Phylogeny of the lamprey genus *Lethenteron* Creaser and Hubbs 1922 and closely related genera using the mitochondrial cytochrome *b* gene and nuclear gene introns

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Abstract

The phylogeny of lampreys is controversial, because they possess few taxon-distinctive morphological characters. This is especially true of the relationships among the genus *Lethenteron* and the closely related genera *Eudontomyzon* and *Lampetra*. Thus, the first objective of this thesis was to use DNA sequences of the mitochondrial cytochrome *b* gene and two nuclear gene introns to infer the phylogeny among these three genera. I found that: 1) *Lethenteron* plus *Eudontomyzon morii* without *Lethenteron ninae*, *Lethenteron zanandreai*, and *Lethenteron* sp. S (a distinct cryptic species in the *Lethenteron reissneri* complex) was monophyletic; 2) *Lampetra* from the Pacific drainage of North America and *Lampetra aepyptera* should each be separated, as distinct genera, from *Lampetra* (including *Lethenteron ninae* and *Lethenteron zanandreai*) from the Atlantic drainage of Eurasia; and 3) the remaining *Eudontomyzon* and the Atlantic *Lampetra* clustered together in all analyses. The second objective of this thesis was to resolve the relationship among closely related *Lethenteron* species. Lampreys are either parasitic or non-parasitic, and each non-parasitic (satellite) species is believed to have been derived independently from the parasitic (stem) ancestor. In the phylogenetic analysis, the parasitic Arctic lamprey *Lethenteron camtschaticum* and its four satellite species were not reciprocally monophyletic. Since network methods are generally more useful for closely related haplotypes than bifurcating trees, a haplotype network of these

five *Lethenteron* species was generated using the cytochrome *b* gene sequences; *Lethenteron appendix* showed haplotype frequency distribution differences but there was little support for recognizing the other four taxa as distinct species.

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Chapter 1 General Introduction

Lampreys (order Petromyzontiformes) are jawless eel-like vertebrates with seven pairs of external lateral gill openings. They belong to phylum Chordata, subphylum Craniata, superclass Petromyzontomorphi, and class Petromyzontida (Nelson 2006). According to Renaud (2011), there are 40 species in three families and ten genera of extant lampreys in the world; among the 40 species, 36 from family Petromyzontidae are found in the Northern Hemisphere (see Section 1.1).

The taxonomic status of several lamprey genera and species has been controversial. Over the past several decades, there have been different views on the division of genera, particularly among *Eudontomyzon* Regan 1911, *Lampetra* Bonnaterre 1788 and *Lethenteron* Creaser and Hubbs 1922 (see Section 1.2). Likewise, the placement of some species continues to be uncertain. The Lombardy brook lamprey *Lethenteron zanandreai* (Vladykov 1955), for example, was originally placed in *Lampetra* (Vladykov 1955), but was later placed in *Lethenteron* by Hubbs and Potter (1971); different authors have followed Vladykov's classification (Vladykov and Kott 1979a, b; Docker et al. 1999; Caputo et al. 2009; Lang et al. 2009), whereas others have followed Hubbs and Potter's (Potter 1980; Tutman et al. 2009; Renaud 2011). In this thesis, the following issues have been studied: 1) the phylogenetic relationships of the lamprey genus *Lethenteron* and the closely related genera *Lampetra* and *Eudontomyzon*; and 2) relationships among

Lethenteron species.

Compared to bony vertebrates, lampreys possess few morphological characters for species identification and phylogenetic reconstruction. Molecular methods have been used to help resolve the phylogeny of lampreys (e.g., using mitochondrial gene sequences; Docker et al. 1999; Yamazaki et al. 2006; Lang et al. 2009). This thesis uses the mitochondrial cytochrome *b* (cyt *b*) gene (Chapter 2), as well as the nuclear transporter associated with antigen processing (TAP) and SRY-related high mobility group box D (soxD) gene introns (Chapter 3) to help resolve the phylogenetic relationships among *Lethenteron* species and clarify the relationships among *Lethenteron*, *Lampetra* and *Eudontomyzon*.

1.1 Lampreys and satellite species

All lampreys are restricted to the regions north and south of the 30° latitudes in both hemispheres, which corresponds with the annual 20 °C isotherm (Renaud 2011). This distribution appears to be determined by the temperature requirements of the developing embryos, which vary in different species and is below 25 °C in all reported cases (Clemens et al. 2010). Among the ten extant genera of lampreys, two (*Mordacia* Gray 1851 and *Geotria* Gray 1851) are in the Southern Hemisphere, and eight (*Lethenteron*, *Lampetra*, *Entosphenus* Gill 1862, *Eudontomyzon*, *Tetrapleurodon* Creaser and Hubbs 1922, *Caspiomyzon* Berg 1906, *Ichthyomyzon* Girard 1858 and *Petromyzon* L.)

are found north of the equator. All Northern Hemisphere genera are classified in the family Petromyzontidae; *Mordacia* and *Geotria* are placed in Mordaciidae and Geotriidae, respectively (Nelson 2006).

Lampreys undergo a radical metamorphosis from a larva, called an ammocoete, to an adult (Renaud 2011). In lamprey morphology, some standard counts and measurements have been used to distinguish lamprey species and to infer relationships among species and genera. The counts include the dentition of the oral disc and the number of trunk myomeres, oral papillae, oral fimbriae, and velar tentacles (Hubbs and Trautman 1937; Vladykov 1955; Potter 1968; McPhail and Lindsey 1970; Khidir and Renaud 2003). The measurements include the total body length, relative eye length, disc length, prebranchial length, branchial length, trunk length, and tail length (Hubbs and Trautman 1937; Vladykov 1955). However, some of these characters (e.g., dentition, oral papillae, eye length, disc length) are only present in metamorphosed lampreys. Only a few characters, such as pigmentation (Renaud 1982; Richards et al. 1982), trunk myomere number (Hubbs and Trautman 1937; Vladykov 1955), and dorsal and caudal fin arrangements (Vladykov and Kott 1980), are applied to both larvae and post-metamorphic lampreys.

The adult feeding type of lamprey species is either parasitic or non-parasitic. All ammocoetes are blind and feed on microscopic organisms and detritus. After metamorphosis, non-parasitic lampreys do not feed at all, spawning and dying within

6-10 months of metamorphosis, whereas parasitic lampreys feed on actinopterygian fishes for several years (Docker 2009). The non-parasitic lampreys are collectively called brook lampreys; unlike most parasitic lampreys that migrate to the ocean, lakes, or larger rivers to feed, most non-parasitic lampreys remain within their natal streams. Researchers hypothesized that non-parasitic lamprey species have been derived independently from a parasitic species (Hubbs and Trautman 1937; Zanandrea 1959; Potter 1980). The non-parasitic species and the parasitic descendant of its parasitic ancestor have been called “paired species” (Zanandrea 1959). The term “satellite species” is used for several cases in which more than one non-parasitic (satellite) species is paired with a single parasitic (stem) species (Vladykov and Kott 1979a). For example, the Alaskan brook lamprey *Lethenteron alaskense* Vladykov and Kott 1978, the American brook lamprey *Lethenteron appendix* (DeKay 1842), the Siberian lamprey *Lethenteron kessleri* (Anikin 1905) and the Far Eastern brook lamprey *Lethenteron reissneri* (Dybowski 1869) were believed to be the satellite species of the Arctic lamprey *Lethenteron camtschaticum* (Tilesius 1811) (Potter 1980; Lang et al. 2009; Docker 2009), which means all of the former four have been derived from a *Lethenteron camtschaticum*-like ancestor.

The satellite and stem species are morphologically similar and differ from each other mostly in terms of the characters associated with their respective modes of life in the adult stage (Renaud 2011). For example, *Lethenteron kessleri* is distinguished from *Lethenteron camtschaticum* only by its smaller adult size, fewer eggs (Holčík 1986), and

its life history: *Lethenteron kessleri* is considered a non-parasitic freshwater resident while *Lethenteron camtschaticum* typically has an anadromous parasitic life history (Renaud 2011).

To date, there is no clear answer to the question of whether paired species (satellite and stem species) are distinct species that are reproductively isolated and show reciprocal monophyly or whether they are merely different forms within the same species (Docker 2009; Renaud 2011). One hypothesis is that the contrasting life history types distinguish paired species from each other as different species (Hubbs and Trautman 1937). The other hypothesis is that life history type is not a valid criterion of species differentiation, and different feeding forms only reflect differences in trophic type (McPhail and Lindsey 1970). At the heart of this disagreement are the conflicting suggestions that: 1) size differences at spawning lead to immediate reproductive isolation between the life history types (i.e., that they may be good biological species); but that 2) different populations of each non-parasitic species have been derived independently (at different times or different locations) from the parasitic ancestor (i.e., that they are not good phylogenetic species); see Docker (2009).

When these two contradicting hypotheses were tested using molecular data, the results varied with different paired species and different methods (Docker et al. 2012; Espanhol et al. 2007; Mateus et al. 2011; Mateus et al. 2013). Molecular methods to test inter-taxon relationships include phylogenetic analysis of DNA sequence data (Docker et

al. 2012; Espanhol et al. 2007; Mateus et al. 2011, 2013), restriction fragment length polymorphism (RFLP) (Docker et al. 2012), and microsatellite data (Docker et al. 2012). Phylogenetic analysis divides species based on the phylogenetic concept of species: the smallest biological entities that are diagnosable and/or monophyletic (Mayden 1997). If stem and satellite species are reciprocally monophyletic (i.e., each being monophyletic), they are different phylogenetic species; if each of them does not form a monophyly, they are the same phylogenetic species. For example, the parasitic silver lamprey *Ichthyomyzon unicuspis* Hubbs and Trautman 1937 and the non-parasitic northern brook lamprey *Ichthyomyzon fossor* Reighard and Cummins 1916 were found to not be reciprocally monophyletic using 4213 bp of mitochondrial DNA (mtDNA), supporting them as ecotypes of a single species (Docker et al. 2012). In the case of the parasitic European river lamprey *Lampetra fluviatilis* (L.) and the non-parasitic European brook lamprey *Lampetra planeri* (Bloch 1784), Mateus et al. (2013) suggested they were reciprocally monophyletic based on the single-nucleotide polymorphisms (SNPs) widely spread in the whole genome using the restriction site-associated DNA sequencing (RADseq); thus, they were different phylogenetic species. However, they were not reciprocally monophyletic using the cyt *b* gene and have also been considered ecotypes of the same species (Espanhol et al. 2007; Mateus et al. 2011).

Differences in sequences, including DNA barcodes (e.g., 648 bp of the mitochondrial cytochrome *c* oxidase subunit I (COI) gene), RFLP and microsatellite

markers, are often found between different species and are tools for species identification (e.g., Hebert et al. 2003; Wolf et al. 2000; Hansen et al. 2001). However, DNA barcoding may not distinguish among all species. April et al. (2011), for example, reported that 13 of 27 lamprey species were barcode-indistinguishable. However, they could be different species since there may be diagnostic differences at other loci in the genome of these barcode-indistinguishable species. Thus, DNA barcoding is often used to provide clues of species division rather than to resolve the separation or combination of species. Significant differences in allele frequencies using RFLP or microsatellite markers would suggest reproductive isolation, which is the biological concept of species, that is, where a species consists of an interbreeding natural population that is reproductively isolated from other such groups (Mayr and Ashlock 1991). When using phylogenetic analysis to test the existence of a phylogenetic species, there is the possibility of incomplete lineage sorting causing the lack of reciprocal monophyly using selected genes (e.g., Pestano et al. 2003; Marijnissen et al. 2006). Phylogenetic analyses are sometimes combined with other methods to test whether incomplete lineage sorting causes the lack of reciprocal monophyly (e.g., to test allele frequencies using RFLP or microsatellites). In the case of *Ichthyomyzon unicuspis* and *Ichthyomyzon fossor*, congruent with the phylogenetic result, not only were there no fixed species-specific differences (i.e., they were barcode-indistinguishable), no significant differences were found between these two species from the same location using RFLP assays of mtDNA or nuclear microsatellite

markers, which was probably the result of contemporary gene flow between them (Docker et al. 2012). Although Filcek et al. (2005) distinguished the two species from separate lake basins using nuclear microsatellite markers, the difference may correspond to the geographical isolation rather than the divergence of life history type. Thus, *Ichthyomyzon unicuspis* and *Ichthyomyzon fossor* from the same locality were suggested to be the same species by Docker et al. (2012).

It will be helpful to test the hypotheses about the relationship between paired species using different species, and preferably multiple individuals from multiple localities for each species. Relationships among the closely related stem (*Lethenteron camtschaticum*) and satellite (*Lethenteron alaskense*, *Lethenteron appendix*, *Lethenteron kessleri* and *Lethenteron reissneri*) species within *Lethenteron* are the interests of this study. Previous studies have not provided clear answers to the relationship between each satellite species and *Lethenteron camtschaticum*. Yamazaki et al. (2006) distinguished *Lethenteron camtschaticum* and *Lethenteron kessleri* plus *Lethenteron reissneri* as different biological species using nuclear allozyme alleles (i.e., fixed differences suggest reproductive isolation), while the other two satellite species were not tested. Previous phylogenetic studies (Docker et al. 1999; Lang et al. 2009) contained relatively few samples for each species and did not resolve the relationships. Docker et al. (1999) included two *Lethenteron camtschaticum* from a single locality and two *Lethenteron appendix* from a single locality. Although the two species were reciprocally monophyletic, the result only

represented one population from each species. Lang et al. (2009) used a single individual for each species and the relationships among them were unresolved. It is impossible to test reciprocal monophyly with one individual per species.

Chapter 2 of this thesis discusses the relationship among *Lethenteron camtschaticum* and its putative satellite species *Lethenteron alaskense*, *Lethenteron appendix*, *Lethenteron kessleri* and *Lethenteron reissneri* using the cyt *b* gene. Speciation was tested with the phylogenetic concept – whether the taxa are reciprocally monophyletic. Samples from multiple localities were collected for each species except *Lethenteron alaskense*, including *Lethenteron camtschaticum* from the Pacific drainage of Asia, White Sea basin and Beaufort Sea basin; *Lethenteron kessleri* from the White Sea basin and Kara Sea basin; *Lethenteron reissneri* from the Onon River basin and Amur River basin; *Lethenteron appendix* from the Great Lakes basin and Atlantic drainage of North America; and one *Lethenteron* sp. (presumably *Lethenteron alaskense*) sample from Northwest Territories, Canada. With a larger sample size and better coverage of the geographical range, this study should provide more precise results than previous studies (e.g., Lang et al. 2009) on these species. Multiple individuals for each species also allows more detailed relationships (i.e., population-level relationships) to be resolved using a network approach (Bandelt et al. 1999) besides phylogenetic analysis. For different non-parasitic satellite species, different relationships with the stem *Lethenteron camtschaticum* have been discovered in the haplotype network. The network shows the haplotype frequency

distributions in species and geographical regions. Each substitution in the cyt *b* gene among haplotypes is indicated in the network. Thus fixed differences among species or populations are easily discovered (see Section 2.3.3). Relationships between other stem and satellite species will be discussed in the general discussion (Chapter 4) based on the cyt *b* and nuclear gene phylogenies.

1.2 Phylogenetic status of the genus *Lethenteron*

The relationships among *Lethenteron*, *Lampetra*, *Entosphenus* and *Eudontomyzon* have not been clear. Potter (1980) suggested that *Lampetra*, *Lethenteron* and *Entosphenus* were three subgenera within the genus *Lampetra* based on the similarity of the general plan of their dentition and the overlap of other morphological characters, while some authors (Vladykov and Follett 1967; Vladykov and Kott 1979b) considered them as three distinct genera based only on their dentition. Vladykov and Kott (1979b) put *Lethenteron*, *Lampetra* and *Eudontomyzon* under the subfamily Lampetrinae and put *Entosphenus* and *Tetrapleurodon* in the subfamily Entospheninae. Bailey (1980) put *Lampetra*, *Lethenteron*, *Entosphenus*, *Eudontomyzon*, and *Tetrapleurodon* all within the genus *Lampetra* as subgenera. These five putative subgenera share the unique derived character of a supraoral lamina with a wide central bridge and lateral cusps (Gill et al. 2003). *Tetrapleurodon* and *Eudontomyzon* differ from *Entosphenus*, *Lampetra* and *Lethenteron* in the presence of labial teeth on all fields of the oral disc, with the latter three having the

exolateral teeth (exolaterals) absent (Vladykov and Follett 1967; Gill et al. 2003). Recent taxonomic lists (Nelson 2006; Renaud 2011) consider them as separate genera following the phylogenetic analysis result of Gill et al. (2003) using morphological characters of living parasitic species. Nelson (2006) put all the five genera in the subfamily Lampetrinae along with *Caspiomyzon* (Table 1.1). Page et al. (2013), in their list of fishes from the United States, Canada, and Mexico, also recognized *Lethenteron*, *Entosphenus* and *Tetrapleurodon* as distinct genera from *Lampetra* based on Gill et al. (2003); *Eudontomyzon* is found only in Europe and was not explicitly discussed in this list.

The genera *Eudontomyzon*, *Lampetra* and *Lethenteron* are related more closely to each other than to *Entosphenus* according to several previous phylogenetic studies using molecular or morphological characters (Docker et al. 1999; Gill et al. 2003; Lang et al. 2009); this is consistent with Vladykov and Kott (1979b) placing these three genera together into the subfamily Lampetrinae. Molecular phylogenetic analysis using partial cyt *b* and NADH dehydrogenase subunit 3 (ND3) genes suggested that *Lethenteron* and *Lampetra* (including the Kern brook lamprey *Lampetra hubbsi* (Vladykov and Kott 1976), which was referred to as *Entosphenus hubbsi* following the original description) formed one clade while *Entosphenus* was a distinct genus from them; the *Eudontomyzon* species were not included in this analysis (Docker et al. 1999). *Lampetra* was paraphyletic, with *Lampetra* from the Atlantic drainages (in North America and Europe) being more closely related to *Lethenteron* than to *Lampetra* from the Pacific coast of North America (Docker

et al. 1999). Gill et al. (2003), based on a morphological analysis of the 18 extant parasitic lamprey species, suggested that *Lampetra* was monophyletic and was more closely related to *Eudontomyzon* than to *Lethenteron*, while *Entosphenus* was distinct from these three genera. Lang et al. (2009) used partial cyt *b* gene sequences from nearly all recognized lamprey species. They discovered that *Lampetra* is paraphyletic: *Lampetra* from the Atlantic drainages of Eurasia and North America is sister to *Eudontomyzon* [not considering the metamorphosing sample of the Korean lamprey *Eudontomyzon morii* (Berg 1931)]; *Lethenteron* excluding *Lethenteron* sp. S is sister to them; *Lampetra* from the Pacific coast of North America (including *Lampetra hubbsi*) is sister to all other *Lampetra* plus *Eudontomyzon* and *Lethenteron* (excluding *Lethenteron* sp. S). Different sampling and characters (mtDNA or morphological characters) led to different placements of genera in these studies. Docker et al. (1999) sampled only two *Lethenteron* species and five *Lampetra* species (including *Lampetra hubbsi*) with the genus *Eudontomyzon* missing. Gill et al. (2003) excluded all the non-parasitic lampreys. Although Lang et al. (2009) included nearly all the lamprey species, a single sample was used for most species. Given that intraspecific variation may often be as great as, or greater than, variation between the stem and satellite species (e.g., Boguski et al. 2012), their results may be biased due to the incomplete geographic sampling.

Morphologically, *Eudontomyzon*, *Lampetra* and *Lethenteron* are distinguished from *Entosphenus* by three endolateral teeth (endolaterals) on each side of the oral disc and a

U-shaped transverse lingual lamina with greatly enlarged median cusp (Vladykov and Follett 1967; Gill et al. 2003; summarized in Table 1.2). *Lethenteron* differs from *Lampetra* and *Eudontomyzon* by the presence of a single, either complete or incomplete, row of posterial circumoral teeth (posterials) (Vladykov and Follett 1967; Naseka et al. 2009). *Eudontomyzon* is distinguished from *Lampetra* and *Lethenteron* by 1-4 rows of posterials and the presence of exolaterals (Vladykov and Follett 1967; Gill et al. 2003; Renaud 2011; Table 1.2).

The phylogenetic status of *Lethenteron* and the placement of some *Lethenteron* species are uncertain (Docker et al. 1999; Gill et al. 2003; Blank et al. 2008; Lang et al. 2009). Thus, the phylogenetic questions of this genus are not isolated from other genera. Therefore, the relationships among *Lethenteron* and closely related genera have been discussed in this thesis. Based on the previous result (Docker et al. 1999; Gill et al. 2003; Lang et al. 2009) that *Lethenteron* was more closely related to *Eudontomyzon* and *Lampetra* (or Pacific *Lampetra* and Atlantic *Lampetra*) than to any other genera, this thesis studied the relationships among these genera using the mitochondrial cyt *b* gene and two nuclear gene introns.

1.3 Species of *Lethenteron*

The most recent taxonomic list included seven species (one parasitic and six non-parasitic species) in the genus *Lethenteron* (Renaud 2011; Table 1.3). The

relationships among species in *Lethenteron* have been controversial. Relevant issues about the phylogenetic status of species in this genus will be discussed below.

1.3.1 Lethenteron camtschaticum, Lethenteron appendix *and* Lethenteron alaskense

The relationships among the parasitic *Lethenteron camtschaticum*, and two of the presumed non-parasitic satellite species (see Section 1.1), *Lethenteron appendix* and *Lethenteron alaskense*, are unresolved. For example, the taxonomic status and the feeding type of the lamprey populations in the Naknek River system, Alaska, have long been controversial. Wilimovsky (1954) recognized both the typical parasitic anadromous form and the freshwater resident form occurring in this area as subspecies of *Lethenteron appendix.* The freshwater resident form was considered non-parasitic. Some authors (Hubbs and Lagler 1958; Quast and Hall 1972; Robins et al. 1980; Page and Burr 1991) accepted the hypothesis considering the freshwater resident form as non-parasitic *Lethenteron appendix*, although the distribution of *Lethenteron appendix* was previously thought to be restricted to the Mississippi River, Great Lakes basins and Atlantic drainage of North America (Fig. 1.1). Although Hubbs and Trautman (1937) suggested that the presence of metamorphosing ammocoetes with maturing gonads was evidence for the existence of non-parasitic lampreys, Heard (1966) failed to find any metamorphosing ammocoetes with maturing gonads in the Naknek River system and considered both the anadromous and freshwater forms to be parasitic *Lethenteron camtschaticum*. Parasitism

in fresh water was further supported by lamprey scars observed on juvenile sockeye salmon *Oncorhynchus nerka* (Walbaum 1792) in this river system. Furthermore, immature specimens were morphologically identical with the immature parasitic lampreys in the same river system and, after being kept alive over winter in pens in Brooks Lake, they resembled the freshwater form when they were almost mature (Heard 1966).

McPhail and Lindsey (1970) considered Heard's (1966) evidence insufficient to support the conclusion that the freshwater form was parasitic and treated the freshwater form as a non-parasitic form of *Lethenteron camtschaticum*. Vladykov and Kott (1978) first described this freshwater form as a distinct non-parasitic species *Lethenteron alaskense* and considered it to be another satellite species of *Lethenteron camtschaticum*. *Lethenteron alaskense* differs from *Lethenteron camtschaticum* by its non-functional intestinal tract (in metamorphosed specimens, *Lethenteron camtschaticum* has a functional intestinal tract until sexual maturation, while *Lethenteron alaskense* has a non-functional intestinal tract during the whole period following metamorphosis), weaker dentition, smaller oral disc and smaller size in the post-metamorphic and the spawning stage, and is separated from *Lethenteron appendix* by the dentition, the number of velar tentacles, the pigmentation, the size of adult and the geographical distribution (Vladykov and Kott 1978). Many authors (Potter 1980; Bailey 1980; Mecklenburg 2002; Renaud 2011) then treated *Lethenteron alaskense* as a distinct species that is morphologically

distinguishable from *Lethenteron camtschaticum* and *Lethenteron appendix*.

However, the question of whether the freshwater form in the Naknek River system is parasitic or non-parasitic does not appear to be resolved. The evidence provided by Heard (1966) that this form is parasitic seems strong, but *Lethenteron alaskense* was described as a non-parasitic species based on the characteristics of metamorphosed specimens (Vladykov and Kott 1978). Since the functional intestinal track and the sharpness of teeth present in sexually immature parasitic lampreys (i.e., during their period of feeding) will become non-functional and weak when sexually mature, it can be misleading to infer the feeding type of lampreys with only morphological characters. The uncertainty of the parasitism of *Lethenteron alaskense* has contributed to the controversies about whether it is a distinct species from the parasitic *Lethenteron camtschaticum*.

Even if the life history of *Lethenteron alaskense* is determined, there will still be the question of whether life history is a valid parameter of species delimitation. Can there be forms with different life histories within the species *Lethenteron camtschaticum*? Interestingly, *Lethenteron camtschaticum* was found to have different life history strategies. Kucheryavyi et al. (2007) reported three forms of *Lethenteron camtschaticum* in the Utkholok River (Western Kamchatka) with different life history strategies, typical anadromous (parasitic), anadromous early maturing forma praecox (mainly males, parasitic) and resident (non-parasitic). After a four-year ammocoete stage and metamorphosis in the Uthkolok River, the typical anadromous and the praecox forms

become parasitic and migrate downstream to the sea, while the resident form remains in the river (and is non-parasitic). Before migrating back to the river and spawning, the praecox form presumably spends several months to a year feeding in the sea, while the typical anadromous form likely feeds in the sea for around two years, thus attaining a larger size without overlap with the other two forms. These three forms spawn jointly and thus were considered to be the same species (Kucheryavyi et al. 2007). The authors speculated that the environment, food supply, and the type of food consumed at early developmental stages determined the life history strategy. Yamazaki et al. (2011) revealed contemporary and long-term gene flow between the sympatric freshwater non-parasitic and anadromous parasitic forms of *Lethenteron camtschaticum* in the Ohno River, Japan using polymorphic microsatellite loci. This finding also suggests that life history type is not a valid diagnostic character to distinguish lamprey species. Yamazaki et al. (2011) provided two possible explanations for the gene flow between the two forms: 1) the gene exchange may happen through sneaking behavior in males (reported in *Lethenteron appendix* and *Lampetra fluviatilis/Lampetra planeri*, by Cochran et al. (2008) and Hume et al. (2013), respectively), so that body size differentiation does not cause reproductive isolation; or 2) life history types are polymorphic forms in one population. This evidence suggests that different life history types may not distinguish sympatric lampreys as different biological species and that morphology, which will be convergent based on life history, can also be unreliable.

As to the relationship between *Lethenteron appendix* and *Lethenteron camtschaticum*, which are not sympatric (Fig. 1.1, 1.2), these two species were found by Lang et al. (2009) to have the most sequence differences (1.15%) in the cyt *b* gene among *Lethenteron camtschaticum* and its closely related satellite species (*Lethenteron appendix*, *Lethenteron alaskense*, *Lethenteron kessleri* and *Lethenteron reissneri*). More differences were also found between *Lethenteron appendix* and the other three satellite species than that among the other three (Lang et al. 2009). However, only one individual for each species used by Lang et al. (2009) did not resolve the relationships among *Lethenteron appendix* and the other *Lethenteron* species. Docker et al. (1999) reported fixed differences in mitochondrial cyt *b* (2 bp, 0.52%) and ND3 (2 bp, 0.57%) genes. However, a study by April et al. (2011) with a larger sample size (24 *Lethenteron appendix* and six *Lethenteron camtschaticum* from several localities) using 620 bp of the COI gene discovered no fixed differences between them.

Interestingly, Manion and Purvis (1971) reported five giant individuals of *Lethenteron appendix*. These giant individuals resembled others in the same species morphologically, except for their larger size, higher myomere count, larger oral disc and sharper teeth. The giant *Lethenteron appendix* was considered parasitic based on the body size, the morphological adaptations of parasitic life (e.g., large oral disc and sharp teeth), and the absence of extremely large ammocoetes among over one hundred thousand sampled (which means they probably reached their large size after feeding) (Manion and

Purvis 1971). Three other giant individuals were reported from the Great Lakes (Vladykov and Kott 1980; Cochran 2008). This could be an additional evidence for the existence of different life history types within one species. The coexistence of two life history types in each of *Lethenteron camtschaticum* and *Lethenteron appendix* throw doubt on the morphological method of species classification: what characters are diagnostic characters to distinguish between species, and what characters only distinguish between different ecotypes or morphs? Although *Lethenteron camtschaticum* and *Lethenteron appendix* are morphologically different (Vladykov and Kott 1978), they may or may not be genetically different and reciprocally monophyletic. The geographic isolation would interrupt the contemporary gene flow between them. However, that does not mean that the intrinsic reproductive isolation is complete.

In this thesis, given the previous lack of resolution through conventional phylogenetic analysis, the relationships among *Lethenteron camtschaticum*, *Lethenteron alaskense*, *Lethenteron appendix*, *Lethenteron kessleri* and *Lethenteron reissneri* have been studied through a median-joining network using the cyt *b* gene (Chapter 2). The network shows the detailed haplotype frequency distribution in each species from each locality with a larger sample size and a better coverage of geographic range of these species than previous studies (Docker et al. 1999; Lang et al. 2009). This thesis includes a discussion of the interspecies relationships based on the analyses of multiple populations from multiple localities, not among single individuals or single populations of each

species.

