

The Phylogeny of *Oncorhynchus* (Euteleostei: Salmonidae) Based on Behavioral and Life History Characters

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There is no consensus between morphological and molecular data concerning the relationships within the Pacific basin salmon and trout clade *Oncorhynchus*. In this paper we add another source of characters to the discussion. Phylogenetic analysis of 39 behavioral and life history traits produced one tree structured (*O. clarki* (*O. mykiss* (*O. masou* (*O. kisutch* (*O. tshawytscha* (*O. nerka* (*O. keta*, *O. gorbusha*)))))). This topology is congruent with the phylogeny based upon Bayesian analysis of all available nuclear and mitochondrial gene sequences, with the exception of two nodes: behavior supports the morphological data in breaking the sister-group relationship between *O. mykiss* and *O. clarki*, and between *O. kisutch* and *O. tshawytscha*. The behavioral traits agreed with molecular rather than morphological data in placing *O. keta* as the sister-group of *O. gorbusha*. The behavioral traits also resolve the molecular-based ambiguity concerning the placement of *O. masou*, placing it as sister to the rest of the Pacific basin salmon. Behavioral plus morphological data placed *Salmo*, not *Salvelinus*, as more closely related to *Oncorhynchus*, but that placement was only weakly supported and awaits collection of missing data from enigmatic species such as the lake trout, *Salvelinus namaycush*. Overall, the phenotypic characters helped resolve ambiguities that may have been created by molecular introgression, while the molecular traits helped resolve ambiguities introduced by phenotypic homoplasy. It seems reasonable therefore, that systematists can best respond to the escalating biodiversity crisis by forming research groups to gather behavioral and ecological information while specimens are being collected for morphological and molecular analysis.

THE Salmoninae is a group of euteleost fishes naturally distributed in the cold temperate waters of the Northern Hemisphere (Groot, 1996). Together with their sister-group Thymallinae (Grayling; Wilson, 1997) and the basal Coregoninae (Whitefish, Cisco, Inconnu, and Sheefish), they form the commercially important Salmonidae. There are at least four monophyletic salmonine genera: *Hucho* (Huchen and Taimen), *Salmo* (Atlantic trout and salmon), *Oncorhynchus* (Pacific trout and salmon), and *Salvelinus* (char). The remaining extant species form a paraphyletic assemblage extending across Siberia and China to southeastern Europe. The exact composition, affiliations, and biology of these “archaic trout” (*Salmothymus*, *Acantholingua*, *Platysalmo*, *Brachymystax*) have not been completely resolved, although morphological data place them between *Thymallus* and the more derived salmonines (Stearley and Smith, 1993).

Although biologists have been studying the taxonomy and systematics of salmonines for decades, there is still no consensus about how many species exist in the subfamily and how those species are related to one another. One major point of contention involves identifying the sister-group of *Oncorhynchus*, with some data indicating *Salmo* (morphology: Stearley and Smith, 1993; Wilson and Li, 1999; molecular:

Phillips and Oakley, 1997) and other data supporting *Salvelinus* (molecular: Crespi and Fulton, 2004). Within *Oncorhynchus*, three subgroups have generally been recognized: the Pacific trout (e.g., *O. mykiss* [Rainbow/Steelhead] + *O. clarki* [Cutthroat]; but see Stearley, 1992; Stearley and Smith, 1993), and the Pacific salmon comprising *O. kisutch* (Coho) + *O. tshawytscha* (Chinook), plus *O. gorbusha* (Pink) + *O. nerka* (Sockeye) + *O. keta* (Chum; allozymes: Utter et al., 1973; Osinov, 1999; morphology: Stearley, 1992; ecology: Smith and Stearley, 1989; mtDNA: Shedlock et al., 1992; Domanico and Phillips, 1995; Oohara et al., 1997; both nuclear and mtDNA: Domanico et al., 1997; Kitano et al., 1997; Phillips and Oakley, 1997; growth hormone introns: Oakley and Phillips, 1999; all data sets combined: Crespi and Fulton, 2004). The relative position of these three groups to one another remains controversial, although most authors agree that the Pacific trout, whether monophyletic or not, are basal to the Pacific salmon. Also controversial is the position of *O. masou* (the Masu Salmon) within the genus and the relationships among *O. gorbusha*, *O. nerka*, and *O. keta*. Even more ambiguity is found in *Salvelinus*; only the sister-group relationship between *S. alpinus* (Arctic Char) and *S. malma* (Dolly Varden) has been repeatedly recognized (morphology: Ca-

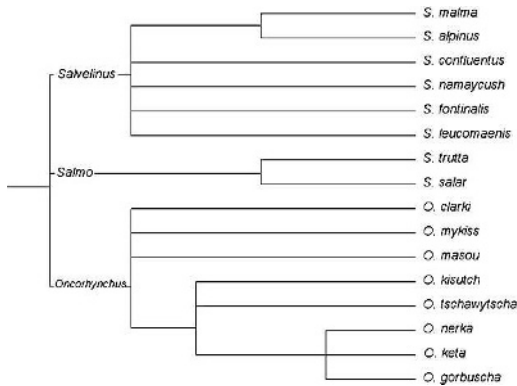


Fig. 1. Consensus tree produced when topologies from previous studies are combined to show areas of congruence and debate concerning the relationships within and among the salmonine genera *Salmo*, *Salvelinus*, and *Oncorhynchus*.

vender, 1980; Behnke, 1984; Stearley, 1992; molecular: Phillips and Oakley, 1997; Crespi and Fulton, 2004; allozymes: Osinov, 1999). If we were to draw a consensus tree based solely upon the topologies shown in preceding studies, the resulting tree would show a substantial lack of resolution (Fig. 1).

Salmonines have attracted the attention of ecologists, conservationists, and evolutionary biologists for decades because of their complicated life histories (Hendry and Stearns, 2004 and references therein). Although they all reproduce in freshwater, some species are anadromous and spend part of their growth phase in salt water, returning to their ancestral breeding grounds to spawn, whereas others complete their entire life cycle in freshwater. A general synopsis of salmonine breeding would roughly resemble the following pattern: females search for space to excavate a series of nests in which they will successively deposit eggs. Once a location is chosen, they create depressions in the gravel substrate with powerful beats of their caudal fin ("digging"). Periodically, they test nest progress by lying on the gravel with their bodies arched and their anal fin pressed into the gravel ("probing"). Males fight to establish a dominance hierarchy, which is maintained within the groups clustering around a nest-building female. The dominant male blocks access to the female and, at the same time, courts her by approaching laterally and vibrating his body intensely from head to tail ("quivering"). When the female signals her readiness to spawn by lying over the nest with her mouth agape and body trembling, the dominant male moves beside her and the two simultaneously release gametes while trembling and gaping. Other males may attempt to rush in

and fertilize the eggs at this time (for an extensive discussion see Esteve [2005a] and references therein). Both males and females are polygamous, spawning whenever possible with many partners during the breeding season.

