority rule consensus gene trees obtained using MrBayes for the individual loci, (A) cyt *b*, (B) S7, and (C) RAG1, and (D) the *BEAST species tree. Asterisks represent posterior support greater than or equal to 0.95. Posterior probability values below 0.70 are not shown. The numbers of variable sites and parsimony-informative sites within the *Rhinichthys cataractae* species group are given in parentheses. Abbreviations in parentheses next to locality names of *R. cataractae* are USA states or Canadian provinces for the locality. Color codes (purple, blue, ivory, red, green) for the *R. cataractae* species group correspond to those in Fig. 1.

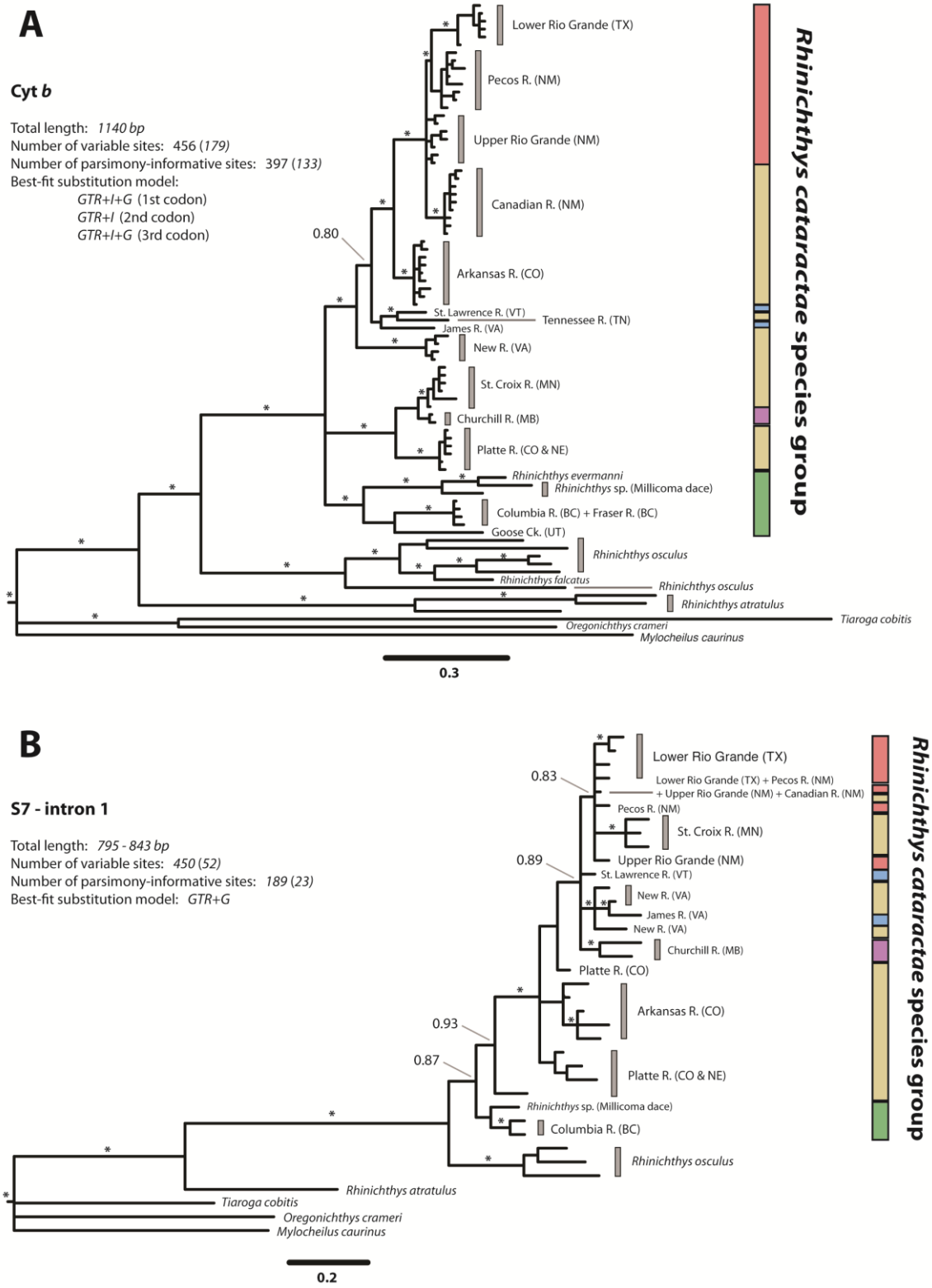
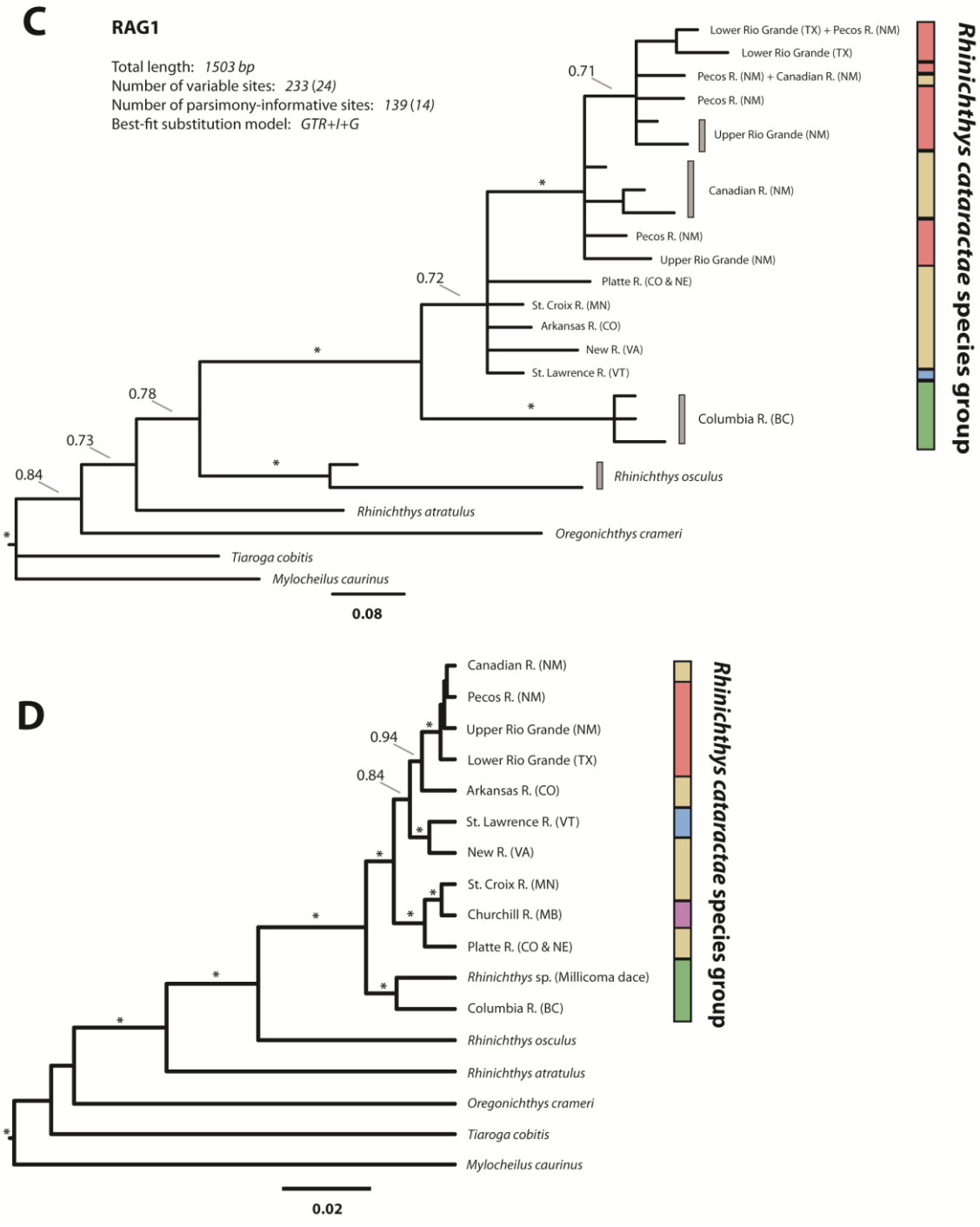

