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Phylogeography of the longnose dace (*Rhinichthys cataractae*) species group in northwestern North America — the Nooksack dace problem

Eric B. Taylor, J.D. McPhail, and J.A. Ruskey

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Abstract: The longnose dace (*Rhinichthys cataractae* (Valenciennes, 1842); Cyprinidae) is one of the most widespread freshwater fishes in North America, and across its range there have been several divergent forms described that are of uncertain taxonomic status. One of these forms, the Nooksack dace, is found in southwestern British Columbia and adjacent portions of western Washington, and is distinguished from longnose dace by a lower number of lateral-line scales. We sequenced a total of approximately 1400 base pairs (bp) of mitochondrial DNA (mtDNA) and noted that the longnose dace found west of the Continental Divide and Nooksack dace constituted reciprocally monophyletic clades that differed from each other by between 2% and 3% sequence divergence. Sequence analysis at two nuclear loci (the S7 ribosomal protein intron 1 (*S7*) and recombination activation gene 1 (*RAG1*)), however, showed no consistent difference between longnose dace and Nooksack dace and several alleles were shared between them. By contrast, consistent differences at both mtDNA and nuclear DNA loci were resolved between *R. cataractae* samples from east and west of the Continental Divide. The Nooksack dace does not appear to warrant separate taxonomic status from the longnose dace, but the mtDNA differences support its recognition as an important component of the evolutionary and biogeographic legacy of *R. cataractae*.

Key words: phylogeography, cryptic species, Chehalis fauna, *Rhinichthys*, DNA sequencing.

Résumé : Le naseux des rapides (*Rhinichthys cataractae* (Valenciennes, 1842); cyprinidés) est un des poissons d'eau douce les plus répandus en Amérique du Nord et, à l'échelle de son aire, plusieurs formes divergentes ont été décrites dont le statut taxonomique demeure incertain. Une de ces formes, le naseux de la Nooksack, est présente dans le sud-ouest de la Colombie-Britannique et des régions attenantes de l'ouest de Washington, et se distingue du naseux des rapides par un nombre plus faible d'écailles le long de la ligne latérale. Nous avons séquencé un total d'environ 1400 paires de bases (pb) d'ADN mitochondrial (ADNmt) et constaté que les naseux des rapides présents à l'ouest de la ligne continentale de partage des eaux et le naseux de la Nooksack constituent des clades réciproquement monophylétiques qui se distinguent l'un de l'autre par une divergence de séquence de 2 % à 3 %. L'analyse de séquence en deux loci nucléaires (l'intron 1 de la protéine ribosomique S7 (*S7*) et le gène 1 de l'activation de la recombinaison (*RAG1*)) n'a toutefois démontré aucune différence cohérente entre le naseux des rapides et le naseux de la Nooksack, et plusieurs allèles leur étaient communs. En revanche, des différences cohérentes en des loci tant mitochondriaux que d'ADN nucléaire ont été décelées entre des échantillons de *R. cataractae* provenant des versants est et ouest, respectivement, de la ligne continentale de partage des eaux. Le naseux de la Nooksack ne semble pas justifier un statut taxonomique distinct de celui du naseux des rapides, mais des différences sur le plan de l'ADNmt appuient sa reconnaissance comme composante importante du patrimoine évolutif et biogéographique de *R. cataractae*. [Traduit par la Rédaction]

Mots-clés : phylogéographie, espèces cryptiques, faune de Chehalis, *Rhinichthys*, séquençage de l'ADN.

Introduction

Cryptic species are defined as those that are difficult or impossible to distinguish by morphology and that may have been incorrectly lumped as a single species (Sáez and Lozano 2005). This does not mean that they are literally identical; cryptic species may be morphologically distinguishable with the use of tools such as scanning electron microscopy and statistical morphometrics, and may rely upon nonvisual cues to identify conspecifics (e.g., Feulner et al. 2006). Cryptic species may signal general morphological stasis: species diversification, decoupled from ecological and (or) morphological change (Bond et al. 2001). The rate of identification of cryptic species has increased enormously since the advent of the polymerase chain reaction (PCR) and affordable DNA sequencing and they are an important component of taxonomically undescribed biodiversity (Bickford et al. 2007). The discovery of cryptic species can have important conservation implications: in many cases, a species thought to have a wide distribution has been shown to be a cryptic species complex, with each species having a very small distribution and therefore being at much greater risk of extinction (e.g., Ravaoarimanana et al. 2004). Detection of cryptic species has increased diversity estimates within some groups by over 100% (e.g., Funk et al. 2012) and can, therefore, have a huge effect on biodiversity estimates, making understanding the distributions of cryptic species important for identifying global biodiversity hotspots (Trontelj and Fišer 2009).

The genus *Rhinichthys* or "riffle dace" (Pisces: Cyprinidae) contains seven to nine species endemic to North America. The genus has a curious distribution with one widespread species, the long-

Received 25 January 2015. Accepted 26 May 2015.