The preceding discussion illustrates some of the many complex characters involved in salmonine courtship. Given the diversity of these traits within salmonids, it is surprising that no one has incorporated them into a phylogenetic analysis for the family or any of the subfamilies. In this paper we present such an attempt, combining behavioral and life history traits gleaned from extensive video-analysis of courtship in 13 salmonid species and from the literature. We were interested in answering three questions: First, do behavioral traits produce a well-supported phylogeny for the genus *Oncorhynchus*? Second, if so, how does this phylogenetic pattern compare with topologies based upon morphological and molecular data? We will address this question in part by combining the behavioral and morphological evidence in a total evidence analysis *sensu* Kluge (1998). Neither of us is a molecular systematist, so we leave to others the task of adding the extensive molecular database to the morphology and behavior. Third, do behavioral data support *Salmo* or *Salvelinus* as the sister-group to *Oncorhynchus*? Our overall goal in presenting this study is to add another source of characters to the growing database for salmonine systematics.

MATERIALS AND METHODS

The ingroup.—The 11 species of *Oncorhynchus* are distributed throughout the north Pacific and associated shorelines, including river systems associated with those shorelines, and drainages into inland areas. Few behavioral characters have been recorded for inland species, the endangered Gila Trout (*O. gilae*), the vulnerable Mexican Golden Trout (*O. chrysogaster*), and the critically endangered Apache Trout (*O. apache*), so those species were not included in our analysis at this time. The ingroup thus consists of eight species: *Oncorhynchus clarki*, *O. mykiss*, *O. masou*, *O. kisutch*, *O. nerka*, *O. tshawytscha*, *O. keta*, and *O. gorbuscha*.

Outgroup.—*Thymallus arcticus* (Arctic Grayling), *Hucho hucho* (Huchen), *Salvelinus namaycush* (Lake Trout), *S. alpinus*, *S. malma*, *S. confluentus* (Bull Trout), *S. fontinalis* (Brook Trout), *Salmo trutta* (Brown Trout), and *S. salar* (Atlantic Salmon) were used as outgroups. We constructed composite outgroups (Maddison et al., 1984; Wiley et al., 1991; Swofford and Maddison, 1992)

TABLE 1. SPECIES, LOCATION, AND DATE OF VIDEO RECORDINGS^a.

Species	Location (river, region, country)	Date
<i>Thymallus arcticus</i>	Red Rod Creek, Montana, USA	May 2003
<i>Salmo salar</i>	Cares, Asturias, Spain	Dec. 1993, 1998, 1999
	Pigueña, Asturias, Spain	Dec. 1995
	Farrar, Highlands, Scotland	Nov.–Dec. 1996
	Nansa, Cantabria, Spain	Dec. 1997
	Findhorn, Highlands, Scotland	Oct.–Nov. 1999
<i>S. trutta</i>	Vall Ferrera, Pyrenees Mountains, Spain	Nov. 1995, 1998
	Cares, Asturias, Spain	Nov.–Dec. 1997
	Dobra, Asturias, Spain	Dec. 1997
<i>Salvelinus confluentus</i>	Sauk South Fork, Washington, USA	Oct. 2002
<i>S. fontinalis</i>	Hell Roaring Creek, Montana, USA	Oct. 2002
<i>S. malma</i>	Steep Creek, Alaska, USA	Aug. 2002
<i>Oncorhynchus clarki</i>	Big Beef Creek Washington, USA	Dec. 2002
<i>O. gorbuscha</i>	Skykomish, Washington, USA	Sep. 2001
	Weaver Creek, British Columbia, Canada	Oct. 2001
<i>O. keta</i>	Big Beef Creek, Washington, USA	Nov.–Dec. 2001
	Marx Creek, Alaska, USA	Sep. 2003
<i>O. kisutch</i>	Chehalis, British Columbia, Canada	Feb.–Mar. 2001
	Big Beef Creek Washington, USA	Nov.–Dec. 2001, Dec. 2002
<i>O. nerka</i>	Cedar, Washington, USA	Oct.–Dec. 2000, Jan. 2003
	Steep Creek, Alaska, USA	Aug. 2002
<i>O. tshawytscha</i>	Steep Creek, Alaska, USA	Aug. 2002
	Yakima, Washington, USA	Sep. 2002

^a Additional tapes provided by the following researchers: *S. confluentus* (J. Glasgow, D. Bickford, D. Chaplin); *O. mykiss* and *O. tshawytscha* (B. Berejikian); *O. masou* (T. Yamamoto).

based upon two hypothesized relationships (Crespi and Fulton, 2004) among those species: (*Thymallus* (*Hucho* (*Salvelinus* (*Salmo*, *Oncorhynchus*)))) and (*Thymallus* (*Hucho* (*Salmo* (*Salvelinus*, *Oncorhynchus*))))). The data matrix was analyzed separately with each composite outgroup. We were particularly interested in exploring whether varying the placement of *Salmo* and *Salvelinus* with respect to *Oncorhynchus* would affect hypotheses of phylogenetic relationships within that clade.

The data set.—Hi-8 mm and mDV video cameras mounted in acrylic underwater housings were used to monitor spawning salmonids in the wild and in semi-natural spawning channels (localities and filming dates are provided in Table 1). The camera was placed in the river at about 0.3–1.0 m from a developing redd. The video signal was transmitted via cable to a color monitor and a remote commander allowed manual focus and zoom from the bank. A black and white high sensitivity CCTV camera along with a 900-watt power light with an infrared (800 nm) filter was used to film salmonids during complete darkness. This set-up does not disturb natural behavior (Gaudemar and Beall, 1999; Grant et al., 2002). Approximately 300 hrs of video data

were analyzed at a later date using frame-by-frame playback whenever necessary, recording the structure, duration, and frequency of each behavior.

In addition to personal observations, data were collected from the literature for particular species (for a summary of references used by species, see Esteve, 2005b). Characters are described in Appendix 1. Character coding was conservative. When we did not have enough data to make a decision, we used the (?) code. We only coded “absence” when we did not observe a behavior over at least 10 h of observation. We checked for non-independence of traits by asking whether traits within the same category (e.g., female nest digging) had identical entries in the data matrix for all species. If so, we combined any traits in which the descriptions were so close that we might have mistakenly subdivided one trait into two or more units.

Phylogenetic analysis.—The data matrix (Table 2) of 39 behavioral and life history traits was entered into PAUP (vers. 4.0b2*, D. L. Swofford, Sinauer Associates, Inc., Sunderland, MA, 1998) and examined using the exhaustive search algorithm. Autapomorphies were included because they represent evolutionary change even though they

TABLE 2. DATA MATRIX. COg (composite outgroup), sister to *Oncorhynchus* is 1 (*Salmo*) or 2 (*Salvelinus*); “—” = inapplicable.