A
Cyt b
Total length: 1140 bp
Number of variable sites: 456 (179)
Number of parsimony-informative sites: 397 (133)
Best-fit substitution model:
GTR+I+G (1st codon)
GTR+I (2nd codon)
GTR+I+G (3rd codon)
Lower Rio Grande (TX)
Pecos R. (NM)
Upper Rio Grande (NM)
Canadian R. (NM)
0.80
Arkansas R. (CO)
St. Lawrence R. (VT)
Tennessee R. (TN)
James R. (VA)
New R. (VA)
St. Croix R. (MN)
Churchill R. (MB)
Platte R. (CO & NE)
Rhinichthys evermanni
Rhinichthys sp. (Millicoma dace)
Columbia R. (BC) + Fraser R. (BC)
Goose Ck. (UT)
Rhinichthys osculus
Rhinichthys falcatus
Rhinichthys osculus
Rhinichthys atratulus
Tiaroga cobitis
Oregonichthys crameri
Mylocheilus caurinus
0.3
Rhinichthys cataractae species group
B
S7 - intron 1
Total length: 795 - 843 bp
Number of variable sites: 450 (52)
Number of parsimony-informative sites: 189 (23)
Best-fit substitution model: GTR+G
Lower Rio Grande (TX)
0.83
Lower Rio Grande (TX) + Pecos R. (NM)
+ Upper Rio Grande (NM) + Canadian R. (NM)
Pecos R. (NM)
St. Croix R. (MN)
0.89
Upper Rio Grande (NM)
St. Lawrence R. (VT)
New R. (VA)
James R. (VA)
New R. (VA)
Churchill R. (MB)
Platte R. (CO)
Arkansas R. (CO)
0.93
0.87
Platte R. (CO & NE)
Rhinichthys sp. (Millicoma dace)
Columbia R. (BC)
Rhinichthys osculus
Rhinichthys atratulus
Tiaroga cobitis
Oregonichthys crameri
Mylocheilus caurinus
0.2
Rhinichthys cataractae species group