1.3.2 Lethenteron camtschaticum *and* Lethenteron kessleri

Lethenteron camtschaticum was recognized by Berg (as cited in Renaud 2011) in 1931 as three subspecies *japonicum*, *septentrionalis* and *kessleri*. *Lethenteron kessleri* was subsequently considered a separate species from *Lethenteron camtschaticum*, while the former two were treated as synonyms of *Lethenteron camtschaticum* (Poltorykhina 1974; Holčík 1986; Renaud 2011). According to the concept of subspecies, the breeding ranges of two subspecies of the same species do not overlap geographically. If two discrete breeding populations coexist in the same locality, they are full biological species (Mayr and Ashlock 1991). The reason why Poltorykhina (1974) separated *Lethenteron kessleri* from *Lethenteron camtschaticum* as a full species was that their populations are sympatric (Fig. 1.2, 1.3) and suggested to be reproductively isolated with different life histories and adult body sizes. In the case of *Lethenteron camtschaticum septentrionalis*, its range and that of other *Lethenteron camtschaticum* populations (*Lethenteron camtschaticum camtschaticum*) are disjunctive (Fig. 1.1). *Lethenteron camtschaticum septentrionalis* was considered a synonym of *Lethenteron camtschaticum* (referring to *Lethenteron camtschaticum camtschaticum* in this thesis if not indicated) because they "have virtually no difference" (Holčík 1986). *Lethenteron camtschaticum septentrionalis* is smaller in size than *Lethenteron camtschaticum* (Berg as cited in Vladykov and Kott

1978). Other previous studies comparing the two putative subspecies either morphologically or genetically are not found.

In this thesis, attempts were made to determine whether *Lethenteron camtschaticum septentrionalis* is genetically distinct from *Lethenteron camtschaticum*. The cyt *b* sequences of *Lethenteron camtschaticum septentrionalis* collected from the same river of its type locality (Onega River, Russia) and another locality were compared with the sequences of *Lethenteron camtschaticum* from seven different localities.

Few molecular markers have been found to distinguish *Lethenteron kessleri* from its putative stem species *Lethenteron camtschaticum* (Okada et al. 2010). Yamazaki and Goto (1998) recognized a fixed allele of malate dehydrogenase 3 (MDH3) that diagnostically distinguished *Lethenteron kessleri* from *Lethenteron camtschaticum* even when they occurred sympatrically. Yamazaki et al. (2006) then found the same allele as *Lethenteron kessleri* in *Lethenteron reissneri*, distinguishing the complex of these two species from *Lethenteron camtschaticum*. Lang et al. (2009) showed *Lethenteron kessleri* to be in a single clade with *Lethenteron camtschaticum*, *Lethenteron reissneri*, and *Lethenteron alaskense* using partial cyt *b* sequences from one individual of each species. Sequences of *Lethenteron kessleri* and *Lethenteron alaskense* were identical, while *Lethenteron kessleri*, *Lethenteron reissneri* and *Lethenteron camtschaticum* had few differences in the cyt *b* sequences (Lang et al. 2009). These differences may not be fixed to species since only one individual for each species was used. Yamazaki et al. (2006)

found one fixed substitution between *Lethenteron camtschaticum* and *Lethenteron kessleri* in a 384 bp region of the cyt *b* gene using *Lethenteron camtschaticum* from the Pacific Ocean basin in Russia (Magadan, one locality) and Japan (Hokkaidō, two localities) and *Lethenteron kessleri* from the Pacific Ocean basin (Amur River system, Sakhalin, Hokkaidō, one locality each) and Arctic Ocean basin (Ob River system, Lena River system, one locality each) in Russia and Japan. No fixed differences were found in 1009 bp of the COI gene. Although Yamazaki et al. (2006) included the main basins in the range of *Lethenteron kessleri*, the geographic sampling of *Lethenteron camtschaticum* was incomplete with localities from the Arctic Ocean basin in Eurasia and North America missing. Also, results based on one individual per locality may not represent the diversity found in populations. When multiple individuals from each locality and different localities are included, the fixed difference in the cyt *b* gene may not be found between the two species. Previous studies with wider geographic sampling certainly indicate that intraspecific variation in widespread lamprey species could be as great as, or greater than, the difference between paired and satellite species (e.g., Lorion et al. 2000; Docker et al. 2012; Boguski et al. 2012).

Ioganzen (as cited in Holčík 1986) reported *Lethenteron kessleri* attached to fish, although *Lethenteron kessleri* is likely non-parasitic (Holčík 1986). As with different life history forms observed in *Lethenteron camtschaticum* (Kucheryavyi et al. 2007; Yamazaki et al. 2011) and probably in *Lethenteron appendix* (Manion and Purvis 1971;

Vladykov and Kott 1980; Cochran 2008; see Section 1.3.1), the *Lethenteron kessleri* attached to fish was possibly a rare parasitic form within the species. *Lethenteron kessleri* is distinguished from *Lethenteron camtschaticum* mainly by the type of life history and the morphological characters related to the mode of life (Poltorykhina 1974). If life history is not a valid species-specific character, the divergence of *Lethenteron kessleri* from *Lethenteron camtschaticum* based on the morphological characters would be doubtful.

In this thesis, the relationship between *Lethenteron kessleri* and *Lethenteron camtschaticum* was studied through a median-joining network using the cyt *b* gene. As with the other satellite species of *Lethenteron camtschaticum* (Section 1.3.1), the recency of their divergence suggests that there may have been insufficient time to accumulate genetic differences in certain genes (e.g., mitochondrial genes) to form reciprocal monophyly (Pestano et al. 2003; Marijnissen et al. 2006).

1.3.3 Lethenteron reissneri

Lethenteron reissneri (*sensu lato*) in Japan and South Korea was found to have two partly sympatric “forms”, a southern and a northern form, differing from the *Lethenteron reissneri* (*sensu stricto*) from the type locality. The southern and northern forms were distinguished by allele substitutions at 11 loci of allozymes and were then believed to be reproductively isolated (Yamazaki and Goto 1996; Yamazaki and Goto 2000). Analysis

based on mtDNA showed that the southern form, the northern form and *Lethenteron reissneri* (*s.s.*) were reciprocally monophyletic, so that the southern and northern forms were elevated as two separate species from *Lethenteron reissneri* (*s.s.*), *Lethenteron* sp. S and *Lethenteron* sp. N, by Yamazaki et al. (2003). Molecular data using the cyt *b* gene suggested that *Lethenteron* sp. S is highly divergent; it was excluded from the clades of *Lethenteron*, *Lampetra*, *Eudontomyzon* or *Entosphenus* (Lang et al. 2009). *Lethenteron* sp. N was recovered as sister to the clade of *Lethenteron camtschaticum* and its satellite species plus the parasitic *Eudontomyzon morii* (Lang et al. 2009). These two species have not yet been formally described and thus are not listed in recent taxonomic lists (e.g., Renaud 2011).

Despite their pronounced genetic distinctiveness, no significant morphological differences have been found between *Lethenteron* sp. N and *Lethenteron* sp. S (Yamazaki and Goto 1997). *Lethenteron* sp. N and *Lethenteron* sp. S both have lower numbers of trunk myomeres relative to *Lethenteron reissneri* (*s.s.*), though the ranges of myomere numbers overlap (Yamazaki and Goto 1997; Vladykov and Kott 1979a).

Lethenteron reissneri (*s.l.*) is morphologically distinguishable from *Lethenteron camtschaticum* and its other closely related satellite species, although the differences are few. Compared with *Lethenteron camtschaticum*, *Lethenteron reissneri* (*s.l.*) has a lower number of trunk myomeres, weaker dentition (Iwata et al. 1985; Yamazaki and Goto 1997) and an unpigmented second dorsal fin (Renaud 2011). *Lethenteron reissneri* (*s.l.*) is

distinguished from *Lethenteron kessleri* by the poorly developed posterials, the blunt supraoral lamina, the smaller eye diameter, and the lower number of trunk myomeres (Iwata et al. 1985). The other two satellite species of *Lethenteron camtschaticum*, *Lethenteron alaskense* and *Lethenteron appendix,* are restricted to North America, distinct from the range of *Lethenteron reissneri* (*s.l.*) (Renaud 2011). Compared to these two species, *Lethenteron reissneri* has lower trunk myomere count and different coloration (Iwata et al. 1985; Vladykov and Kott 1978; Renaud 2011).

Two other proposed *Lethenteron* species have been described in Japan, sympatric with *Lethenteron reissneri* (*s.l.*). *Lethenteron matsubarai* Vladykov and Kott 1978 (type locality: Shokotsu River, northern Hokkaidō, Japan) was considered a synonym of *Lethenteron kessleri* by Iwata et al. (1985) based on a comparison between samples of both species from Hokkaidō, Japan. Hubbs and Potter (1971) provisionally recognized *Lethenteron mitsukurii* (Hatta 1901) (type locality: Hondo, Hokkaidō, Japan) from southern Japan as a species, which was treated as a synonym of *Lethenteron reissneri* by Vladykov and Kott (1978) based on the myomere count. Both proposed species possess low myomere numbers. Their phylogenetic relationships to *Lethenteron* sp. N and *Lethenteron* sp. S have not been tested, but samples of these two proposed species were not available for this study.

Since the same allele of MDH3 as *Lethenteron kessleri* from the Ob, Lena, middle Amur River systems, Sakhalin, Russia, and Hokkaidō, Japan (Yamazaki and Goto 1998;

Yamazaki et al. 2006), was found in *Lethenteron reissneri* (*s.s.*) from upper Amur River system, they were suggested as one species, bearing the name *Lethenteron reissneri* (Yamazaki et al. 2006). However, the suggestion was not adopted in the recent taxonomic list since the materials for the allozyme allele analysis were ammocoetes (Renaud 2011). This allele distinguished *Lethenteron reissneri* and *Lethenteron kessleri* from *Lethenteron camtschaticum* diagnostically (Yamazaki and Goto 1998; Yamazaki et al. 2006). Other genetic markers distinguishing *Lethenteron reissneri* (*s.s.*) from *Lethenteron camtschaticum* and other satellite species are not reported.

The cyt *b* gene sequences of the northern and southern forms of *Lethenteron reissneri* were retrieved from the NCBI Nucleotide database (GenBank) and were included in the maximum parsimony and Bayesian analyses in Chapter 2.

1.3.4 Lethenteron ninae

The Western Transcaucasian brook lamprey *Lethenteron ninae* Naseka, Tuniyev, and Renaud 2009, a non-parasitic lamprey, was described from the rivers of Western Transcaucasia, the Black Sea basin, Russia and Abkhazia. *Lethenteron ninae* was distinguished as a new species, distinct from the Turkish brook lamprey *Lampetra lanceolata* Kux and Steiner 1972 and the Ukrainian brook lamprey *Eudontomyzon mariae* (Berg 1931) from Black Sea basin, and placed in *Lethenteron* mainly based on the following characters: exolaterals absent; posterials present in one incomplete row or

occasionally absent (Naseka et al. 2009). *Lethenteron ninae* differs from *Lethenteron zanandreai*, the only other *Lethenteron* from western Eurasia, by a slightly higher myomere count in all stages and a dark blotch on the second dorsal fin in adults (Naseka et al. 2009). Interestingly, the distribution (Black Sea basin) of *Lethenteron ninae* is different from any other *Lethenteron* species (Fig. 1.1 – 1.7), and is nearer to that of *Lampetra lanceolata* and *Eudontomyzon mariae* (Naseka et al. 2009; Renaud 2011). The relationship between *Lethenteron ninae* and other species has not been tested phylogenetically; given its recent description, it was not included in the Lang et al. (2009) study. Thus, the hypothesis that *Lethenteron ninae* belongs to *Lethenteron* was tested in this thesis using mitochondrial and nuclear genes (Section 2.3.1, 2.3.2 and 3.3).

1.3.5 Lethenteron zanandreai *and* Eudontomyzon morii

It is controversial whether the non-parasitic *Lethenteron zanandreai* belongs to the genus *Lethenteron*. Some authors (Hubbs and Potter 1971; Potter 1980) suggested that this species should be put in the genus *Lethenteron* based on the presence of a single row of posterials, despite that Vladykov (1955) first described this species as *Lampetra* without posterials. Renaud (2011) agreed with Hubbs and Potter (1971) and reported two samples with two and five posterials each. However, Tutman et al. (2009) reported zero posterials in samples from the Hutovo Blato wetland, Neretva River basin (Adriatic Sea watershed) in Bosnia and Herzegovina (although the authors used the name "*Lethenteron*

zanandreai"). Molecular evidence based on mtDNA sequences suggested that *Lethenteron zanandreai* was in the clade of the genus *Lampetra* (Docker et al. 1999; Caputo et al. 2009; Lang et al. 2009). *Lethenteron zanandreai* is distributed along the Adriatic coast from central Italy to Bosnia and Herzegovina (Tutman et al. 2009). The distribution of *Lethenteron zanandreai* has no overlap with any other *Lethenteron* species (Fig. 1.1 – 1.7), although it does overlap with *Lampetra fluviatilis*. Vladykov and Kott (1979a) considered *Lethenteron zanandreai* as a satellite species of the parasitic *Lampetra fluviatilis* based on the number of trunk myomeres and geographical distribution. However, Hubbs and Potter (1971) considered it to be an ancient southern relict of *Lethenteron camtschaticum*; in this scenario, *Lethenteron zanandreai* diverged from a *Lethenteron camtschaticum*-like ancestor (which is no longer extant or no longer sympatric) in southern Europe at an earlier time.

On the other hand, some species considered to belong to other genera may be in the genus *Lethenteron*. *Eudontomyzon morii* is parasitic and freshwater resident (Hubbs and Potter 1971). The ancestor of this species is controversial. Berg (1931, as cited in Hubbs and Potter 1971) considered it derived from *Lethenteron camtschaticum*, while Hubbs and Potter (1971) treated it as *Eudontomyzon* based on the dentition and considered *Eudontomyzon* from the Black Sea basin to have been derived from *Lampetra fluviatilis*. *Eudontomyzon morii* was put in *Eudontomyzon* in the recent taxonomic list (Renaud 2011) based on the morphological phylogeny by Gill et al. (2003). The result of the cyt *b* gene

sequence analysis placed *Eudontomyzon morii* in the clade of the genus *Lethenteron* using, however, a single metamorphosing individual (Lang et al. 2009). Since the dentition character used to distinguish species is for the adults, the identification of this immature specimen is uncertain. Data from adult individuals of this species are needed to provide more evidence. One feature of the dentition of *Lethenteron* is a single row of posterials. Interestingly, *Eudontomyzon morii* possesses only one row of posterials while more than one row of posterials was found in other *Eudontomyzon* species (Vladykov and Follett 1967; Renaud 2011).

To better resolve the above issues, the cyt *b* gene and nuclear introns of several individuals of *Lethenteron zanandreai* from Vipava River, Slovenia were sequenced, and the cyt *b* sequence of *Eudontomyzon morii* was retrieved from GenBank for a more geographically comprehensive phylogenetic analysis (Section 2.2.1). Nuclear genes were not used in previous studies on the placement of *Lethenteron zanandreai* (Docker et al. 1999; Caputo et al. 2009; Lang et al. 2009), and as mentioned above, previous studies have used very few individuals of each species. The placement of these two species will be discussed in the following chapters.

1.4 Mitochondrial cyt *b* gene and nuclear gene introns

1.4.1 Mitochondrial cyt b *gene*

Animal mtDNA is an extrachromosomal genome with the size of about 16 kb

containing 13 protein-coding, two rRNA and 22 tRNA genes typically (Boore 1999). The mtDNA is often used as a material for the phylogeny of animals. In general, the mtDNA evolves five to ten times more rapidly than nuclear DNA with regard to nucleotide substitutions (Brown et al. 1979). The substitution rate of mitochondrial tRNA genes is about 100 times that of nuclear tRNA genes; the ratio for small rRNA is about 20; the silent substitution rate of mitochondrial protein coding genes is 22 times that of nuclear protein coding genes (Pesole et al. 1999). Thus, mtDNA is more likely to resolve the relationships among closely related species than nuclear DNA. Furthermore, given that mtDNA is relatively consistent in gene content and arrangement in vertebrates (Boore 1999), it is easy to amplify and sequence even without taxon-specific sequence information. It has been used widely for molecular phylogenetic research on poorly-studied taxa such as lampreys. For example, there have been studies based on cyt *b* (Docker et al. 1999; Lorion et al. 2000; Yamazaki et al. 2006; Espanhol et al. 2007; Blank et al. 2008; Boguski 2009; Caputo et al. 2009; Lang et al. 2009; Mateus et al. 2011; Boguski et al. 2012; Docker et al. 2012), ND3 (Docket et al. 1999; Blank et al. 2008; Martin and White 2008; Docker et al. 2012), COI gene (Yamazaki et al. 2003; Yamazaki et al. 2006; Blank et al. 2008; Hubert et al. 2008; Boguski 2009; Caputo et al. 2009; Docker et al. 2012), and the non-coding mtDNA regions, NC1 and NC2 (Blank et al. 2008; Martin and White 2008; Okada et al. 2011; Docker et al. 2012). Lang et al. (2009) investigated the systematics of lampreys using 1133 bp of cyt *b* sequences from all

genera and nearly all recognized species. However, the subspecies *Lethenteron camtschaticum septentrionalis* and the new species *Lethenteron ninae* was not included in Lang et al. (2009)'s study, and they included only a few representatives of each species (in most cases, only a single individual per species).

In Chapter 2 of this thesis, not only samples of *Lethenteron camtschaticum septentrionalis* and *Lethenteron ninae* were analyzed, but also a larger sample size for each selected species from several locations were obtained. Samples of the same species from different locations help obtain a more accurate result. Since different individuals, especially ones from different locations, may possess different haplotypes and sometimes cause different placements in phylogeny with other species, a single individual may not represent the species. Examples can be found in studies using multiple samples from several locations: the placements of the Klamath lamprey *Entosphenus similis* Vladykov and Kott 1979, the Pacific lamprey *Entosphenus tridentatus* Gairdner and Richardson 1836 and the Pit–Klamath brook lamprey *Entosphenus lethophagus* (Hubbs 1971) varied with individuals and locations (see Fig. 4 in Blank et al. 2008); similar is the placement of the river lamprey *Lampetra ayresii* (Günther 1870) and the western brook lamprey *Lampetra richardsoni* Vladykov and Follett 1965 (see Fig. 3 in Boguski et al. 2012). If only one individual for each is used for the phylogeny of these species, there would be several possible phylogenetic relationships depending on the selection of individuals. None of the phylogenetic relationships based on one individual per species reflects the

placement of the species, which is actually not monophyletic. Especially for relationships among closely related species, where the sequence differences are subtle among species, differences may be revealed only in the haplotype frequency distribution in populations. In this study, samples were included from each main river/sea/ocean basin in the range of each *Lethenteron* species where samples were available. Some localities/areas have not been sampled in previous studies (Docker et al. 1999; Lang et al. 2009; Yamazaki et al. 2006): *Lethenteron appendix* from the Lake Huron and Lake Michigan basins, and streams in Maine and Delaware, USA, in the Atlantic Ocean basin; *Lethenteron camtschaticum* from the Beaufort Sea basin; presumptive *Lethenteron kessleri* from the Dvinnitsa River system in the White Sea basin (this is a new locality for this species, thus the identification of samples from this locality is uncertain); and *Lethenteron* sp. (presumably *Lethenteron alaskense*) from Northwest Territories, Canada (see Fig. 1.1 - 1.7).

A cyt *b* median-joining network (Section 2.3.3) was generated with samples of *Lethenteron camtschaticum* and its satellite species from several locations. Several network analytical methods (e.g., median-joining network) represent the relationships among haplotypes with a network minimizing the number of mutations (Bandelt et al. 1999; Posada and Crandall 2001). A median-joining network (Bandelt et al. 1999) combines the minimum-spanning trees (Foulds et al. 1979) within a single network and adds median vectors (which represent missing intermediates) to it with a parsimony

criterion. Network methods are more useful for closely related haplotypes than bifurcating trees (Posada and Crandall 2001) and are thus highly appropriate for examining relationships among closely related *Lethenteron* species. If the divergence among haplotypes is low (e.g., *Lethenteron* stem and satellite species; see Section 2.3.4) then few characters are informative for the phylogenetic tree methods, while each substitution among haplotypes is shown in the median-joining network. In a phylogenetic tree, the ancestors of the existent haplotypes are represented with internodes. However, for recently diverged haplotypes, one of the existent haplotypes is possibly the ancestor of other haplotypes and, thus, a bifurcating tree with all haplotypes on the tips of terminal branches is not an appropriate model for their relationships. Also one ancestral haplotype may have multiple descendants with different mutations; thus, the relationships among them should be multifurcations rather than bifurcations. The loops in a network represent the possibility of parallel, convergent or reversing mutations, while bifurcating tree methods (e.g., maximum parsimony algorithm) select a tree minimizing these assumptions. These phenomena may not be important for more divergent taxa, but make a difference among closely related haplotypes. Thus, the networks may reflect the geographic pattern and subtle relationships among populations which may not be resolved using phylogenetic trees (e.g., Guzmán et al. 2011; Dai et al. 2012; Jeratthitikul et al. 2013).

1.4.2 Nuclear gene introns

Previous lamprey phylogenetic analyses were limited to mitochondrial genes. When a phylogeny is estimated from a set of DNA sequences, the gene tree may not be congruent with the species tree due to incomplete lineage sorting (Pamilo and Nei 1988). The mtDNA is maternally inherited in lampreys and most other vertebrates (Hutchison et al. 1974; Giles et al. 1980; Gyllensten et al. 1985). The mitochondrial cyt *b* gene analyses reflect the genetic information from only the female ancestors, providing no information of hybridizations or recombinations. With a higher rate of substitution (Pesole et al. 1999), mtDNA is more likely to resolve the relationships among closely related species than nuclear DNA but this higher rate of substitution also means mtDNA is more affected by superimposed substitutions (multiple substitutions at one site) than nuclear DNA in recovering deep-level relationships (Springer et al. 2001). Most nuclear DNA in vertebrates evolves slower, and contains the information from two parents, and thus can supplement mtDNA analyses. Thus, in the present study, besides the cyt *b* gene, two nuclear gene introns, the TAP and soxD introns, from the selected materials were sequenced and analyzed. The structure of the TAP gene in the sea lamprey *Petromyzon marinus* L. was described by Uinuk-ool et al. (2003). Thus, the locations and lengths of the introns were known prior to the study. Primers were designed for the second intron, which is 949 bp in length (i.e., a reasonable length for the polymerase chain reaction (PCR) amplification but longer than four of the other 12 introns, thus potentially

containing more informative sites). SoxD introns have been amplified in *Lethenteron camtschaticum* and *Lethenteron kessleri* (Okada et al. 2010); primers modified from those designed by Okada et al. (2010) were used here. Maximum parsimony and Bayesian trees of each intron (Section 3.3.1 and 3.3.2) and the combined dataset of two introns (Section 3.3.3) have been inferred.

Sequencing nuclear gene regions in non-model organisms is more challenging than mitochondrial genes. With little knowledge on the gene content and sequences of a particular nuclear genome, primers amplifying specific targets are hard to design. This thesis, however, took advantage of the recently sequenced *Petromyzon marinus* genome (Smith et al. 2013). In order to design primers that were likely to amplify the desired introns in all lamprey species of interest, sequences of the same gene from other taxa were aligned with the *Petromyzon marinus* sequence to find regions in exons that were conserved over taxa (Palumbi and Baker 1994; see Section 3.2.2). These primers, however, sometimes amplify multiple products (e.g., the primer pairs initially designed for 40S ribosomal protein subunit 23 (RPS23) and Mannose-Binding Lectin-Associated Serine Protease A (MASP-A); Section 3.2.2). In this thesis, primers designed for TAP2 and soxD introns were specific and worked in most species. Another challenge, however, was amplification efficiency. Numerous mitochondria, each containing one mitochondrial genome, are found in one cell, but there is only one nucleus containing one nuclear genome per cell. Thus, even if conserved primers for nuclear genes are designed, the

efficiency and yield of amplification and the quality of sequence generally does not compare with that of mtDNA. Thus, in this study, only 21 samples for the TAP2 and 10 for the soxD gene introns were sequenced, while 72 samples were sequenced for the cyt *b* gene. A further challenge with nuclear DNA (given its lower rate of substitution than the mtDNA in coding and non-coding regions) is that, even with sequences of the same length, it usually generates trees with lower resolution than mtDNA. In this thesis, as expected, there were fewer variable characters in the nuclear introns than in the cyt *b* gene (Section 2.3 and 3.3). Although this meant that the relationship among closely related species could not be resolved, phylogenetic analyses using nuclear introns provided ideas about the relationships among the genera *Eudontomyzon*, *Lampetra* and *Lethenteron* (Chapter 3). The similarities and differences between the cyt *b* and nuclear trees will be discussed in the General Discussion (Chapter 4).

1.5 Objectives

As mentioned in previous sections, the relationships among the genera *Eudontomyzon*, *Lampetra* and *Lethenteron* and among *Lethenteron camtschaticum* and its satellite species are controversial. The placements of some species from these genera are uncertain. In this study, the following questions were intended to be tested:

1) The division of genera: Are *Eudontomyzon*, *Lampetra* and *Lethenteron* reciprocally monophyletic in the cyt *b* and nuclear intron trees? Are *Lampetra* from the

Pacific drainage and that from the Atlantic drainage each reciprocally monophyletic? How many main clades are there and which species do they contain?

2) The evolutionary relationships among genera: What are the relationships among the main clades found in each tree? Which genera/groups are most closely related?

3) The placement of new or problematic species: Are *Lethenteron ninae* and *Lethenteron zanandreai* in the genus *Lethenteron* or *Lampetra* (Atlantic *Lampetra*)? Is *Lethenteron* sp. N in *Lethenteron* and what is its relationship to *Lethenteron camtschaticum* and other satellite species? Is *Lethenteron* sp. S distinct from any genera included in this study and what is its relationship to them? Is *Eudontomyzon morii* in *Lethenteron* or *Eudontomyzon*?

4) The congruence of results: Do the results of phylogenetic analyses using four datasets (cyt *b*, TAP intron, soxD intron and combined dataset of TAP and soxD introns) agree with each other on questions 1), 2), and 3)? What are the agreements among all the datasets and methods?

5) The relationships among *Lethenteron camtschaticum* and its closely related satellite species: Is *Lethenteron camtschaticum septentrionalis* a synonym of *Lethenteron camtschaticum* or another species based on the phylogenetic species concept? Do they possess distinct haplotypes? Are *Lethenteron alaskense*, *Lethenteron appendix*, *Lethenteron kessleri* and *Lethenteron reissneri* separate phylogenetic species from *Lethenteron camtschaticum*? What are the haplotype frequency distributions of these

species? Are there fixed sequence differences among species?

6) The relationship of satellite species to the stem: Are the satellite species studied in this thesis the same species as their stems or distinct from the stems by the phylogenetic species concept?

To answer the above questions, maximum parsimony and Bayesian analyses were conducted using the cyt *b* gene, the TAP2 intron, the soxD intron and the combined nuclear dataset. A haplotype network of *Lethenteron camtschaticum* (including *Lethenteron camtschaticum septentrionalis*), *Lethenteron alaskense*, *Lethenteron appendix*, *Lethenteron kessleri*, *Lethenteron reissneri* were also generated using the cyt *b* sequences.

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1.7 Tables and figures

Table 1.1 Classification of genera or subgenera *Lampetra*, *Lethenteron*, *Entosphenus*, *Eudontomyzon* and *Tetrapleurodon* by different authors. Filled circle means a recognized genus. Open circle connected to a filled circle means a subgenus under that genus. Filled circles connected together with broken lines means genera under the same subfamily. Vladykov and Kott (1979b) put *Lampetra*, *Lethenteron* and *Eudontomyzon* in the subfamily Lampetrinae, and put *Entosphenus* and *Tetrapleurodon* in the subfamily Entospheninae. Nelson (2006) put all five genera in the subfamily Lampetrinae.

	Vladykov and Follett (1967)	Vladykov and Kott (1979b)	Potter (1980)	Bailey (1980)	Nelson (2006)	Renaud (2011)
Lampetra	●	●	○ ●	○ ●	●	●
Lethenteron	●	●	○	○	●	●
Entosphenus	●	●	○	○	●	●
Eudontomyzon	●	●	●	○	●	●
Tetrapleurodon	●	●	●	○	●	●

Table 1.2 Morphological characters distinguishing *Entosphenus*, *Eudontomyzon*, *Lampetra* and *Lethenteron*. "X" means the possession of the character; characters follow Vladykov and Follett (1967), Gill et al. (2003) and Renaud (2011).

	Entosphenus	*Eudontomyzon*	*Lampetra*	*Lethenteron*
Transverse lingual lamina:				
A. Transverse lingual lamina U-shaped		X	X	X
B. Transverse lingual lamina weakly w-shaped	X			
Median cusp of transverse lingual lamina:				
A. Transverse lingual lamina with a greatly enlarged median cusp		X	X	X
B. Transverse lingual lamina with the median cusp only slightly enlarged	X			
Endolaterals:				
A. 3 endolaterals		X	X	X
B. 4 endolaterals	X			
Exolaterals:				
A. Exolaterals present		X		
B. Exolaterals absent	X		X	X
Posterials:				
A. 1-4 rows of posterials		X		
B. A single row of posterials	X			X
C. Posterials absent			X	

Table 1.3 Information about *Lethenteron* species. The scientific name, common name, first description, feeding type and distribution follow the most recent taxonomic list by Renaud (2011). Details about the disputes related to taxonomy have been discussed in Section 1.3. P = parasitic; N = non-parasitic. See Fig. 1.1 – 1.7 for maps showing the distribution of each species.