Species	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
<i>Thymallus arcticus</i>	?	0	1	0	—	?	1	0	0	—	0	0	0	0	—	0	0	0	0	0
<i>Hucho hucho</i>	0	?	1	?	?	0	0	0	?	?	0	?	0	?	—	1	?	0	0	0
<i>Salmo trutta</i>	0	0	0	0	1	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0
<i>S. salar</i>	0	1	0	0	0	0	0	0	0	0	0	0	0	0	1	0	1	0	0	1
<i>Salvelinus namaycush</i>	0	?	0	?	—	0	1	0	?	—	0	0	0	?	—	0	?	0	0	1
<i>S. alpinus</i>	0	1	1	0	?	0	0	0	?	0	0	?	0	1	0	0	0	0	0	0
<i>S. malma</i>	0	?	1	0	?	0	0	0	?	?	0	0	0	1	0	0	0	0	0	0
<i>S. confluentus</i>	0	1	0	0	0	0	0	0	1	?	0	0	0	1	0	0	0	0	0	0
<i>S. fontinalis</i>	0	0	1	0	0	0	0	0	1	?	0	0	0	1	0	0	0	0	0	0
<i>Oncorhynchus clarki</i>	0	?	0	0	?	0	0	0	?	0	0	0	0	1	0	?	1	0	0	1
<i>O. mykiss</i>	0	1	0	0	0	0	0	0	1	?	0	0	0	0	1	1	1	0	0	1
<i>O. masou</i>	0	?	0	?	?	0	0	0	?	?	0	?	0	?	0	1	1	0	0	1
<i>O. tshawytscha</i>	0	1	0	1	1	1	0	1	?	0	1	0	0	0	1	1	1	0	0	1
<i>O. kisutch</i>	0	1	0	1	1	1	0	1	1	0	1	0	0	0	1	1	1	0	0	1
<i>O. nerka</i>	0	1	0	1	1	1	0	1	1	1	0	1	0	0	1	1	1	1	1	1
<i>O. keta</i>	1	1	0	1	1	1	0	2	1	1	1	0	1	0	1	1	1	1	1	1
<i>O. gorbuscha</i>	1	1	0	1	1	1	0	2	1	1	1	1	1	0	1	1	1	1	1	1
COg 1	0	1	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0
COg 2	0	1	0	0	0	0	0	0	1	0	0	0	0	1	0	0	0	0	0	0

Species	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	
<i>Thymallus arcticus</i>	0	0	1	—	0	1	—	0	0	0	0	1	—	1	0	0	0	?	?	0
<i>Hucho hucho</i>	0	0	1	1	0	?	1	0	1	?	1	1	?	?	?	?	?	?	?	?
<i>Salmo trutta</i>	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
<i>S. salar</i>	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
<i>Salvelinus namaycush</i>	0	0	1	—	0	—	—	0	?	?	1	0	—	?	0	—	1	?	?	?
<i>S. alpinus</i>	0	0	0	1	0	?	1	0	1	?	1	0	1	?	?	1	1	0	?	?
<i>S. malma</i>	0	0	0	1	0	1	1	0	0	0	1	0	1	0	?	1	1	0	0	?
<i>S. confluentus</i>	0	0	0	1	0	0	1	0	0	0	1	0	0	0	0	1	1	0	0	0
<i>S. fontinalis</i>	0	0	0	1	0	1	0	0	0	0	0	0	0	0	0	1	1	1	0	0
<i>Oncorhynchus clarki</i>	0	0	0	0	0	?	0	0	0	0	0	1	0	0	?	0	0	?	?	?
<i>O. mykiss</i>	0	1	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0
<i>O. masou</i>	0	1	0	0	1	?	0	1	?	?	0	0	?	?	?	0	0	0	0	0
<i>O. tshawytscha</i>	0	1	0	0	1	0	0	1	0	1	0	0	1	0	0	0	0	1	0	0
<i>O. kisutch</i>	0	1	1	0	1	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0
<i>O. nerka</i>	1	1	1	0	1	1	0	1	0	1	0	0	0	1	0	0	0	1	1	1
<i>O. keta</i>	1	1	1	0	1	1	0	1	0	1	0	0	0	1	1	0	0	1	1	1
<i>O. gorbuscha</i>	1	1	1	0	1	1	0	1	0	1	0	0	0	1	0	0	1	1	1	1
COg 1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
COg 2	0	0	0	1	0	0	1	0	0	0	1	0	0	0	0	0	0	0	0	0

are not phylogenetically informative (de Queiroz and Wimberger, 1993), and they may one day need to be reevaluated as missing data from other species becomes available. Although bootstrap analysis was developed for molecular data, we used it as a heuristic device to assign rough values for nodal support based on 1,000,000 random repetitions.

Strong and Lipscomb (1999) recommended scoring inapplicable characters as “?” in NONA (vers. 2, P. Goloboff, Tucumán, Argentina, 1999), using the option “eliminate semi-strict branches” to remove nodal support based on

ambiguous resolutions of “?”. PAUP does not have this option, which makes it difficult to choose the least problematic way to code these states, given that all protocols are flawed in one way or another. Fortunately, all of the “inapplicable” states were present in only two of the outgroup taxa, *Salvelinus namaycush* and/or *Thymallus arcticus*. Given the relationships among the outgroups, this did not create any ambiguity for character optimization when *Oncorhynchus* was the ingroup. When the larger group *Oncorhynchus* + *Salmo* + *Salvelinus* was examined, we coded inapplicable characters in two ways: as

missing (“?”) and as a distinct character state, then compared the resulting topologies, watching carefully for nonsense groups formed when an inapplicable character state functioned as a synapomorphy.

All characters were run unweighted and unordered (Fitch, 1971) and the parsimony option “collapse branches if minimum length is zero” was chosen. The above protocols were repeated for total evidence analyses adding the 119 morphological traits described in Stearley and Smith (1993) to the behavioral data matrix. When the larger group *Oncorhynchus* + *Salmo* + *Salvelinus* was examined, the matrix was run using Branch and Bound and 100,000 bootstrap iterations.

RESULTS

Relationships within Oncorhynchus.—Exhaustive search of the behavioral data matrix generated only one tree (Fig. 2A). There was no difference in the topology of this tree, and only very minor differences in tree statistics, when either *Salmo* or *Salvelinus* was used as the sister-group to *Oncorhynchus* for computing the composite outgroup values. When the outgroup configuration was (*Thymallus* (*Hucho* (*Salvelinus* (*Salmo*))), 25 characters were parsimony informative, CI = 83.78 (excluding uninformative characters 81.25), RC = 73.09, RI = 87.23. When the outgroup configuration was (*Thymallus* (*Hucho* (*Salmo* (*Salvelinus*))), 25 characters were parsimony informative, CI = 84.62 (excluding uninformative characters 81.25), RC = 73.81, RI = 87.23.