Fig. 2 Continued



distinct groups (a southeastern and a northeastern group), were recovered among samples of *R. cataractae* from drainages east of the Continental Divide in the gene-tree analysis using cyt *b*. The southeastern group is comprised of individuals from the Atlantic slope (St. Lawrence and James rivers), the southern tributaries to the Mississippi River (New, Tennessee, Arkansas, and Canadian rivers), and the Rio Grande drainage; while the northeastern group is comprised of individuals from the Arctic slope (Churchill River) and northern tributaries to the Mississippi River (St. Croix and Platte rivers) (Fig. 2A). Notably, the southeastern and northeastern groups of *R. cataractae* differed from each other by an average of 5.1% sequence divergence for cyt *b* (3.7% after accounting for variation within groups), which is only slightly smaller than the average sequence divergence between the southeastern group of *R. cataractae* and the western clade of the *R. cataractae* species-group (6.2%, or 4.2% after accounting for variation within groups). The gene-tree analyses of the S7 and RAG1 genes did not recover the northeastern and southeastern groupings of *R. cataractae* as recovered in the cyt *b* gene-tree analyses, with all members of the eastern clade grouping together within a poorly resolved polytomy (Fig. 2B-C). During the species-tree analysis, a sister group relationship between the northeastern and southeastern groups was recovered with moderate statistical support (BPP=0.82; Fig. 2D).

Within the eastern clade, individuals of *R. cataractae* from the Canadian River were found to be most closely related to populations of *R. cataractae* in the Rio Grande drainage in all gene-tree and species-tree analyses (Fig. 2). A clade comprised of populations in the Canadian River and the Rio Grande drainage also was recovered

during gene-tree analyses of cyt *b* and RAG1 (Fig. 2A,C). One unique haplotype of the S7 gene was shared by all individuals collected from the Canadian River (ten individuals), the majority of individuals from the Pecos River and the lower Rio Grande (nine and ten individuals, respectively), and four individuals from the upper Rio Grande. The majority of individuals in the Canadian and Pecos rivers (seven and eight individuals, respectively) also shared a unique haplotype for the RAG1 gene. Genetic distances among populations in the Canadian River and the Rio Grande drainage were minimal, ranging from 0.8-1.7% for cyt *b* (or 0.4-1.5 after accounting for variation within populations), 0.0-0.1% for S7, and 0.1-0.2% for RAG1 (or 0.0-0.1 after accounting for variation within populations).

Divergence time estimations based on a fossil calibration and the substitution rate for cyt *b* resulted in similar mean divergence times toward the tips of the phylogeny, although the divergence time estimate based on substitution rate inferred significantly wider, highest posterior densities (HPD) (Fig. 3). The most recent common ancestor of the *R. cataractae* species group was estimated to have diverged from the most recent common ancestor of *R. osculus* around 6.03 MYA (7.41-4.83 MYA, 95% HPD; Fig. 3A), based on the fossil calibration, or 6.07 MYA (10.53-2.89 MYA, 95% HPD; Fig. 3B). based on the substitution rate for cyt *b*. The most recent common ancestor of the *R. cataractae* species group and *R. osculus* was estimated to have originated in a Pacific drainage, flowing to the West of the Continental Divide, with a probability of 96% (Fig. 4). The ancestral area reconstruction analysis also reconstruted the ancestral area of the most recent common ancestor of the *R. cataractae* species group as Pacific, with a