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nose dace (*Rhinichthys cataractae* (Valenciennes, 1842)), found from northern Mexico, north to Labrador in the east and the lower Mackenzie River in the northwest, and south to the middle Columbia River in Oregon (Lee et al. 1980). The other species have much more limited distributions: the blacknose dace (*Rhinichthys atratulus* (Hermann, 1804)) from the Atlantic coastal drainages in Georgia northwest to the Nelson River system in Manitoba; the speckled dace (*Rhinichthys osculus* (Girard, 1856)) from western coastal and interior drainages in Sonora, Mexico, north to the Kettle River (Columbia River) in Canada; the leopard dace (*Rhinichthys falcatus* (Eigenmann and Eigenmann, 1893)) from the Columbia and Snake river systems in the United States to the upper Fraser River system in Canada; the Umatilla dace (*Rhinichthys umatilla* (Gilbert and Evermann, 1894)) endemic to the Columbia River system in Canada and the United States; the Umpqua dace (*Rhinichthys evermanni* Synder, 1908) has the smallest range and is endemic to the Umpqua River and tributaries in coastal Oregon; the loach minnow (*Rhinichthys cobitis* (Girard, 1856)) is found in streams and small rivers throughout the Gila River and San Pedro River systems in Arizona and New Mexico, the United States, and Sonora, Mexico (Lee et al. 1980). A potential eighth species, the western blacknose dace (*Rhinichthys obtusus* Agassiz, 1854), is of uncertain validity (see Fraser et al. 2005; Kraczkowski and Chernoff 2014) and a ninth taxon, the Las Vegas dace (*Rhinichthys deaconi* Miller, 1984), is extinct (Miller 1984). The species has been considered to comprise three species groups: the longnose dace group (longnose dace and Umpqua dace), the blacknose dace group (the blacknose dace), and the speckled dace group (speckled, leopard, and Las Vegas dace) (e.g., see Hubbs et al. 1974; Miller 1984). Given that the longnose dace group has such a wide distribution, it is surprising that only two species are recognized within this species group—the widely distributed longnose dace and the much more narrowly distributed Umpqua dace.

The presence of cryptic variation and its taxonomic implications have long been recognized as issues within the *R. cataractae* species group (e.g., Hubbs 1926; McPhail 1967; Bartnik 1972; Kim and Conway 2014). For instance, McPhail (1967) first noted the occurrence of a divergent form of the longnose dace, commonly referred to as the "Nooksack" dace, from western Washington and southwestern British Columbia that consistently differed from the longnose dace in scale-count traits (see also Bisson and Reimers 1977; Ruskey and Taylor 2015). The Nooksack dace has a horseshoe-shaped geographic distribution that is centered around the Chehalis River of western Washington (Fig. 1). It extends from the northwestern edge of the Olympic Peninsula (Quillayuite River), south to the Chehalis and Willipa rivers, north up the east side of Puget Sound to the Nooksack River and the lower Fraser River in British Columbia (McPhail 1967). The Chehalis River Valley was a Pleistocene glacial refuge that was independent from the much larger lower Columbia or Pacific refuge (McPhail and Lindsey 1986). McPhail (1967) hypothesized that the Nooksack dace diverged from the longnose dace at some point in the Pleistocene as a result of its isolation in the Chehalis Refuge. Furthermore, Bisson and Reimers (1977) described another morphologically divergent form of longnose dace, the Umpqua dace, from the Umpqua River on the Oregon coast. McPhail and Taylor (2009) used molecular sequence data to argue the Umpqua dace and the undescribed "Millicoma" dace (found in the Coos River, Oregon) are sister species that together form an Oregon coastal clade embedded within the *R. cataractae* species group. Furthermore, Kim and Conway (2014) used a multigene approach to suggest that a primary division within the *R. cataractae* group should be made between those that are found east of the North American Continental Divide and those west of the Continental Divide (and including the longnose, Umpqua, and Millicoma dace). Interestingly, Bartnik (1972) demonstrated that longnose dace east and west of the Continental Divide, although morphologically similar to one another, had divergent reproductive behaviour and ecology supporting their designation as distinct subspecies, *Rhinichthys cataractae cataractae* (Valenciennes in Cuvier and Valenciennes, 1842) (eastern longnose dace) and *Rhinichthys cataractae dulcis* (Girard, 1856) (western longnose dace), respectively. Furthermore, the speckled dace in Oregon contains several divergent lineages, some of which may represent cryptic species (Pfrender et al. 2004; Ardren et al. 2010; Hoekzema and Sidlauskas 2014).

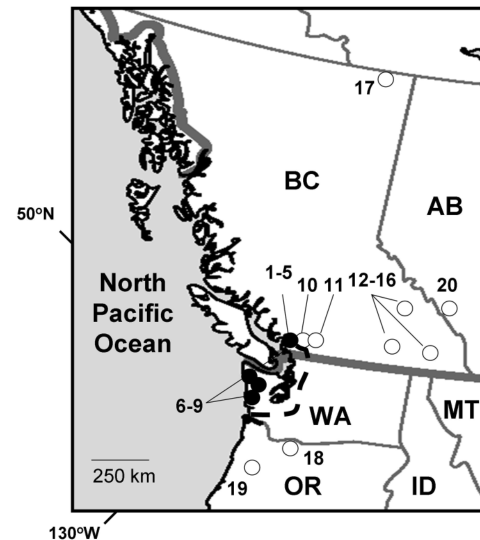

Fig. 1. Map of various localities sampled for Nooksack dace (solid circles) and longnose dace (*Rhinichthys cataractae*) (open circles) in western Canada and the United States. Numerals refer to numbered localities shown in Table 1. Only a subset of localities is shown to represent the general geographic distribution of Nooksack dace and longnose dace, and owing to close spatial proximity of some localities, not every numeral is accompanied by a symbol. Other sample localities, for longnose dace in eastern North America and outgroup taxa, are detailed in Table 1. AB, Alberta; BC, British Columbia; ID, Idaho; MT, Montana; OR, Oregon; WA, Washington. The broken heavy line represents the approximate northern, eastern, and southern range limits of the Nooksack dace.

In this study, we assessed the distinctiveness of the Nooksack dace as another component of potentially cryptic species diversity within the western lineage of longnose dace. Specifically, we tested the hypothesis that the Nooksack dace, as suggested by its morphological distinctiveness and presumptive origin in a glacial refuge distinct from those used by the longnose dace, would form a monophyletic clade within the western group of *R. cataractae* both at mitochondrial DNA (mtDNA) and nuclear DNA (nDNA) loci. We further predicted that the Nooksack dace and longnose dace would be divergent from one another at a level consistent with the timing of one or more of the Pleistocene glaciations (i.e., diverged at least 100 000 to 150 000 years ago; see April et al. 2013). Resolution of the relationship between the longnose dace and the Nooksack dace is important for a more complete understanding of the nature and origins of *R. cataractae* in North America (Kim and Conway 2014), especially because the Nooksack dace is recognized as a wildlife species that is distinct from the longnose dace and is listed as Endangered under Canada's *Species at Risk Act* (COSEWIC 2007).