Adding the morphological database to the behavioral traits produced two trees differing only in the postulated sister-group of *O. gorbuscha* (consensus tree, Fig. 2B). Once again there were no differences in the tree topologies when either *Salmo* (53 characters parsimony informative, CI = 83.33 [excluding uninformative characters 78.08], RC = 69.73, RI = 83.67) or *Salvelinus* (54 characters parsimony informative, CI = 83.81 [excluding uninformative characters 77.33], RC = 69.56, RI = 83.00) was used as the sister-group to *Oncorhynchus* to compute the composite outgroup.

Relationship among Salmo, Salvelinus, and Oncorhynchus.—Running the larger behavioral data matrix using *Thymallus* and *Hucho* to reconstruct the composite outgroup produced one tree placing a monophyletic *Salvelinus* in a polytomy with a paraphyletic *Salmo* and *Oncorhynchus* (Fig. 3A). There were only minor differences in

the tree statistics when inapplicable characters were scored as another state (37 parsimony informative characters, CI = 72.31 [not including autapomorphies 71.43], RC = 61.18, RI = 84.62) or as missing (37 parsimony informative characters, CI = 69.87 [not including autapomorphies 67.86], RC = 58.36, RI = 84.62).

Adding the 119 morphological traits produced four trees differing only in the position of *O. gorbuscha* and *Salvelinus fontinalis* (Fig. 3B). There was no difference in the topologies of those trees whether inapplicable characters were scored as another state or as missing (“?”), although there were minor differences in the tree statistics (missing: 98 parsimony informative characters, CI = 68.05 [without autapomorphies 65.82], RC = 58.17, RI = 85.48; new state: 98 parsimony informative characters, CI = 69.32 [without autapomorphies 67.27], RC = 59.26, RI = 85.48).

DISCUSSION

The characters.—One of the central tenets of phylogenetic systematic inference is that each character used in the analysis must have experienced “independent, heritable transformation events” (Grant and Kluge, 2004:26; transformation series of Hennig, 1966). In other words, characters are differentiated on the basis of their unique history. Independence is thus a hypothesis that is postulated *a priori* during the process of character description, then evaluated *a posteriori* by comparison with other characters. All of the traits used in this study met the historical criterion for independence; in no case did two or more traits within the same category (e.g., female nest digging) have identical patterns of origin and elaboration/loss. So, for example, females in some species dig nests (character 7) but never show displacement digging (character 5; e.g., *Salmo salar*, *Salvelinus fontinalis*, *S. confluentus*, *Oncorhynchus mykiss*), while many species with different age classes (character 1) also have precociously maturing parr (character 23), except *Oncorhynchus kisutch* and *O. nerka*.

Characters 36 and 37 (undulating before and after spawning) are particularly interesting because they demonstrate the kind of complexity that can arise during the process of character description. The behavior itself (undulation) appears identical, which implies that its structure (pattern of muscle contractions) is controlled by the same set of genetic instructions regardless of when it is performed. What makes the two traits different is a shift in both timing (pre-spawning vs. post-spawning) and function (clean the nest of debris vs. settle eggs into interstices). The