probability of 90% (Fig. 4). Subsequently, a divergence time of 2.18 MYA (2.99-1.56 MYA, 95% HPD; Fig. 3A), based on the fossil calibration, or 2.74 MYA (4.76-1.32 MYA, 95% HPD; Fig. 3B), based on the substitution rate for the cyt *b* gene, was inferred between the eastern and western clades of the *R. cataractae* species-group. Within the eastern clade, a divergence time of 1.43 MYA (1.94-0.95 MYA, 95% HPD; Fig. 3A), based on the fossil calibration, or 1.88 MYA (3.29-0.86 MYA, 95% HPD; Fig. 3B), based on the substitution rate for the cyt *b* gene, was inferred between the southeastern and northeastern groups of *R. cataractae*. The group of *R. cataractae* comprised of populations in the Canadian River, the Pecos River, and the upper and lower Rio Grande was estimated to have diverged from the population in the Arkansas River around 0.82 MYA (1.12-0.47 MYA, 95% HPD; Fig. 3A), based on the fossil calibration, or 1.01 MYA (1.80-0.45 MYA, 95% HPD; Fig. 3B), based on the substitution rate for cyt *b*. The population in the lower Rio Grande and populations in the remaining Rio Grande drainage (including the Pecos River) plus the Canadian River were estimated to have separated around 0.34 MYA (0.50-0.17 MYA, 95% HPD; Fig. 3A), based on the fossil calibration, or 0.44 MYA (0.80-0.18 MYA, 95% HPD; Fig. 3B), based on the substitution rate for cyt *b*. The divergence time inferred between populations of *R. cataractae* in the Pecos and Canadian rivers was 0.17 MYA (0.31-0.09 MYA, 95% HPD; Fig. 3A), based on the fossil calibration, or 0.24 MYA (0.47-0.09 MYA, 95% HPD; Fig. 3B), based on the substitution rate for cyt *b*.

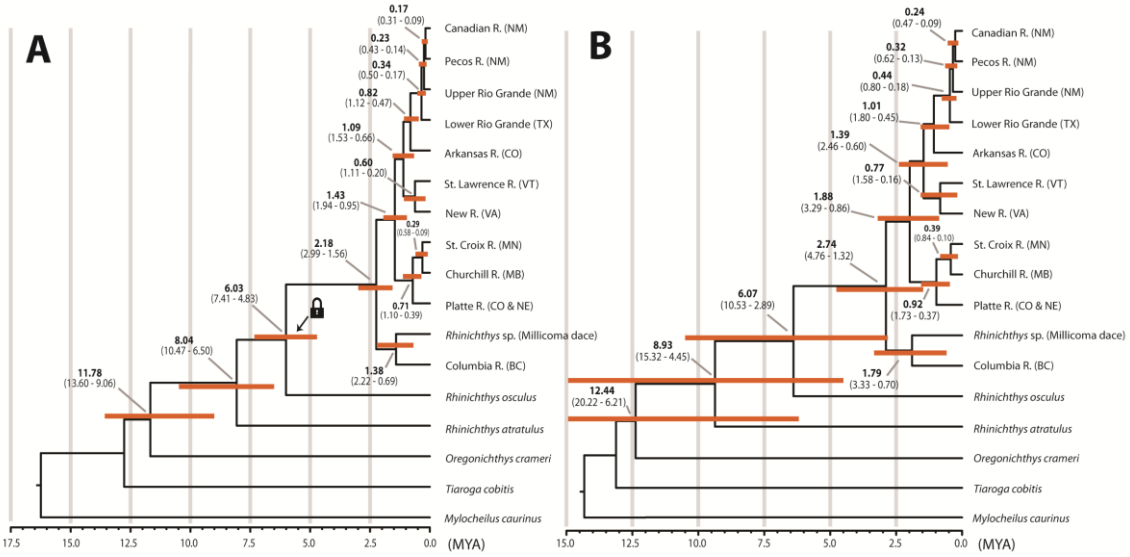


Fig. 3 Time-calibrated species tree phylogeny and divergence time estimates for nodes based on the fossil calibration (A) and the substitution rate for the cyt *b* gene (Zardoya and Doadrio, 1999) (B). Bars on nodes represent the 95% highest posterior density of divergence-time estimates. Each population of *Rhinichthys cataractae* is shown as a name of the population. Fossil calibration point is indicated by a lock-sign (A). Parts of the bars extending beyond 15 MYA are not shown (B). MYA, million years ago.