Scientific name	Common name	First described by	Feeding type	Distribution	Disputes related to taxonomy
Lethenteron camtschaticum	Arctic lamprey	Tilesius 1811	P	Varanger Fiord and Pasvik River, Norway; White Sea basin, Barents Sea basin to the Pechora River, Arctic Ocean basin and its rivers, Anadyr Territory, Kamchatka Peninsula, Iturup and Sakhalin Islands, Amur and Suchan Rivers, Russian Federation; Northeast People's Republic of China; to the southern extremity of the Korean Peninsula; Hokkaidō Island and Honshū Island, Japan; Kenai Peninsula, Brooks Lake, Naknek River basin, Nushagak and Yukon Rivers, Bering Sea, Beaufort Sea, and Anderson River, Mackenzie River basin, Alaska (USA) and Canada (see Fig. 1.2).	Is the subspecies *Lethenteron camtschaticum septentrionalis* distributed in the White Sea basin and Barents Sea basin a full phylogenetic species or a synonym of *Lethenteron camtschaticum*?
Lethenteron alaskense	Alaskan brook lamprey	Vladykov and Kott 1978	N	Naknek River basin and Yukon River basin, Alaska (USA) and Mackenzie River basin, Canada (see Fig. 1.4).	1) Is *Lethenteron alaskense* ecotype of *Lethenteron camtschaticum* or a separate species? 2) Is *Lethenteron alaskense* a distinct species from *Lethenteron appendix*?
Lethenteron appendix	American brook lamprey	DeKay 1842	N	Lake Superior basin, Lake Huron basin, Lake Michigan basin, Lake Erie basin, Lake Ontario basin, Mississippi River basin, St. Lawrence River basin, Atlantic Slope basins, Canada and USA (see Fig. 1.1).	Is *Lethenteron alaskense* a distinct species from *Lethenteron appendix*?
Lethenteron kessleri	Siberian lamprey	Anikin 1905	N	Kolyma and Anadyr River basins, Russian Federation; Upper Irtysh River basin, Kazakhstan; Hokkaidō Island, Japan (see Fig. 1.3).	Is *Lethenteron kessleri* a distinct species from *Lethenteron camtschaticum* or an ecotype within the same species?

Table 1.3 Continued.

Scientific name	Common name	First described by	Feeding type	Distribution	Disputes related to taxonomy
Lethenteron ninae	Western Transcaucasian brook lamprey	Naseka, Tuniyev, and Renaud 2009	N	Black Sea basin: Shakhe and Mzymta Rivers, and Chakhtsutsyr Brook, Russian Federation; Bzyb' and Mokva Rivers, Abkhazia and Georgia (see Fig. 1.5).	Does *Lethenteron ninae* belong to the genus *Lethenteron*?
Lethenteron reissneri	Far Eastern brook lamprey	Dybowski 1869	N	Amur River basin, Sakhalin Island, Russian Federation; Honshū Island and Hokkaidō Island, Japan (see Fig. 1.6).	Are the northern form (*Lethenteron* sp. N) and southern form (*Lethenteron* sp. S) found in Japan (See 1.3.3) closely related to *Lethenteron reissneri* and other *Lethenteron* species? Do they belong to the genus *Lethenteron*?
Lethenteron zanandreai	Lombardy brook lamprey	Vladykov 1955	N	Adige River basin, Italy; Croatia (see Fig. 1.7).	Does *Lethenteron zanandreai* belong to the genus *Lethenteron* or *Lampetra*?

Fig. 1.1 Distribution and collection sites for *Lethenteron appendix*. Pink area is the distribution range. Blue dots are the collection sites. See Table 2.1 and Fig. 2.4 for detailed locality information of samples.

Fig. 1.2 Distribution and collection sites for *Lethenteron camtschaticum* (including *Lethenteron camtschaticum septentrionalis*). Pink area and blue dots are the distribution range and collection sites of *Lethenteron camtschaticum camtschaticum*. Green area and red squares are the distribution range and collection sites of *Lethenteron camtschaticum septentrionalis*. See Table 2.1 and Fig. 2.4 for detailed locality information of samples.

Fig. 1.3 Distribution and collection sites for *Lethenteron kessleri*. Pink area is the distribution range. Blue dots are the collection sites. See Table 2.1 and Fig. 2.4 for detailed locality information of samples.

Fig. 1.4 Distribution of and collection sites for *Lethenteron alaskense*. Pink area is the distribution range. Blue dot is the collection site of *Lethenteron alaskense* (GQ206178, Lang et al. 2009). Red square is the collection site of *Lethenteron* sp. (presumably *Lethenteron alaskense*). See Table 2.1, 2.2 and Fig. 2.4 for detailed locality information of samples.

Fig. 1.5 Distribution and collection sites for *Lethenteron ninae*. Pink area is the distribution range. Blue dots are the collection sites. See Table 2.1 and Fig. 2.4 for detailed locality information of samples.

Fig. 1.6 Distribution and collection sites for *Lethenteron reissneri* (*s.l.*). Pink area is the distribution range. Blue dots are the collection sites. See Table 2.1 and Fig. 2.4 for detailed locality information of samples.

Fig. 1.7 Distribution and collection sites for *Lethenteron zanandreai*. Pink area is the distribution range. Blue dot is the collection site. See Table 2.1 and Fig. 2.4 for detailed locality information of samples.

Chapter 2 Phylogeny of *Lethenteron* and closely related genera using the cytochrome *b* gene

2.1 Introduction

Compared to bony vertebrates, lampreys possess few morphological characters for species identification and phylogeny reconstruction. Adult dentition has traditionally been used for such purposes, but not without difficulty. In particular, the phylogenetic status of *Lethenteron* Creaser and Hubbs 1922 and closely related genera *Eudontomyzon* Regan 1911 and *Lampetra* Bonnaterre 1788 are uncertain, and different authors have suggested different divisions of genera based on morphological characters (e.g., Vladykov and Kott 1979b; Potter 1980; Bailey 1980; Nelson 2006). Molecular studies using mitochondrial DNA (mtDNA) that include *Lethenteron* and closely related genera suggested different generic divisions yet again; the above three genera were not monophyletic and *Lampetra* species from the Pacific drainage of North America were placed into a different clade than those from the Atlantic drainages of North America and Eurasia (Docker et al. 1999; Blank et al. 2008; Lang et al. 2009). Due largely to different taxonomic or geographic sampling, however, previous molecular studies disagree with each other on the relationships among genera. In this chapter, all the seven *Lethenteron* species listed in Renaud (2011), three *Eudontomyzon* and seven *Lampetra* species were sampled from multiple localities over their distribution ranges where samples were available, especially

for the *Lethenteron* species. The mitochondrial cytochrome *b* (cyt *b*) gene was used for the phylogenetic analyses to resolve the relationships among genera.

Relationships among the Arctic lamprey *Lethenteron camtschaticum* (Tilesius 1811) and its closely related satellite species, the Alaskan brook lamprey *Lethenteron alaskense* Vladykov and Kott 1978, the American brook lamprey *Lethenteron appendix* (DeKay 1842), the Siberian lamprey *Lethenteron kessleri* (Anikin 1905) and the Far Eastern brook lamprey *Lethenteron reissneri* (Dybowski 1869), are also examined in this chapter. Although morphologically distinguishable, previous studies using mtDNA involving at least some of these taxa (Yamazaki et al. 2006; Lang et al. 2009; April et al. 2011) generally show them to be a single species by the phylogenetic species concept (i.e., they lack diagnostic differences or are not reciprocally monophyletic; Mayden 1997). Since network methods are more useful for resolving relationships among closely related haplotypes than bifurcating trees (Posada and Crandall 2001; see Section 1.4.1), the relationships within the genus *Lethenteron* were examined using median-joining network analysis.

2.1.1 The relationships among Eudontomyzon, Lampetra *and* Lethenteron

As summarized in Section 1.2 and Table 1.2, *Eudontomyzon*, *Lampetra* and *Lethenteron* are three morphologically similar genera possessing few differences in their dentition. Their different posterial circumoral teeth (posterials) are important features to

distinguish them: *Lethenteron* possesses a single row of posterials, either complete or incomplete; *Eudontomyzon* typically has 1-4 rows of posterials; *Lampetra* has no posterials (Vladykov and Follett 1967; Gill et al. 2003; Naseka et al. 2009; Renaud 2011). Exolateral teeth (exolaterals) are absent in *Lampetra* and *Lethenteron* and present in *Eudontomyzon* (Vladykov and Follett 1967; Gill et al. 2003). In several previous taxonomic studies, *Lampetra* and *Lethenteron* were lumped together as subgenera in the same genus (Bailey 1980; Potter 1980) or genera in the same subfamily (Vladykov and Kott 1979b; Nelson 2006), while *Eudontomyzon* was excluded from the group of the former two (Potter 1980) or put together with them (Vladykov and Kott 1979b; Bailey 1980; Nelson 2006).

Previous phylogenetic analyses using morphological characters (Gill et al. 2003) or molecular data (Lang et al. 2009) did not support the generic division suggested by Potter (1980). In the morphological study of Gill et al. (2003), *Eudontomyzon* and *Lampetra* were reciprocally monophyletic and sister to each other and *Lethenteron* was sister to the group of the former two. One of the most surprising disagreements between morphological and molecular results was on the genus *Lampetra*, which was not monophyletic using partial mitochondrial cyt *b* and NADH dehydrogenase 3 (ND3) genes (Docker et al. 1999; Blank et al. 2008; Lang et al. 2009) but was monophyletic using morphological data (Gill et al. 2003). The molecular results suggested dividing *Lampetra* into two parts, Atlantic *Lampetra* from the Atlantic drainages of Eurasia and

North America and Pacific *Lampetra* from the Pacific drainage of North America (Docker et al. 1999; Blank et al. 2008; Lang et al. 2009). These results were surprising because these two groups of *Lampetra* are morphologically very similar; in fact, the river lamprey *Lampetra ayresii* (Günther 1870), a parasitic lamprey from the Pacific drainage of North America, was initially treated as a synonym of the European river lamprey *Lampetra fluviatilis* (L.). *Lampetra ayresii* was redescribed by Vladykov and Follett (1958) as a species distinct from *Lampetra fluviatilis* based on slight differences in body proportions, caudal fin shape, pigmentation, and average trunk myomere count. Similarly, the non-parasitic western brook lamprey *Lampetra richardsoni* Vladykov and Follett 1965, from the Pacific drainage of North America, showed only subtle morphological differences from the non-parasitic European brook lamprey *Lampetra planeri* Bloch 1784. These similarities in morphology could be due to phenotypic convergence (i.e., similar features that were not found in their most recent common ancestor; Patterson 1988) or conserved features from their common ancestor.

Docker et al. (1999) did not include *Eudontomyzon* and recovered Pacific *Lampetra* as a sister group to Atlantic *Lampetra* plus *Lethenteron*. Blank et al. (2008) included only the Ukrainian brook lamprey *Eudontomyzon mariae* (Berg 1931) and discovered that Pacific *Lampetra* is sister to Atlantic *Lampetra* plus *Eudontomyzon mariae* while *Lethenteron* is sister to the former three plus *Entosphenus* Gill 1862 (although the authors interpreted the result that *Eudontomyzon mariae* should be placed in *Lampetra*). Lang et

al. (2009) reported Pacific *Lampetra* was sister to Atlantic *Lampetra* plus *Eudontomyzon* and *Lethenteron*.

Although *Lethenteron* is the focus of this study, the genera *Lampetra* and *Eudontomyzon* are included, so that the phylogenetic status of *Lethenteron* could be discussed. Also, to resolve the placement of some species, such as the Western Transcaucasian brook lamprey *Lethenteron ninae* Naseka, Tuniyev, and Renaud 2009, the Lombardy brook lamprey *Lethenteron zanandreai* (Vladykov 1955), and the Korean lamprey *Eudontomyzon morii* (Berg 1931), *Lampetra* and *Eudontomyzon* have to be included (see the forthcoming section). In this chapter, 77 cyt *b* gene sequences from these three genera were used to test the monophyly of genera/groups and to resolve the relationships among them. The genetic distances between groups were estimated using Kimura's two-parameter (K2P) distance.

2.1.2 The placement of species

The placement of some species is difficult to determine based only on morphology. Morphological characters are sometimes ambiguous or variable in lamprey species, especially a non-parasitic lamprey. One example is in *Lethenteron ninae*, in which posterials are present in one incomplete row or occasionally absent (Naseka et al. 2009). *Lethenteron* usually possess one complete or incomplete row of posterials (Vladykov and Follett 1967). The variation of posterials in *Lethenteron ninae* adds to the uncertainty of

its placement in *Lethenteron*. For *Lethenteron zanandreai*, different authors had different descriptions on the posterials, present in one complete or incomplete row (Hubbs and Potter 1971; Renaud 2011) or absent (Vladykov 1955; Tutman et al. 2009). Thus this species was suggested as *Lethenteron* (Hubbs and Potter 1971; Potter 1980; Renaud 2011) or *Lampetra* (Vladykov 1955; Vladykov and Kott 1979b) by different researchers. Some of the morphological features of different genera overlap; for example, in *Eudontomyzon* species, posterials are present in 1-4 rows and rarely absent in *Eudontomyzon mariae*, while posterials are typically present in one row in *Lethenteron* and absent in *Lampetra* (Vladykov and Follett 1967; Renaud 1982; Renaud 2011). The situation may throw doubt on the placement of some species, for example, *Eudontomyzon morii*, which possesses one row of posterials (Renaud 1982; Renaud 2011), like most *Lethenteron* species. However, based on the morphological phylogeny, Gill et al. (2003) suggested that *Eudontomyzon morii* belongs in *Eudontomyzon* with the Carpathian lamprey *Eudontomyzon danfordi* Regan 1911, sharing the feature that exolaterals are present. Interestingly, *Lethenteron ninae*, *Lethenteron zanandreai* and *Eudontomyzon morii* are all allopatric from other species in their genera and are partly sympatric or parapatric with (i.e., in the same river system) some species in other genera (e.g., *Lethenteron ninae* is partly sympatric with *Eudontomyzon mariae*; *Lethenteron zanandreai* is partly sympatric with *Lampetra fluviatilis* and *Lampetra planeri*, and *Eudontomyzon morii* occurs near *Lethenteron camtschaticum* and *Lethenteron reissneri* (*s.l.*) in the Amur River system;

Vladykov and Kott 1979b; Renaud 2011).

Several molecular studies using the cyt *b* gene suggested *Lethenteron zanandreai* belongs in *Lampetra*. Docker et al. (1999) used 267 bp of the cyt *b* sequence of *Lethenteron zanandreai* from Tagliavini et al. (1994) to construct a neighbor-joining tree with other *Entosphenus, Lampetra* and *Lethenteron* samples and found it was not in the same clade with other *Lethenteron* species. Caputo et al. (2009) reported *Lethenteron zanandreai* formed a clade with *Lampetra fluviatilis* and *Lampetra planeri* rather than *Lethenteron camtschaticum*, *Lethenteron kessleri* and *Lethenteron reissneri* in a neighbor-joining tree using partial cyt *b* (384 bp) and cytochrome *c* oxidase subunit I (COI, 603 bp) genes. Lang et al. (2009) confirmed that *Lethenteron zanandreai* was in the Atlantic *Lampetra* (Section 2.1.1) clade rather than the *Lethenteron* clade using 1133 bp of the cyt *b* gene from almost all the existent lamprey species (although generally with only one individual per species). In this chapter, the hypothesis that *Lethenteron zanandreai* belongs in the genus *Lampetra* (Atlantic *Lampetra*) rather than *Lethenteron* was tested using complete 1191 bp of the cyt *b* gene with seven individuals of *Lethenteron zanandreai* sampled. Lang et al. (2009) also included a metamorphosing *Eudontomyzon morii* sample and assigned it to *Lethenteron* rather than *Eudontomyzon*, which disagreed with the placement by Gill et al. (2003). In this chapter, the *Eudontomyzon morii* sequence from Lang et al. (2009) will be included to confirm this placement within a more comprehensive sample set. To date, molecular phylogenetic

studies on the recently described *Lethenteron ninae* are lacking. This chapter includes seven *Lethenteron ninae* cyt *b* sequences (two from the type locality) to test the placement of *Lethenteron ninae*.

Some species [e.g., *Lethenteron* sp. N, *Lethenteron* sp. S and *Lethenteron reissneri* (*s.s.*)] are distinct from each other genetically, but difficult to distinguish morphologically due to the lack of diagnostic characters (Yamazaki and Goto 1996; Yamazaki and Goto 1997; Yamazaki and Goto 2000; Yamazaki et al. 2003; Section 1.3.3). Lang et al. (2009) reported *Lethenteron* sp. N sister to *Lethenteron* plus *Eudontomyzon morii*, and *Lethenteron* sp. S sister to the clade of *Eudontomyzon*, *Lampetra* and *Lethenteron*. Okada et al. (2010) found special repeat sequences in the non-coding region 2 (NC2) in the *Lethenteron* sp. S samples from Kamo River, Upper Shougawa, Toyama, Japan, that were distinct from *Lethenteron* sp. N, *Lethenteron camtschaticum* and *Lethenteron kessleri*, in agreement with the hypothesis that *Lethenteron* sp. S is more divergent from *Lethenteron camtschaticum* than other *Lethenteron* species (Lang et al. 2009). This chapter will use the cyt *b* sequences of *Lethenteron* sp. N (Shougawa River, Japan, 1133 bp) from Lang et al. (2009) and *Lethenteron* sp. S (Senju River, Japan, 1191 bp) from Okada et al. (2010) to resolve their relationships to other *Lethenteron* species. *Lethenteron reissneri* (*s.l.*) from Shougawa River (i.e., sympatric with *Lethenteron* sp. N) was also included in this study. Also included was *Lethenteron* sp. S from a different locality (Naktong River, Republic of Korea). With most other species sampled from multiple localities, the

phylogenetic analysis presented here is the most comprehensive to date.

2.1.3 Lethenteron camtschaticum *and closely related satellite species*

Lethenteron alaskense, *Lethenteron appendix*, *Lethenteron kessleri* and *Lethenteron reissneri* (*s.s.*) are considered closely related satellite species of *Lethenteron camtschaticum* (Docker 2009). The relationships among these species, however, are uncertain (see Section 1.3.1 - 1.3.3). Lang et al. (2009) failed to resolve the relationships among them using phylogenetic analysis with 1133 bp of the cyt *b* gene. Few differences in the cyt *b* sequences were found among these species. The boundaries between *Lethenteron camtschaticum* and *Lethenteron alaskense*, *Lethenteron camtschaticum* and *Lethenteron kessleri*, *Lethenteron camtschaticum septentrionalis* and *Lethenteron camtschaticum camtschaticum* were not clear (see Section 1.3.1, 1.3.2). The geographical pattern of haplotypes of *Lethenteron appendix* and *Lethenteron reissneri* and their relationships to other satellite species of *Lethenteron camtschaticum* were not resolved in phylogenetic studies using bifurcating trees (e.g., Lang et al. 2009).

Haplotype networks have recently been used for intraspecific population phylogeny or dealing with the relationships among closely related taxa (Guzmán et al. 2011; Dai et al. 2012; Jeratthitikul et al. 2013). Guzmán et al. (2011) and Jeratthitikul et al. (2013) used median-joining networks (Bandelt et al. 2000) to show the geographical patterns of haplotypes within species, which were unclear in the phylogenetic trees. Dai et al. (2012)

found it difficult to resolve the relationships among three closely related pine moth species (the genus *Dendrolimus* Germar 1812) using phylogenetic trees and resolved them with haplotype networks. A haplotype network would resolve the relationships among *Lethenteron camtschaticum* (two subspecies) and its closely related satellite species more precisely than a bifurcating phylogenetic tree, and would be able to show the geographic pattern of haplotypes of each species (Posada and Crandall 2001). This chapter includes a median-joining network of *Lethenteron camtschaticum* and its satellite species. Except for *Lethenteron alaskense*, of which only one cyt *b* sequence was obtained from Lang et al. (2009), at least four individuals from at least two different localities were included for each species. One specimen from Northwest Territories was identified as *Lethenteron* but not to species, which could be *Lethenteron alaskense* or *Lethenteron camtschaticum* judging from the distribution of *Lethenteron* species (Renaud 2011). Multiple individuals will reduce the bias caused by individual mutation and help resolve the complicated relationships among haplotypes of species.

2.1.4 Mitochondrial DNA and cyt b *gene*

The limitation of morphological characters as the basis of lamprey taxonomy has been discussed in previous sections. Molecular phylogenetic analysis may provide useful information where the morphological characters are ambiguous or overlapping (see Section 2.1.2). The mtDNA of animals is a useful material for examining the molecular

phylogeny for several reasons, most notably because it has a higher substitution rate than that of single-copy nuclear DNA (Brown et al. 1979; Brown et al. 1982; Pesole et al. 1999), allowing for better resolution among closely related taxa, and its haploid nature and conserved gene content and arrangement (Boore 1999) allow it to be easily amplified and sequenced in virtually any eukaryote.

The lamprey mitochondrial genome contains about 16.2 kb of 37 genes and two non-coding regions; 13 of the genes are protein-coding (Lee and Kocher 1995; Delarbre et al. 2000; Hwang et al. 2013a; Hwang et al. 2013b). The complete sea lamprey *Petromyzon marinus* L. (Lee and Kocher 1995), *Lampetra fluviatilis* (Delarbre et al. 2000), *Lethenteron camtschaticum* (Hwang et al. 2013a) and *Lethenteron reissneri* (Hwang et al. 2013b) mitochondrial genomes are available. Based on the available information of lamprey mitochondrial genomes, previous researchers sequenced different mitochondrial genes and used them to address phylogenetic questions in lampreys, including cyt *b* (Docker et al. 1999; Lorion et al, 2000; Yamazaki et al. 2006; Espanhol et al. 2007; Blank et al. 2008; Boguski 2009; Caputo et al. 2009; Lang et al. 2009; Mateus et al. 2011; Boguski et al. 2012; Docker et al. 2012), ND3 (Docket et al. 1999; Blank et al. 2008; Martin and White 2008; Docker et al. 2012), COI (Yamazaki et al. 2003; Yamazaki et al. 2006; Blank et al. 2008; Hubert et al. 2008; Boguski 2009; Caputo et al. 2009; Docker et al. 2012), ATPase subunit 6/8 (Espanhol et al. 2007; Mateus et al. 2011; Docker et al. 2012), and the non-coding regions (Blank et al. 2008; Martin and White

2008; Okada et al. 2010; Docker et al. 2012).

As a protein-coding gene, the cyt *b* gene has a low rate of replacement (non-synonymous) substitutions and relatively high rate of synonymous substitutions, which is appropriate for species or population level phylogenetic analysis (Irwin et al. 1991). This study obtained 69 complete and three partial cyt *b* sequences from 11 lamprey species using primers from Boguski (2009), Lang et al. (2009) and Docker (unpublished), as well as primers newly designed from cyt *b* sequences obtained with those primers. In this chapter the cyt *b* gene was used to infer phylogenetic trees with two phylogenetic methods and a median-joining network.

2.2 Materials and Methods

2.2.1 Samples

Lamprey muscle tissue samples were provided by Dr. C. B. Renaud (Canadian Museum of Nature), Dr. A. M. Naseka (Russian Academy of Sciences), and Dr. M. F. Docker (University of Manitoba). The tissues were from specimens collected from Europe, southeastern Russia, Japan, the west and northeast of the U.S., and Yukon Territory and Northwest Territories, Canada (Table 2.1). The species were identified by Dr. C. B. Renaud and Dr. A. M. Naseka using morphological methods (see Renaud 2011; Section 1.2 and 1.3). Ten species from *Eudontomyzon*, *Lampetra*, and *Lethenteron* were collected: *Eudontomyzon danfordi*, *Eudontomyzon mariae* [including the synonym

Eudontomyzon vladykovi (Oliva and Zanandrea 1959)], *Lampetra fluviatilis*, *Lethenteron camtschaticum* (including the subspecies *Lethenteron camtschaticum camtschaticum* and *Lethenteron camtschaticum septentrionalis*), *Lethenteron appendix*, *Lethenteron alaskense*, *Lethenteron kessleri*, *Lethenteron reissneri*, *Lethenteron ninae*, *Lethenteron zanandreai* as well as *Petromyzon marinus* as the outgroup. Three specimens of *Lethenteron appendix* from the Great Lakes (the only one of this genus in this basin) and one *Lethenteron* sp. specimen from the Northwest Territories (presumably *Lethenteron alaskense*), Canada, were larvae; all others were adult lampreys to facilitate species identification. Two *Eudontomyzon vladykovi* (a synonym of *Eudontomyzon mariae*), two *Lampetra fluviatilis* and two *Lethenteron ninae* specimens were from the type localities. *Lampetra fluviatilis* and *Lethenteron zanandreai* included different coloration patterns (indicated in Table 2.1) to test whether the coloration patterns represent distinct taxa.

The tissue samples were preserved in 100% ethanol. DNA was extracted from the tissue samples as described below (Section 2.2.2) or, for the *Lethenteron camtschaticum* specimen from Liaohe River, China, the DNA extraction was sent by Dr. W. Li (Michigan State University).

For the phylogenetic analysis, additional sequences were retrieved from the NCBI Nucleotide database (GenBank): 24 cyt *b* sequences of 20 species from genera *Eudontomyzon*, *Lampetra*, *Lethenteron* and *Petromyzon* (as the outgroup), including *Lampetra richardsoni* and the Pacific brook lamprey *Lampetra pacifica* Vladykov 1973

from the type localities (Table 2.2).

2.2.2 DNA extraction, amplification, and sequencing

DNA was extracted using the DNeasy Blood and Tissue Kit (Qiagen) following the spin-column protocol in the instructions with the option of adding 3 µL of 100 mg/mL RNase A to 200 µL lysate and incubating for 10 min before adding buffer AL. Polymerase chain reaction (PCR) was used for the amplification of the cyt *b* gene. Overlapping fragments of the cyt *b* gene with the upstream or downstream regions were amplified using primers listed in Table 2.3. For each sample, one of the three internal reverse primers, Cytb-518-R (M. F. Docker, unpublished), Cytb-513-R and Cytb-606-R, was paired with Glu-F (Boguski 2009) to amplify about 800-900 bp of the 5' end of the cyt *b* gene (from position 44 of the tRNA-Glu gene to position 539, 533, and 627, respectively, of the cyt *b* gene, inclusive). The reverse primers Cytb-513-R and Cytb-606-R were newly designed since Cytb-518-R would not amplify the target piece in all the samples. The primer pair Cytb-361-F (M. F. Docker, unpublished) and Phe1612H (Lang et al. 2009) was used to amplify about 1000 bp of the 3' end of the cyt *b* gene (from position 361 of the cyt *b* gene to the position 37 of tRNA-Phe gene). The partial sequences were assembled to obtain the complete 1191 bp of cyt *b* sequences.

Each 30 µL PCR reaction contained 1X PCR buffer (Invitrogen) (20 mM Tris-HCl pH 8.4; 50 mM KCl), 2.5 mM $MgCl_2$, 0.2 mM of each deoxynucleotide triphosphate

(dNTP), 0.4 µM of each primer, and 0.02 U of Go*Taq*® DNA polymerase (Promega) or 0.02 U of *Taq* DNA Polymerase (Invitrogen). Reactions were initially denatured at 96 °C for 3 min. Amplifications were carried out in 30 cycles: denaturation at 96 °C for 30 sec, primer annealing at 60 °C, 58 °C, and 55 °C for 30 sec for 10 cycles each, extension at 72 °C for 2 min, and additional extension at 72 °C for 5 min.

Precipitation with 50% isopropanol and 0.5 M sodium acetate was conducted to purify the PCR products, which were then used as templates in the sequencing reactions. The sequencing reactions were performed using the Applied Biosystems 3500 Genetic Analyzer and BigDye® Terminator v3.1 Cycle Sequencing Kits (Applied Biosystems Inc.). Sequencing was done by Robarts Research Institute DNA Sequencing Facility using Applied Biosystems 3730 Analyzer, or in the Docker laboratory using Applied Biosystems 3500 Genetic Analyzer (Applied Biosystems Inc.). Each sample was sequenced with the primers Cytb-361-F, Phe1612H, and one of the internal primers Cytb-518-R, Cytb-513-R, and Cytb-606-R (Table 2.3). The electropherograms (ABI files) of the resulting sequences were viewed and edited with the software Chromas Lite v2.01 (Conor McCarthy, Griffith University, Australia), exported into FASTA files, and assembled by eye. The upstream and downstream regions of the cyt *b* gene were trimmed and the 1191 bp of the cyt *b* gene were aligned by eye for the phylogenetic analyses.

2.2.3 Phylogenetic analyses

A dataset of 79 cyt *b* sequences from *Lethenteron*, *Lampetra*, *Eudontomyzon* and the outgroup *Petromyzon marinus* (indicated in Table 2.1 and 2.2) was used for a maximum parsimony analysis and a Bayesian analysis (as described below). To resolve the relationships among six closely related *Lethenteron* species or subspecies (*Lethenteron camtschaticum*, *Lethenteron camtschaticum septentrionalis*, *Lethenteron alaskense*, *Lethenteron appendix*, *Lethenteron kessleri*, and *Lethenteron reissneri*), 42 cyt *b* sequences of these species from 22 different locations (indicated in Table 2.1 and 2.2) were used for a median-joining network.