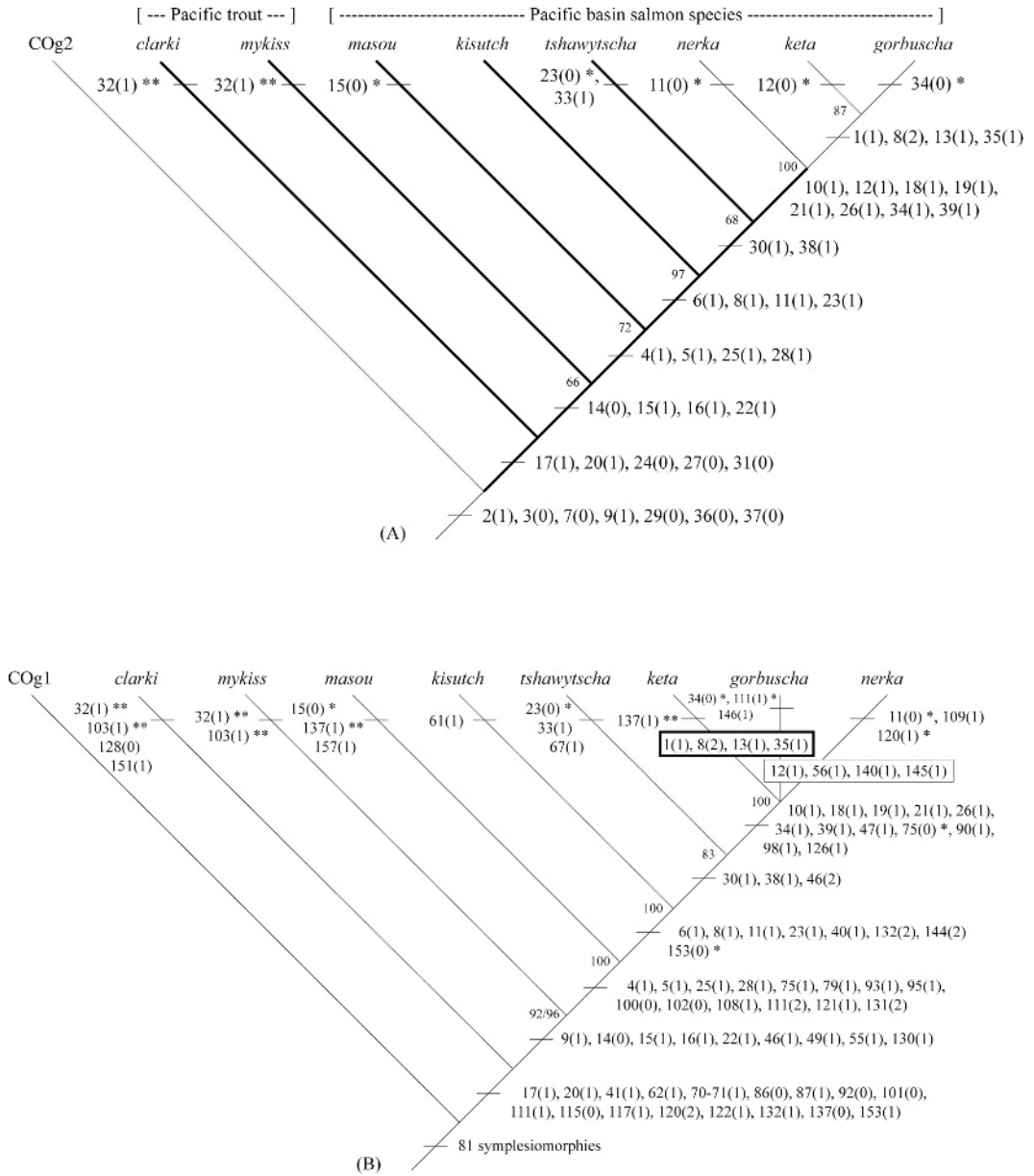


Fig. 2. (A) Single tree recovered for *Oncorhynchus* based on 39 behavioral and life-history characters. Bold lines = congruence with morphological data. Note that the “Pacific trout” are paraphyletic. (B) Consensus of two equally parsimonious trees based on 158 behavioral + morphological traits. For descriptions of morphological traits and data matrix see Stearley and Smith (1993). Characters in boxes represent traits shared between *O. gorbuscha* and either *O. keta* (bold box, all behavioral traits) or *O. nerka* (mainly morphological traits). Numbers = bootstrap values from 1,000,000 replicates. COg1 = composite outgroup with *Salmo* as sister-group to *Oncorhynchus*. ** = convergent traits; * = reversal to plesiomorphic state.

observation that *Salvelinus namaycush* has lost pre-spawning undulation (females do not make a nest), but retains the post-spawning behavior, indicates that the two traits are decoupled to some extent. It is thus possible that the two traits

share a common genetic system for trait production, a different system controlling timing and have been subject to a different set of selection pressures during character evolution. We hypothesized *a priori* that this represented

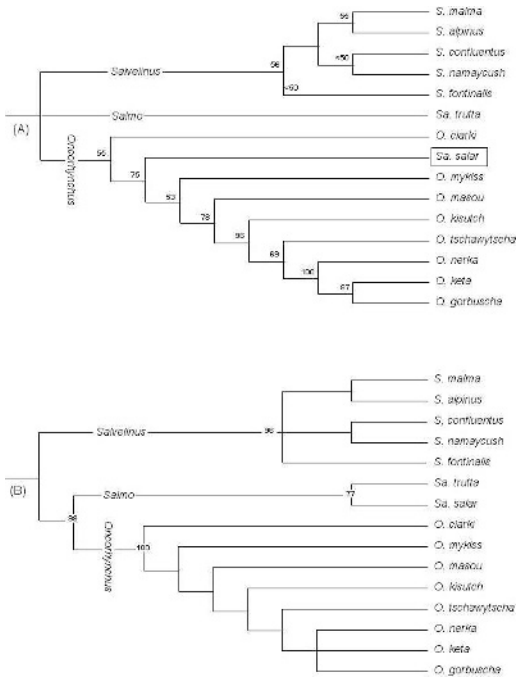


Fig. 3. (A) Single tree recovered for *Oncorhynchus*, *Salmo*, and *Salvelinus* based on 39 behavioral and life history traits. *Salmo salar* is misplaced within *Oncorhynchus*. (B) Consensus of four equally parsimonious trees based on 158 behavioral + morphological traits. Numbers are bootstrap values from 100,000 replicates.

enough independence to code the two traits separately, and the change in *S. namaycush* corroborated that hypothesis. This does not mean that the story ends here. If the two traits have been subjected to selection for different functions, then we may be able to measure subtle differences in structure and/or performance parameters such as frequency or intensity that distinguish nest cleaning from egg deposition. Using that information to refine the initial character descriptions and codings begins the process of reciprocal illumination that Kluge (2003) called cycles of empirical testing and retesting.

Relationships within Oncorhynchus.—The 39 behavioral traits produced a robust hypothesis of phylogenetic relationships within *Oncorhynchus*. That hypothesis is congruent with the phylogeny based upon Bayesian analysis of all available nuclear and mitochondrial gene sequences (Crespi and Fulton, 2004) with two exceptions. First, a sister-group relationship was not recovered between *O. kisutch* and *O. tshawytscha*. The placement of *O. tshawytscha* as sister-group to the

O. nerka + *O. keta* + *O. gorbuscha* clade, rather than to *O. kisutch*, is supported by the morphological data (Stearley and Smith, 1993). Although that placement is only supported by three synapomorphies (dermethmoid with widely divergent posterior wings, loss of both a series of, and intense, male quivering; Fig. 2B), the group *O. kisutch* + *O. tshawytscha* occurred with a frequency of less than 1% in 1,000,000 bootstrap iterations on both the behavior and behavior + morphology trees. The molecular tree of Crespi and Fulton showed relatively strong support for a sister-group relationship between these two species; however, that support was based upon congruence among MHC, VIT, ITS2, *CYTb*, GHIC genes, coupled with a lack of congruence among sequences from CO3, 16S, ITS1, GH2C, and ND3. Based on this, Crespi and Fulton (2004) concluded that the sister-group status of the two taxa was “not entirely unambiguous”, and our data support that conclusion.

Second, behavioral traits, like morphology, do not support the monophyly of the Pacific trout clade, *Oncorhynchus clarki* and *O. mykiss*; that topology is recovered less than 1% of the time in the behavioral database (1,000,000 bootstrap iterations), while Stearley and Smith (1993) uncovered no synapomorphies for these two species in their 119 morphological traits. *Oncorhynchus mykiss* is instead placed as the sister-group to the remaining species of *Oncorhynchus* based on four morphological (A-shaped dermethmoid, square lateral ethmoids, frontals with expanded shelf above orbit, square, stubby ceratohyal) and five behavioral characters (Fig. 2B).

Why might there be a disagreement between the phenotypic and genotypic data? Many salmonid species, particularly *Salvelinus* and the various “trout,” including *Salmo trutta* and the inland/Pacific trout (e.g., *Oncorhynchus apache*, *O. gilae*, *O. mykiss*, *O. clarki*), hybridize quite easily with one another. That hybridization is often followed by genetic introgression (Chevassus, 1979; McLennan, in press and references therein), which can confound phylogenetic reconstruction in three general ways. First, it may be difficult to correctly classify individuals from introgressed populations based on either genetic or phenotypic markers depending upon the number of F1 hybrids, backcrosses, and F2s in the population and degree of asymmetry in the introgression (Epifanio et al., 1999; Weigel et al., 2002). Second, if introgression occurs between distant relatives, then the phenotypic signal may be weakened by increased homoplasy in the data set (Funk, 1985; see Feliner et al. [2001] for additional references and an extensive discussion

of the subtleties of this dynamic). If, on the other hand, such interactions occur between close relatives, then the distinctive status of each species may be obscured by the “exchange” of autapomorphic traits producing spurious synapomorphies.