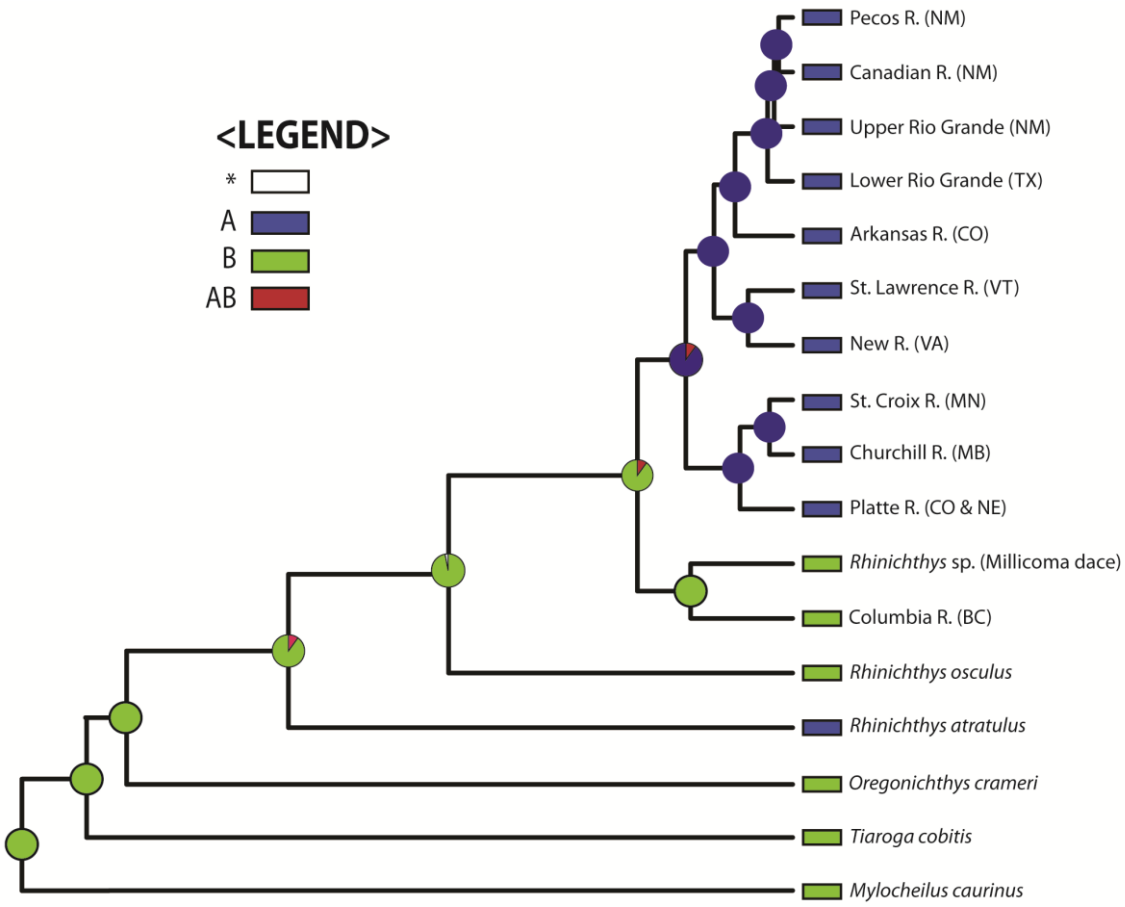


Fig. 4 Reconstruction of ancestral areas for *Rhinichthys* and closely related species obtained from a Bayesian Binary MCMC (BBM) analysis implemented in RASP. For each node, a circle with color corresponding to the geographic area represents the inferred ancestral area during the BBM analysis (A, east of the Great Continental Divide; B, west of the Great Continental Divide; *, null distribution). The proportions of the color represent the probability calculated for the corresponding geographic area. Squares with color indicate the current distribution of terminal taxa. Each population of *R. cataractae* is shown as a name of the population at terminal nodes.

4. DISCUSSION

4.1 Non-Monophyly of *Rhinichthys cataractae*

Species delineation across *Rhinichthys cataractae sensu lato* has been controversial for almost a century (Hubbs, 1926; Sigler and Miller, 1963; Bartnik, 1972; Bisson and Reimers, 1977; Lee et al., 1980). Bartnik (1972) recognized two subspecies in Canada, *R. c. cataractae* and *R. c. dulcis*, and argued that they differed in time of spawning (diurnal vs. nocturnal) and male nuptial color. Gilbert (1998) and Scharpf (2005) recognized three subspecies of *R. cataractae*, including *R. c. cataractae* (referred to as the "eastern subspecies"), *R. c. dulcis* ("western subspecies"), and *R. c. smithi* ("Banff longnose dace"). *Rhinichthys c. cataractae* and *R. c. smithi* occur to the east of the Continental Divide, and *R. c. dulcis* reportedly occurs to the west of the Continental Divide. The type locality (Platte River drainage, Wyoming) and several records of the subspecies in northern Canada are located east of the Continental Divide (Girard, 1856; Baxter and Simon, 1970; Lindsey and McPhail, 1986). *Rhinichthys c. smithi* was known only from small hot springs in the Banff National Park in Canada (Saskatchewan River drainage in the Hudson Bay slope) and has been considered extinct since 1981 (Renaud and McAllister, 1988). Mitochondrial data obtained from historical museum specimens suggests that the Banff longnose dace represented a unique lineage within *R. c. dulcis* (Macullo, 2008; see below).