The maximum parsimony analysis was conducted in PAUP* (Swofford 2002). PAUPRat (http://mercury2.iab.uaf.edu/derek_sikes/software2.htm; Nixon 1999) was used for the maximum parsimony tree search. The ratchet was executed in PAUP* using ten independent runs each with 200 iterations. For each iteration, 15% of the characters were perturbed to produce a parsimony tree. The bootstrap supporting values greater than 80 were estimated using 10,000 “fast-stepwise” addition replicates of bootstrapping and mapped onto the strict consensus maximum parsimony tree. Two *Petromyzon marinus* were used as outgroups. Gaps were treated as missing data.

For Bayesian analysis, MrModelTest 2.3 (Nylander 2004) was used to select the models for each partition of the dataset defined by codon positions with the Akaike Information Criterion (AIC). Bayesian analysis was run for 5,000,000 generations with

every 5,000 generations sampled in MrBayes 3.2.1 (Huelsenbeck and Ronquist 2001; Ronquist and Huelsenbeck 2003). Appropriate mixing was assessed using the average standard deviation of split frequencies (<0.01). The priors for the phylogenetic model were set as default. The gamma shape parameter, proportion of invariable sites, character state frequencies, and substitution rate of the General Time Reversible (GTR) model (Tavaré 1986) were unlinked across partitions. Stationarity of negative log-likelihood values was evaluated by plotting these values against generation. The 50% majority rule consensus of the posterior distribution of trees was inferred with the burnin period of 25% discarded.

The relationships among *Lethenteron camtschaticum* and its satellite species were not resolved in the maximum parsimony tree or the Bayesian tree. A median-joining network including 42 individuals of these species was generated using Network 4.6.1.1 (Bandelt et al. 1999) with the connection cost criterion to show the relationships on the level of population. All characters were equally weighted. The value of epsilon was zero. The transitions/transversions weight was set to 1:1.

2.3 Results

2.3.1 Maximum parsimony analysis

The length of all complete cyt *b* gene sequences included in the maximum parsimony analysis was 1191 bp. No gaps occurred in the sequence alignment. All the

sequences from GenBank sequenced by Lang et al. (2009) were partial cyt *b* sequences of 1-1133bp. Among 350 variable characters, 290 characters were parsimony informative. Through each independent run of the ratchet, 201 trees were discovered. Ten runs of the ratchet have discovered 2010 maximum parsimony trees with the equal length of 653 steps. The strict consensus tree of the 2010 most parsimonious trees is shown in Fig. 2.1.

Excluding *Lethenteron* sp. S, *Lethenteron zanandreai*, and *Lethenteron ninae*, the genus *Lethenteron* plus *Eudontomyzon morii* formed a monophyletic group ("Le" in Fig. 2.1) with the bootstrap supporting value of 99%. The genus *Eudontomyzon* excluding *Eudontomyzon morii* was monophyletic ("Eu" in Fig. 2.1) with the supporting value of 97%. *Lethenteron zanandreai* and *Lethenteron ninae* were in the same clade with the Turkish brook lamprey *Lampetra lanceolata* Kux and Steiner 1972, *Lampetra fluviatilis*, *Lampetra planeri*, and the least brook lamprey *Lampetra aepyptera* (Abbott 1860). Even if *Lethenteron zanandreai* and *Lethenteron ninae* were placed within *Lampetra*, the genus *Lampetra* remained paraphyletic. The *Lampetra* species (including *Lethenteron zanandreai* and *Lethenteron ninae*) from western Eurasia and the Atlantic Ocean drainage basin of North America formed one monophyletic group ("ALa" in Fig. 2.1) while the *Lampetra* species from the Pacific Ocean drainage basin of North America formed another monophyletic group ("PLa" in Fig. 2.1). The supporting value for clade PLa was 100% while the supporting value for clade ALa was only 62%. If excluding *Lampetra aepyptera*, which was not in clade ALa in the Bayesian tree (Section 2.3.2), the

supporting value for ALa was 76%. Atlantic *Lampetra* and *Eudontomyzon* were sister taxa. Pacific *Lampetra* was sister to Atlantic *Lampetra* plus *Eudontomyzon*. *Lethenteron* was sister to Pacific *Lampetra* plus *Eudontomyzon* plus Atlantic *Lampetra* [PLa + (Eu + ALa)]. *Lethenteron* sp. S was sister to all these four groups.

Lethenteron camtschaticum (referring to *Lethenteron camtschaticum camtschaticum* if not indicated), *Lethenteron camtschaticum septentrionalis*, *Lethenteron reissneri*, *Lethenteron appendix*, *Lethenteron alaskense*, and *Lethenteron kessleri* were not reciprocally monophyletic. The relationships among these species or subspecies were unresolved in the cyt *b* tree. All individuals of these six species or subspecies, excluding the *Lethenteron reissneri* from Shougawa River, Honshu Island, Japan, formed a clade with the supporting value of 81%.

Eudontomyzon morii (GQ206163) was sister to this *Lethenteron* group. The *Lethenteron reissneri* (*s.l.*) and *Lethenteron* sp. N from Shougawa River, Honshu Island, Japan, were sister to the *Lethenteron* group plus *Eudontomyzon morii*.

Lethenteron reissneri from Shougawa River, Honshu Island, Japan, and the *Lethenteron* sp. N from the same locality (GQ206182) formed a clade with the bootstrap supporting value of 100%.

Eudontomyzon danfordi was monophyletic with 100% bootstrap support. The typical *Eudontomyzon mariae* and the synonym *Eudontomyzon vladykovi* were not reciprocally monophyletic. *Eudontomyzon danfordi* and the typical *Eudontomyzon mariae* plus the

synonym *Eudontomyzon vladykovi* were sister taxa with 53% bootstrap support, while *Eudontomyzon stankokaramani* (Karaman 1974), another synonym of *Eudontomyzon mariae*, was sister to them with 97% bootstrap support.

Lampetra fluviatilis and *Lampetra planeri* formed a clade and were not reciprocally monophyletic. The different coloration patterns of *Lampetra fluviatilis* (one individual each) were both in this clade. The branching order within this clade was not resolved.

Lethenteron zanandreai from Vipava River, Slovenia, was monophyletic and was in the ALa rather than the Le group. The different coloration patterns (two individuals each) were not reciprocally monophyletic. *Lethenteron zanandreai* was sister to *Lethenteron ninae* plus *Lampetra lanceolata*.

Although described as a *Lethenteron* species (Naseka et al. 2009), *Lethenteron ninae* was in the ALa group and was paraphyletic. The individuals from Mokva River, Georgia, and the ones from Myzmta River, Russia, formed a clade with *Lampetra lanceolata* with 99% bootstrap support. The *Lethenteron ninae* from Bzyb River, Georgia, and Shakhe River, Russia, were sister to this clade.

2.3.2 Bayesian analysis

The Bayesian analysis used the same character matrix as the maximum parsimony analysis. MrModelTest 2.3 (Nylander 2004) suggested: the General Time Reversible (GTR) model (Tavaré 1986) for the first codon position with a parameter for invariable

sites (I = 0.5049) and a gamma distribution shape parameter (Γ = 0.6155); the Hasegawa-Kishino-Yano (HKY) model (Hasegawa et al. 1985) for the second codon position with a parameter for invariable sites (I = 0.8855) and equal rates for all sites; and the GTR model for the third codon position with a gamma distribution shape parameter (Γ = 1.8051). The 50% majority rule consensus of 750 trees with the posterior probabilities is shown in Fig. 2.2.

The four main clades (Le, PLa, Eu, and ALa) discovered in the maximum parsimony tree were also recovered in the Bayesian tree and colored with the same colors. The difference between the Bayesian tree and the maximum parsimony tree was that the phylogenetic relationships among *Lampetra aepyptera*, ALa and Eu were unresolved in the Bayesian tree while *Lampetra aepyptera* formed a clade with ALa in the maximum parsimony tree. However, the supporting value of clade ALa with *Lampetra aepyptera* in the maximum parsimony tree was low (62 %). Except for this, the relationships among *Lethenteron, Eudontomyzon* and *Lampetra* species shown in the Bayesian tree agreed with that in the maximum parsimony tree.

2.3.3 Median-joining network

The relationships among *Lethenteron camtschaticum, Lethenteron camtschaticum septentrionalis, Lethenteron alaskense, Lethenteron appendix, Lethenteron reissneri,* and *Lethenteron kessleri* were not resolved in the maximum parsimony tree or the Bayesian

tree. A median-joining network of these six species and subspecies from several geographic regions is shown in Fig. 2.3. The sizes of the nodes are proportional to the frequencies of the haplotypes. The lengths of the links estimate the number of substitutions between haplotypes. The information of samples and the frequency of each haplotype are listed in Table 2.4 and 2.5. The locations are marked on the map in Fig. 2.4.

The median-joining network showed 20 cyt *b* haplotypes among 42 individuals. The largest haplotype H1 contained 14 individuals from *Lethenteron camtschaticum* (five of 15 individuals from five locations), *Lethenteron camtschaticum septentrionalis* (all four individuals from two locations), *Lethenteron alaskense* (the only individual), and *Lethenteron kessleri* (three of eight individuals from two locations), providing no evidence for these lampreys separating out along subspecies or species lines. An additional nine haplotypes were observed in the remaining 10 *Lethenteron camtschaticum* specimens, and an additional four haplotypes were found in the remaining five *Lethenteron kessleri*. The unidentified larval *Lethenteron* sp. from Martin River, NT, Canada, was either *Lethenteron camtschaticum* or *Lethenteron alaskense*. The partial sequence (1-482 bp of the cyt *b* gene) of this sample showed no difference from H1.

Three haplotypes (H17, H18 and H19) were discovered in *Lethenteron appendix* (eight individuals from seven locations). These haplotypes contained only *Lethenteron appendix* and were all in haplogroup HG502. That means there was one substitution fixed to species between *Lethenteron camtschaticum* and *Lethenteron appendix* at position 502

of the cyt *b* gene. The average percentage of different sites was 0.30% (0.25% synonymous) between *Lethenteron camtschaticum* and *Lethenteron appendix*; 0.31% (0.23% synonymous) between *Lethenteron appendix* and *Lethenteron kessleri*; 0.14% (0.14% synonymous) between *Lethenteron appendix* and *Lethenteron alaskense*; and 0.25% (0.24% synonymous) between *Lethenteron appendix* and *Lethenteron reissneri*. Unlike *Lethenteron camtschaticum* and *Lethenteron kessleri*, the haplotypes of *Lethenteron appendix* corresponded to the distribution of the populations. The five individuals from the Great Lakes formed a haplogroup (HG309) differing from all other haplotypes at position 309 of the cyt *b* gene, while the three individuals from Maine and Delaware, USA (Atlantic Ocean drainage of North America) were another haplotype (H17). H17 and H18 had 3 bp (0.25%) differences, H17 and H19 had 4 bp (0.34%) differences, and H18 and H19 had 3 bp (0.25%) differences. *Lethenteron reissneri* had three haplotypes. Two individuals from Vishnevka Reservoir, Russia were haplotype H15, 1 bp (0.08%) different from *Lethenteron camtschaticum*. Two individuals from Barh River, Mongolia, were haplotype H16, 2 bp (0.17%) different from *Lethenteron camtschaticum*. These two haplotypes were in haplogroup HG502 with *Lethenteron appendix*. Interestingly, another haplotype of *Lethenteron reissneri* (H20), containing only one individual from Vishnevka Reservoir, Russia, was on a different lineage from H15 (3bp, 0.25% differences from H20) and H16 (4bp, 0.34% differences from H20) which was directly out of H1 (2bp, 0.17% differences from H20). The average percentage of different sites was 0.22% (0.19%

synonymous) between *Lethenteron reissneri* and *Lethenteron camtschaticum*; 0.25% (0.24% synonymous) between *Lethenteron reissneri* and *Lethenteron appendix*; 0.24% (0.17% synonymous) between *Lethenteron reissneri* and *Lethenteron kessleri*; 0.09% (0.09% synonymous) between *Lethenteron reissneri* and *Lethenteron alaskense*.

2.3.4 Genetic distances

Between-species genetic distances were calculated with percent K2P distance (Kimura 1980) and are listed in Table 2.6. Except *Petromyzon marinus* and *Lethenteron* sp. S, all species were divided into four groups Le, Eu, PLa and ALa based on the maximum parsimony analysis (Section 2.3.1). Within-group and between-group percent K2P distances among Le, Eu, PLa and ALa are listed in Table 2.7.

Lethenteron camtschaticum and its satellite species plus *Eudontomyzon morii* were closely related (within-group mean K2P distance = 0.69%). Among the satellite species, *Lethenteron* sp. N was most divergent from *Lethenteron camtschaticum* (mean K2P = 3.56%; minimum K2P = 3.53%). Other satellite species were more closely related to *Lethenteron camtschaticum*. The mean K2P distance between *Lethenteron appendix* and *Lethenteron camtschaticum* was 0.38%, which was more distant than *Lethenteron reissneri*, *Lethenteron kessleri* and *Lethenteron alaskense* to *Lethenteron camtschaticum*. *Lethenteron reissneri* diverged from *Lethenteron camtschaticum* by 0.24% on average. Both *Lethenteron kessleri* and *Lethenteron alaskense* were only 0.06% mean K2P

distance to *Lethenteron camtschaticum*. Interestingly, *Eudontomyzon morii* was closely related to *Lethenteron camtschaticum* (mean K2P = 1.06%; minimum K2P = 1.00%) while the minimum distance between it and other *Eudontomyzon* species was 7.17%.

The mean K2P distance between *Lethenteron ninae* and *Lethenteron lanceolata*, which were not reciprocally monophyletic, was only 0.19% while the minimum was zero. The mean distance between *Lethenteron ninae* and *Lethenteron zanandreai* was 1.63%. The distance between *Lethenteron ninae* and *Lampetra fluviatilis* was 3.91% while that between *Lethenteron ninae* and other *Lethenteron* (excluding *Lethenteron zanandreai*) species were at least 7.79%. The mean distance between *Lethenteron zanandreai* and *Lampetra fluviatilis* was 2.94% while the distance between *Lethenteron zanandreai* and other *Lethenteron* (excluding *Lethenteron ninae*) species were at least 6.47%.

In terms of between-group distance, groups ALa and Eu were more closely related to each other than to Le or PLa. The mean K2P distance between ALa and Eu was 4.89% (range 4.30-5.97%). In contrast, the mean distances between PLa and Le, PLa and Eu, PLa and ALa were 7.95, 8.20, and 8.23%, respectively, and the mean distances between Le and Eu and Le and ALa were 7.47 and 7.46%, respectively (Table 2.7).

2.4 Discussion

2.4.1 Division of genera based on the cyt b *phylogenetic analyses*

Previous morphological and molecular studies suggested different divisions of

genera among *Lethenteron*, *Eudontomyzon* and *Lampetra*. Morphological studies always treat them as three genera (Vladykov and Follett 1967; Vladykov and Kott 1979b; Gill et al. 2003; Renaud 2011) or subgenera (Potter 1980; Bailey 1980). However, several molecular studies suggested *Lampetra* from the Pacific Ocean drainage and Atlantic Ocean drainages were each monophyletic, but were not sister to each other, that is, they did not together form a clade (Docker et al. 1999; Blank et al. 2008; Lang et al. 2009). This study supports the division by the molecular researchers, dividing *Lampetra* into two distinct clades, "Pacific *Lampetra*" and "Atlantic *Lampetra*". The congruence of the current results to previous studies is not surprising, given the use of mitochondrial DNA sequence in all studies (and, in many cases, use of the same gene). However, it is important to verify these results with a more comprehensive data set. Lang et al. (2009), for example, did not include *Lampetra pacifica* or *Lethenteron ninae*, and often relied on only a single representative of each species.

The four clades in the phylogenetic trees Le, Eu, PLa and ALa generally correspond to the genera/group *Lethenteron*, *Eudontomyzon*, Pacific *Lampetra* and Atlantic *Lampetra*, but there are some exceptions. For the first three clades, the bootstrap support value (BS) and the posterior probability (PP) were relatively high: Le (BS = 99.2%; PP = 1), Eu (BS = 96.83%; PP = 1) and PLa (BS = 100%; PP = 1). Le (genus *Lethenteron*) includes *Lethenteron camtschaticum*, *Lethenteron alaskense*, *Lethenteron appendix*, *Lethenteron kessleri*, *Lethenteron reissneri*, and *Lethenteron* sp. N, but also *Eudontomyzon morii*. Eu

(genus *Eudontomyzon*) includes *Eudontomyzon danfordi* and *Eudontomyzon mariae* (including the synonyms *Eudontomyzon stankokaramani* and *Eudontomyzon vladykovi*). PLa (Pacific *Lampetra*) includes *Lampetra ayresii*, *Lampetra richardsoni*, *Lampetra pacifica* and the Kern brook lamprey *Lampetra hubbsi* (Vladykov and Kott 1976). The division of these three genera/groups was congruent with Lang et al. (2009) using the same gene although *Lampetra pacifica* was not included in Lang et al. (2009). The inclusion of *Lampetra hubbsi* in Pacific *Lampetra* is consistent with previous molecular studies (Docker et al. 1999; Lang et al. 2009; Boguski et al. 2012), although this species was initially described as *Entosphenus hubbsi* based on the dentition (Vladykov and Kott 1976) and, in their most recent List of Common and Scientific Names of Fishes from the United States, Canada, and Mexico, was only recently referred to as *Lampetra* by the American Fisheries Society (Page et al. 2013).

As to the group ALa, the placement of *Lampetra aepyptera* in this group is supported by the maximum parsimony analysis (BS = 61.87%) but not the Bayesian analysis. The clade ALa excluding *Lampetra aepyptera* is supported in both trees although the bootstrap supporting value (75.8%) is lower than that of other three clades. The probability for ALa without *Lampetra aepyptera* is one. Thus in this discussion, ALa (Atlantic *Lampetra*) includes *Lampetra fluviatilis*, *Lampetra planeri*, *Lampetra lanceolata*, *Lethenteron zanandreai* and *Lethenteron ninae* (but does not include *Lampetra aepyptera*). Both the “black” and “grey” *Lampetra fluviatilis* (see Table 2.1)

are in the clade of *Lampetra fluviatilis* plus *Lampetra planeri*. Although this clade contains two clades in the Bayesian tree, they are poorly supported and not diagnosable; the structure in the clade is not resolved in the maximum parsimony tree. The "monotonous" and "spotted" *Lethenteron zanandreai* (see Table 2.1) are in the clade of *Lethenteron zanandreai*, showing no diagnostic difference in the cyt *b* gene from each other. Thus, these samples with different colorations should be treated as the same species.

In Lang et al. (2009), ALa (*Lampetra fluviatilis*, *Lampetra planeri*, *Lampetra lanceolata*, *Lethenteron zanandreai*) and *Lampetra aepyptera* (as *Okkelbergia aepyptera*) are reciprocally monophyletic and sister to each other in both maximum parsimony and Bayesian trees. However the bootstrap value for this sister relationship was lower than 85% (not shown) and the probability was lower than 0.95 (not shown). Docker et al. (1999) reported *Lampetra aepyptera* sister to *Lampetra fluviatilis* in the neighbor-joining tree using the cyt *b* and ND3 genes with 97% bootstrap support, and clustered with *Lampetra fluviatilis*, *Lampetra planeri* and *Lethenteron zanandreai* in another tree using 267 bp of the cyt *b* gene with 57% bootstrap support. Blank et al. (2008) also suggested *Lampetra aepyptera* was sister to ALa (*Lampetra fluviatilis* and *Lampetra planeri*) with 100% probability using 384 bp of cyt *b* gene. Blank et al. (2008) used the same two *Lampetra aepyptera* ammocoetes from Docker et al. (1999) while one adult *Lampetra aepyptera* from Lang et al. (2009) was used in this thesis. The support value varied with different

samples from different localities. Also, the inclusion of more ALa species (e.g., *Lethenteron zanandreai*) may decrease the support of the *Lampetra aepyptera* plus ALa clade.

Lampetra aepyptera is the only *Lampetra* species with both exolaterals and posterials present (Renaud 2011) and was once put in *Okkelbergia* as the only species in this genus by Hubbs and Potter (1971). Since the morphological characteristics in this species are "poorly developed," Hubbs and Potter (1971) considered the generic placement of it to be "somewhat dubious," and put it in the "provisional and noncommittal" genus *Okkelbergia*. Renaud (2011) "provisionally" placed it in *Lampetra* and stated that further study may support placing it in *Okkelbergia*. The Bayesian analysis in this chapter supports the separation of *Lampetra aepyptera* from Atlantic *Lampetra*. More *Lampetra aepyptera* samples from other localities and sequences of other genes, especially nuclear genes (see Chapter 3), may provide more evidence for this species as a distinct genus from *Lampetra*. Regardless of whether *Lampetra aepyptera* is sister to ALa or form a clade with Eu and ALa with the relationships to them unresolved, the results are not against the hypothesis that *Lampetra aepyptera* is descended from a *Lampetra fluviatilis*-type ancestor (Docker et al. 1999) rather than a *Lampetra ayresii*-type (Vladykov and Kott 1979a) or a *Lethenteron camtschaticum*-type (Bailey 1980; Potter 1980) ancestor.

Besides *Lampetra aepyptera*, there is another species, *Lethenteron* sp. S, which

could not be put in any of the Le, Eu, ALa, and PLa groups. *Lethenteron* sp. S is sister to all the four groups with the bootstrap value of 99% and the probability of 0.96. This result is in agreement with Lang et al. (2009). Yamazaki et al. (2006) also reported that *Lethenteron* sp. S was sister to the genus *Lethenteron*. If *Lethenteron* sp. S is to be described as a new species, as suggested by Yamazaki et al. (2003), it may be appropriate to put it in a new monotypic genus. However, the present evidence for the placement of this species (Lang et al. 2009; this thesis) is based on only one gene, the mitochondrial cyt *b* gene. Sequences of nuclear genes from *Lethenteron* sp. S and species from other genera would be useful to provide more evidence for its phylogenetic status.

The genetic distinctiveness of *Lethenteron* sp. S is somewhat surprising, however, given its morphological similarity to *Lethenteron* sp. N and *Lethenteron reissneri* (*s.s.*). Differences in the frequency distribution of morphological characters have been reported among these three taxa, but all the characteristics overlap in the former two (Yamazaki and Goto 1997). *Lethenteron* sp. N and *Lethenteron* sp. S thus appear to be cryptic species (i.e., two or more distinct species that were classified as a single species due to their morphological similarity). Other cryptic lamprey species have been reported in Atlantic and Pacific *Lampetra*: Boguski et al. (2012) suggested four unrecognized non-parasitic *Lampetra* spp. from the Pacific drainage of North America based on the phylogeny using the cyt *b* gene; Mateus et al. (2013) described three new cryptic species from Portugal which were previously treated as *Lampetra planeri*, but reciprocally

monophyletic using the cyt *b* gene (Mateus et al. 2011). The true number of lamprey species is more than that in the recent taxonomic list (Renaud 2011).

Genetically highly divergent cryptic species were also reported in other taxa besides lampreys (e.g., bonefishes *Albula* spp.; Colborn et al. 2001; the neotropical skipper butterfly *Astraptes fulgerator* (Walch 1775); Hebert et al. 2004; Vesicomyid clams *Vesicomya* spp.; Goffredi et al. 2003). These cryptic species are morphologically indistinguishable and sometimes sympatric, and are generally revealed by molecular studies (Yamazaki and Goto 1996; Yamazaki and Goto 2000; Colborn et al. 2001; Hebert et al. 2004; Goffredi et al. 2003). Thus, morphologically similar taxa are not necessarily recently diverged (Bickford et al. 2007). Selection pressures (e.g., extreme environmental conditions) could reduce or eliminate the changes in morphology during speciation (Bickford et al. 2007) or cause phenotypic convergence in distinct taxa (Wake 1991). Even if these morphologically similar populations are sympatric, they could be reproductively isolated (Yamazaki and Goto 2000). Thus, non-visual characters, such as chemical mating signals and DNA barcodes, should be taken into consideration in taxonomic studies.

2.4.2 Relationships among genera

Although the status of *Okkelbergia*/*Lampetra aepyptera* is still uncertain (see Section 2.4.1), the other four genera/groups, Le, Eu, ALa and PLa, are well supported.

Since *Lethenteron* sp. S is sister to all these groups, this section will focus on the relationships among *Lethenteron*, *Eudontomyzon*, Pacific *Lampetra* and Atlantic *Lampetra*.

As summarized in Fig. 2.5, previous phylogenetic analyses using morphological (Gill et al. 2003) and molecular (Docker et al. 1999; Blank et al. 2008; Lang et al. 2009) data discovered varied relationships among these four groups. Even though the cyt *b* gene was used in all the molecular studies, including this thesis, the generic phylogenies were incongruent reciprocally. However, it is an agreement among molecular analyses using the cyt *b* gene (Blank et al. 2008; Lang et al. 2009; this thesis) that ALa and Eu are more closely related to each other than to other groups. Morphological phylogenetic analysis using only parasitic species by Gill et al. (2003) suggested Atlantic and Pacific *Lampetra* were sister taxa. Morphological characters, especially the dentition, often vary in non-parasitic lampreys, and are less divergent in parasitic lampreys (Renaud 2011). One explanation is that the dentition of non-parasitic lampreys is often weaker than in parasitic lampreys (i.e., smaller and with fewer teeth or cusps) and is less subject to selection. The dentition in parasitic species corresponds with their feeding biology (e.g., blood-feeding versus flesh-feeding lampreys possess different dentition; Potter and Hilliard 1987; Renaud et al. 2009). The fact that all the parasitic species in *Eudontomyzon* (*Eudontomyzon danfordi* and *Eudontomyzon morii*), *Lampetra* (*Lampetra fluviatilis* and *Lampetra ayresii*) and *Lethenteron* (*Lethenteron camtschaticum*) mainly feed on muscle

tissues is related to some common characteristics in their dentition (e.g., the lack of complete rows of teeth in the lateral and posterior fields; Renaud et al. 2009). The selection power of feeding biology may reduce the divergence of dentition in parasitic lampreys (or result in convergent evolution), while the dentition of non-parasitic lampreys evolves free from this selection. Thus, the result including only parasitic lampreys (Gill et al. 2003) may not represent the relationships among genera, since most genera consist of both parasitic and non-parasitic species. In terms of genetic distance, ALa and Eu are more closely related to each other than to any other groups. Interestingly, groups ALa and Eu are both endemic to the Atlantic Ocean drainage (including Mediterranean Sea and Black Sea drainages) of Eurasia, while Le and PLa are both allopatric from them (Renaud 2011). Non-parasitic lampreys in Eu and ALa (e.g., *Eudontomyzon mariae* and *Lampetra planeri*) have been found to be morphologically similar, with only diagnostic differences in number and arrangement of velar tentacles (Holčík and Delić 2000). Rembiszewski (as cited in Holčík and Delić 2000) reported hybrids between *Eudontomyzon* and *Lampetra planeri* in 1968, although it was suspected that this represented misidentification of *Eudontomyzon mariae* with varied dentition (Holčík and Delić 2000). The lack of diagnostic morphological characters distinguishing species from Eu and ALa correspond to their close relationship revealed using the cyt *b* gene. The close relationship between groups ALa and Eu discovered in the cyt *b* trees will be tested again using nuclear genes (see Chapter 3).

It is controversial whether group Le or PLa diverged earlier from the other three groups. Docker et al. (1999) suggested that PLa is more divergent from ALa than Le with a low support value of 62%. Lang et al. (2009) also suggested that PLa was sister to the other three groups with the bootstrap support under 85% and the posterior probability under 0.95. However, Blank et al. (2008) reported that Le was sister to the other three groups plus *Entosphenus* with 100% posterior probability. This study agrees with Blank et al. (2008) although not including *Entosphenus*. However, the support value of Le being sister to the other three groups is low (BS < 50%; P = 0.82). All the support values or posterior probabilities are low (Docker et al. 1999; Lang et al. 2009; this thesis), except the posterior probability in the Bayesian analysis by Blank et al. (2008). Blank et al. (2008) used uniform model (GTR model with gamma) for the whole dataset, while Lang et al. (2009) and this thesis used separate models for each codon position. Also, different sampling may cause the observed differences in posterior probability values. In terms of genetic distance, the distances between PLa and ALa/Eu are both slightly higher than that between Le and ALa/Eu, which contradicts the phylogenetic analyses results. Since the differences in genetic distances are subtle, PLa and Le probably diverged from ALa plus Eu at a similar time.

In conclusion, ALa and Eu are most closely related to each other among the four groups, while PLa and Le are less closely related to them. In the next chapter, the relationships among these four groups will be further discussed using nuclear gene

phylogenetic analyses.

2.4.3 Placement of species

2.4.3.1 Lethenteron ninae *and* Lethenteron zanandreai

Lethenteron zanandreai and *Lethenteron ninae* are both in the ALa rather than Le clade in the maximum parsimony and Bayesian trees. The genetic distances from these two species to those of the Le group are significantly larger than that to the ALa species (Section 2.3.4). Although several authors (Hubbs and Potter 1971; Potter 1980; Renaud 2011) believed that *Lethenteron zanandreai* possesses a single row of posterials and treated it as a *Lethenteron* species, the molecular studies using the cyt *b* gene (Docker et al. 1999; Caputo et al. 2009; Lang et al. 2009; this thesis) disagreed with this placement and put it in genus *Lampetra* as Vladykov (1955) suggested in the original description. However, the controversial morphological descriptions and the molecular analyses using the same gene (cyt *b*) are insufficient as evidence to determine the placement of *Lethenteron zanandreai*. The evidence based on nuclear genes will be provided in next chapter.