The extent of introgression depends, in part, upon the genetic system. The mitochondrial genome is expected to introgress more rapidly than nuclear genes, particularly when hybridization events are rare (Rognon and Guyomard, 2003). For example, the leuciscine cyprinid, *Scardinius dergle*, clusters with other members of its genus based on morphological and nuclear data, but cytochrome *b*, places it soundly within the distantly related leuciscine genus *Squalius*. Mitochondrial DNA introgression is thought to have occurred very rapidly between these two cyprinids following dam construction in 1962 (Freyhof et al., 2005). Hybridization/introgression is much more extensive between *O. mykiss* and *O. clarki* (Bettles et al., 2005 and references therein) than between *Scardinius* and *Squalius*. Of 86 populations surveyed by Weigel et al. (2002), 17 displayed more than 50% introgression, and 28 were scored from 1–49%. Interactions between the two species are postulated to have been occurring since at least the last Pleistocene glaciation (Brown et al., 2004) and possibly the Pliocene (Smith et al., 2002), enough time, given the level of hybridization, for both mtDNA (*Cytb*, DLoop, ND3, CO3) and nuclear genes (GH1c, GH2c) to produce the congruent patterns obtained by Crespi and Fulton (2004).

If these results do indeed reflect genetic introgression and not descent from a most recent common ancestor, why do phenotypic traits not show a similar pattern of relatedness? The most obvious answer is that mitochondrial introgression is generally expected to introduce a larger suite of correlated homoplasies into a study than phenotypic introgression because the mitochondrion is transferred as a unit, but its genes are treated as independent characters in phylogenetic analyses (Smith, 1992). Genetic sequences are also more straightforward to score than phenotypic traits, which often represent the endpoint of a complex interaction among different genes, and between those genes and environmental conditions during development. “Phenotypic” introgression can thus completely replace one character state with another, produce an intermediate form of both states, a continuum between the two, or have no effect (Smith, 1992). In other words, it may be more difficult to detect and score introgressed phenotypic traits, particularly when introgression produces a continuum of states. Overall, then, the

monophyly of the Pacific trout, which may reflect the influence of false synapomorphies created from a history of hybridization and introgression between *O. mykiss* and *O. clarki*, is not supported by our analysis.

The remaining *Oncorhynchus* species (the Pacific basin salmon) appear to be far more resistant to hybridization across major conspecific lineages and species; indeed, hybridization is generally described as rare and introgression very limited (Rosenfield et al., 2000), possibly because of the complicated life histories displayed by these fishes (Utter, 2000, 2004). It is thus interesting that the molecular data place *O. gorbuscha* as sister to *O. keta*, while morphology postulates an *O. gorbuscha*–*O. nerka* (*O. nerka* + fossil species, *O. rastrusus*) connection. Stearley and Smith (1993) proposed that the molecular results might reflect introgression between *O. gorbuscha* and *O. keta*; however, as noted above, hybridization is rare in these fish (*O. keta* and *O. gorbuscha*: Simon and Noble, 1968), and there is no evidence for introgression between these species in nature. More importantly, the relationship is supported by nuclear genes, which require an extensive history of introgression between species to be maintained in the populations (Rognon and Guyomard, 2003). Behavioral traits support the molecular data (Fig. 2A), so it seems more plausible that the morphological traits linking *O. gorbuscha*–*O. nerka* in Stearley and Smith’s analysis are homoplastic. One character (>25 gillrakers), is homoplastic throughout the salmonids and might bear re-coding. The other two (dorsal hump in breeding males, frontals contact epiotics in large individuals) may be either truly homoplastic or miscoded, should behavior + molecular data be giving us the “correct” picture of phylogenetic relationships in this group.

The behavioral traits place *Oncorhynchus masou* as the sister to the remaining Pacific basin salmon. The bootstrap value was moderate for this node based on four synapomorphies (the loss of the frontal display, 4[1]; the origin of female displacement digging, 5[1]; post-spawning nest defense, 24[1]; and semelparity, 28[1]). It is important to note, however, that since the first two behavioral traits were coded as “missing,” the PAUP algorithm assigned them to this node based on the distribution of other traits. Until we can collect more behavioral data for *O. masou*, this assignment remains a hypothesis, subject to falsification. The placement of *O. masou* agrees with the morphology-based analysis, and together the two datasets identified 14 synapomorphies for the node connecting the two clades (with the preceding caveat about

missing data: Fig. 2B). The molecular data are less robust, placing *O. masou* either as sister to a monophyletic *O. clarki* + *O. mykiss* (Bayesian analysis of all molecular data) or in an unresolved position within the Pacific basin salmon (maximum parsimony of all molecular data).

Crespi and Fulton (2004) preferred the Bayesian analysis for two reasons. First, the bootstrap values were higher for that configuration. No gene on its own, however, produced a monophyletic *O. masou* + *O. mykiss* + *O. clarki* under maximum parsimony, maximum likelihood, or Bayesian analyses. According to the trees presented by Crespi and Fulton, *O. masou* was variously placed as sister to *O. mykiss*, *O. tshawytscha*, *O. rhodurus*, all other species of *Oncorhynchus*, in a polytomy within *Oncorhynchus* or in a larger polytomy of *Oncorhynchus*, *Salmo*, and *Salvelinus*. Given this, the molecular results may be an artifact of minimal support for the group *O. masou* + *O. mykiss* coupled with stronger support for *O. mykiss* + *O. clarki*, which may itself reflect a long history of introgression as discussed previously. Second, Crespi and Fulton cited previous research indicating that *O. masou* was the “most trout-like” of the salmon species. According to our study, three of the traits used as evidence for the *O. masou*’s “troutness” are symplesiomorphies (Fig. 2A: feed during spawning migration, 6[0]; freshwater residence times of 1–2+ years, 8[0]; freshwater populations common in the species, 11[0]), while one, a semelparous life cycle (28[1]), appears to have been miscoded in the past, as suggested by Crespi and Fulton. The final piece of behavioral evidence for the existence of an *O. masou* + *O. mykiss* + *O. clarki* clade comes from the observation that male *O. masou* interbreed in the lab more easily with female *O. mykiss* than with other female *Oncorhynchus* (Chevassus, 1979). The ability to interbreed does not reliably indicate degree of relatedness because that ability is symplesiomorphic (Rosen, 1979; Cracraft, 1983). Conceptual point aside, three of the six cases of natural hybridization within *Oncorhynchus* involve *O. mykiss* (with *O. clarki*: Baumsteiger et al., 2005; *O. apache*: Brown et al., 2004; *O. gilae*: Dowling and Childs, 1992), which implies that there is something special about *O. mykiss* (not *O. masou*) that is allowing individuals to interbreed more successfully with close relatives than can other Pacific trout or salmon.

In conclusion then, neither the bootstrap values, nor the perception that *O. masou* is the “most trout-like” of the Pacific basin salmon species are strong arguments for preferring the Bayesian topology over the tree based on maximum parsimony of all available molecular

data. That latter topology, which places *O. masou* in a polytomy within the Pacific salmon clade, is congruent with both the morphological and behavioral analyses, which resolve the ambiguity by placing *O. masou* as sister to the remaining salmon species.