Monophyly of *R. cataractae* was not supported in any of the gene-tree analyses or the species-tree analysis. This likely is due to placement of western populations of *R.*

cataractae (Columbia and Fraser rivers), which were consistently recovered as the sister group to the "Millicoma dace", a species with pending description (E. Taylor, Pers. Comm.) endemic to the Coos River drainage (Pacific slope drainage), in the gene- and species-tree analyses. Monophyly of *R. cataractae* also was violated by placement of *R. evermanni*, another Pacific slope congener (endemic to the Umpqua River, Oregon; Page and Burr, 2011), which was recovered as the closest relative of the "Millicoma dace," in the cyt *b* gene-tree analysis. Non-monophyly of *R. cataractae* (due to the position of *R. evermanni* and "Millicoma dace") has been recovered in gene-tree analyses for additional mitochondrial and nuclear genes not investigated here (E. Taylor, Pers. Comm.). Taken together, these results may warrant recognition of separate species status for the populations of *R. cataractae* found in Pacific slope drainages west of the Continental Divide.

4.2 Pre-Glacial Colonization of *Rhinichthys cataractae* across the Continental Divide

Divergence between eastern and western clades of the *Rhinichthys cataractae* species-group was estimated to have occurred during the Pliocene to early-Pleistocene, roughly 2.18 MYA (2.99-1.56 MYA) or 2.74 MYA (4.76-1.32 MYA). These results are not compatible with a hypothesis of westward post-glacial dispersal (Bernatchez and Wilson, 1998; Rempel and Smith, 1998) and predate the hypothesized post-glacial or late-Pleistocene dispersal of *Catostomus catostomus* (McPhail and Taylor, 1999), *Salvelinus confluentus* (Taylor et al., 1999) and *Lota lota* (Van Houdt et al., 2005) across the Continental Divide. A mid-Pleistocene dispersal of *Salvelinus namaycush* (Wilson

and Hebert, 1998) also is not consistent with my results, because my estimation of divergence time for separation between the eastern and western clades of *R. cataractae* species group also predates the mid-Pleistocene.

Interestingly, Hershler et al. (2008) suggested a Pacific slope origin with Pliocene expansion east of the Continental Divide for the freshwater gastropods *Pyrgulopsis anguina*, *P. bedfordensis*, and *P. blainica*. In this case, timing of colonization from west to east was estimated to be approximately 3.6-2.5 MYA (Hershler et al., 2008), roughly consistent with my estimation of divergence time for separation between the eastern and western clades of the *R. cataractae* species group. Their hypothesis of a Pacific slope origin for the gastropods also is consistent with results from my ancestral area reconstruction for the *R. cataractae* species-group. The Continental Divide is hypothesized to have moved slightly east and west of its present location in the states of Wyoming and Montana multiple times between the Miocene and Pleistocene (Galloway et al. 2011). It is conceivable that such large scale movement of the western North American Cordillera may have altered drainage basins in the region during the Pliocene to early-Pleistocene, which may have opened up dispersal routes facilitating moving of aquatic fauna from western slope to eastern slope drainages. Along these lines, initial colonization of the east by ancestral populations of *R. cataractae* may be attributed to stream capture of the headwaters of Pacific-slope rivers by eastern flowing rivers, during the Pliocene to the early-Pleistocene.

While central and north-central Canada have been the focus for the hypothesis of post-glacial colonization across the Continental Divide by North American freshwater

fishes, representatives of *R. cataractae* from a large portion of this region (e.g., Saskatchewan and Mackenzie rivers) were unavailable for this study. Published reports on populations of *R. cataractae* in these areas suggest that secondary contact between the eastern and western lineages of *R. cataractae* may have occurred during relatively recent times in the Saskatchewan and Mackenzie rivers drainages. Macullo (2008) proposed that the western lineage of *R. cataractae* (referred to as "*R. c. smithi"*) in the Cave and Basin Marsh of Banff National Park in Canada (Saskatchewan River drainage), which has reportedly gone extinct, was the result of eastward post-glacial colonization from Pacific drainages to Arctic drainages. Haplotypes of the mitochondrial cyt *b* and control region obtained from museum specimens of "*R. c. smithi*" (collected in 1891-1892) exhibit a much smaller sequence divergence from haplotypes of "*R. c. dulcis*" in the Fraser River (Pacific slope) than the haplotypes of the eastern lineage of *R. c. cataractae*. Interestingly, haplotypes analyzed from the extant populations of *R. cataractae* in the Cave and Basin Marsh (the only localities known for "*R. c. smithi*") were placed in the eastern lineage of *R. cataractae*. Consequently, Macullo (2008) concluded that the western lineage of *R. cataractae* may have been replaced by the eastern lineage in the Cave and Basin Marsh. In the Mackenzie River (Arctic slope), both phenotypic "forms" of *R. cataractae* (*R c. cataractae* and *R. c. dulcis*) are known to occur (Lindsey and McPhail, 1986). Further phylogeographic investigation of populations of *R. cataractae* in central and north-central Canada should be conducted before firm conclusions regarding the role that post-glacial colonization has played in

facilitating the present sympatry of eastern and western lineages in this region are reached.