Materials and methods

Sample acquisition

Various species and populations of dace were sampled between 1993 and 2012 and comprised a mixture of field collections and sequences obtained from the GenBank database (Table 1, Fig. 1; available from http://www.ncbi.nlm.nih.gov/genbank/). The areas

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Table 1. Taxon, locality, drainage, and geographic coordinates for samples sequenced at cytochrome *b* (*cytb*) and NADH-2 (*ND2*) mitochondrial DNA genes, as well as S7 ribosomal protein intron 1 (*S7*) and recombination activation gene 1 (*RAG1*) nuclear loci, in various taxa of the North American genus *Rhinichthys*.

			Coordinates		Haplotype or allele number			
Taxon	Locality	Drainage	Latitude (°N)	Longitude (°W)	*cytb*	*ND2*	*S7*	*RAG1*
Rhinichthys cataractae (Nooksack dace)	Alouette R.[1]	Lower Fraser R., BC	49.208889	122.601389	2, 3, 6 (2)	1, 4	1 (2), 3, 6 (2)	2 (2)
	Brunette R.[2]	Lower Fraser R., BC	49.201111	122.870833	3 (5)	NS	4	NS
	Bertrand Cr.[3]	Nooksack R., WA	49.030556	122.530278	2, 3, 12 (2)	8	1, 2, 7	NS
	Kanaka Cr.[4]	Lower Fraser R., BC	49.206111	122.533611	3 (2), 7	NS	NS	NS
	Coquitlam R.[5]	Lower Fraser R., BC	49.234167	122.753889	3, 7 (3)	NS	1	NS
	Chehalis R.[6]	Chehalis R., WA	46.968056	123.595556	3	NS	NS	NS
	Willipa R.[7]	Chehalis R., WA	46.731111	123.731944	1, 2, 4 (2), 5	1 (2), 2 (2), 3	1 (2), 4, 5	1, 6
	Porter Cr.[8]	Chehalis R., WA	46.967778	123.275556	14, 15 (2)	10, 11	1, 2	6
	West Fork Satsop R.[9]	Chehalis R., WA	47.043333	123.524444	16 (2), 17	12, 13	1 (2)	6
Rhinichthys cataractae (longnose dace)	Norrish Cr.[10]	Lower Fraser R., BC	49.176111	122.148333	7	NS	1	NS
	Coquihalla R.[11]	Lower Fraser R., BC	49.369722	121.396111	7	NS	NS	NS
	Little Sand Cr.[12]	Upper Columbia R., BC	49.350556	115.296111	7 (3)	5	NS	3
	Kootenay L.[13]	Upper Columbia R., BC	49.625350	116.934167	8 (2)	6	NS	6
	Beaver Cr.[14]	Upper Columbia R., BC	49.108056	117.554167	8	NS	5	4
	Blueberry Cr.[15]	Upper Columbia R., BC	49.250278	117.955833	8, 9	6	7, 8	2
	Columbia L.[16]	Upper Columbia R., BC	52.102222	115.818889	11	4	4	6
	Petitot R.[17]	Laird R., BC	59.648611	122.924167	7 (2)	NS	NS	NS
	Bridge Cr.[18]	Lower Columbia R., OR	44.733333	120.307510	NS	NS	6	NS
	Coast Fork R.[19]	Lower Columbia R., OR	43.615278	123.081111	7	NS	4	NS
	Upper Kananaskis Reservoir[20]	South Saskatchewan R., AB	50.613601	115.122853	13 (2)	NS	11 (2)	NS
	Churchill R..	Hudson Bay, MB	58.675833	94.184722	13 (3)	9	12	NS
	St. Marguerite R.	St. Lawrence R., QC	48.251944	69.880000	10 (2)	7	10 (2)	5
	Rock Cr.	Mississippi R., MN	45.716000	92.886389	NS	NS	13 [KF640194]	7 [KF640168]
	Big Stoney Cr.	Mississippi R., VI	37.420833	80.599722	NS	NS	NS	8 [KF640173]
	Blue Cr.	Missouri R., NE	41.340556	102.201667	NS	NS	NS	9 [JX443315]
Rhinichthys evermanni (Umpqua dace)	Umpqua R.	Umpqua R., OR	43.310833	123.217778	Reve [EU780890]	Reve	1	Reve
Rhinichthys sp. ("Millicoma" dace)	Coos R.	Coos R., OR	43.443889	123.993056	Mill [EU871709]	Mill	Mill	Mill [KF640204]
Rhinichthys osculus (speckled dace)	Cottonwood Cr.	Malheur R., OR	42.632944	120.506944	Rosc [JX442983]	NS	NS	Rosc [GU136359]
	Rock Creek Springs	Colorado R., CO	39.699722	107.070833	NS	NS	Rosc [GU134265]	NS
	Hay Cr.	Goose L., OR and CA*	42.184444	120.707222	NS	Rosc [EU158229]	NS	NS
Rhinichthys falcatus (leopard dace)	Lower Fraser R.	Lower Fraser R., BC	49.203880	121.785556	Rfal	Rfal	Rfal	Rfal
Rhinichthys atratulus (blacknose dace)	Lewis Cr.	Richelieu R., VT and QC	44.276111	73.183611	Ratr [KF640095]	NS	Ratr [KF640179]	NS
	Blackberry R.	Housatonic R., CT	42.000819	73.219711	NS	Ratr [JN569207]	NS	NS
	South Fork Zumbro R.	Mississippi R., MN	43.907000	92.703000	NS	NS	NS	Ratr [KF640174]
Mylocheilus caurinus (peamouth chub)	Billingsley Cr.	Snake R., ID	42.839166	114.904444	NS	NS	NS	Mcau [JX443336]