Relationship among Salmo, Salvelinus, and Oncorhynchus.—Although we did not have enough data to examine relationships within *Salvelinus*, we were interested in asking whether behavioral characters might help resolve the dispute concerning the sister-group to *Oncorhynchus*. The answer to this question is fairly simple: not on their own. The behavioral characters provided very little insight, aside from supporting morphology and molecular data in hypothesizing a sister-group relationship between *S. malma* and *S. alpinus* (Cavender, 1980; Crespi and Fulton, 2004).

Aside from missing data, there are two problematic taxa in our analysis. The first taxon, *Salvelinus namaycush*, has a dramatically different life history from that displayed by other trout, char, and salmon. Individuals only spawn at night, females do not dig or defend nests, do not probe the substrate prior to spawning, and do not cover their eggs afterwards (Martin and Oliver, 1980; Gunn, 1995; Fleming, 1998). At the moment, *S. namaycush* is primarily a mosaic of missing, inapplicable, and plesiomorphic traits, so the behavioral characters are of little help in resolving its placement vis à vis other species of *Salvelinus*. Removing it from the analysis decreases the number of equally parsimonious trees from four to two by placing *Salmo salar* back with *S. trutta*. We are attempting to videotape lake trout spawning to fill in as many of the missing traits as possible.

Salmo salar, the second problem taxon, clusters within *Oncorhynchus* between *O. clarki* and *O. mykiss*. Problem taxa may be hybrid species or may have experienced introgression with other species (Funk, 1985). No other phylogenetic analysis has suggested that *S. salar* belongs with *Oncorhynchus*, and it seems unlikely that only behavioral traits would reflect past hybridization or introgression events. It is more likely that the problem stems from the data themselves; most of the traits diagnosing *S. salar* are homoplastic with species of *Oncorhynchus*, possibly due to the convergent evolution of an anadromous life history. If, as hypothesized, anadromy originated in the ancestor of the Pacific basin salmon (Stearley, 1992; Esteve, 2005b), it did so following the loss of male territoriality (20[1]) and male aggression towards novel female intruders (17[1]). These characters are also present in *S.*

salar (as are over-wintering in the sea, 22[1], and migrating long distances, 15[1]), indicating that the energy saved through decreasing male–male and male–female aggressive territorial interactions may have been one of the factors that promoted the energetically expensive life style of anadromy (59–85% of total reserves: Fleming, 1998; Healey et al., 2003) when it originated. Some of those savings may have been transferred to intense competition for mates in males (the Pacific salmon and *S. salar*) and prolonged nest guarding in females (Pacific salmon only). *Salmo salar* is characterized by only two morphological traits, one of which is convergent with *Oncorhynchus* (deciduous vomeral teeth, 82[1]), and one of which is an autapomorphy (X-shaped spots, 116[1]). Overall then, *Salmo salar* is misplaced on our tree primarily because of misleading information from behavioral traits, which may be involved with the convergent origin of anadromy.

Behavior, like morphology (Stearley and Smith, 1993; Wilson and Li, 1999) and some genes (ITS: Phillips and Oakley, 1997), supports a closer relationship between *Oncorhynchus* and *Salmo* than between *Oncorhynchus* and *Salvelinus*; however, that support is not robust. Combining morphology with the behavioral data strengthens that hypothesis (Fig. 3B), whether the inapplicable traits in lake trout are scored as missing (“?”) or as a different state (“3”). Nevertheless, these results must be treated with caution until the molecular characters, many of which posit a relationship between *Salvelinus* and *Oncorhynchus*, are added to the total database.

In summary then, behavior and life history provided additional characters for resolving the relationships within *Oncorhynchus*, and may eventually provide more data for a larger investigation of the Salmoninae once missing data have been recorded. Our analysis of relationships with *Oncorhynchus* was largely congruent with the topology based on nuclear and mitochondrial data, and almost identical with the morphology-based tree. Interestingly the behavioral data were weakest in three areas that have traditionally been problematic for salmonid systematics, the sister-group status of *O. clarki* to *O. mykiss*, the sister-group status of *O. kisutch* to *O. tshawytscha*, and the sister group of *Oncorhynchus*. This begs the question of the mechanisms underlying the loss of phylogenetic resolution in only particular areas of an overall strong, multilevel data set.

The salmonids are a commercially important, evolutionarily fascinating group of fishes, one in which many species are currently threatened by the negative impacts of anthropogenic intervention (Epifanio and Nielsen, 2001). The effects of widespread introgression and small population

sizes are making it increasingly difficult to reconstruct robust phylogenetic relationships within the group, particularly using mitochondrial DNA sequences. Crespi and Fulton (2004) recommended that additional nuclear DNA markers be sequenced to aid in the resolution of relationships within this family. To this we add one further recommendation: all data, from gene sequences to their actualization in phenotypic characters, should be combined. Our analysis has demonstrated the importance of using incongruencies between data sets to help detect anomalies peculiar to different types of data. So, for example, knowing that molecular data are more susceptible to introgression than are phenotypic traits in fishes allows researchers to use the phylogenetic patterns based on behavioral/morphological traits to highlight possible instances of introgression in the genetic database (e.g., *O. clarki* and *O. mykiss*). Homoplastic evolution of similar traits under identical selective regimes, on the other hand, is more likely to be a problem for phenotypic data (e.g., *Salmo salar*). Patterns based on selectively neutral molecular traits will help researchers identify nodes reflecting the influence of such correlated phenotypic homoplasy. Of course this kind of “reciprocal illumination” is not perfect—phenotypic traits may be affected by introgression and not all genes are selectively neutral—but it does give us a place to begin when attempting to explain incongruent results. Incorporating different types of data in one analysis is thus doubly advantageous; it increases the phylogenetic signal in the dataset and it highlights *a posteriori* cases in which a subset of the data may be producing a false picture of relationships (Smith, 1992).

ACKNOWLEDGMENTS

Underwater recordings were done with the help of C. Grisolia, P. Gener, F. Melero, L. Lawhorn, R. Lladós, N. Peña, G. George, M. López, J. Shellberg, G. Boltz, N. Peterson, V. Esteve, A. da Silva, V. Ewert, C. García de Leániz, L. Cordero, A. González, B. Ernst, C. Mintz-Vera, M. D. de Souza, B. James, J. Gil, E. Biosca, and Á. Aguirre. A. de Sostoa and I. Doadrio provided useful comments on an earlier version of this manuscript. Comments from R. Mayden and G. Smith contributed greatly to the final form of the paper. Fieldwork was partially funded by the Ministerio de Agricultura, Pesca y Ganadería, Spain and by the University of Washington Center of Streamside Studies. Further work was funded by an NSERC discovery grant to DAM.