Lethenteron ninae plus one *Lampetra lanceolata* form a clade sister to *Lethenteron zanandreai*. The placement of *Lethenteron ninae* in the ALa clade disagrees with Naseka et al. (2009), where *Lethenteron ninae* was described as a *Lethenteron* species. Naseka et al. (2009) pointed out that *Lethenteron ninae* and *Lethenteron zanandreai* share the

feature of possessing one row of posterials, which was not found in other western Eurasian lampreys (e.g., other species from ALa). It is interesting that these two species are distributed nearer to other ALa and Eu species than to any *Lethenteron* species (Renaud 2011) despite possessing the posterials considered characteristic of *Lethenteron*. Although the phylogenetic trees suggest that these two species are descendants of a *Lampetra fluviatilis*-type parasitic ancestor, their relationships to *Lampetra fluviatilis* is not as close as *Lampetra planeri*. In terms of genetic distances, the distances between *Lethenteron ninae* (K2P = 3.91%)*/Lethenteron zanandreai* (K2P = 2.94%) and *Lethenteron fluviatilis* are greater than that between *Lampetra planeri* and *Lampetra fluviatilis* (K2P = 0.09%).

Lethenteron zanandreai was often referred to as "relict species" in previous lamprey studies (Hubbs and Potter 1971; Docker et al. 1999; Docker 2009). Hubbs and Potter (1971) used this term for the non-parasitic lampreys that occur at or near the extreme southern limits of distribution of the Northern Hemisphere lampreys (e.g., *Lethenteron zanandreai*, *Lampetra aepyptera* and *Lampetra hubbsi*). The parasitic "stem species" of these non-parasitic species are often controversial, which presumably represent more ancient derivation of non-parasitism (Docker 2009). *Lethenteron ninae* also appears to be a relict species, which is also distributed in the Black Sea basin (about 43° N), which is more consistent with its placement in Atlantic *Lampetra* (this thesis). Based on the phylogenetic trees using the cyt *b* gene, *Lethenteron zanandreai*, *Lethenteron ninae* and

Lethenteron lanceolata have derived from the *Lampetra fluviatilis*-type ancestor together.

Lethenteron ninae was paraphyletic and formed a clade with *Lampetra lanceolata*, which means they are not separate species by the phylogenetic species concept (Mayden 1997). The K2P distance between these two species ranges from zero to 0.45%. These two species are both non-parasitic and are distinguished from each other by the number of posterials, cusps on the transverse lingual lamina, anterials, oral fimbriae and the pigmentation in ammocoetes (Naseka et al. 2009). One adult (paratype) and three larval *Lampetra lanceolata* samples from the type locality (Iyidere River, Trabzon, Turkey) were compared with *Lethenteron ninae* (Naseka et al. 2009). This study includes no adult samples from the type locality, but an adult *Lampetra lanceolata* sample from near the type locality (Ykizdere Brook, Black Sea basin, Turkey) was used for the nuclear TAP2 intron (the second intron of transporter associated with antigen processing gene), while the sequencing for other genes failed. The *Lampetra lanceolata* sequence (GQ206176; Lang et al. 2009) used in this chapter was from a locality (Chakhtsutsyr River, Southern Federal District, Russia) far from its type locality and in the range of *Lethenteron ninae* (Renaud 2011). Very little research has been done on *Lampetra lanceolata*. Thus the identification of this sample may need to be verified. It remains to be seen whether *Lampetra lanceolata* from other localities, such as Chakhtsutsyr River, Russia, is morphologically different from ones from its type locality. Molecular data of *Lampetra lanceolata* from the type locality are desired to test the monophyly of this

species. In the next chapter, nuclear TAP2 intron sequence from the *Lampetra lanceolata* sample from near the type locality will provide more evidence for the close relationship between *Lethenteron ninae* and *Lampetra lanceolata*.

2.4.3.2 Eudontomyzon morii

The sample tentatively identified (see below) as the freshwater resident parasitic lamprey *Eudontomyzon morii* is in the Le rather than the Eu clade; this is congruent, of course, with Lang et al. (2009) since both studies used the same *Eudontomyzon morii* cyt *b* sequence. Few morphological studies on a limited number of individuals have been conducted on this species (Ma and Yu 1959; Renaud 1982; Khidir and Renaud 2003; Renaud et al. 2009). The morphological phylogenetic tree inferred by Gill et al. (2003) disagreed with the cyt *b* gene trees inferred by Lang et al. (2009) and this thesis. The former suggested *Eudontomyzon morii* and *Eudontomyzon danfordi* are sister taxa. However, *Eudontomyzon morii* is the only *Eudontomyzon* species endemic to eastern Asia, while other *Eudontomyzon* species, such as *Eudontomyzon danfordi* and *Eudontomyzon mariae*, are endemic to Europe (Vladykov and Kott 1979b; Renaud 2011). *Eudontomyzon morii* is distributed in the Yalu River system, China and North Korea (Renaud 2011). Near the region of *Eudontomyzon morii*, *Lethenteron camtschaticum* occurs in Mutan River and Tumen River in northeast China (Renaud 2011), and *Lethenteron reissneri* (*s.l.*) occurs in the Amur River system, Russia (*Lethenteron reissneri s.s.*; Yamazaki et al. 2006)

and northeast China (*Lethenteron reissneri s. l*, Wang et al. 2004), and Naktong River, South Korea (*Lethenteron* sp. S, Yamazaki et al. 2006). The molecular results are consistent with the geographic distribution while the morphological results contradict it. *Eudontomyzon morii* possesses the feature of *Eudontomyzon* in dentition that both exolaterals and posterials are present; thus, it was put in *Eudontomyzon* in morphological studies (Hubbs and Potter 1971; Gill et al. 2003).

The individual used by Lang et al. (2009) and this thesis was from Liaohe River, a separate river from the Yalu River in northeast China, and was a metamorphosing individual with uncertain identification. As mentioned in Section 1.1, many diagnostic characters in lampreys (e.g., dentition, oral papillae, eye length and disc length) are only for metamorphosed individuals. Furthermore, larval and metamorphosing *Eudontomyzon morii* have not been described. Thus, this specimen is possibly misidentified. Lang et al. (2009) suspected that this specimen belonged to a *Lethenteron reissneri*-like species based on its placement in the cyt *b* gene tree. Like *Lethenteron* sp. N, this *Eudontomyzon morii* individual appears to belong in *Lethenteron* but is more divergent from *Lethenteron camtschaticum* (1.00% ≤ K2P ≤ 1.27%) than the closely related satellite species (0 ≤ K2P ≤ 1.09%). This individual is also different from *Lethenteron* sp. N with the K2P of 3.52%. If this sample is not *Eudontomyzon morii*, these results may suggest an unknown species. However, no taxonomic revision should be suggested without including DNA sequences from adult *Eudontomyzon morii* from the Yalu River system.

2.4.3.3 Lethenteron *sp. N and* Lethenteron *sp. S*

It is impossible to distinguish *Lethenteron* sp. N, *Lethenteron* sp. S and *Lethenteron reissneri* (*s.s.*) from each other morphologically when they occur sympatrically. All the morphological characters overlap in these species, so that they are not diagnostic (Yamazaki and Goto 1997). The distribution range of *Lethenteron* sp. N and *Lethenteron* sp. S are within that of *Lethenteron reissneri* (*s.s.*), and the former two were sympatric in the northern part of Honshu Island, Japan. *Lethenteron* sp. N occurs in Hokkaidō Island and the northern part of Honshu Island, Japan, while *Lethenteron* sp. S is distributed in Honshu, Shikoku and Kyushu Islands, Japan, and the southern part of Korean Peninsula (Yamazaki et al. 2003). In this study, the tissue samples from *Lethenteron* sp. N, *Lethenteron* sp. S and *Lethenteron reissneri* (*s.s.*) are all identified as *Lethenteron reissneri*, referring to *Lethenteron reissneri* (*s.l.*). All the *Lethenteron reissneri* (*s.l.*) individuals except one from Shougawa River, Honshu Island, Japan, were clustered with *Lethenteron camtschaticum* and its other closely related satellite species. These individuals are likely *Lethenteron reissneri* (*s.s.*). The one from Shougawa River, Japan, where *Lethenteron* sp. N and *Lethenteron* sp. S are sympatric (Yamazaki and Goto 1996; Yamazaki et al. 2003; Yamazaki et al. 2006), is sister to *Lethenteron* sp. N from the same locality (Lang et al. 2009) with the bootstrap support of 100% and the posterior probability of one. This individual is likely *Lethenteron* sp. N.

Within the clade of Le, compared with *Lethenteron alaskense*, *Lethenteron appendix*,

Lethenteron kessleri and *Lethenteron reissneri* (*s.s.*), *Lethenteron* sp. N is more divergent from *Lethenteron camtschaticum*. The K2P distance in the cyt *b* gene between *Lethenteron* sp. N and *Lethenteron camtschaticum* is larger than those between *Lethenteron camtschaticum* and other species in the Le clade, which is in agreement with Yamazaki et al. (2006), who reported the Nei's genetic distance of 11 allozyme loci between *Lethenteron* sp. N to *Lethenteron camtschaticum* larger than that between *Lethenteron camtschaticum* and *Lethenteron kessleri*, and *Lethenteron camtschaticum* and *Lethenteron reissneri* (*s.s.*). Compared with species from ALa, Eu, and PLa, *Lethenteron* sp. N is relatively closely related to *Lethenteron camtschaticum* (see Table 2.6). The placement of *Lethenteron* sp. N in the cyt *b* gene trees is congruent with that by Lang et al. (2009). *Lethenteron* sp. N is likely a descendant of a *Lethenteron camtschaticum*-type ancestor diverged earlier than any other species in the clade Le. Similar to the relict species (see Section 2.4.3.1) *Lethenteron zanandreai* and *Lethenteron ninae*, non-parasitic *Lethenteron* sp. N may represent a more ancient derivation of non-parasitism from the *Lethenteron camtschaticum*-type ancestor than the closely related satellite species.

As mentioned in Section 2.4.1, *Lethenteron* sp. S is sister to all the four groups. Using a different individual from a different locality (AB565771; Okada et al. 2010), the current study result verified that of Lang et al. (2009). The K2P distances to other species of the complete cyt *b* gene from *Lethenteron* sp. S are only smaller than that from the

outgroup *Petromyzon marinus*. The average Nei's genetic distance from *Lethenteron* sp. S to *Lethenteron camtschaticum* (1.270%), *Lethenteron kessleri* (1.269%), *Lethenteron reissneri* (1.308%), and *Lethenteron* sp. N (1.297%) are significantly larger than that among the latter four (no larger than 0.470%) using allozyme loci data (Yamazaki et al. 2006). *Lethenteron* sp. S differs from *Lethenteron* sp. N by 11 loci of allozymes (Yamazaki and Goto 1996). The differences in the nuclear genome among *Lethenteron* sp. S and other lamprey species have not been studied. Based on the mitochondrial gene analyses, *Lethenteron* sp. S is highly divergent from any parasitic species. For non-parasitic species deeply diverged from the possible parasitic stem, their stem cannot be unambiguously resolved. For example, *Lethenteron* sp. S could be a very ancient non-parasitic derivative from the parasitic common ancestor of genus *Eudontomyzon*, *Lampetra* and *Lethenteron*, or a recent (or older) non-parasitic derivative from an unknown parasitic stem. The unknown parasitic stem, which may be extinct, probably resembles *Lethenteron camtschaticum* morphologically but is different genetically.

2.4.4 Lethenteron camtschaticum *and its closely related satellite species*

2.4.4.1 "Subspecies" of Lethenteron camtschaticum

This thesis includes four individuals that, based on distribution, would traditionally have been considered the subspecies *Lethenteron camtschaticum septentrionalis* by Berg (1931, as cited in Renaud 2011); individuals from two localities were included to analyze

the relationship of this tentative subspecies to other *Lethenteron camtschaticum* populations. However, more recently, the abuse of the term "subspecies" has been criticized by many authors (Mayr 1982; McKitrick and Zink 1988; Frost and Hillis 1990; Frost and Kluge 1994) since the recognition of many subspecies were based on insignificant differences among populations. Mayr and Ashlock (1991) provided a definition of subspecies: "A subspecies is an aggregate of phenotypically similar populations of a species inhabiting a geographic subdivision of the range of that species and differing taxonomically from other populations of that species." The authors pointed out that the lack of a standard for distinguishing populations as subspecies (e.g., the range of genetic distance between two subspecies within a species) made it difficult to use this term appropriately.

Burbrink et al. (2000) used the mitochondrial cyt *b* gene and control region 1 (NC1) to resolve the phylogenetic relationships among eight North American rat snake *Elaphe obsoleta* (Say 1823) subspecies and concluded that these subspecies were not evolutionary entities (i.e., were not reciprocally monophyletic) and should be eliminated. In contrast, common carp *Cyprinus carpio* L. subspecies *Cyprinus carpio carpio* and *Cyprinus carpio haematopterus* were found to be reciprocally monophyletic with the percent genetic distance of 1.46±0.36 (Zhou et al. 2003). However, since they are diagnosable by restriction fragment length polymorphism (RFLP) of NADH dehydrogenase subunit 5 and 6 (ND5 and ND6) segment, and they are reciprocally

monophyletic (Zhou et al. 2003), these two subspecies are actually two full species by the phylogenetic concept of species. Phylogenetic species is defined as the smallest evolutionary entities that are diagnosable and monophyletic, while there is no phylogenetic concept for subspecies. Thus, the phylogenetic analysis could only test whether *Lethenteron camtschaticum septentrionalis* and *Lethenteron camtschaticum camtschaticum* are two full species or the same species, but provides no evidence for intraspecific taxonomy.

Since the lack of reciprocal monophyly may arise from incomplete lineage sorting or contemporary gene flow (Funk and Omland 2003), incipient species may not be phylogenetic species based on certain gene(s). Despite a lack of reciprocal monophyly using mtDNA data, Mulcahy (2008) suggested keeping the subspecies of the western North American nightsnake *Hypsiglena torquata* (Günther 1860), since they "may represent incipient species that may not yet have achieved reciprocal monophyly, but possess unique morphologies, and are geographically discrete." This statement means that: 1) subspecies are morphologically distinguishable; 2) they are geographically isolated; and 3) they will eventually achieve reciprocal monophyly. The former two conditions can be observed, while the latter cannot be tested in the short term. Thus, to keep subspecies fulfilling conditions 1) and 2) may be misleading, because conditions 1) and 2) may not cause 3). When subspecies are not reciprocally monophyletic, it may be more appropriate to call them geographic populations than to call them subspecies.

In the phylogenetic trees, *Lethenteron camtschaticum septentrionalis* and *Lethenteron camtschaticum camtschaticum* are not reciprocally monophyletic, and they show little or no genetic difference. In the haplotype network, the only haplotype discovered in *Lethenteron camtschaticum septentrionalis* is shared by *Lethenteron camtschaticum camtschaticum*. Thus, it appears that *Lethenteron camtschaticum septentrionalis* is the same species as the *Lethenteron camtschaticum* from other regions, and it would be more appropriate to refer it as "*Lethenteron camtschaticum* from the White Sea basin."

The lack of variation in the cyt *b* gene in *Lethenteron camtschaticum* from the White Sea basin may be explained by its geographical isolation from other *Lethenteron camtschaticum* populations. Other *Lethenteron camtschaticum* populations show variations in cyt *b* sequences (10 haplotypes in 15 individuals from seven locations). Populations from the Pacific Ocean drainage of Asia, such as populations from Sakhalin Island and Ussuri Bay, have several haplotypes in the same locality, which may suggest gene flow from *Lethenteron camtschaticum* populations from North America across the Bering Strait.

Haplotype H1, with the total frequency of 14/42 (33.3%), is the most common haplotype in the network. It is predicted that the most common haplotype is the oldest one (Posada and Crandall 2001); thus, H1 should be the oldest haplotype and at the root of the network. H1 includes 9/19 (47.4%) of the *Lethenteron camtschaticum* samples

(including those from the White Sea basin). Eight of nine other haplotypes are from the Pacific Ocean basin of Asia, and the remaining one is from the Beaufort Sea basin; most of these haplotypes contain a single individual or, in the case of H9, two individuals. The substitutions between haplotypes are relatively new according to the theory that the expected rank of the alleles by age is the same as the rank of alleles by frequency (Posada and Crandall 2001). Besides the lack of migration from other geographic regions, the other cause of the single haplotype observed in the White Sea basin population could be the relatively recent expansion of this species to this region, allowing less time for new mutations.

2.4.4.2 Closely related satellite species of Lethenteron camtschaticum

Non-parasitic lampreys *Lethenteron alaskense*, *Lethenteron appendix*, *Lethenteron kessleri*, *Lethenteron reissneri* (*s.s.*) and parasitic *Lethenteron camtschaticum* are clustered in one clade and are not reciprocally monophyletic. The clade of these five species was also discovered by Lang et al. (2009) using the same gene. Compared with *Lethenteron* sp. N, which is also apparently derived from a *Lethenteron camtschaticum*-type ancestor but more divergent from *Lethenteron camtschaticum*, the former four non-parasitic species are considered closely related satellite species of *Lethenteron camtschaticum* in this thesis.

Lethenteron appendix is the only closely related satellite species distributed in a

region disjunctive to that of *Lethenteron camtschaticum* or any of its other satellite species. This is consistent with the observation that the sequence divergence between *Lethenteron camtschaticum* and *Lethenteron appendix* (K2P ranges from 0.18 to 1.09%) is higher than that between *Lethenteron camtschaticum* and other closely related satellite species (K2P ranges from 0.00 to 0.45%) with which it co-occurs. It is worth noting, however, that the sequence divergence between *Lethenteron camtschaticum* and *Lethenteron appendix* is still lower than that between most sister species (2%, Avise and Walker 1999). Although not reciprocally monophyletic in the phylogenetic trees, *Lethenteron appendix* and *Lethenteron camtschaticum* are in different haplogroups in the network, and a fixed difference was found at site 502 of the cyt *b* gene. They may be found reciprocally monophyletic with phylogenetic analysis using more genes. However, even if they are found to be distinct phylogenetic species, they have diverged recently. Using the substitution rate for the cyt *b* gene of 29.2×10^{-9} substitutions per site per year (Pesole et al. 1999), *Lethenteron camtschaticum* and *Lethenteron appendix* diverged approximately 130,000 years ago.

As a non-migrating lamprey, the evolution of *Lethenteron appendix* shows a geographical pattern. The Great Lakes basin populations (HG309) differ from the Atlantic Ocean drainage populations (H17) by site 309 and 728 of the cyt *b* gene. Two Atlantic Ocean drainage populations were collected from Maine and Delaware, USA, which are more than 800 km apart. *Lethenteron appendix* probably resided in the Atlantic Coastal

and Mississippi refugia during the Wisconsinan glacial period (80,000 – 10,000 years ago), and recolonized in the north (the Great Lakes basin and the Atlantic coast) following the glacial period through the dispersal routes of Chicago, Lower Peninsula of Michigan, Mohawk and Champlain (Mandrak and Crossman 1992). Haplotype H17 likely was present before the end of the Wisconsinan glacial period, survived in the Atlantic coastal refugium (near present day Delaware) during the glacial period, and dispersed to the Atlantic coast near present day Maine through the dispersal route of Champlain. The population structure of *Lethenteron appendix* using other genes, including the nuclear genes, with samples from all five lake basins in the Great Lakes and samples from the Mississippi River basin would be an interesting topic for future studies.

Lethenteron reissneri is the second most divergent non-parasitic species (K2P ranges from 0.18 to 0.45%) among the closely related satellite species of *Lethenteron camtschaticum*. The Mongolian population possesses one haplotype (H16), and the Russian population possesses two haplotypes (H15 and H20). H15 and H16 are on the same lineage while H20 (containing only one individual) is on a different lineage. Excluding the only individual of H20, *Lethenteron reissneri* is in HG502 with *Lethenteron appendix*. After diverging from the parasitic *Lethenteron camtschaticum*-like ancestor, the common ancestor of *Lethenteron reissneri* and *Lethenteron appendix* may have partly migrated across the Bering Strait. The individual in H20 is a different lineage which descended directly from the most common haplotype, H1. That would suggest that

Lethenteron reissneri haplotypes have derived from the ancestor haplotype (H1) multiple times through different pathways; thus *Lethenteron reissneri* is polyphyletic and not a good phylogenetic species. However, *Lethenteron reissneri* was reported to be reproductively isolated from *Lethenteron camtschaticum* based on one fixed allozyme allele difference (Yamazaki et al. 2006), which means they are separate species by the biological concept. However, Yamazaki et al. (2006) sampled *Lethenteron reissneri* from two locations near each other in upper Amur River system, and *Lethenteron camtschaticum* from only the Pacific Coast of Russia and Japan, which are not sympatric. This allozyme allele difference may not be diagnostic for these two species where they are sympatric. It is thus possible that *Lethenteron reissneri* and *Lethenteron camtschaticum* are the same biological species. Yamazaki et al. (2006) combined *Lethenteron reissneri* and *Lethenteron kessleri* into one species since they are identical in 11 allozyme allele loci. If *Lethenteron reissneri* is the same species as *Lethenteron camtschaticum*, the allozyme allele difference between *Lethenteron camtschaticum* and *Lethenteron kessleri* would be non-diagnostic. Thus all three could be the same species.

All the *Lethenteron alaskense* and *Lethenteron kessleri* populations possess the haplotype H1, which is shared by seven *Lethenteron camtschaticum* populations (including the two White Sea basin populations). The genetic distances between *Lethenteron camtschaticum* and these two species are small (0-0.27%). This study provides no evidence that *Lethenteron alaskense* and *Lethenteron kessleri* are separate

species from *Lethenteron camtschaticum*. Although no fixed difference has been found in mtDNA, a fixed allele of MDH3 distinguishes *Lethenteron kessleri* from *Lethenteron camtschaticum* with hundreds of samples from their sympatric range (Yamazaki and Goto 1998; Yamazaki et al. 2006). However, since *Lethenteron camtschaticum* is widely distributed, a more complete sampling for *Lethenteron camtschaticum* may lead to different results with the allozyme allele analysis.

Interestingly, haplotypic variation was discovered in the freshwater resident populations of *Lethenteron kessleri* (i.e., there were five haplotypes in eight individuals from two locations). The diversity of haplotypes in populations of *Lethenteron kessleri* may reflect recent or contemporary gene flow from the anadromous *Lethenteron camtschaticum*.

The view that *Lethenteron alaskense* is a synonym of *Lethenteron appendix* (Wilimovsky 1954; Hubbs and Lagler 1958; Quast and Hall 1972; Robins et al. 1980; Page and Burr 1991) is not supported since *Lethenteron appendix* is a distinct lineage from *Lethenteron alaskense* and *Lethenteron camtschaticum* in the network. However, the divergence between *Lethenteron camtschaticum* and *Lethenteron alaskense* is low. This thesis could not refute the hypothesis that *Lethenteron alaskense* is a freshwater form of *Lethenteron camtschaticum* (Heard 1966; McPhail and Lindsey 1970). Since the haplotype of *Lethenteron alaskense* is also shared by *Lethenteron kessleri*, it is possible that they are both freshwater forms of *Lethenteron camtschaticum*. The morphological

differences among *Lethenteron camtschaticum* and these two non-parasitic species are all related to their life history type, which could be polymorphic characters in the same species. However, analyses on other genes are needed to support them as one single species or separate species.

Two of three haplotypes of *Lethenteron reissneri* are in the same haplogroup with *Lethenteron appendix*, which means that these two species may be more closely related to each other than to other species. However, the genetic distance between them is 0.41%, larger than that between *Lethenteron reissneri* or *Lethenteron appendix* and *Lethenteron camtschaticum* or other closely related satellite species. The sequences from Lang et al. (2009) are not included in the network, except one *Lethenteron alaskense*. The *Lethenteron reissneri* collected in Slavanaya River, Iturup Island, Russia, from Lang et al. (2009) is different from any other *Lethenteron reissneri* sequences, which may have affected the genetic distance. Also, one haplotype of *Lethenteron reissneri* on a different lineage increases the genetic distance between *Lethenteron reissneri* and *Lethenteron appendix*. At least, some haplotypes of *Lethenteron reissneri* are closely related with *Lethenteron appendix* despite their nonadjacent distribution ranges. In future studies, the relationships among *Lethenteron reissneri*, *Lethenteron appendix* and *Lethenteron camtschaticum* may be further resolved with multiple samples from more localities (e.g., *Lethenteron reissneri* and *Lethenteron camtschaticum* from Japan, and *Lethenteron appendix* from all five lakes of the Great Lakes and the Mississippi River basin).

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2.6 Tables and Figures

Table 2.1 List of tissue samples for the cyt *b* gene analyses. "(?) " after the species name means the identification of the specimen is uncertain. If not indicated, the specimens are all adults. Samples of *Lampetra fluviatilis* and *Lethenteron zanandreai* include populations with different colorations from the same location; the color features are indicated after the species names for these samples. "(T)" after the country name means the collection locality is the type locality.

Species	Year of collection	Basin	Stream	Country	Latitude	Longitude	Collector(s)	Individuals included in the trees	Individuals included in the network
Eudontomyzon danfordi	2003	Mediterranean Sea basin	Lehotsky Brook at Muránska Dlhá Lúka, Muránska planina National Park	Slovakia	48°47′11″N	20°01′47″E		2	
Eudontomyzon danfordi	2010	Black Sea basin	Borzhava River, Tissa River drainage, Danube River system	Ukraine	48°29'20.87"N	23°13'13.11"E	Talabishka E.	1	
Eudontomyzon danfordi	2003	Mediterranean Sea basin	Rimava River at Tisovec, Muránska planina National Park	Slovakia	48°47′11″N	20°01′47″E		1	
Eudontomyzon mariae	2003	Volga River basin	Chardym River	Russia	52° 39' 54" N	45° 46' 24" E		1	
Eudontomyzon mariae (?)	2010	Baltic Sea basin	Stream, Nida River at Kielce, Vistula River system	Poland	50°45'2.23"N	20°37'7.09"E	Naseka A. M., Nowak M.,	2	
Eudontomyzon mariae (?)	2011	Black Sea basin	Stryy River at Verkne Vysotskoye, Dniester River system	Ukraine	48°57'14.90"N	23° 4'9.55"E	Naseka A. M., Talabishka E.	2	
Eudontomyzon vladykovi *	2009	Black Sea basin	Stream, Krka River drainage, Sava River drainage, Danube River system	Slovenia (T)	45°49'4.49"N	15°19'57.01"E	Naseka A. M., Kapla A.	2	

Table 2.1 Continued.

Species	Year of collection	Basin	Stream	Country	Latitude	Longitude	Collector(s)	Individuals included in the trees	Individuals included in the network
Lampetra fluviatilis	2007	Baltic Sea basin	Neva River, from a shop, St. Petersburg	Russia (T)	59°56'58.00"N	30°19'54.42"E	Naseka A. M.	2	
Lampetra fluviatilis (?)	2007	Black Sea basin	Sea of Azov at Shirokino	Ukraine	47°05'N	37°50'E	Naseka A. M., Diripasko O. A.	1	
Lampetra fluviatilis (black)	2010	Baltic Sea basin, Ladoga Lake basin	Syas' River at Kolchanovo	Russia	60°1'6.65"N	32°35'2.14"E	Naseka A. M.	1	
Lampetra fluviatilis (grey)	2010	Baltic Sea basin, Ladoga Lake basin	Syas' River at Kolchanovo	Russia	60°1'6.65"N	32°35'2.14"E	Naseka A. M.	1	
Lethenteron appendix	2011	Great Lakes basin, Lake Michigan basin	Pigeon River at Sturgeon Valley Road, Cheboygan, Michigan	USA	45°16'2"N	86°12'18"W		1	1
Lethenteron appendix		Great Lakes basin, Lake Michigan basin	Betsie River	USA	44°36'41"N	84°27'8"W		1	1
Lethenteron appendix		Great Lakes basin, Lake Michigan basin	Jambo Creek	USA	44°15'52"N	87°40'51"W		1	1
Lethenteron appendix		Atlantic Ocean basin	Delaware	USA	38°57'3"N	75°30'52"W		1	1
Lethenteron appendix		Atlantic Ocean basin	Maine	USA	45°13'15"N	69°18'46"W		2	2
Lethenteron appendix (Ammocoete)		Great Lakes basin, Lake Ontario basin	Oshawa Creek	Canada	43°53'26"N	78°51'11"W		1	1
Lethenteron appendix (Ammocoete)		Great Lakes basin, Lake Huron basin	Maple River	USA	45° 30'37"N	84°47'33"W			1

Table 2.1 Continued.