Note: All are single samples unless indicated otherwise in parentheses. Shown are the haplotype or allele numbers depicted in Figs. 2*a*, 2*b*, 3*a*, and 3*b*. AB, Alberta; BC, British Columbia; CA, California; CO, Colorado; CT, Connecticut; ID, Idaho; MB, Manitoba; MN, Minnesota; NE, Nebraska; OR, Oregon; QC, Quebec; VI, Virginia; VT, Vermont; WA, Washington. NS, not sequenced; Ratr, *R. atratulus*; Reve, *R. evermanni*; Mill, Millicoma dace; Rfal, *R. falcatus*; Rosc, *R. osculus*; Mcau, *M. caurinus*; all other haploypes or alleles are *R. cataractae*. Accession numbers for sequences obtained from GenBank are given in brackets in the last four columns of the table. Underlined localities with *R. cataractae* are east of the Continental Divide. Superscripts accompanying locality names (where applicable) refer to localities in Fig. 1.

*An endorheic basin.

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sampled focused on British Columbia and Washington, but included samples from across North America from *R. cataractae*, and outgroup taxa *R. evermanni*, *Rhinichthys* sp. "Millicoma" dace (hereafter referred to as Millicoma dace), *R. atratulus*, *R. falcatus*, and *R. osculus*. Sequences obtained from GenBank were selected such that our results would be directly compared with the previous studies of McPhail and Taylor (2009) and Kim and Conway (2014).

Sequencing

Genomic DNA was extracted using QIAquick spin columns, eluted in 150 μL of AE buffer supplied by the manufacturer, and stored at –20 °C until analysis. We used PCR to amplify fragments of four genes: the mitochondrial DNA cytochrome *b* (*cytb*) and NADH subunit 2 (*ND2*) genes, as well as the single-copy nuclear DNA encoded S7 ribosomal protein gene intron 1 (*S7*) and the recombination activation gene 1 (*RAG1*). A total of 52 individuals were sequenced for *cytb*, after which we sequenced at least 1 individual of each unique *cytb* haplotype: 17 for *ND2*, 26 for *S7*, and 18 for *RAG1*. Amplifications were carried out in 50 μL total volumes in the presence of (final concentrations) 1× New England Biolabs ThermoPol Buffer (20 mmol/L Tris–HCl; 10 mmol/L $(NH_4)_2SO_4$; 10 mmol/L KCl; 2 mmol/L $MgSO_4$; 0.1% Triton X-100), 100 μmol/L of each dNTP, 0.2 μmol/L of each primer, 1.5 units of *Taq* polymerase, and between 200 and 1000 ng of template DNA. The *cytb* gene amplification comprised a 630 base pair (bp) long fragment using the primers HD and GluDG (Palumbi 1996; Dowling et al. 2002). Amplification cycling conditions consisted of an initial denaturation at 95 °C for 3 min; 5 cycles of 95 °C denaturation for 45 s, 55 °C annealing for 30 s, and 72 °C extension for 30 s; 30 cycles of 92 °C denaturation for 45 s, 52 °C annealing for 30 s, and 72 °C extension for 30 s; and a final extension step at 72 °C for 10 min. The PCR for the *ND2* gene amplified a ~1050 bp fragment using the primers nd2-H and nd2-L of Tipton et al. (2011). The *S7* (~850 bp) and *RAG1* (~1400 bp) fragments were amplified using the primers S7RPEX1F and S7RPEX2R (Chow and Hazama 1998) and R1 2533F and R1 4078R (López et al. 2004), respectively. Amplified fragments were checked for quality (e.g., single-clear fragment) and quantity on 1.5% agarose gels stained using SYBR® Safe DNA gel stain and viewed under ultraviolet light. Amplified fragments were purified using QIAquick PCR Purification columns and cycle-sequenced on an Applied Biosystems Inc. 37390S sequencer using dGTP BigDye® Terminator version 3.0 chemistry using the HD (*cytb*), nd2-H (*ND2*), COF (*COI*), S7RPEX1F (*S7*), and R1 2533F (*RAG*) primers.

Data analyses

All sequences were aligned using Bioedit version 7.0.5.3 (updated from Hall 1999) and have been deposited in GenBank under submission numbers KP694016-29 (*S7*), KP694030-47 (*cytb*), KP744099-114 (*ND2*), and KP744115-122 (*RAG1*). Following sequence alignment, jModeltest2 version 2.1.2 was used to estimate the best-fit model among 88 models of sequence evolution for each set of sequences using the corrected Akaike's information criterion (AIC_c; Darriba et al. 2012). Recombination in the *S7* and *RAG1* sequences was tested using the Φ test implemented in Splitstree version 4.12.8 (Huson and Bryant 2006). Phylogenetic analyses using unique sequences for each DNA segment were subsequently conducted on haplotypes or alleles using maximum likelihood employing the appropriate substitution models accompanied by 1000 bootstrap replicates using MEGA version 5.1 (Tamura et al. 2011).

We estimated divergence time for longnose dace and Nooksack dace using the Bayesian framework implemented in BEAST version 2.1.3.0 (updated from Drummond et al. 2012) and concatenated sequences for *cytb* and *ND2* (total of 1453 bp). To allow for different evolutionary rates among lineages, we used the log-normal relaxed molecular clock model under a calibrated Yule model of speciation. We used the HYK substitution model with a gamma category count of 4. We used a single, fossil-based calibration point for a specimen of *R. osculus* with an estimated age of about 4.5 million years (Smith and Dowling 2008; see also Kim and Conway 2014). Consequently, the origin of the *R. osculus* lineage was treated as a minimum age, using a log-normal distribution with a mean of 1.95, SD of 1.2, and an offset of 4.5. The BEAST analysis was replicated four times with each Markov chain Monte Carlo analysis running for 40 000 000 generations, sampled every 1 000 generations, and disregarded the first 4 000 000 steps as a burn-in period that resulted in acceptable effective sample sizes (>200) and acceptance probabilities for all parameters of >0.25.