4.3 Pleistocene Diversification of *Rhinichthys cataractae* in the Rio Grande Drainage

The upper reaches of the Rio Grande are hypothesized to have formed 4 to 5 MYA and to have flowed into the ancient Paleo-Lake Cabeza de Vaca (Mack et al., 1997), which covered large parts of northwestern Chihuahua and southwestern New Mexico (Smith and Miller, 1986). During this period, the ancient lower Rio Grande and the lower Pecos River are hypothesized to have flowed southwest into the Gulf of Mexico (Thomas, 1972; Echelle and Echelle, 1978; Richardson and Gold, 1999). The upper Rio Grande is hypothesized to have stopped flowing into Lake Cabeza de Vaca when it diverted to its current route, through the Lower Rio Grande Valley, approximately 2.25 MYA (Gustavson, 1991). Echelle and Echelle (1998) and McPhee et al. (2008) hypothesized that these geo-historical events played a major role in the evolutionary history of the Rio Grande ichthyofauna. However, the timing of such geo-historical events are well before the mid- to late-Pleistocene diversification of *Rhinichthys cataractae* within the Rio Grande drainage, and are unlikely to have shaped the current distribution of *R. cataractae* within the Rio Grande drainage.

The population of *R. cataractae* in the Arkansas River (Mississippi River drainage) was recovered as sister to a lineage comprised of populations of *R. cataractae* in the Canadian River and the Rio Grande drainage in the cyt *b* gene-tree and species-tree analyses. Divergence between the population of *R. cataractae* in the Arkansas River and

the most recent common ancestor of the lineage consisting of populations in the Canadian River and the Rio Grande drainage was estimated to have occurred around 0.82 MYA (1.12-0.47 MYA) or 1.01 MYA (1.80-0.45 MYA). These results are consistent with the reported mid-Pleistocene (0.94 MYA) divergence between populations of the cutthroat trout, *Oncorhynchus clarkii,* in the Arkansas River and the Rio Grande drainage (Loxterman and Keeley, 2012). It is possible that populations of *R. cataractae* in the Rio Grande drainage and the adjacent Canadian River are the result of stream capture events between the Arkansas River and the upper Rio Grande. Previous studies have provided some evidence for stream capture events between the Arkansas River and the San Luis Valley (now upper Rio Grande drainage) in south-central Colorado during the Pleistocene (Knepper and Marrs, 1971; Hanna and Harmon, 1989), which coincides with the divergence time estimates inferred between the population in the Arkansas River and the most recent common ancestor of the lineage comprised of populations in the Rio Grande drainage and Canadian River.

The Raton-Clayton Volcanoes located between the upper Arkansas and upper Canadian rivers are hypothesized to have been active during the Pleistocene (Calvin, 1987; Wisniewski and Pazzaglia, 2002). It is possible that the Raton-Clayton Volcanoes may have interrupted possible connections between the upper Arkansas and upper Canadian rivers, facilitating the divergence between the two lineages. However, the Canadian River does not appear to have been utilized as a colonization route for *R. cataractae* from the Arkansas River to the Rio Grande drainage, because the first connection between the Canadian River and the Rio Grande drainage (Pecos River) is

hypothesized to have occurred during the late-Pleistocene (0.13–0.01 MYA) (Thomas, 1972). A divergence time of 0.34 MYA (0.50-0.17 MYA) or 0.44 MYA (0.80-0.18 MYA) between the population in the lower Rio Grande and populations in the upper Rio Grande, Pecos River, and Canadian River indicates that the ancestral population of *R. cataractae* colonized the Rio Grande drainage prior to the first hypothesized connection between the Canadian River and the Rio Grande drainage.