Species	Year of collection	Basin	Stream	Country	Latitude	Longitude	Collector(s)	Individuals included in the trees	Individuals included in the network
Lethenteron camtschaticum **	2011	Pacific Ocean basin	Stream without name, Ussuri Bay, Sea of Japan, Vladivostok area	Russia	43°11'43.93"N	132° 6'47.21"E	Naseka A. M., Shedko M.	1	3
Lethenteron camtschaticum	2011	Pacific Ocean basin	Klyuch River, Ussuri Bay, Sea of Japan, Vladivostok area	Russia	43°18'16.05"N	132°15'8.18"E	Naseka A. M., Shedko M.	1	1
Lethenteron camtschaticum	2011	Pacific Ocean basin	Belaya River at Sokol, Sakhalin Island , Sea of Japan	Russia	47°14'35.62"N	142°46'20.88"E	Naseka A. M.	2	4
Lethenteron camtschaticum	2011	Pacific Ocean basin	Stream at Sokol, Belaya River system, Sakhalin Island, Sea of Japan	Russia	47°14'31.85"N	142°46'9.62"E	Naseka A. M.	4	4
Lethenteron camtschaticum	2012	Beaufort Sea basin	Shingle Point, YT	Canada	68° 56'8"N	137°13'30"W	Renaud C. B., Naseka A. M., Alfonso N. R.		1
Lethenteron camtschaticum	2004	Beaufort Sea basin	Issijak Site	Canada	68° 56'8"N	137°13'30"W			1
Lethenteron camtschaticum (DNA extraction, no tissue)		Pacific Ocean basin	Northeast China	P. R. China	47° 45'15"N	130°15'14"E		1	1
Lethenteron camtschaticum septentrionalis		White Sea basin	Onega River, Porog	Russia	59° 16'7"N	33°24'34"E		1	2
Lethenteron camtschaticum septentrionalis (?)	2011	Arctic Ocean basin, White Sea basin	Keret River at Keret	Russia	66°16'32.57"N	33°33'45.22"E	Zhidkov Z. B.	2	2
Lethenteron kessleri	2010	Arctic Ocean basin	Biya River at Biysk, Ob River system, Altayski Kray	Russia	52°33'6.16"N	85°19'36.67"E	Naseka A. M., Ostroshabov A.	1	3

Table 2.1 Continued.

Species	Year of collection	Basin	Stream	Country	Latitude	Longitude	Collector(s)	Individuals included in the trees	Individuals included in the network
Lethenteron kessleri (?)	2010	Arctic Ocean basin, White Sea basin	Stream at Chekshino, Dvinnitsa River system, Sukhona River system, Severnaya Dvina River system, Vologodskaya obl.	Russia	59°39'14.83"N	40°38'8.79"E	Naseka A. M.	1	5
Lethenteron ninae	2009	Black Sea basin	Shakhe River, Krasnodarskiy Kray	Russia (T)	43°48'18.48"N	39°40'45.31"E	Mosyagina M. B.	2	
Lethenteron ninae	2009	Black Sea basin	Mzymta River, Krasnodarskiy Kray	Russia	43°29'50.94"N	39°59'25.66"E	Mosyagina M. B.	2	
Lethenteron ninae	2006	Black Sea basin	Bzyb River at Inkiti	Georgia	43°11'37.99"N	40°17'34.17"E	Naseka A. M., Tuniyev S. B.	1	
Lethenteron ninae	2007	Black Sea basin	Mokva River at Ajazhvi	Georgia	42°46'28.50"N	41°28'54.77"E	Naseka A. M., Tuniyev S. B.	2	
Lethenteron reissneri	2002	Pacific Ocean basin	Shougawa River, Toyama Prefecture, Honshu Island	Japan	36°40'26"N	137°2'2"E		1	
Lethenteron reissneri	2011	Pacific Ocean basin	Stream at Yevseyevka, Spasovka River system, Vishnevka Reservoir, Amur River system, Khanka Lake basin, Spassk-Dalniy area	Russia	44°24'58.78"N	132°55'12.27"E	Naseka A. M.	1	3
Lethenteron reissneri	2006	Pacific Ocean basin	Barh River, Onon, Khentii	Mongolia	48°58'13"N	111°46'54"E	Sabaj M.	2	2
Lethenteron sp. (Ammocoete)	2012	Beaufort Sea basin	Martin River, Near bridge of MacKenzie Hwy, NT	Canada	61°53'35.85"N	121°36'45.52"W	Renaud C. B., Naseka A. M., Alfonso N. R.		1
Lethenteron zanandreai	2011	Adriatic Sea basin	Vipava River at Male Zable	Slovenia	45°52'22.81"N	13°50'53.88"E	Naseka A. M.	2	
Lethenteron zanandreai (monotonous)	2011	Adriatic Sea basin	Vipava River at Male Zable	Slovenia	45°52'22.81"N	13°50'53.88"E	Naseka A. M.	2	

Table 2.1 Continued.

Species	Year of collection	Basin	Stream	Country	Latitude	Longitude	Collector(s)	Individuals included in the trees	Individuals included in the network
Lethenteron zanandreai (spotted)	2011	Adriatic Sea basin	Vipava River at Male Zable	Slovenia	45°52'22.81"N	13°50'53.88"E	Naseka A. M.	2	
Petromyzon marinus	2011	Great Lakes basin	Deer Creek, Jordan River	USA	45°10'0"N	85°6'40"W		1	

*_Eudontomyzon stankokaramani_ and _Eudontomyzon vladykovi_ were considered synonyms of _Eudontomyzon mariae_ in Renaud (2011), since _Eudontomyzon mariae_ exhibits a broad geographic distribution with clear disjunctions and wide variation in a number of taxonomic characters, and sufficient adult specimens from across the range has not been collected. Holčík and Šorić (2004) elevated _Eudontomyzon stankokaramani_ as a species. _Eudontomyzon vladykovi_ was elevated by Kottelat and Freyhof (2007).

**In this thesis, _Lethenteron camtschaticum_ refers to _Lethenteron camtschaticum camtschaticum_ if not indicated (the _Lethenteron camtschaticum_ excluding _Lethenteron camtschaticum septentrionalis_).

Table 2.2 List of sequences from GenBank for the cyt *b* gene analyses. "(?) " after the species name means the identification of the specimen is uncertain. If not indicated, the specimens are all adults. "(T)" after the country name means the collection locality is the type locality. All the sequences are included in the phylogenetic trees. The *Lethenteron alaskense* (GQ206178) is also included in the network.

Species	**GenBank accession No. of cyt *b* sequence(s)**	**Basin**	**Stream**	**Country**	**Latitude**	**Longitude**	**Reference**
Eudontomyzon danfordi	GQ206158	Mediterranean Sea basin	Zdychava River	Slovakia	48°44'17"N	20°8'22"E	Lang et al. 2009
Eudontomyzon mariae	GQ206162	Black Sea basin	Ivianka River	Ukraine	50° 18' 00" N	23° 46' 00" E	Lang et al. 2009
Eudontomyzon morii (?)	GQ206163	Pacific Ocean basin	Liaohe River, Liaoning Province	P. R. China	41°58'4"N	122°51'17"E	Lang et al. 2009
Eudontomyzon stankokaramani *	GQ206189	Mediterranean Sea basin	Zeta River	Montenegro	42°27'52" N	19° 15' 40" E	Lang et al. 2009
Eudontomyzon vladykovi *	GQ206161	Mediterranean Sea basin	Studenec Brook	Slovakia	49°1'0"N	20°46'0"E	Lang et al. 2009
Lampetra aepyptera	GQ206173	Atlantic Ocean basin	Carver Creek, Missouri	USA	37°24'28"N	90°41'47"W	Lang et al. 2009
Lampetra ayresii	GU120868 GU120867	Pacific Ocean basin	Near San Francisco	USA	37°47′ N	122°25′ W	Boguski et al. 2012
Lampetra fluviatilis	NC001131	Atlantic Ocean basin	Estuary of the Garonne River	France	45°2'29"N	0°36'24"E	Delarbre et al. 2000
Lampetra hubbsi **	GU120869 GU120870	Pacific Ocean basin	Merced River	USA	37°20'57"N	120°58'32"W	Boguski et al. 2012
Lampetra lanceolata	GQ206176	Black Sea basin	Chakhtsutsyr River	Russia	43°37'41"N	40°9'57"E	Lang et al. 2009
Lampetra pacifica	GU120799	Pacific Ocean basin	Clackamas River	USA (T)	45°23'44"N	122°33'45"W	Boguski et al. 2012
Lampetra planeri	GQ206149	Mediterranean Sea basin	Kalte Moldau, Bavaria	Germany	48°49'23"N	13°46'7"E	Lang et al. 2009

Table 2.2 Continued.

Species	GenBank accession No. of cyt *b* sequence(s)	Basin	Stream	Country	Latitude	Longitude	Reference
Lampetra richardsoni	GU120737	Pacific Ocean basin	Smith Creek, BC	Canada (T)	49°53'15"N	119°38'28"W	Boguski et al. 2012
Lethenteron alaskense	GQ206178	Beaufort Sea basin	Lower Chena River near Fairbanks, Alaska	USA	64°47'44"N	147°54'43"W	Lang et al. 2009
Lethenteron appendix	GQ206179	Atlantic Ocean basin	Tennessee	USA	35°33'32"N	86°33'1"W	Lang et al. 2009
Lethenteron camtschaticum	GQ206180	Pacific Ocean basin	Ishikari River, Hokkaido Island	Japan	43°31'0"N	142°3'41"E	Lang et al. 2009
Lethenteron kessleri	GQ206183	Arctic Ocean basin	Upper Yenisey River	Russia	56° 0'58.91"N	93° 7'19.83"E	Lang et al. 2009
Lethenteron reissneri	GQ206181	Pacific Ocean basin	Slavanaya River, Iturup Island	Russia	45°3'56.74"N	147°45'38.23" E	Lang et al. 2009
Lethenteron sp. N	GQ206182	Pacific Ocean basin	Shougawa River, Honshu Island	Japan	36°40'26"N	137°2'2"E	Lang et al. 2009
Lethenteron sp. S	AB565771	Pacific Ocean basin	Senju River	Japan	36°8'10"N	139°22'6"E	Okada et al. 2010
Lethenteron zanandreai	GQ206184	Adriatic Sea basin	Vipava River	Slovenia	45°52'22.81"N	13°50'53.88"E	Lang et al. 2009
Petromyzon marinus	NC001626	Atlantic Ocean basin	Cocheco River at Dover, NH	USA	43°11'48"N	70°52'28"W	Lee and Kocher 1995

**Eudontomyzon stankokaramani* and *Eudontomyzon vladykovi* were considered synonyms of *Eudontomyzon mariae* in Renaud (2011), since *Eudontomyzon mariae* exhibits a broad geographic distribution with clear disjunctions and wide variation in a number of taxonomic characters, and sufficient adult specimens from across the range has not been collected. Holčík and Šorić (2004) elevated *Eudontomyzon stankokaramani* as a species. *Eudontomyzon vladykovi* was elevated by Kottelat and Freyhof (2007).

***Lampetra hubbsi* was put in *Entosphenus* in the original description by Vladykov and Kott (1976). However, recently American Fisheries Society (Page et al. 2013) has put it in *Lampetra* based on the molecular studies by Docker et al. (1999) and Lang et al. (2009).

Table 2.3 Primers used for the amplification and sequencing of the cyt *b* gene

Primer name	Primer sequence (5'-3')	Primer complement	Approximate product size	Reference
Glu-F	CACCGTTGTAGAATTCAACTATAAG	Cytb-518-R	800 bp	Boguski 2009
		Cytb-513-R	800 bp	
		Cytb-606-R*	900 bp	
Cytb-518-R	GTTAGGGTGGCGTTTGATACTG	Glu-F	800 bp	Docker, unpublished
Cytb-513-R	GTGGCGTTTGATACTGAGAAG	Glu-F	800 bp	This study
Cytb-606-R	AGATCCTGTTTGATGAAGGAAT	Glu-F	900 bp	This study
Cytb-361-F	GTCATTTTATTTGCATTAACTGC	Phe1612H	1000 bp	Docker, unpublished
Phe1612H	CTTCAGTGCTCTGCTTTAATG	Cytb-361-F	1000 bp	Lang et al. 2009

*Cytb-518-R amplifies the desired gene fragment in *Eudontomyzon danfordi*, *Eudontomyzon mariae* (including the synonym *Eudontomyzon vladykovi*), *Lampetra fluviatilis*, *Lethenteron camtschaticum* (including *Lethenteron camtschaticum septentrionalis*), *Lethenteron kessleri*, *Lethenteron ninae*, *Lethenteron reissneri*; Ctyb-513-R works with the same species as well as *Lethenteron* sp. (presumably *Lethenteron alaskense*), *Lethenteron appendix*, *Lethenteron zanandreai* and *Petromyzon marinus*; Cytb-606-R works with all the *Eudontomyzon* and *Lethenteron* species.

Table 2.4 Samples for the median-joining network using the cyt *b* gene (Fig. 2.3). Unless indicated as ammocoetes (i.e., larvae), all the individuals are adult lampreys. "(?)" means the identification of the specimen is uncertain.

Species	Label	Basin	Stream	No. of individuals
Lethenteron alaskense				**1**
	01	Beaufort Sea basin	Lower Chena River, Alaska, USA	1
Lethenteron appendix				**8**
	02	Atlantic Ocean basin	Maine, USA	2
	03	Great Lakes basin	Pigeon River, Lake Michigan basin, USA	1
	04	Great Lakes basin	Betsie River, Lake Michigan basin, USA	1
	05	Great Lakes basin	Jambo Creek, Lake Michigan basin, USA	1
	06	Atlantic Ocean basin	Delaware, USA	1
(Ammocoete)	07	Great Lakes basin	Oshawa Creek, Lake Ontario basin, Canada	1
(Ammocoete)	08	Great Lakes basin	Maple River, Lake Huron basin, USA	1
Lethenteron camtschaticum				**15**
	09	Pacific Ocean basin	Belaya River, Sakhalin Island , Russia	4
	10	Pacific Ocean basin	Stream at Sokol, Sakhalin Island, Russia	4
	11	Pacific Ocean basin	Stream without name, Ussuri Bay, Russia	3
	12	Pacific Ocean basin	Klyuch River, Ussuri Bay, Russia	1
	13	Beaufort Sea basin	Shingle point, YT, Canada	1
	14	Beaufort Sea basin	Issijak site, Canada	1
	15	Pacific Ocean basin	Northeast China	1
Lethenteron camtschaticum septentrionalis				**4**
	16	White Sea basin	Onega River, Porog, Russia	2
(?)	17	White Sea basin	Keret River at Keret, Russia	2
Lethenteron kessleri				**8**
	18	Kara Sea basin	Biya River, Ob River system, Russia	3
(?)	19	White Sea basin	Stream at Chekshino, Dvinnitsa River system, Russia	5
Lethenteron reissneri				**5**
	20	Pacific Ocean basin	Vishnevka Reservoir, Amur River system, Russia	3
	21	Pacific Ocean basin	Barh River, Onon, Khentii, Mongolia	2
***Lethenteron* sp.**				**1**
(Ammocoete)	22	Beaufort Sea basin	Martin River, NT, Canada	1

Table 2.5 Frequencies of cyt *b* haplotypes for each population. All species are *Lethenteron*; *camtschaticum = Lethenteron camtschaticum camtschaticum*; *septentrionalis = Lethenteron camtschaticum septentrionalis.* In the Locality column, R. = River, L. = Lake, and Cr. = Creek.

Haplotype / Label	H 1	H 2	H 3	H 4	H 5	H 6	H 7	H 8	H 9	H 10	H 11	H 12	H 13	H 14	H 15	H 16	H 17	H 18	H 19	H 20	Species	Locality
01	1																				*alaskense*	Lower Chena R., Alaska, USA
02																	2				*appendix*	Maine, USA
03																		1			*appendix*	Pigeon R., L. Michigan, USA
04																		1			*appendix*	Betsie R., L. Michigan, USA
05																		1			*appendix*	Jambo Cr., L. Michigan, USA
06																	1				*appendix*	Delaware, USA
07																			1		*appendix*	Oshawa Cr., L. Ontario, Canada
08																		1			*appendix*	Maple R., L. Huron, USA
09	1						1		1	1											*camtschaticum*	Belaya R., Sakhalin Island , Russia
10	1			1		1			1												*camtschaticum*	Stream at Sokol, Sakhalin Island, Russia
11	1				1						1										*camtschaticum*	Stream without name, Ussuri Bay, Russia
12								1													*camtschaticum*	Klyuch R., Ussuri Bay, Russia
13	1																				*camtschaticum*	Shingle point, YT, Canada
14		1																			*camtschaticum*	Issijak site, Canada
15	1																				*camtschaticum*	Northeast China
16	2																				*septentrionalis*	Onega R., Porog, Russia
17	2																				*septentrionalis*	Keret R. at Keret, Russia
18	2													1							*kessleri*	Biya R., Ob R., Russia
19	1		1									2	1								*kessleri*	Stream at Chekshino, Dvinnitsa R., Russia
20															2					1	*reissneri*	Vishnevka Reservoir, Amur R., Russia
21																2					*reissneri*	Barh R., Onon, Khentii, Mongolia
22	1																				sp.	Martin R., NT, Canada
Total	14	1	1	1	1	1	1	1	2	1	1	2	1	1	2	2	3	4	1	1		

Table 2.6 Between-taxon percent Kimura 2-parameter (K2P) distances of the cyt *b* gene; range (lowest-highest values) above the diagonal, mean is given below the diagonal. Four groups Le, PLa, Eu and ALa are marked out above and beside the labels. *Le.* = *Lethenteron*; *La.* = *Lampetra*, *Eu.* = *Eudontomyzon*, and *Pe.* = *Petromyzon*.

			Le								PLa			
		Species	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
Le	**1**	***Le. camtschaticum***		0.00-0.27	0.00-0.27	0.18-0.45	0.00-0.27	0.18-1.09	3.53-3.62	1.00-1.27	8.31-8.41	8.01-8.11	7.68-7.78	7.47-7.58
	2	***Le. camtschaticum septentrionalis***	0.06		0.00-0.00	0.18-0.18	0.00-0.00	0.18-0.81	3.53-3.53	1.00-1.00	8.31-8.31	8.01-8.01	7.68-7.68	7.47-7.47
	3	***Le. alaskense***	0.06	0.00		0.18-0.18	0.00-0.00	0.18-0.81	3.53-3.53	1.00-1.00	8.31-8.31	8.01-8.01	7.68-7.68	7.47-7.47
	4	***Le. reissneri* (*s.s.*)**	0.24	0.18	0.18		0.18-0.18	0.18-1.00	3.52-3.62	1.00-1.18	8.21-8.31	7.91-8.10	7.58-7.88	7.38-7.48
	5	***Le. kessleri***	0.06	0.00	0.00	0.18		0.18-0.81	3.53-3.53	1.00-1.00	8.31-8.31	8.01-8.01	7.68-7.68	7.47-7.47
	6	***Le. appendix***	0.38	0.32	0.32	0.41	0.32		3.43-4.00	1.00-1.64	8.20-8.60	7.90-8.29	7.57-8.17	7.37-7.76
	7	***Le.* sp. N**	3.56	3.53	3.53	3.55	3.53	3.55		3.52-3.52	8.20-8.20	7.49-7.49	7.99-7.99	7.37-7.37
	8	***Eu. morii***	1.06	1.00	1.00	1.04	1.00	1.14	3.52		8.62-8.62	8.31-8.31	8.08-8.08	7.78-7.78
PLa	**9**	***La. ayresii***	8.34	8.31	8.31	8.28	8.31	8.38	8.20	8.62		3.05-3.05	3.24-3.24	1.46-1.46
	10	***La. pacifica***	8.04	8.01	8.01	8.03	8.01	8.11	7.49	8.31	3.05		2.67-2.67	2.86-2.86
	11	***La. hubbsi***	7.71	7.68	7.68	7.75	7.68	7.80	7.99	8.08	3.24	2.67		3.05-3.05
	12	***La. richardsoni***	7.50	7.47	7.47	7.45	7.47	7.55	7.37	7.78	1.46	2.86	3.05	
Eu	**13**	***Eu. danfordi***	7.49	7.46	7.46	7.43	7.46	7.51	7.55	7.57	8.06	8.41	8.81	8.38
	14	***Eu. mariae***	7.40	7.37	7.37	7.31	7.37	7.42	7.68	7.38	8.27	7.74	8.30	7.92
	15	***Eu. vladykovi* ***	7.47	7.44	7.44	7.36	7.44	7.49	7.86	7.55	8.20	7.90	8.44	7.82
	16	***Eu. stankokaramani* ***	7.83	7.80	7.80	7.76	7.80	7.72	7.80	7.70	7.58	7.39	7.78	7.27
ALa	**17**	***La. fluviatilis***	7.00	6.97	6.97	7.02	6.97	7.07	7.48	6.67	8.01	8.01	8.21	8.01
	18	***La. planeri***	6.99	6.96	6.96	7.00	6.96	7.06	7.47	6.66	7.99	7.99	8.20	7.99
	19	***La. lanceolata***	8.35	8.32	8.32	8.47	8.32	8.42	8.21	8.01	8.33	8.33	8.42	8.33
	20	***Le. ninae***	8.13	8.10	8.10	8.25	8.10	8.20	8.08	7.79	8.19	8.19	8.38	8.19
	21	***Le. zanandreai***	7.20	7.17	7.17	7.21	7.17	7.06	6.55	6.66	8.23	8.02	8.54	8.23
	22	***La. aepyptera***	7.37	7.34	7.34	7.36	7.34	7.26	7.03	7.04	8.49	8.38	8.49	8.38
	23	***Le.* sp. S**	11.77	11.74	11.74	11.71	11.74	11.74	12.31	11.40	11.75	12.20	12.08	11.08
	24	***Pe. marinus***	15.04	15.02	15.02	15.01	15.02	14.89	13.95	14.30	15.01	14.54	14.51	14.66

Table 2.6 Continued

			Eu				ALa							
		Species	**13**	**14**	**15**	**16**	**17**	**18**	**19**	**20**	**21**	**22**	**23**	**24**
Le	**1**	***Le. camtschaticum***	7.28-7.69	7.06-7.58	7.37-7.58	7.80-7.90	6.85-7.36	6.96-7.06	8.32-8.42	7.80-8.42	7.08-7.28	7.34-7.44	11.74-11.85	14.95-15.20
	2	***Le. camtschaticum septentrionalis***	7.28-7.59	7.06-7.47	7.37-7.48	7.80-7.80	6.85-7.25	6.96-6.96	8.32-8.32	7.80-8.32	7.08-7.18	7.34-7.34	11.74-11.74	14.96-15.08
	3	***Le. alaskense***	7.28-7.59	7.06-7.47	7.37-7.48	7.80-7.80	6.85-7.25	6.96-6.96	8.32-8.32	7.80-8.32	7.08-7.18	7.34-7.34	11.74-11.74	14.96-15.08
	4	***Le. reissneri* (*s.s.*)**	7.17-7.58	6.96-7.47	7.26-7.47	7.69-7.79	6.85-7.45	6.95-7.16	8.31-8.52	7.79-8.52	7.07-7.38	7.34-7.44	11.62-11.73	14.95-15.09
	5	***Le. kessleri***	7.28-7.59	7.06-7.47	7.37-7.48	7.80-7.80	6.85-7.25	6.96-6.96	8.32-8.32	7.80-8.32	7.08-7.18	7.34-7.34	11.74-11.74	14.96-15.08
	6	***Le. appendix***	7.07-8.29	7.06-8.18	7.17-8.18	7.59-8.29	6.85-7.75	6.96-7.45	8.32-8.82	7.80-8.82	6.87-7.47	7.14-7.84	11.52-12.27	14.49-15.17
	7	***Le.* sp. N**	7.49-7.69	7.47-8.00	7.78-7.89	7.80-7.80	7.37-7.77	7.47-7.47	8.21-8.21	7.90-8.21	6.47-6.57	7.03-7.03	12.31-12.31	13.89-14.00
	8	***Eu. morii***	7.39-7.70	7.17-7.49	7.48-7.59	7.70-7.70	6.55-6.95	6.66-6.66	8.01-8.01	7.49-8.01	6.57-6.68	7.04-7.04	11.40-11.40	14.25-14.36
PLa	**9**	***La. ayresii***	8.00-8.21	8.20-8.62	8.09-8.31	7.58-7.58	7.89-8.29	7.99-7.99	8.33-8.33	8.01-8.33	8.21-8.32	8.49-8.49	11.75-11.75	14.95-15.07
	10	***La. pacifica***	8.22-8.54	7.48-8.21	7.79-8.01	7.39-7.39	7.89-8.29	7.99-7.99	8.33-8.33	8.01-8.33	8.01-8.11	8.38-8.38	12.20-12.20	14.48-14.60
	11	***La. hubbsi***	8.62-8.94	7.88-8.51	8.29-8.62	7.78-7.78	8.09-8.50	8.20-8.20	8.42-8.42	8.32-8.42	8.52-8.63	8.49-8.49	12.08-12.08	14.45-14.56
	12	***La. richardsoni***	8.31-8.52	7.88-8.09	7.68-7.99	7.27-7.27	7.89-8.29	7.99-7.99	8.33-8.33	8.01-8.33	8.21-8.32	8.38-8.38	11.08-11.08	14.60-14.71
Eu	**13**	***Eu. danfordi***		2.02-3.53	2.20-2.86	2.39-2.68	4.59-5.18	4.69-4.99	5.28-5.58	4.79-5.58	4.31-4.70	4.58-4.77	11.85-12.07	15.45-15.81
	14	***Eu. mariae***	2.42		0.27-3.24	2.49-3.05	4.39-5.37	4.49-4.98	5.47-5.97	4.98-5.97	4.30-5.19	4.57-5.46	11.50-12.05	14.73-16.15
	15	***Eu. vladykovi* ***	2.59	2.02		2.48-2.58	4.49-5.07	4.59-4.88	5.37-5.67	4.88-5.67	4.30-4.60	4.77-4.86	11.16-11.61	15.45-15.80
	16	***Eu. stankokaramani* ***	2.56	2.63	2.55		4.40-4.68	4.49-4.49	4.98-4.98	4.49-4.98	4.40-4.50	4.38-4.38	10.74-10.74	15.33-15.45
ALa	**17**	***La. fluviatilis***	4.87	4.67	4.72	4.49		0.00-0.36	4.00-4.38	3.52-4.38	2.76-3.14	4.09-4.37	11.30-11.72	15.09-15.54
	18	***La. planeri***	4.87	4.67	4.72	4.49	0.09		4.10-4.10	3.62-4.10	2.86-2.95	4.18-4.18	11.41-11.41	15.20-15.20
	19	***La. lanceolata***	5.46	5.66	5.51	4.98	4.11	4.10		0.00-0.45	1.73-1.83	4.96-4.96	11.75-11.75	15.14-15.26
	20	***Le. ninae***	5.25	5.44	5.32	4.77	3.91	3.89	0.19		1.46-1.83	4.67-4.96	11.42-11.75	15.14-15.61
	21	***Le. zanandreai***	4.57	4.61	4.48	4.49	2.94	2.94	1.75	1.63		3.80-3.90	11.30-11.41	14.43-14.66
	22	***La. aepyptera***	4.63	4.82	4.80	4.38	4.18	4.18	4.96	4.84	3.89		12.69-12.69	14.59-14.71
	23	***Le.* sp. S**	11.91	11.70	11.35	10.74	11.42	11.41	11.75	11.61	11.39	12.69		15.61-15.73
	24	***Pe. marinus***	15.58	15.45	15.62	15.39	15.22	15.20	15.20	15.35	14.58	14.65	15.67	

**Eudontomyzon stankokaramani* and *Eudontomyzon vladykovi* were considered synonyms of *Eudontomyzon mariae* in Renaud (2011), since *Eudontomyzon mariae* exhibits a broad geographic distribution with clear disjunctions and wide variation in a number of taxonomic characters, and sufficient adult specimens from across the range has not been collected. Holčík and Šorić (2004) elevated *Eudontomyzon stankokaramani* as a species. *Eudontomyzon vladykovi* was elevated by Kottelat and Freyhof (2007).

Table 2.7 Between-group and within-group mean and range of K2P% distance of the cyt *b* gene for groups Le, Eu, PLa and ALa. Le includes *Lethenteron camtschaticum*, *Lethenteron camtschaticum septentrionalis*, *Lethenteron alaskense*, *Lethenteron reissneri*, *Lethenteron kessleri*, *Lethenteron appendix*, *Lethenteron* sp. N and *Eudontomyzon morii*; Eu includes *Eudontomyzon danfordi* and *Eudontomyzon mariae* (including the synonyms *Eudontomyzon vladykovi* and *Eudontomyzon stankokaramani*); PLa includes *Lampetra ayresii*, *Lampetra pacifica*, *Lampetra hubbsi* and *Lampetra richardsoni*; ALa includes *Lampetra fluviatilis*, *Lampetra planeri*, *Lampetra lanceolata*, *Lethenteron ninae*, *Lethenteron zanandreai*, and *Lampetra aepyptera*. Ranges are given above the diagonal; mean K2P% distances are below the diagonal.