Results

Phylogenetic relationships

Our *cytb* sequencing covered 433 bp and resolved 17 unique haplotypes among the 52 *R. cataractae* individuals examined. Mean uncorrected *p*-distances between *R. cataractae* and *R. falcatus*, *R. osculus*, *R. atratulus*, *R. evermanni*, and Millicoma dace were 0.081, 0.095, 0.090, 0.040, and 0.041, respectively. The longnose dace haplotypes sampled from west of the Continental Divide differed from those sampled east of the Continental Divide by a mean of 0.040 (0.025 net) *p*-distance.

Phylogenetic analysis for the *cytb* sequences suggested that *R. cataractae* was paraphyletic with respect to *R. evermanni* and the Millicoma dace (Fig. 2*a*). The *cytb* tree resolved a clade consisting of *R. evermanni* and the Millicoma dace, which was sister to a clade consisting of all *R. cataractae* from west of the Continental Divide. The samples from east of the Continental Divide formed a clade (99% support) that was sister to all "western" *R. cataractae* group plus *R. evermanni* and the Millicoma dace (Fig. 2*a*). Within the clade consisting of all western *R. cataractae* group, there was one subclade (97% bootstrap support) containing all *R. cataractae* sampled from areas tributary to the upper and lower Fraser River and the upper Columbia River (all considered to be localities with fish resembling *R. cataractae* morphologically). Another subclade consisted of *R. evermanni* and the Millicoma dace (100% bootstrap support), and a third subclade (65% bootstrap support) comprised fish sampled from tributaries of the Chehalis, Nooksack, and lower Fraser rivers, and from the Willipa River (all considered to be localities containing only the Nooksack dace morphologically—the Nooksack dace clade; Fig. 2*a*). Three tributaries of the lower Fraser River (Kanaka Creek and the Coquitlam and Alouette rivers) were the only ones to contain haplotypes from both the western *R. cataractae* and the Nooksack dace clades (Table 1). The western longnose dace and the Nooksack dace differed from each other by 0.026 (0.021 net) *p*-distance.

We sequenced each *cytb* haplotype at the *ND2* gene (1020 bp, *N* = 1 fish per haplotype) and found that *p*-distances between *R. cataractae* and *R. falcatus*, *R. osculus*, *R. atratulus*, *R. evermanni*, and the Millicoma dace were 0.142, 0.140, 0.168, 0.070, and 0.063, respectively. Among the longnose dace, there were 13 distinct haplotypes (i.e., seven of the distinct *cytb* haplotypes were the same when sequenced at *ND2*, two fish with identical *cytb* sequences had different *ND2* sequences; Table 1). Longnose dace sampled from west of the Continental Divide differed from those sampled east of the Continental Divide by 0.072 (0.050 net) *p*-distance and western longnose dace and Nooksack dace differed from one another by 0.030 (0.023 net) *p*-distance. Phylogenetic analysis of the *ND2* sequences also indicated that *R. cataractae* was paraphyletic with respect to *R. evermanni* and the Millicoma dace because the eastern longnose dace clade (100% bootstrap support) was sister to a lineage consisting of western longnose dace and *R. evermanni* and the Millicoma dace (Fig. 2*b*). The *ND2* sequences resolved the same distinction between western longnose dace and Nooksack dace as with *cytb*, but with much higher levels of bootstrap support (96%–100%; Fig. 2*b*).

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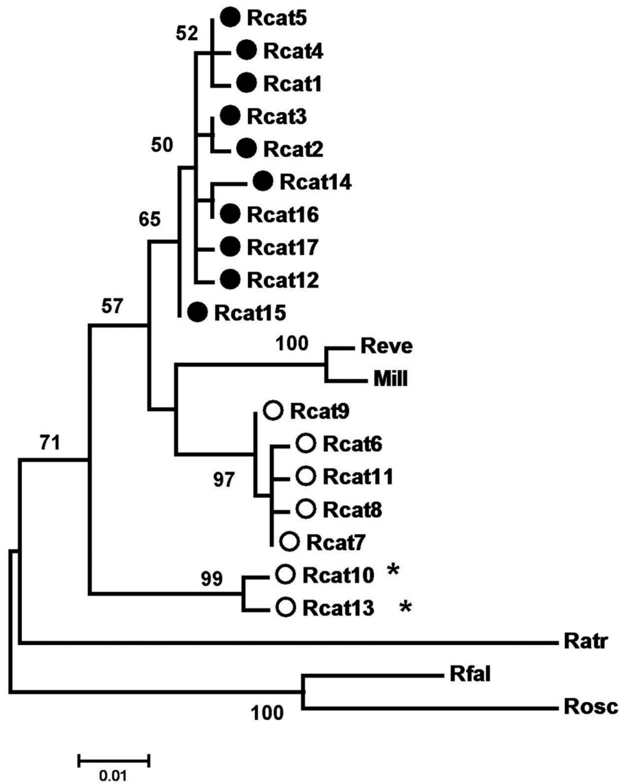
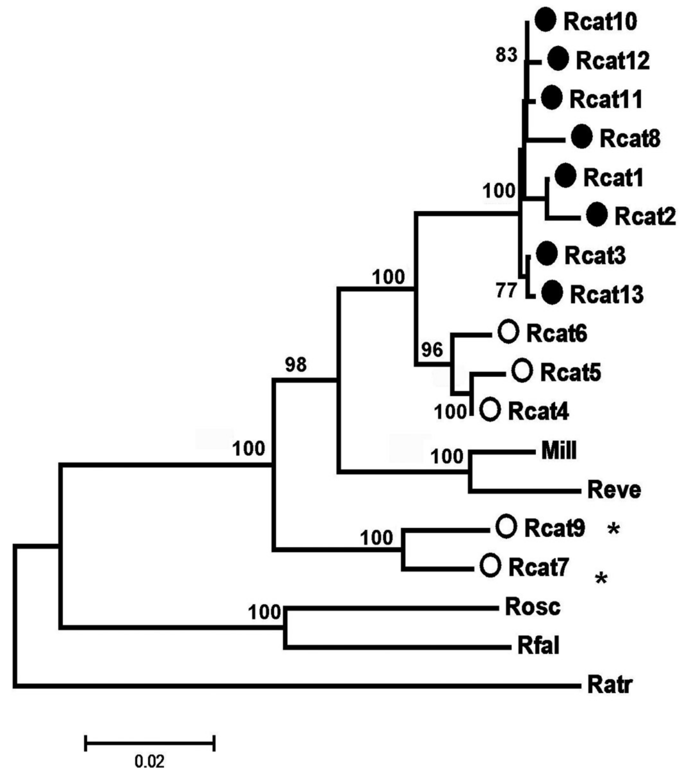
Fig. 2. Maximum-likelihood-derived trees for (*a*) cytochrome *b* and (*b*) NADH-2 mitochondrial DNA haplotypes of various taxa of the genus *Rhinichthys*. Numerals at branch points represent bootstrap percentages from 1000 pseudoreplications with those with less than 50% support not shown. Also included for each tree is the best-fit substitution model for each sequence. The locations and taxon for each haplotype are defined in Table 1. Longnose dace (*Rhinichthys cataractae*) haplotypes are indicated by open circles. Nooksack dace haplotypes are indicated by solid circles. Haplotypes from dace east of the Continental Divide are accompanied by asterisks. Rcat, *R. cataractae*; Ratr, *Rhinichthys atratulus* (blacknose dace); Reve, *Rhinichthys evermanni* (Umpqua dace); Rfal, *Rhinichthys falcatus* (leopard dace); Rosc, *Rhinichthys osculus* (speckled dace); Mill, *Rhinichthys* sp. ("Millicoma" dace).