Thomas (1972) hypothesized that the upper Rio Grande had once flowed directly into the lower Pecos River during the late-Pleistocene. My results provide support for this hypothesis as the population of *R. cataractae* in the upper Rio Grande was inferred to be more closely related to the population in the Pecos River than to the population in the lower Rio Grande. Active gene flow may have occurred between ancestral populations of *R. cataractae* in the Pecos River and the upper Rio Grande during the direct connection between the Pecos River and the upper Rio Grande. The majority of the nuclear S7 and RAG1 haplotypes were shared between populations in the Canadian and Pecos rivers, which also indicates that recent gene flow may have occurred between these two populations. The Pecos River was hypothesized to have captured the upper Canadian River during the late-Pleistocene (0.13-0.01 MYA), followed by the loss of portions of the upper Pecos River back to the Canadian River (Thomas, 1972). Divergence time inferred between the populations of *R. cataractae* in the Pecos and Canadian rivers roughly overlapped with the hypothesized timing of the stream capture event, which supports the hypothesis that the divergence between the two populations is the result of a late-Pleistocene stream capture event.

Rhinichthys cataractae is absent from the middle Rio Grande and the lower Pecos River. A similar distribution pattern for two other cyprinids, *N. jemezanus* and *N. braytoni*, in the Rio Grande drainage has been hypothesized to be directly attributed to recent anthropogenic disturbance within the region (Lee et al., 1980). If the absence of *R. cataractae* in the middle Rio Grande between southern New Mexico and Presidio and in the lower Pecos River downstream of southeastern New Mexico is the result of anthropogenic disturbance, one would expect populations in the upper and lower Rio Grande and the Pecos River to have been separated for only a short period of time (i.e., post-historic). The divergence time estimates of 0.34 MYA (0.50-0.17 MYA) or 0.44 MYA (0.80-0.18 MYA) between the population of *R. cataractae* in the lower Rio Grande and the populations in the upper Rio Grande, Pecos, and Canadian rivers are incompatible with a hypothesis of recent anthropogenic disturbance. I hypothesize that inhospitable habitat conditions for *R. cataractae* may have have existed in the middle Rio Grande and the lower Pecos prior to anthropogenic disturbance, preventing gene flow between populations in the lower Rio Grande and the remaining populations in the Rio Grande drainage. The absence of other riffle-dwelling fishes in the upper and middle Rio Grande and the lower Pecos that are sympatric with *R. cataractae* in the lower Rio Grande (e.g., *Cycleptus* sp., *Ictalurus lupus*, *Percina macrolepida*) also can be explained by this hypothesis. Put simply, historic habitat conditions in the middle Rio Grande and middle Pecos may have prevented upstream colonization by rheophilic species of fishes, resulting in their present day absence in the upper Rio Grande and upper Pecos. Recent anthropogenic disturbance has, and continues to have, a major impact on the

ichthyofauna of the Rio Grande drainage, with significant declines in fish diversity throughout the system (Hubbs et al., 1977; Edwards et al., 2002; McGarvey, 2011). Despite the magnitude of this anthropogenic disturbance, the absence of certain riffle-dwelling fishes, such as *R. cataractae*, in the middle Rio Grande and the lower Pecos are not so easily attributed to recent human influence within the drainage.

5. CONCLUSIONS

I suggest a monophyletic *Rhinichthys cataractae* species-group contains *R. evermanni*, *R.* sp. "Millicoma dace" and a non-monophyletic *R. cataractae*. The *R. cataractae* species group consisted of two well-supported clades, an eastern clade (containing individuals of *R. cataractae* from east of the Continental Divide; Arctic, Atlantic, and Gulf slope drainages) and a western clade (containing *R. evermanni*, *R.* sp. "Millicoma dace" and individuals of *R. cataractae* from west of the Continental Divide). The two clades were estimated to have diverged during Pliocene to early-Pleistocene with a direction of colonization from the west of the Continental Divide eastward. These results are not compatible with a hypothesis of westward post-glacial dispersal for fishes with a trans-continental distribution. *Rhinichthys cataractae* likely entered the Rio Grande drainage during the early- to mid-Pleistocene via stream capture events between the Arkansas River (Mississippi River drainage) and headwaters of the Rio Grande. Divergence time inferred between sister-group populations of *R. cataractae* in the Pecos (Rio Grande drainage) and Canadian (Mississippi River drainage) rivers was roughly consistent with the hypothesized timing of stream capture events between the two rivers during the late-Pleistocene. The population of *R. cataractae* in the lower Rio Grande appears to have separated from other populations in the Rio Grande drainage (upper Rio Grande and Pecos River) and Canadian River 0.50-0.17 MYA, based on the fossil calibration, or 0.80-0.18 MYA, based on the substitution rate for cyt *b*, which is well before initiation of recent and significant anthropogenic disturbance within the Rio

Grande drainage. Although this anthropogenic disturbance has, and continues to have, caused significant declines in fish diversity throughout the Rio Grande drainage, the absence of *R. cataractae* in the middle Rio Grande and the lower Pecos River is not so easily attributed to recent human influence within the drainage.

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