	Within-group mean K2P%	**Within-group K2P% range**	**Le**	**Eu**	**PLa**	**ALa**
Le	0.69	0.00-4.00		6.96-8.29	7.37-8.62	6.47-8.82
Eu	2.42	0.00-3.53	7.47		7.27-8.94	4.30-5.97
PLa	2.04	0.00-3.24	7.95	8.20		7.89-8.63
ALa	2.22	0.00-4.96	7.46	4.89	8.23	

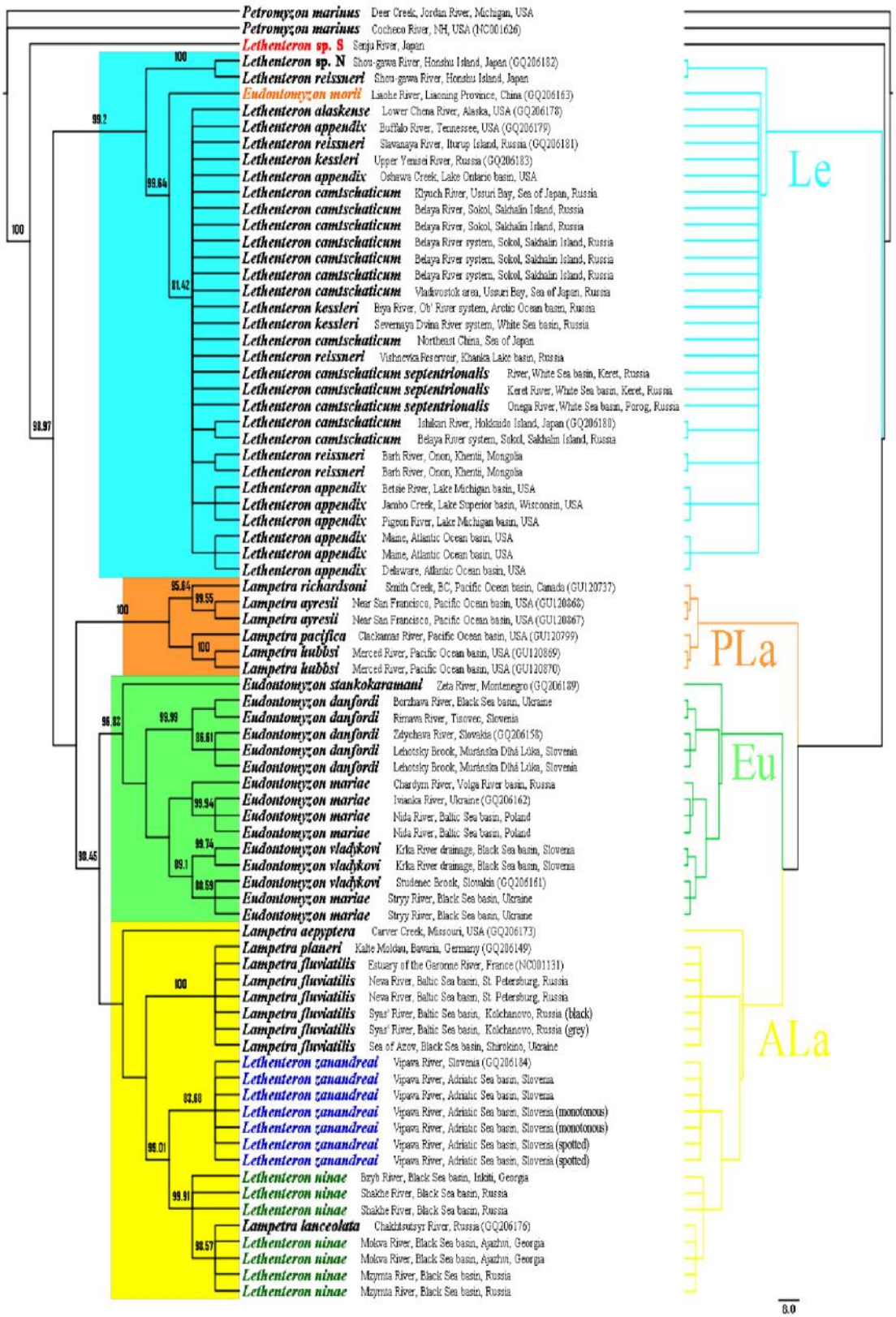

Petromyzon marinus Deer Creek, Jordan River, Michigan, USA
Petromyzon marinus Cocheco River, NH, USA (NC001626)
Lethenteron sp. S Senju River, Japan
Lethenteron sp. N Shou-gawa River, Honshu Island, Japan (GQ206182)
Lethenteron reissneri Shou-gawa River, Honshu Island, Japan
Eudontomyzon morii Liaohe River, Liaoning Province, China (GQ206163)
Lethenteron alaskense Lower Chena River, Alaska, USA (GQ206178)
Lethenteron appendix Buffalo River, Tennessee, USA (GQ206179)
Lethenteron reissneri Slavanaya River, Iturup Island, Russia (GQ206181)
Lethenteron kessleri Upper Yenisei River, Russia (GQ206183)
Lethenteron appendix Oshawa Creek, Lake Ontario basin, USA
Lethenteron camtschaticum Khyuch River, Ussuri Bay, Sea of Japan, Russia
Lethenteron camtschaticum Belaya River, Sokol, Sakhalin Island, Russia
Lethenteron camtschaticum Belaya River, Sokol, Sakhalin Island, Russia
Lethenteron camtschaticum Belaya River system, Sokol, Sakhalin Island, Russia
Lethenteron camtschaticum Belaya River system, Sokol, Sakhalin Island, Russia
Lethenteron camtschaticum Belaya River system, Sokol, Sakhalin Island, Russia
Lethenteron camtschaticum Vladivostok area, Ussuri Bay, Sea of Japan, Russia
Lethenteron kessleri Erya River, Ob' River system, Arctic Ocean basin, Russia
Lethenteron kessleri Severnaya Dvina River system, White Sea basin, Russia
Lethenteron camtschaticum Northeast China, Sea of Japan
Lethenteron reissneri Vishnevka Reservoir, Khanka Lake basin, Russia
Lethenteron camtschaticum septentrionalis River, White Sea basin, Keret, Russia
Lethenteron camtschaticum septentrionalis Keret River, White Sea basin, Keret, Russia
Lethenteron camtschaticum septentrionalis Onega River, White Sea basin, Porog, Russia
Lethenteron camtschaticum Ishikari River, Hokkaido Island, Japan (GQ206180)
Lethenteron camtschaticum Belaya River system, Sokol, Sakhalin Island, Russia
Lethenteron reissneri Barh River, Onon, Khentii, Mongolia
Lethenteron reissneri Barh River, Onon, Khentii, Mongolia
Lethenteron appendix Betsie River, Lake Michigan basin, USA
Lethenteron appendix Jambo Creek, Lake Superior basin, Wisconsin, USA
Lethenteron appendix Pigeon River, Lake Michigan basin, USA
Lethenteron appendix Maine, Atlantic Ocean basin, USA
Lethenteron appendix Maine, Atlantic Ocean basin, USA
Lethenteron appendix Delaware, Atlantic Ocean basin, USA
Lampetra richardsoni Smith Creek, BC, Pacific Ocean basin, Canada (GU120737)
Lampetra ayresii Near San Francisco, Pacific Ocean basin, USA (GU120868)
Lampetra ayresii Near San Francisco, Pacific Ocean basin, USA (GU120867)
Lampetra pacifica Clackamas River, Pacific Ocean basin, USA (GU120799)
Lampetra hubbsi Merced River, Pacific Ocean basin, USA (GU120869)
Lampetra hubbsi Merced River, Pacific Ocean basin, USA (GU120870)
Eudontomyzon stankokaramani Zeta River, Montenegro (GQ206189)
Eudontomyzon danfordi Borzhava River, Black Sea basin, Ukraine
Eudontomyzon danfordi Rimava River, Tisovec, Slovenia
Eudontomyzon danfordi Zdychava River, Slovakia (GQ206158)
Eudontomyzon danfordi Lehotsky Brook, Muránska Dlhá Lúka, Slovenia
Eudontomyzon danfordi Lehotsky Brook, Muránska Dlhá Lúka, Slovenia
Eudontomyzon mariae Chardym River, Volga River basin, Russia
Eudontomyzon mariae Ivianka River, Ukraine (GQ206162)
Eudontomyzon mariae Nida River, Baltic Sea basin, Poland
Eudontomyzon mariae Nida River, Baltic Sea basin, Poland
Eudontomyzon vladykovi Krka River drainage, Black Sea basin, Slovenia
Eudontomyzon vladykovi Krka River drainage, Black Sea basin, Slovenia
Eudontomyzon vladykovi Studenec Brook, Slovakia (GQ206161)
Eudontomyzon mariae Stryy River, Black Sea basin, Ukraine
Eudontomyzon mariae Stryy River, Black Sea basin, Ukraine
Lampetra aepyptera Carver Creek, Missouri, USA (GQ206173)
Lampetra planeri Kalte Moldau, Bavaria, Germany (GQ206149)
Lampetra fluviatilis Estuary of the Garonne River, France (NC001131)
Lampetra fluviatilis Neva River, Baltic Sea basin, St. Petersburg, Russia
Lampetra fluviatilis Neva River, Baltic Sea basin, St. Petersburg, Russia
Lampetra fluviatilis Syas' River, Baltic Sea basin, Kolchanovo, Russia (black)
Lampetra fluviatilis Syas' River, Baltic Sea basin, Kolchanovo, Russia (grey)
Lampetra fluviatilis Sea of Azov, Black Sea basin, Sharokino, Ukraine
Lethenteron zanandreai Vipava River, Slovenia (GQ206184)
Lethenteron zanandreai Vipava River, Adriatic Sea basin, Slovenia
Lethenteron zanandreai Vipava River, Adriatic Sea basin, Slovenia
Lethenteron zanandreai Vipava River, Adriatic Sea basin, Slovenia (monotonous)
Lethenteron zanandreai Vipava River, Adriatic Sea basin, Slovenia (monotonous)
Lethenteron zanandreai Vipava River, Adriatic Sea basin, Slovenia (spotted)
Lethenteron zanandreai Vipava River, Adriatic Sea basin, Slovenia (spotted)
Lethenteron ninae Bzyb River, Black Sea basin, Inkiti, Georgia
Lethenteron ninae Shakhe River, Black Sea basin, Russia
Lethenteron ninae Shakhe River, Black Sea basin, Russia
Lampetra lanceolata Chakitsutsyr River, Russia (GQ206176)
Lethenteron ninae Mokva River, Black Sea basin, Ajazhvi, Georgia
Lethenteron ninae Mokva River, Black Sea basin, Ajazhvi, Georgia
Lethenteron ninae Mzymta River, Black Sea basin, Russia
Lethenteron ninae Mzymta River, Black Sea basin, Russia
100
99.2
99.64
100
81.42
98.97
95.84
99.55
100
100
96.82
99.99
86.61
99.94
99.74
98.45
89.1
98.59
100
83.88
99.01
99.91
98.57
Le
PLa
Eu
ALa
0.0

Fig. 2.1 Strict consensus of 2010 maximum parsimony trees (length = 653) resulting from heuristic searches using the Parsimony Ratchet for the cyt *b* gene (1191 bp) dataset. The cladogram is on the left of the species names and the collection locations. Bootstrap support values greater than 80 are above the branches. The proportional tree is on the right. The scale refers to the number of substitutions. Four clades are colored: *Lethenteron* plus *Eudontomyzon morii* (Le) in blue, Pacific *Lampetra* (PLa) in orange, *Eudontomyzon* (Eu) in green, and Atlantic *Lampetra* plus *Lethenteron zanandreai* and *Lethenteron ninae* (Ala) in yellow. The species names are in different colors where the generic names disagree with the clade names.

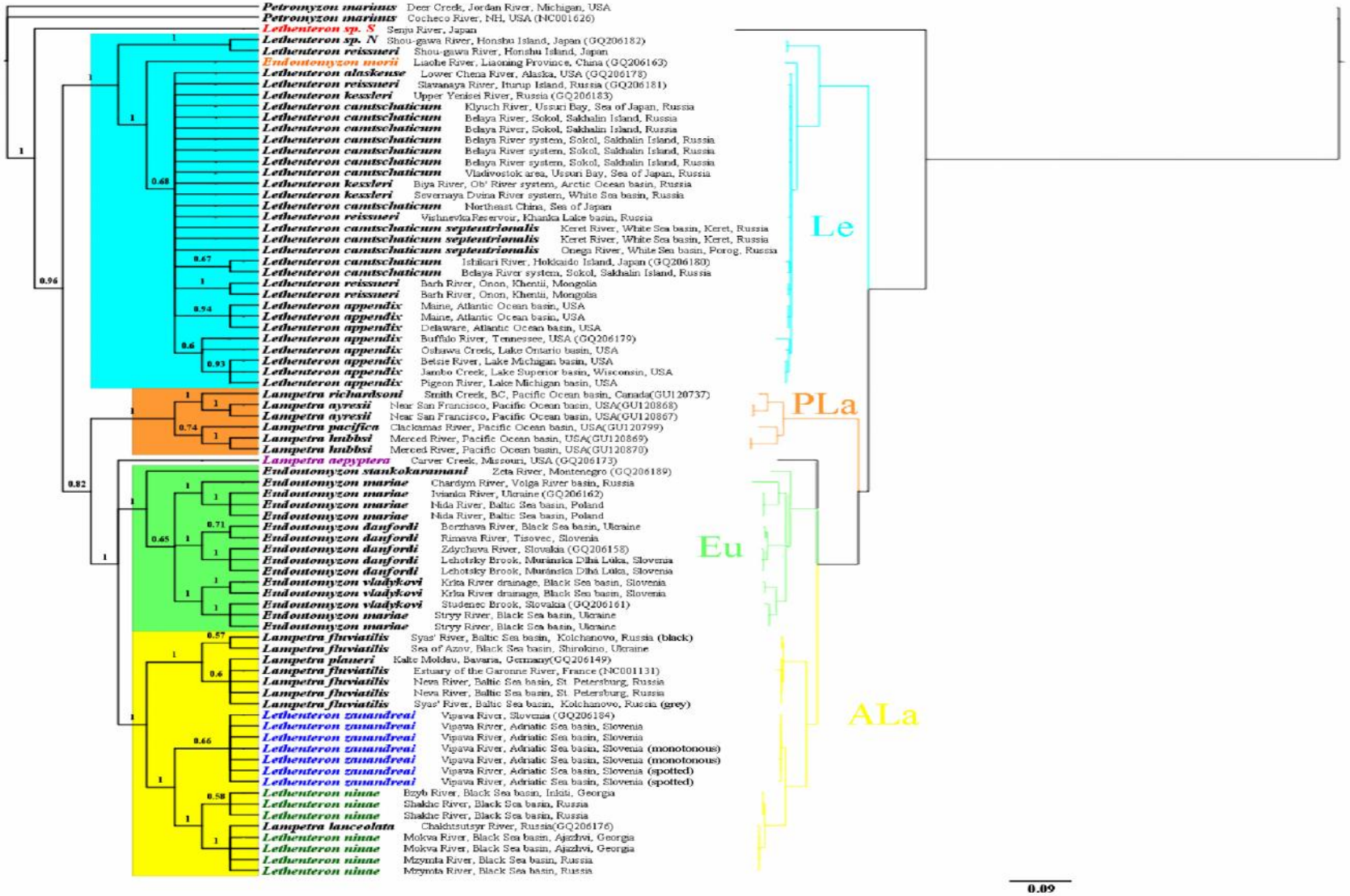
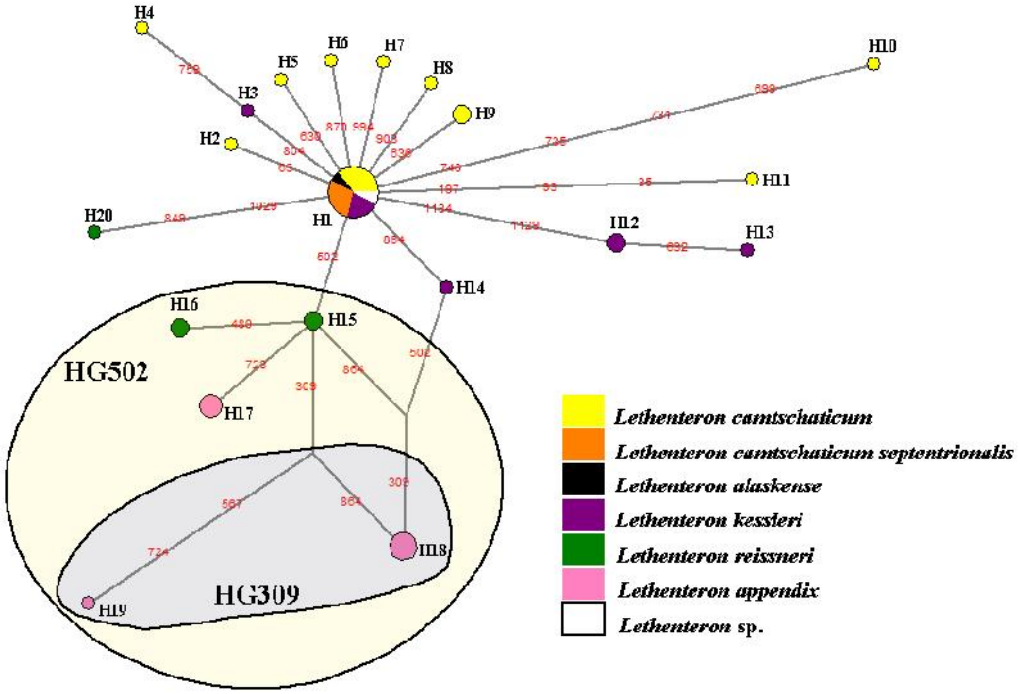

Petromyzon marinus Deer Creek, Jordan River, Michigan, USA
Petromyzon marinus Cocheco River, NH, USA (NC001626)
Lethenteron sp. S Senju River, Japan
Lethenteron sp. N Shou-gawa River, Honshu Island, Japan (GQ206182)
Lethenteron reissneri Shou-gawa River, Honshu Island, Japan
Endotonyzon morii Liaohe River, Liaoning Province, China (GQ206163)
Lethenteron alaskense Lower Chena River, Alaska, USA (GQ206178)
Lethenteron reissneri Slavanaya River, Iturup Island, Russia (GQ206181)
Lethenteron kessleri Upper Yenisei River, Russia (GQ206183)
Lethenteron camtschaticum Klyuch River, Ussuri Bay, Sea of Japan, Russia
Lethenteron camtschaticum Belaya River, Sokol, Sakhalin Island, Russia
Lethenteron camtschaticum Belaya River, Sokol, Sakhalin Island, Russia
Lethenteron camtschaticum Belaya River system, Sokol, Sakhalin Island, Russia
Lethenteron camtschaticum Belaya River system, Sokol, Sakhalin Island, Russia
Lethenteron camtschaticum Belaya River system, Sokol, Sakhalin Island, Russia
Lethenteron camtschaticum Vladivostok area, Ussuri Bay, Sea of Japan, Russia
Lethenteron kessleri Biya River, Ob' River system, Arctic Ocean basin, Russia
Lethenteron kessleri Severnaya Dvina River system, White Sea basin, Russia
Lethenteron camtschaticum Northeast China, Sea of Japan
Lethenteron reissneri Vishnevka Reservoir, Khanka Lake basin, Russia
Lethenteron camtschaticum septentrionalis Keret River, White Sea basin, Keret, Russia
Lethenteron camtschaticum septentrionalis Keret River, White Sea basin, Keret, Russia
Lethenteron camtschaticum septentrionalis Onega River, White Sea basin, Porog, Russia
Lethenteron camtschaticum Ishikari River, Hokkaido Island, Japan (GQ206180)
Lethenteron camtschaticum Belaya River system, Sokol, Sakhalin Island, Russia
Lethenteron reissneri Barh River, Onon, Khentii, Mongolia
Lethenteron reissneri Barh River, Onon, Khentii, Mongolia
Lethenteron appendix Maine, Atlantic Ocean basin, USA
Lethenteron appendix Maine, Atlantic Ocean basin, USA
Lethenteron appendix Delaware, Atlantic Ocean basin, USA
Lethenteron appendix Buffalo River, Tennessee, USA (GQ206179)
Lethenteron appendix Oshawa Creek, Lake Ontario basin, USA
Lethenteron appendix Betsie River, Lake Michigan basin, USA
Lethenteron appendix Jambo Creek, Lake Superior basin, Wisconsin, USA
Lethenteron appendix Pigeon River, Lake Michigan basin, USA
Lampetra richardsoni Smith Creek, BC, Pacific Ocean basin, Canada(GU120737)
Lampetra ayresii Near San Francisco, Pacific Ocean basin, USA(GU120868)
Lampetra ayresii Near San Francisco, Pacific Ocean basin, USA(GU120867)
Lampetra pacifica Clackamas River, Pacific Ocean basin, USA(GU120799)
Lampetra hubbsi Merced River, Pacific Ocean basin, USA(GU120869)
Lampetra hubbsi Merced River, Pacific Ocean basin, USA(GU120870)
Lampetra aepyptera Carver Creek, Missouri, USA (GQ206173)
Endotonyzon stankokaramani Zeta River, Montenegro (GQ206189)
Endotonyzon mariae Chardym River, Volga River basin, Russia
Endotonyzon mariae Ivianka River, Ukraine (GQ206162)
Endotonyzon mariae Nida River, Baltic Sea basin, Poland
Endotonyzon mariae Nida River, Baltic Sea basin, Poland
Endotonyzon danfordi Berzhava River, Black Sea basin, Ukraine
Endotonyzon danfordi Rimava River, Tisovec, Slovenia
Endotonyzon danfordi Zdychava River, Slovakia (GQ206158)
Endotonyzon danfordi Lehotsky Brook, Muránska Dlhá Luka, Slovenia
Endotonyzon danfordi Lehotsky Brook, Muránska Dlhá Luka, Slovenia
Endotonyzon vladykovi Krka River drainage, Black Sea basin, Slovenia
Endotonyzon vladykovi Krka River drainage, Black Sea basin, Slovenia
Endotonyzon vladykovi Studenec Brook, Slovakia (GQ206161)
Endotonyzon mariae Stryy River, Black Sea basin, Ukraine
Endotonyzon mariae Stryy River, Black Sea basin, Ukraine
Lampetra fluviatilis Syas' River, Baltic Sea basin, Kolchanovo, Russia (black)
Lampetra fluviatilis Sea of Azov, Black Sea basin, Shirokino, Ukraine
Lampetra planeri Kalte Moldau, Bavaria, Germany(GQ206149)
Lampetra fluviatilis Estuary of the Garonne River, France (NC001131)
Lampetra fluviatilis Neva River, Baltic Sea basin, St. Petersburg, Russia
Lampetra fluviatilis Neva River, Baltic Sea basin, St. Petersburg, Russia
Lampetra fluviatilis Syas' River, Baltic Sea basin, Kolchanovo, Russia (grey)
Lethenteron zanandreai Vipava River, Slovenia (GQ206184)
Lethenteron zanandreai Vipava River, Adriatic Sea basin, Slovenia
Lethenteron zanandreai Vipava River, Adriatic Sea basin, Slovenia
Lethenteron zanandreai Vipava River, Adriatic Sea basin, Slovenia (monotonous)
Lethenteron zanandreai Vipava River, Adriatic Sea basin, Slovenia (monotonous)
Lethenteron zanandreai Vipava River, Adriatic Sea basin, Slovenia (spotted)
Lethenteron zanandreai Vipava River, Adriatic Sea basin, Slovenia (spotted)
Lethenteron ninae Bzyb River, Black Sea basin, Inkit, Georgia
Lethenteron ninae Shakhe River, Black Sea basin, Russia
Lethenteron ninae Shakhe River, Black Sea basin, Russia
Lampetra lanceolata Chakhtsutsyr River, Russia(GQ206176)
Lethenteron ninae Mokva River, Black Sea basin, Ajazhvi, Georgia
Lethenteron ninae Mokva River, Black Sea basin, Ajazhvi, Georgia
Lethenteron ninae Mzymta River, Black Sea basin, Russia
Lethenteron ninae Mzymta River, Black Sea basin, Russia
Le
PLa
Eu
ALa
0.09

Fig. 2.2 Fifty percent majority rule consensus of 750 Bayesian trees using the cyt *b* gene (1191 bp) dataset. The cladogram is on the left of the species names and the collection locations. Posterior probabilities are above the branches. The proportional tree is on the right. The scale refers to the number of substitutions per site. Four clades are colored: *Lethenteron* plus *Eudontomyzon morii* (Le) in blue, Pacific *Lampetra* (PLa) in orange, *Eudontomyzon* (Eu) in green, and Atlantic *Lampetra* plus *Lethenteron zanandreai* and *Lethenteron ninae* (Ala) in yellow. The species names are in different colors where the generic names disagree with the clade names.

A


H4
H5
H6
H7
H8
H3
H9
H2
H10
H11
H20
H1
H12
H13
H14
H16
H15
HG502
H17
HG309
H18
H19
Lethenteron camtschaticum
Lethenteron camtschaticum septentrionalis
Lethenteron alaskense
Lethenteron kessleri
Lethenteron reissneri
Lethenteron appendix
Lethenteron sp.

B

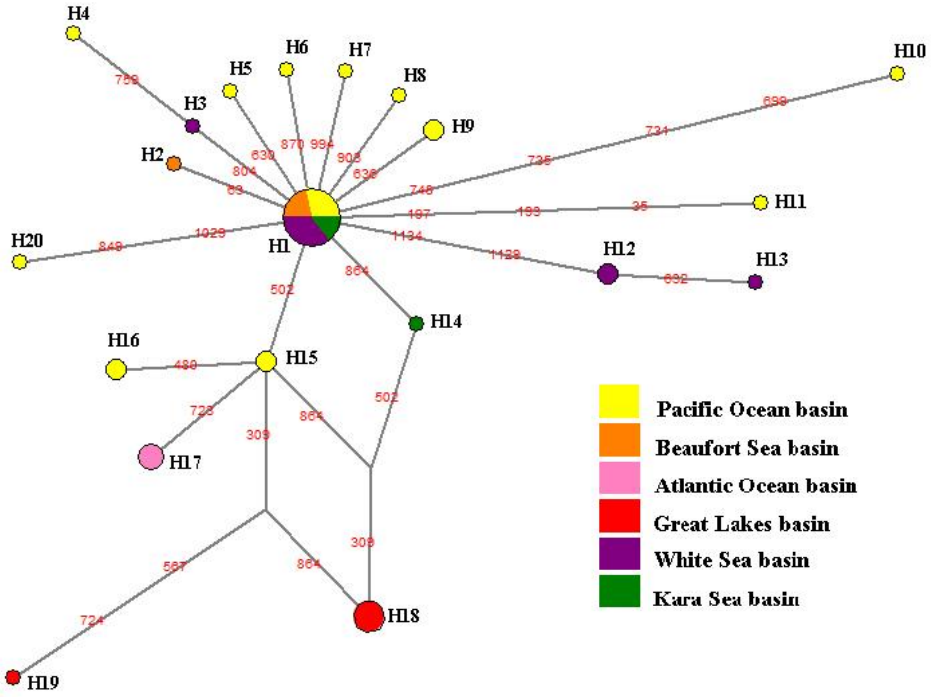

H4
H5
H6
H7
H8
H3
H9
H2
H10
H11
H20
H1
H12
H13
H14
H16
H15
H17
H18
H19
Pacific Ocean basin
Beaufort Sea basin
Atlantic Ocean basin
Great Lakes basin
White Sea basin
Kara Sea basin

Fig. 2.3 Median-joining network of six closely related *Lethenteron* species and subspecies using the cyt *b* gene. Figure A and B are the same network colored differently. Twenty haplotypes (H1–H20) are marked beside the nodes. The sizes of the nodes are proportional to the frequencies of the haplotypes. The lengths of the links estimate the number of substitutions between haplotypes. A: The median-joining network colored by species and subspecies. The haplogroup HG502 (sharing the substitution C - T at 502 bp of cyt *b* gene) includes H15–H19; the haplogroup HG309 (sharing the substitution G - A at 309 bp of cyt *b* gene) includes H18 and H19. B: The median joining network colored by drainage basins of the collection locations.