We resolved 629 bp of the *S7* sequence and found no evidence for recombination ($P > 0.5$). Phylogenetic analysis of the *S7* sequences also suggested that *R. cataractae* was paraphyletic with respect to *R. evermanni* and the Millicoma dace, and also resolved a major split between western and eastern groups of longnose dace (92% bootstrap support; Fig. 3*a*). Two individuals of *R. evermanni* shared allele 1 with a total of 21 longnose dace and Nooksack dace. Furthermore, there was no evidence for a phylogenetic distinction between western longnose dace and Nooksack dace (Fig. 3*a*). In fact, two alleles were shared between western longnose dace and Nooksack dace and, as with *R. evermanni*, allele 1 was widely shared between fish characterized as longnose dace and Nooksack dace (Table 1). Alleles of *S7* in *R. cataractae* (including *R. evermanni* and the Millicoma dace) differed from those in *R. falcatus*, *R. osculus*, and *R. atratulus* by 0.021, 0.024, 0.034 *p*-distance, respectively. The mean *p*-distance between *R. cataractae* sampled east and west of the Continental Divide was 0.013 (0.005 net *p*-distance).

We resolved 956 bp of *RAG1* and there was no evidence for recombination in these sequences ($P > 0.2$). Phylogenetic analysis of the sequences also indicated paraphyly of *R. cataractae* with respect to *R. evermanni* and the Millicoma dace (Fig. 3*b*). A western lineage of *R. cataractae* (including *R. evermanni* and the Millicoma dace) was only weakly supported (50% bootstrap support), and one of the eastern alleles (from Quebec, allele 5) was quite distinct from all other *R. cataractae* and different from them by a mean *p*-distance of 0.007 versus between 0.007 and 0.010 compared with the three other species of *Rhinichthys* (Fig. 3*b*). Furthermore, there was no phylogenetic distinction between longnose dace and Nooksack dace (Fig. 3*b*). Alleles of *RAG1* in *R. cataractae* (including *R. evermanni* and the Millicoma dace) differed from those in *R. falcatus*, *R. osculus*, and *R. atratulus* by 0.012, 0.010, and 0.012 *p*-distance, respectively. The mean difference between *R. cataractae* sampled east and west of the Continental Divide was 0.005 and two alleles were shared between western longnose dace and Nooksack dace (Fig. 3*b*).