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APPENDIX 1

Character descriptions. Inapplicable traits are indicated by “–” to reduce confusion since they were scored in two different ways (“?” or “3”) during the analysis. To see videos of the behavioral traits, go to <http://www.zoo.utoronto.ca/manuesteve/index.html>.

1. Sexual maturation at different ages: 0 = present; 1 = absent.
2. Throat dilation display: Male erects branchiostegal membrane and lowers hyal bones producing a noticeable protrusion in his throat during lateral, flanking, and tail beat displays. 0 = present; 1 = absent.
3. Spawn at night: 0 = present; 1 = absent.
4. Frontal display: Individual orients towards a conspecific slightly head down with depressed dorsal fin. 0 = present; 1 = absent (S. Schroder, pers. comm. for *O. tshawytscha*).
5. Female displacement dig: Female bends her body in a slight C-shape, while beating her caudal fin rapidly from side to side during an agonistic encounter with another female. 0 = absent; 1 = present; – = inapplicable (does not dig; S. Schroder, pers. comm. for *O. tshawytscha*).
6. Feed during spawning. 0 = present; 1 = absent.
7. Female nest dig: Female bends her body in an intense C-shape and beats the gravel substrate with rapid side-to-side flexures of her tail, sweeping gravel away from the area. 0 = present; 1 = absent.
8. Habitat in which fry develop: 0 = all fry freshwater; 1 = most fry freshwater but some marine; 2 = all fry marine.
9. Tail flex display: Female keeps her caudal fin bent laterally to one side while lying over the nest, shaking her anal fin inside the gravel (probing). 0 = absent; 1 = present.
10. Nest take-over: An incoming female challenges a resident female, displaces her during a fight and takes over her nest. 0 = present; 1 = absent; – = inapplicable (no nest building; S. Schroder, pers. comm. for *O. tshawytscha*).
11. Entire breeding populations mature and breed in freshwater with no migration to and from the ocean. 0 = present; 1 = absent. Species in which (i) only a few individuals, rather than the entire population, mature in freshwater (e.g., marginal individuals of *O. kisutch* and *O. nerka* [residuals: Krogius, 1981]), or (ii) individuals remain in freshwater only after being introduced to a non-endemic area (e.g., *Oncorhynchus tshawytscha* in New Zealand, *O. gorbuscha* in the Great Lakes) were scored as trait “absent.”
12. High snout–lateral display: Male orients head-up while performing a lateral display, snout frequently breaks the water surface. 0 = absent; 1 = present (cf. posture display; Healey et al., 2003).
13. Entire populations found spawning in intertidal waters: 0 = absent; 1 = present. Anecdotal evidence indicates that some individuals from one population of *Oncorhynchus tshawytscha* (King Salmon River, Admiralty Island, Alaska) may spawn in tidally influenced parts of the river; however, this has yet to be confirmed, so we coded this trait as absent in *O. tshawytscha*. Additionally, although King Salmon River brood stock has been introduced to many different river systems in southeast Alaska, there are no reports of intertidal spawning in those areas (Halupka et al., 2000).
14. Lateral display: Fish orients parallel to its opponent with body; 0 = held horizontally; 1 = arched with head and tail slightly flexed upwards.
15. Long ocean migrations: 0 = absent (stay in ocean waters close to river’s estuary); 1 = present; “–” = inapplicable (does not go to sea).
16. Male digging: Male digs the gravel with his tail in a manner similar to female nest digging but with fewer tail beats per dig. 0 = absent; 1 = present.

17. Intersexual aggression: Male chases, bites a female intruder. 0 = present; 1 = absent.
18. Multiple redds: Female deposits eggs at two or more noncontiguous locations. 0 = present; 1 = absent (Y. Koseki, pers. comm. for *O. masou*).
19. Mass spawning: 10 or more females nest within 5 standard lengths of one another. 0 = absent; 1 = present.
20. Male territoriality: Males fight to establish and maintain a territory before females appear on the spawning ground. 0 = present; 1 = absent.
21. Nest absence: Female abandons her nest for periods of more than 30 minutes. 0 = present; 1 = absent.
22. Individuals spend at least one full winter in the ocean. 0 = absent; 1 = present.
23. Precocious maturation: Some males in a population become sexually mature at the parr stage. 0 = present; 1 = absent.
24. Post-spawning digging: Female digs the gravel, covering her eggs immediately (within 15 s) after egg deposition. 0 = present; 1 = absent; - = inapplicable (does not dig).
25. Post-spawning nest defense: Female guards nest after her last oviposition. 0 = absent; 1 = present.
26. Stop spawning under midday sun: 0 = yes; 1 = no; - = inapplicable (only spawn at night).
27. Cover eggs after every oviposition: Female, hovering laterally from the upstream rim of the nest, curves her body slightly and beats her caudal fin from side to side, sweeping gravel over the eggs. 0 = present; 1 = absent (the female deposits eggs, pauses, then deposits another batch without covering the eggs); - = inapplicable (does not dig).
28. Semelparity: Fish only spawns once during its lifetime. 0 = absent; 1 = present.
29. Both male and female swim slowly forward during spawning. 0 = absent; 1 = present.
30. Quivering bouts: Male shakes his body, snout touching female, generally alternating sides, in bouts separated by 1–3 seconds until female deposits her eggs. Coded as present when at least three bouts were recorded in 10 seconds. 0 = present; 1 = absent.
31. Sequential spawning: Successive egg depositions, separated by only a few seconds or minutes, in a single nest. 0 = absent; 1 = present.
32. Spawning time of the year: 0 = fall; 1 = spring.
33. Tail bending: Female begins to bend her tail before bending her body during building digs. 0 = present; 1 = absent; - = inapplicable (does not dig).
34. Head-down tail beat: Fish orients head down while beating the caudal fin intensely side to side. 0 = present; 1 = absent.
35. T-display: While engaged in a lateral display, one male turns and swims upstream, orients at a 90° angle to his opponent, then drifts with the current towards that opponent. To be coded as present, the T-display had to be maintained for at least 2 s. 0 = absent; 1 = present.
36. Undulating before spawning: Female cleans the gravel by performing undulating movements with her caudal fin. During this movement the tail is not flexed upwards but remains horizontal, very often touching the gravel (cf. sweeping; Fabricius and Gustafson, 1954). 0 = absent; 1 = present; - = inapplicable (does not dig).
37. Undulating after spawning: Female performs a series of slow and rhythmic swings of her body after spawning, distributing her eggs into the gravel interstices. Normally performed above the substrate without touching the gravel. 0 = absent; 1 = present.
38. Displacement quivering: Male quivers alone, away from the female (cf. trembling; Armstrong and Morrow [1980]). 0 = present; 1 = absent.
39. Winding-probing: Female continually moves her caudal fin laterally from one side to the other while lying motionless over the nest, shaking her anal fin inside the gravel (probing). 0 = present; 1 = absent.