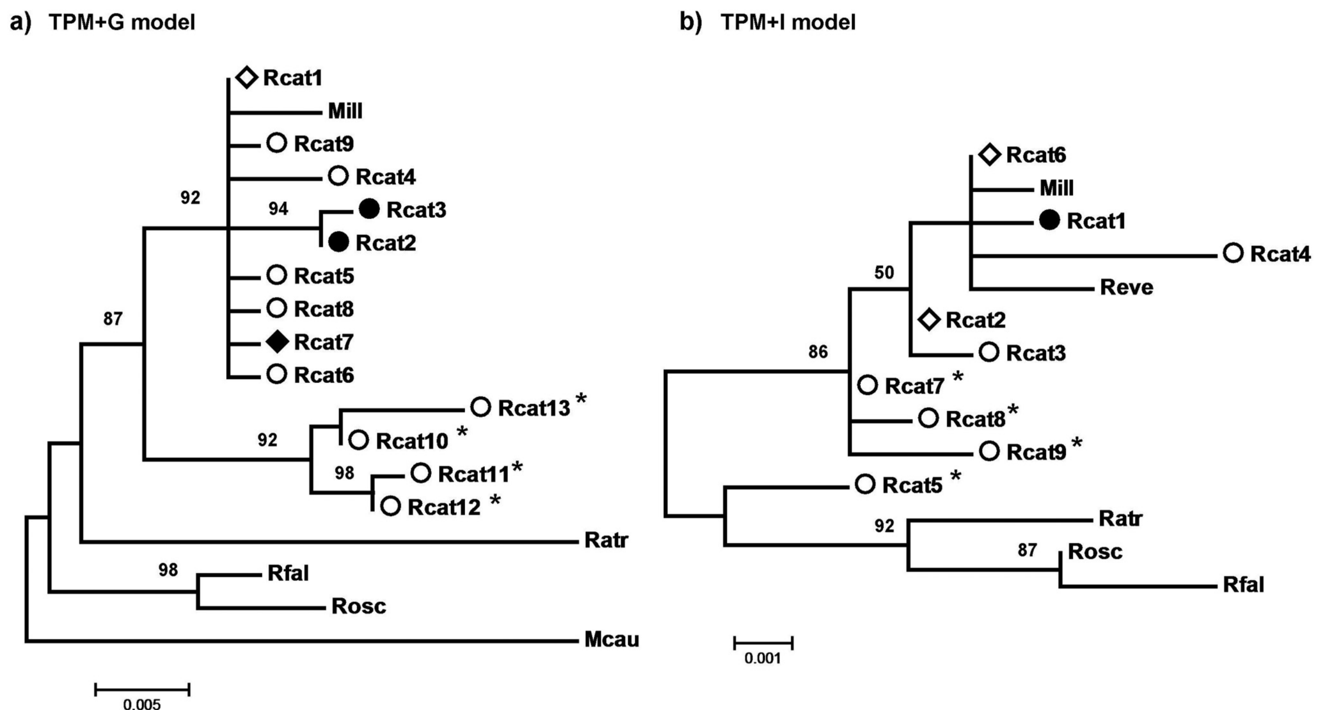
Divergence-time estimation

The fossil-calibrated estimate of divergence between longnose dace and Nooksack dace mtDNA clades using the combined *cytb* and *ND2* data suggested that the two lineages diverged about 1.1 million years ago (mya), but confidence intervals (95% highest posterior density (HPD)) ranged from 0.3 to 2.6 mya. The estimated divergence time between *R. evermanni* and the Millicoma dace mtDNA clades was 0.65 mya (95% HPD = 0.1–1.8 mya) and divergence time between the *R. cataractae* clade east of the Continental Divide and the *R. cataractae* – *R. evermanni* – Millicoma dace west of the Continental Divide was estimated as 2.5 mya (95% HPD = 0.5–6.75 mya).

Discussion

Our analysis resolved a relatively deep mtDNA divergence (2%–3%) between the longnose dace and what has been referred to, from previous biogeographic and morphological analyses, as the Nooksack dace (cf. McPhail 1967; Ruskey and Taylor 2015). Ruskey

Fig. 3. Maximum-likelihood-derived trees for (*a*) S7 ribosomal protein intron 1 and (*b*) the recombination activation gene 1 for various taxa of the genus *Rhinichthys*. Numerals at branch points represent bootstrap percentages from 1000 pseudoreplications with those with less than 50% support not shown. Also included for each tree is the best-fit substitution model for each sequence. Longnose dace (*Rhinichthys cataractae*) alleles are symbolized by open circles. Nooksack dace alleles are indicated by solid circles. Alleles that are shared between longnose dace and Nooksack dace are indicated by a solid diamond. Alleles that are shared among longnose dace, Umpqua dace (*Rhinichthys evermanni*), and Nooksack dace are indicated by open diamonds. Alleles from dace east of the Continental Divide are accompanied by asterisks. The locations and taxon for each allele are defined in Table 1. Rcat, *R. cataractae*; Ratr, *Rhinichthys atratulus* (blacknose dace); Reve, *R. evermanni*; Rfal, *Rhinichthys falcatus* (leopard dace); Rosc, *Rhinichthys osculus* (speckled dace); Mill, *Rhinichthys* sp. ("Millicoma" dace); Mcau, *Mylocheilus caurinus* (peamouth chub).



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and Taylor (2015) examined a larger sample of *R. cataractae*-like dace from across Canada and from Michigan, Oregon, and Washington in the United States using morphology and restriction fragment polymorphism assays of mtDNA (*N* = 322 fish from 12 localities using morphology and 269 fish from 12 localities using mtDNA) and found the Nooksack dace only in portions of the lower Fraser River valley in southwestern British Columbia, south along the eastern portion of Puget Sound, and west to the Chehalis River area of western Washington (see also McPhail 1967). The longnose dace – Nooksack dace divergence was nested within a broader scale divergence between longnose dace east and west of the North American Continental Divide (4%–7%), and is similar in magnitude to divergences between other western regional isolates within the *R. cataractae* group (e.g., *R. evermanni* and the Millicoma dace). Consequently, our results support the idea that considerable cryptic diversity exists within the *R. cataractae* group and that such diversity occurs at different spatial scales (e.g., across North America and both east and west of the Continental Divide; cf. McPhail and Taylor 2009; Kim and Conway 2014). Notwithstanding cryptic diversity revealed by mtDNA, our data also indicated that while divergence at mtDNA was sometimes accompanied by divergence at nuclear loci (e.g., eastern versus western *R. cataractae*), there was no consistent nDNA divergence observed between longnose dace and Nooksack dace, or among *R. cataractae*, *R. evermanni*, or the Millicoma dace within the western assemblage of the *R. cataractae* species group.

North American phylogeography

Kim and Conway (2014) conducted an extensive analysis within the *R. cataractae* group (but did not include the Nooksack dace) and our results are broadly consistent with theirs; paraphyly of *R. cataractae* with respect to *R. evermanni* and the Millicoma dace and a consistent distinction between *R. cataractae* east and west of the Continental Divide. The analysis by Kim and Conway (2014) sampled many more areas east of the Continental Divide and they recovered a major distinction between fish from southeastern North America (Atlantic slope, southern Mississippi River drainage, and Rio Grande River) and those from northeastern North America (Arctic slope and northern tributaries of the Mississippi River). Our estimate of divergence time between western and eastern *R. cataractae* based on combined *cytb* and *ND2* data was similar to that estimated by Kim and Conway (about 2.5 mya), but had wider confidence intervals. Kim and Conway (2014) suggested that the major west–east phylogeographic split within the *R. cataractae* group was associated with vicariant and reconnection events driven by repeated repositioning of the Continental Divide, as well as resultant watershed exchanges during the Miocene through the Pleistocene. Furthermore, the repeated glaciations during the Pleistocene and isolation of *R. cataractae* in separate refugia east and west of the Continental Divide may well have enhanced divergence initiated in earlier times (April et al. 2013; Taylor et al. 2013). Certainly, however, the west–east divergence greatly postdates the last major period of Rocky Mountain orogeny (80–55 mya; English and Johnston 2004). Furthermore, these lineages differ by 2.5% net *p*-distance at mtDNA cytochrome oxidase (E.B. Taylor unpublished data), which is at or slightly above the level associated with significant reproductive isolation inferred from nDNA analyses and with possible recognition as distinct taxa in several North American freshwater fishes (April et al. 2013). Interestingly, Bartnik (1972) reported significant differences in the spawning time, behaviour, and in male nuptial colouration be-

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tween *R. cataractae* sampled from either side of the Continental Divide. Although longnose dace on either side of the Continental Divide have been designated by some as two subspecies (*R. c. cataractae* and *R. c. dulcis*; see Jordan and Evermann 1896; Bartnik 1972), this subspecific distinction is not widely recognized (McPhail and Lindsey 1970) and the magnitude of the molecular differences resolved here and by Kim and Conway (2014), coupled with the phenotypic measures of differentiation, support the idea that *R. cataractae* east and west of the Continental Divide may warrant separate taxonomic status at the species level (Kim and Conway 2014).

Western lineages of *R. cataractae*-like dace

The strong differentiation of the eastern samples of *R. cataractae* also complicates interpretations of the taxonomic relationships of western lineages within the longnose dace because our nDNA analyses indicated that *R. cataractae* was paraphyletic with respect to *R. evermanni* and the Millicoma dace (see also Kim and Conway 2014). This suggests that, at least based on molecular characterization, the three taxa (the Millicoma dace is still awaiting taxonomic description; see Bisson and Reimers 1977) may be better considered to be a single polytypic species, *R. cataractae*. If, however, only the western lineages of longnose dace like samples are examined, then western *R. cataractae* are monophyletic with respect to both *R. evermanni* and Millicoma dace at least for mtDNA. The lack of resolution among western *R. cataractae*, *R. evermanni*, and the Millicoma dace at the two nuclear loci could be attributable to the generally longer coalescence time for these nuclear loci especially if the divergence of all three took place recently during the Pleistocene (Bisson and Reimers 1977; Zink and Barrowclough 2008; McPhail and Taylor 2009).

Similarly, we found no evidence of phyletic distinction between *R. cataractae* and Nooksack dace in the two nuclear genes that we sequenced. Furthermore, Girard and Angers (2011) documented modest variation among eastern populations of *R. cataractae* at single exons of growth hormone and trypsin loci, but we found no distinctions at these loci between small samples (five each) of longnose dace and Nooksack dace (E.B. Taylor, unpublished data). As discussed above for *R. evermanni* and the Millicoma dace, the lack of nuclear sequence differentiation between longnose dace and Nooksack dace, despite their strong mtDNA divergence, does not necessarily mean that they might not be, in fact, distinct taxa because of a lack of resolution at these loci or owing to incomplete lineage sorting. Ruskey (2014) and Ruskey and Taylor (2015), however, studied longnose dace and Nooksack dace at three sites where they occur in sympatry in the lower Fraser River area of southwestern British Columbia. At each of these sites, fish identified as longnose dace and Nooksack dace using mtDNA were not differentiated from one another when assayed at 10 microsatellite DNA loci and individual fish were not diagnosable using a key character, lateral-line scale count, used in the original identification of the Nooksack dace (McPhail 1967). Consequently, the longnose dace and Nooksack dace were members of the same genetic population in sympatry and the presence of both mtDNA lineages in single localities is best interpreted as the result of the retention of an historical divergence owing to the haploid, clonal nature of mtDNA in fish that appear to have freely interbred upon secondary contact in the lower Fraser Valley (Ruskey and Taylor 2015). Consequently, the longnose dace and Nooksack dace appear not to warrant separate taxonomic recognition.

Our work on *R. cataractae* and that of Kim and Conway (2014) and the work of others on related *Rhinichthys* (e.g., Oakey et al. 2004; Pfrender et al. 2004; Smith and Dowling 2008; Hoekzema and Sidlauskas 2014) have indicated that the genus contains a number of cryptic lineages, especially within the mountainous landscape of western North America. The dynamic tectonic history of the area coupled with general features of the life history of *Rhinichthys* (freshwater resident, lithophilic, relatively short generation time, small bodied, and likely of limited dispersal abilities) have probably interacted to promote the evolution of divergent intraspecific lineages and perhaps cryptic species. Despite some continuing taxonomic uncertainties (potential cryptic species in eastern and western lineages, ambiguity of status of *R. evermanni*), conservation of divergent lineages within *Rhinichthys* may be accomplished through their legal recognition as "distinct population segments" and "designatable units" under the *Endangered Species Act* and the *Species at Risk Act* in the United States and Canada, respectively (USFWS and NMFS 1996; COSEWIC 2007).

Acknowledgements

This work was supported by Natural Sciences and Engineering Research Council of Canada (NSERC) Discovery and Equipment grants awarded to E.B.T. We thank M. Pearson, S. Avery-Gomm, J. Witt, J. Mee, and L. Bernatchez for providing a number of samples and two anonymous reviewers for their input.

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