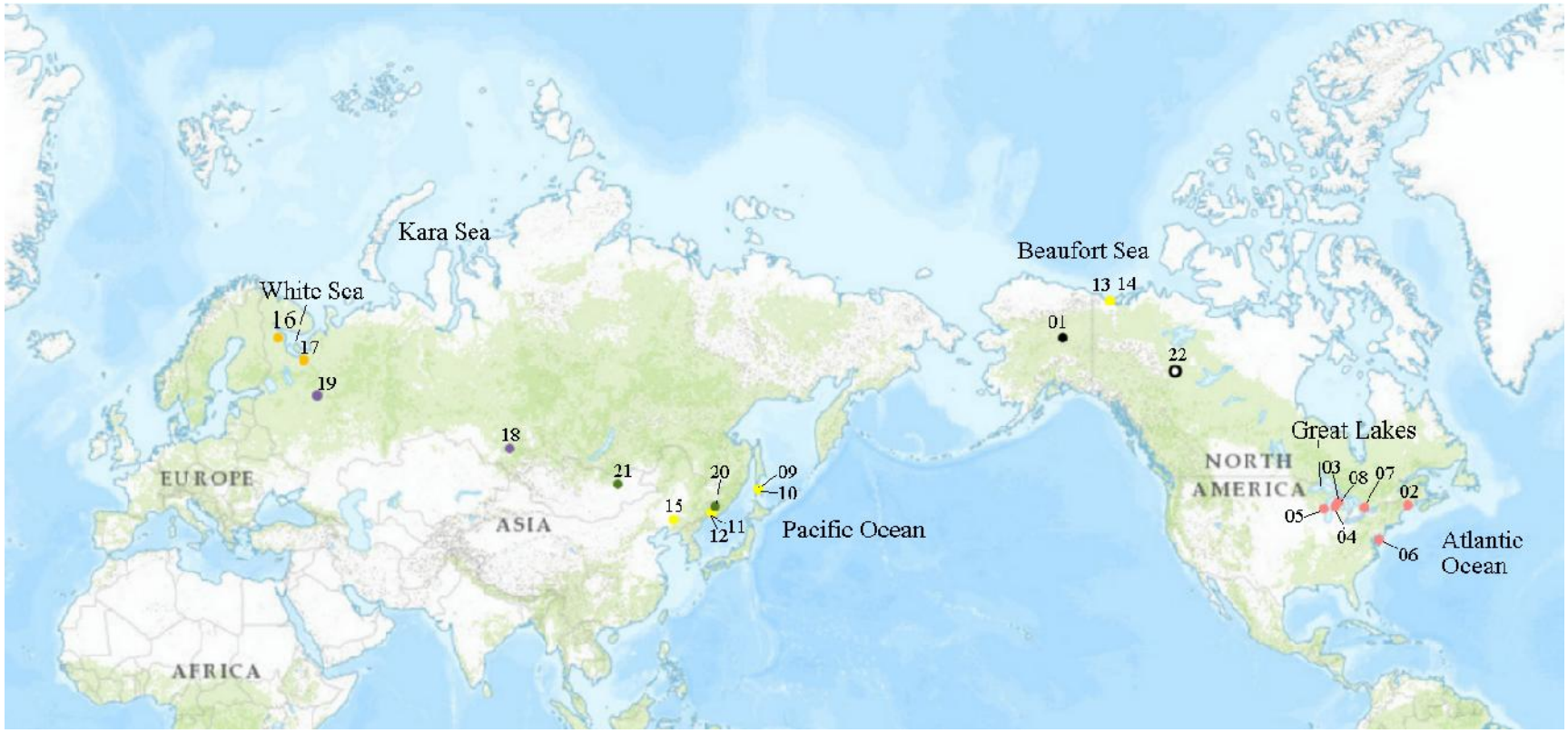
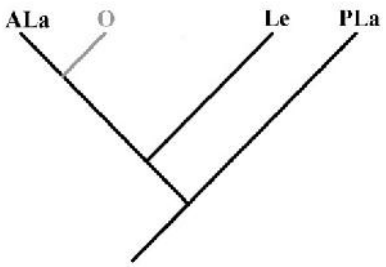


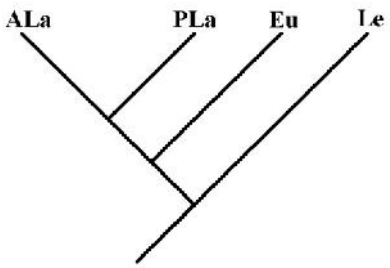
Fig. 2.4 Collection locations of samples for the median-joining network using the cyt *b* gene (Fig. 2.3). The population labels (see Table 2.4) are beside the locations denoted with dots. The dots are colored by species and subspecies.

A.



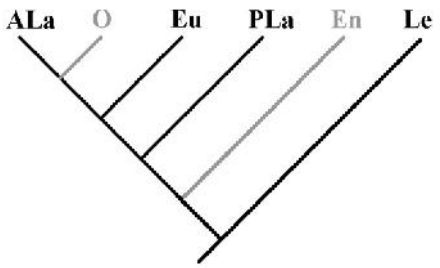
Docker et al. (1999)
Character: cyt *b* (384 bp) + ND3 (351 bp)
Le: *Lethenteron camtschaticum*, *Lethenteron appendix*
ALa: *Lampetra fluviatilis*
PLa: *Lampetra ayresii*, *Lampetra richardsoni*, *Lampetra hubbsi*

B.



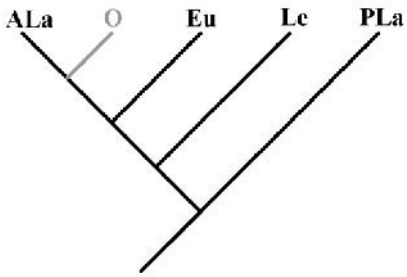
Gill et al. (2003)
Character: morphological characters
Le: *Lethenteron camtschaticum*
Eu: *Eudontomyzon danfordi*, *Eudontomyzon morii*
ALa: *Lampetra fluviatilis*
PLa: *Lampetra ayresii*

C.



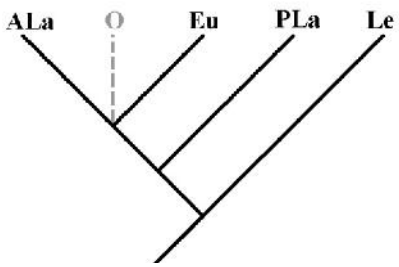
Blank et al. (2008)
Character: cyt *b* (384 bp)
Le: *Lethenteron camtschaticum*, *Lethenteron appendix*, *Lethenteron kessleri*, *Lethenteron reissneri*
Eu: *Eudontomyzon mariae*
ALa: *Lampetra fluviatilis*, *Lampetra planeri*
PLa: *Lampetra ayresii*, *Lampetra richardsoni*, *Lampetra hubbsi*

D.



Lang et al. (2009)
Character: cyt *b* (1133 bp)
Le: *Lethenteron camtschaticum*, *Lethenteron appendix*, *Lethenteron kessleri*, *Lethenteron reissneri*, *Lethenteron alaskense*, *Lethenteron* sp. N, *Eudontomyzon morii*
Eu: *Eudontomyzon danfordi*, *Eudontomyzon mariae* (including synonyms *Eudontomyzon vladykovi* and *Eudontomyzon stankokaramani*)
ALa: *Lampetra fluviatilis*, *Lampetra planeri*, *Lampetra lanceolata*, *Lethenteron zanandreai*
PLa: *Lampetra ayresii*, *Lampetra richardsoni*, *Lampetra hubbsi*

E.



This thesis
Character: cyt *b* (1191 bp)
Le: *Lethenteron camtschaticum*, *Lethenteron appendix*, *Lethenteron kessleri*, *Lethenteron reissneri*, *Lethenteron alaskense*, *Lethenteron* sp. N, *Eudontomyzon morii*
Eu: *Eudontomyzon danfordi*, *Eudontomyzon mariae* (including synonyms *Eudontomyzon vladykovi* and *Eudontomyzon stankokaramani*)
ALa: *Lampetra fluviatilis*, *Lampetra planeri*, *Lampetra lanceolata*, *Lethenteron zanandreai*, *Lethenteron ninae*
PLa: *Lampetra ayresii*, *Lampetra richardsoni*, *Lampetra hubbsi*, *Lampetra pacifica*

Fig. 2.5 Summary of the phylogeny among *Lethenteron* (Le), *Eudontomyzon* (Eu), Pacific *Lampetra* (PLa) and Atlantic *Lampetra* (ALa) according to different authors (Docker et al. 1999; Gill et al. 2003; Blank et al. 2008; Lang et al. 2009; this thesis). The trees are simplified to the generic level. The smallest monophyly including four genera/groups is shown, except that *Eudontomyzon* was not included by Docker et al. (1999). If included, other genera besides those four are in grey. O = *Okkelbergia* (*Lampetra aepyptera*); En = *Entosphenus*. The characters used and the species included in the four [three in Docker et al. (1999)] main groups are indicated under each tree. A: Docker et al. (1999); B: Gill et al. (2003); C: Blank et al. 2008; D: Lang et al. 2009; E: this thesis. The placement of *Okkelbergia* is inconsistent in maximum parsimony and Bayesian trees in this thesis and thus indicated with a broken line.

Chapter 3 Phylogenetic relationships among *Lethenteron* and closely related genera using nuclear genes

3.1 Introduction

As has been the case with many taxa since the advent of molecular phylogenetic methods, morphological and molecular studies in lampreys suggest different divisions of genera and different relationships among them. This has been particularly evident among the closely related genera *Eudontomyzon* Regan 1911, *Lampetra* Bonnaterre 1788, and *Lethenteron* Creaser and Hubbs 1922. All the molecular studies (e.g., Docker et al. 1999; Lang et al. 2009; Boguski et al. 2012), including Chapter 2 of this thesis, agreed that *Lampetra* from the Pacific drainage of North America and *Lampetra* from the Atlantic drainages of Eurasia and North America are distinct genera, despite their highly similar morphology (Vladykov and Follett 1958; Vladykov and Follett 1965; Gill et al. 2003). All previous molecular studies, however, have relied on DNA sequence data from mitochondrial genes, and most used the same gene, the cytochrome *b* (cyt *b*) gene. Conclusions are premature, however, without independent phylogenetic analysis using independent nuclear gene sequences (Degnan and Rosenberg 2006; Pollard et al. 2006). Thus, in this chapter, introns of two nuclear genes, transporter associated with antigen processing (TAP) gene and SRY-related high mobility group box D (soxD) gene, were selected for the phylogenetic analysis. Although nuclear genes also estimate the gene trees rather than the species tree, congruence between the nuclear and mitochondrial gene trees probably represents the true relationships among genera. This is the first generic-level phylogenetic analysis on lampreys using nuclear genes.

3.1.1 Gene selection

3.1.1.1 Nuclear genes vs. mitochondrial genes

In Chapter 2, mitochondrial cyt *b* gene was used as the genetic marker to resolve the relationships among *Lethenteron*, *Eudontomyzon* and Pacific and Atlantic *Lampetra*, as well as the phylogeny within *Lethenteron*. However, phylogeny based on one single gene may not reflect the true relationships among taxa due to incomplete lineage sorting (Pamilo and Nei 1988; Degnan and Rosenberg 2006; Pollard et al. 2006). The lineage sorting hypothesis suggests that genes supporting the same tree (gene tree) cluster in the same lineage, especially for regions with low recombination (Pollard et al. 2006). The nuclear gene trees often differ from mitochondrial gene trees in topology and branch length (e.g., Armstrong et al. 2001; Yu et al. 2008; Wiens et al. 2010). Boguski (2009) used three mitochondrial genes [cyt *b*, NADH dehydrogenase 2 (ND2) and cytochrome *c* oxidase subunit I (COI)] and two nuclear genes (the ninth intron of TAP gene (TAP9) and the internal transcribed spacer region 1 (ITS1) of the rRNA gene) of non-parasitic *Lampetra* from the Pacific drainage of North America, and recovered somewhat different phylogenetic relationships among populations between the nuclear and mitochondrial datasets. Since mitochondrial DNA (mtDNA) and nuclear genes may provide different answers to the same phylogenetic question, which one is the better genetic marker for phylogenetic analyses?

Although mtDNA evolves at a higher rate and thus may provide higher resolution of the phylogeny within *Lethenteron* than nuclear DNA (Brown et al. 1979; Brown et al. 1982; Pesole et al. 1999), the reliability of the mitochondrial gene trees vs. nuclear gene trees has been controversial. Since the coalescence time (the time from the origination of

new alleles or haplotypes to the speciation event) could last longer than the time between speciation events (the internodal distance in the species tree), the branch order of the gene tree may differ from that of the species tree (Moore 1995). The expected coalescence time, which is directly related to the effective population size (N_e), is equal to the number of copies of the gene that are transmitted from generation to generation (N_g) (Moore 1995). Moore (1995) preferred mtDNA over nuclear DNA based on the theory that mtDNA exhibits an expected coalescence time that is one-fourth as long as for the nuclear DNA: in a random mating population with an equal number of males and females, N_g for mitochondrial genes is equal to the number of females ($N_e/2$) since the mitochondrial genome is haploid and transmitted only through females, while N_g for nuclear genes is equal to twice the effective population size ($2N_e$) since the nuclear genome is diploid and transmitted through both sexes. However, Hoelzer (1997) responded that the hypothesis that mtDNA exhibits shorter expected coalescence time than nuclear DNA may not apply to many cases in animal phylogenetics (e.g., in polygynous mating systems, or in species with female philopatry and male dispersal), and suggested using multiple unlinked nuclear genes (Pamilo and Nei 1988) instead of solely mtDNA.

The mitochondrial genome is maternally inherited in almost all vertebrates (one exception is the paternal leakage observed in mice; Gyllesten et al. 1991) and, as such, may not reflect the information of hybridization when estimating the species tree with the gene tree. The lack of recombination in mtDNA means that the mitochondrial genome should be treated as a single locus; all fragments in mtDNA may support the same gene tree, which may not have a congruent topology to the species tree (Hoelzer 1997). Thus,

using different mitochondrial genes is not the same thing as using independent genes, and the phylogeny inferred from multiple unlinked genes may be more reliable than that from only mitochondrial genes.

Springer et al. (2001) compared four mitochondrial genes and eight nuclear genes in inferring the phylogeny of mammals and concluded that nuclear genes are less affected by the superimposed substitutions (i.e., multiple substitutions, including reversal substitutions, at the same site) and thus more fit for deep-level phylogeny reconstruction than mtDNA. Several nuclear genes (e.g., rRNA genes; Mallatt and Sullivan 1998) have been used to resolve the deep-level phylogeny among vertebrates, including lampreys, but such deep-level phylogenies have used highly conserved nuclear genes. Although most nuclear genes may not provide enough information to resolve the phylogeny among closely related *Lethenteron* species, they may be good genetic markers for the generic-level phylogeny. Thus, in this chapter, two nuclear genes were used to resolve the phylogeny among *Lethenteron* and the closely related genera.

3.1.1.2 Introns of TAP and soxD genes

Unlike mtDNA, most nuclear coding genes of higher-level eukaryotes have an exon-intron structure. Exons of nuclear genes are often conserved even among different classes of vertebrates. For example, several coding regions in a TAP family gene in lampreys are conserved in vertebrates, including humans (Uinuk-ool et al. 2003). Protein sequences were used for the phylogeny of vertebrates at the class level (Yu et al. 2008) but the DNA sequence of introns typically provides far more resolution among closely related taxa. Growth hormone introns were used to resolve the relationships among

genera within the subfamily Salmoninae (Oakley and Phillips 1999). Avian ovomucoid intron G was used for phylogeny reconstruction among genera and families in Galliformes (Armstrong et al. 2001). The RNA fingerprint protein 35 intron was sequenced in turtles to resolve the generic level phylogeny (Fujita et al. 2004). In this chapter, two introns of TAP and soxD genes were selected to address the generic relationships of *Lethenteron*, *Eudontomyzon*, and Pacific and Atlantic *Lampetra*.

TAP is a member of ATP-binding cassette transporters (ABC proteins) that transport peptides produced by immunoproteasomes across the membrane of the endoplasmic reticulum (Abele and Tampé 1999). The TAP gene family is found in many vertebrates including lampreys (sea lamprey *Petromyzon marinus* L.; Uinuk-ool et al. 2003). Uinuk-ool et al. (2003) provided the exon-intron structure of the single copy TAP gene (ABCB9 protein coding gene) in *Petromyzon marinus*. By aligning two adjacent exons of this TAP gene from *Petromyzon marinus* and that of other vertebrates, primers amplifying the intron between these two exons can be designed from the conserved regions in the two exons. The ninth intron (1242 bp) of 12 in total was used for resolving the population phylogeny of non-parasitic *Lampetra* (Boguski 2009). In this thesis, a somewhat shorter intron, the second intron (949 bp), of the TAP gene (TAP2) was used for the phylogeny among *Lethenteron* and closely related genera.

The soxD gene family is necessary for formation of the notochord and chondrogenesis. One single soxD gene was identified in lampreys (Uy et al. 2012). Okada et al. (2010) amplified the soxD intron in Arctic lamprey *Lethenteron camtschaticum* (Tilesius 1811) and Siberian lamprey *Lethenteron kessleri* (Anikin 1905) attempting to find sequence differences fixed to species. Substitutions and indels were

found in the sequences from Okada et al. (2010), although not diagnostically distinguishing closely related species such as *Lethenteron camtschaticum* and *Lethenteron kessleri*. One similar region with more than 400 bp of insertion relative to *Lethenteron* has been found in the *Petromyzon marinus* genome (http://www.ensembl.org/Petromyzon_marinus/Info/Index, Smith et al. 2013). The difference between *Petromyzon marinus* and the *Lethenteron* species suggest that fixed indels could be found among different genera. Thus, this soxD intron may resolve relationships among genera.

Maximum parsimony and Bayesian trees are inferred from these two genes individually and from a combined dataset of both genes. The phylogenetic tree using the combined dataset is expected to be more similar to the species tree in terms of topology than single gene trees.

3.1.2 Relationships among genera

The generic relationships among *Lethenteron*, *Eudontomyzon* and *Lampetra* recovered using cyt *b* and other mtDNA sequences (Docker et al. 1999; Blank et al. 2008; Lang et al. 2009; Chapter 2) differed from the morphological phylogeny (Gill et al. 2003). Most notably, the mtDNA phylogeny suggested Atlantic and Pacific *Lampetra* as separate genera, and placed the Western Transcaucasian brook lamprey *Lethenteron ninae* Naseka, Tuniyev, and Renaud 2009 and the Lombardy brook lamprey *Lethenteron zanandreai* (Vladykov 1955) in the Atlantic *Lampetra* clade, and the Korean lamprey *Eudontomyzon morii* (Berg 1931) in the *Lethenteron* clade. Atlantic *Lampetra* was more closely related to *Eudontomyzon* than to *Lethenteron* and Pacific *Lampetra*. The least brook lamprey

Lampetra aepyptera (Abbott 1860) was either a part of Atlantic *Lampetra* or a distinct genus (*Okkelbergia*) containing only this species, as proposed by Hubbs and Potter (1971), and *Lethenteron* sp. S was sister to all four clades. Thus, the first question to answer with the nuclear gene introns is whether the division of genera inferred using the cyt *b* gene is supported. Are *Lethenteron*, *Eudontomyzon*, Pacific *Lampetra*, and Atlantic *Lampetra* (divided as in Section 2.4.1) reciprocally monophyletic? Does *Lampetra aepyptera* (*Okkelbergia*) form a clade with Atlantic *Lampetra*?

3.1.3 Placement of species

As mentioned in the preceding section, mtDNA sequence analysis (Chapter 2) placed *Lethenteron ninae* and *Lethenteron zanandreai* in the Atlantic *Lampetra* clade and not in the *Lethenteron* clade. This chapter will test whether this placement is supported using the nuclear datasets. Previous phylogenetic studies using mtDNA all supported *Lethenteron zanandreai* as a *Lampetra* species (Docker et al. 1999; Caputo et al. 2009; Lang et al. 2009), but this thesis is the first study to examine the phylogeny of the recently described *Lethenteron ninae*.

The last chapter also found *Lethenteron ninae* and Turkish brook lamprey *Lampetra lanceolata* Kux and Steiner 1972 to be closely related to each other, but they were not reciprocally monophyletic. However, this analysis was based on a *Lampetra lanceolata* specimen collected far from its type locality (Lang et al. 2009). In this chapter, the TAP2 intron of one *Lampetra lanceolata* collected from a site closer to the type locality was sequenced. The close relationship between *Lethenteron ninae* and *Lampetra lanceolata* will also be tested using the TAP2 phylogenetic trees.

3.2 Materials and methods

3.2.1 Samples

The 14 species used for nuclear gene analyses are a subset of the species used for the cyt *b* gene analyses (Section 2.2.1, Table 2.1 and 2.2): Carpathian lamprey *Eudontomyzon danfordi* Regan 1911, Ukrainian brook lamprey *Eudontomyzon mariae* (Berg 1931) [including the synonym *Eudontomyzon vladykovi* (Oliva and Zanandrea 1959)], *Lampetra aepyptera*, river lamprey *Lampetra ayresii* (Günther 1870), European river lamprey *Lampetra fluviatilis* (L.), Kern brook lamprey *Lampetra hubbsi* Vladykov and Kott 1976, *Lampetra lanceolata*, Pacific brook lamprey *Lampetra pacifica* Vladykov 1973, American brook lamprey *Lethenteron appendix* (DeKay 1842), *Lethenteron camtschaticum*, *Lethenteron kessleri*, *Lethenteron ninae*, Far Eastern brook lamprey *Lethenteron reissneri* (Dybowski 1869), and *Lethenteron zanandreai*. The TAP2 intron of 21 specimens and the soxD intron of 15 specimens (including five from GenBank) were analyzed, not including the outgroup *Petromyzon marinus*. In the sample list for nuclear gene analyses (Table 3.1), 19 DNA extractions and two tissue samples [the cyt *b* sequences are from the NCBI Nucleotide database (GenBank)] were the same ones used for the cyt *b* analyses. *Lampetra fluviatilis*, *Lampetra pacifica*, and *Lethenteron ninae* from their type localities and *Lampetra aepyptera* and *Lampetra lanceolata* from near the type localities were included. The different coloration patterns of *Lampetra fluviatilis* and *Lethenteron zanandreai* were included.

Three soxD intron sequences of *Lethenteron camtschaticum* and two of *Lethenteron kessleri* from GenBank were used for the phylogenetic analyses (Table 3.2; Okada et al. 2010). The outgroup sequences of *Petromyzon marinus* for TAP2 and soxD introns were

obtained from the *Petromyzon marinus* genome database (http://www.ensembl.org/Petromyzon_marinus/Info/Index; Smith et al. 2013).

3.2.2 Primer design and the selection of sequencing primers

To amplify and select from several potential gene introns, primer pairs were designed from the adjacent exons of the potential introns. The primer pair (Table 3.3, 40SrE1-74F and 40SrE2-39R) for the first intron of 40S ribosomal protein subunit 23 (RPS23) was designed from the conserved exon regions in the alignment of the sea lamprey genome and the mRNA sequences of *Lethenteron camtschaticum* (GenBank Accession No.: DC611396) and Atlantic salmon *Salmo salar* L. (GenBank Accession No.: BT049811). The primer pair (Table 3.3, TAP2a-289F and TAP2b-15R) amplifying the TAP2 was designed from *Petromyzon marinus* sequence (AY171568, Uinuk-ool et al. 2003) and the mRNA sequences of Chinese hamster *Cricetulus griseus* (Milne-Edwards 1867) (XM003510258) and human *Homo sapiens* L. (AB045381). MASPE3-123F and MASPE4-52R for the third intron of Mannose-Binding Lectin-Associated Serine Protease A (MASP-A) were designed from *Lethenteron camtschaticum* MASP-A gene sequence (AB078894) and the mRNA of *Lethenteron camtschaticum* (AB009075) and pig *Sus scrofa* L. (GU810083). Sequences used above for these primer designs were from the GenBank. The primers (Table 3.3, soxD-F and soxD-R) amplifying the soxD intron were modified from the soxD-F and soxD-R primer pair used by Okada et al. (2010). The first eight bases at the 5’ end of Okada et al. (2010)’s soxD-F and the first three bases at the 5’ end of Okada et al. (2010)’s soxD-R were trimmed to lower the annealing temperature to below 65°C.

Polymerase chain reaction (PCR) was performed using one each of *Lethenteron camtschaticum*, *Lethenteron kessleri*, *Eudontomyzon danfordi*, and *Lampetra fluviatilis* samples with four primer pairs soxD-F - soxD-R, TAP2a-289F - TAP2b-15R, 40SrE1-74F - 40SrE2-39R and MASPE3-123F - MASPE4-52R to test the primer pairs. Each 30 µL PCR reaction contained 1X PCR buffer (Invitrogen) (20 mM Tris-HCl pH 8.4; 50 mM KCl), 2.5 mM MgCl2, 0.2 mM of each dNTP, 0.4 µM of each primer, and 0.02 U/µL of Go*Taq*® DNA polymerase (Promega). Reactions were initially denatured at 96 °C for 3 min. Amplifications were carried out in 30 cycles: denaturation at 96 °C for 30 sec, primer annealing at 60 °C, 58 °C, and 55 °C for 30 sec for 10 cycles each, extension at 72 °C for 2 min, and additional extension at 72 °C for 5 min. The electrophoresis image is shown in Fig. 3.1. Obvious double bands were found in *Lampetra fluviatilis* with both RPS23 and MASP-A primer pairs. No multiple bands were observed with the soxD and TAP2 introns primer pairs. The length of the soxD products varied with species, which was presumably due to the length of indels in the introns of different species. The PCR for three samples did not work with the TAP2 primer pairs. PCR conditions were subsequently optimized for the soxD and TAP2 primer pairs (see below), while the RPS23 and MASP-A primer pairs were not used further.

To shorten the PCR products and improve the sequencing quality of the TAP2 intron, five internal primers (TAP2intrF1, TAP2intrF2 TAP2intrF3, TAP2intrR1 and TAP2intrR2) were designed based on sequences initially obtained and then cloned (see Section 3.2.3) from *Eudontomyzon*, *Lampetra* and *Lethenteron*. The forward primers TAP2intrF1 and TAP2inrF2 were paired with TAP2b-15R. The reverse primers TAP2intrR1, TAP2intrR2, and TAP2intrR3 were paired with TAP2a-289F (Table 3.3). TAP2intrF2, TAP2intrF3, and

TAP2intrR2 did not work with many DNA samples from any of the three genera. TAP2intrF1 - TAP2b-15R generated a short PCR fragment approximately 400 bp in length which did not overlap with the fragment amplified with TAP2a-289F - TAP2intrR1. TAP2intrR1, TAP2a-289F and TAP2b-15R were thus used as sequencing primers for the TAP2 intron in all additional samples.

The effort to sequence the soxD intron with primers soxD-F and soxD-R failed in all the *Lethenteron* samples excluding *Lethenteron zanandreai* and *Lethenteron ninae* which were in the Atlantic *Lampetra* rather than the *Lethenteron* clade in the cyt *b* tree (Section 2.3.1 and 2.3.2). Due to the limited time for the Master's project, internal primers for the soxD intron have not been designed. Thus, soxD-F and soxD-R were used as sequencing primers for the soxD intron.

3.2.3 DNA extraction, amplification, and sequencing

Selected 19 DNA extractions from *Eudontomyzon*, *Lampetra* and *Lethenteron* used in the cyt *b* analyses were also used for nuclear gene introns, including two *Eudontomyzon danfordi*, two *Eudontomyzon mariae* (one typical *Eudontomyzon mariae* and one synonym *Eudontomyzon vladykovi*), four *Lampetra fluviatilis* (including those with different coloration patterns), two *Lethenteron appendix*, one *Lethenteron camtschaticum*, one *Lethenteron kessleri*, one *Lethenteron reissneri*, three *Lethenteron ninae* and three *Lethenteron zanandreai* (with different coloration patterns). DNA from some samples (one *Lampetra lanceolata*, one *Lampetra aepyptera*, two *Lampetra pacifica*, one *Lampetra ayresii*, and one *Lampetra hubbsi*) was not extracted for cyt *b* gene sequencing since the cyt *b* sequence from these species were retrieved from the

GenBank. The DNA of these samples was extracted from tissue samples using the DNeasy Blood and Tissue Kit (Qiagen) and the same protocol as that for the cyt *b* analyses (see Section 2.2.2). TAP2 intron was sequenced in one *Eudontomyzon danfordi*, one *Eudontomyzon mariae*, one *Lampetra aepyptera*, one *Lampetra ayresii*, three *Lampetra fluviatilis*, one *Lampetra hubbsi*, one *Lampetra lanceolata*, two *Lampetra pacifica*, two *Lethenteron appendix*, one *Lethenteron camtschaticum*, one *Lethenteron kessleri*, two *Lethenteron ninae*, one *Lethenteron reissneri* and three *Lethenteron zanandreai* (21 specimens in total). SoxD intron was sequenced in one *Eudontomyzon danfordi*, two *Eudontomyzon mariae* (including one *Eudontomyzon vladykovi*), one *Lampetra aepyptera*, one *Lampetra fluviatilis*, one *Lampetra pacifica*, two *Lethenteron ninae* and two *Lethenteron zanandreai* (10 specimens in total).

Based on the conditions tested above (Section 3.2.2), PCR was conducted with primer pairs TAP2a-289F - TAP2b-15R and soxD-F - soxD-R to amplify the complete TAP2 and soxD introns. Each 30 µL PCR reaction contained 1X PCR buffer (Invitrogen) (20 mM Tris-HCl pH 8.4; 50 mM KCl), 5.0 mM MgCl2, 0.2 mM of each dNTP, 0.4 µM of each primer, and 0.02 U/µL of Go*Taq*® DNA polymerase (Promega). Reactions were initially denatured at 96 °C for 8 min. Amplifications were carried out in 40 cycles: denaturation at 96 °C for 30 sec, primer annealing at 60 °C and 58 °C for 30 sec for 10 cycles each, and at 55 °C for 30 sec for 20 cycles, extension at 72 °C for 100 sec, and additional extension at 72 °C for 7 min.

To obtain high quality TAP2 intron sequences and design internal primers from them (Section 3.2.2), the TAP2 intron PCR products from one each of *Eudontomyzon danfordi*, *Eudontomyzon mariae*, *Lampetra fluviatilis*, *Lethenteron camtschaticum*, *Lethenteron*

appendix and *Lethenteron kessleri* were purified with 50% isopropanol and 0.5 M sodium acetate and cloned using the CloneJET PCR Cloning Kit (Fermentas) following the sticky-end cloning protocol. Transformation was conducted with DH5α™ Cells (Life Technologies) following the manufacturer's instructions. Selected colonies were amplified through PCR with the primer pair pJETforward - pJETreverse provided in the CloneJET PCR Cloning Kit (Fermentas). Each 30 µL PCR reaction contained 1X PCR buffer (Invitrogen) (20 mM Tris-HCl pH 8.4; 50 mM KCl), 2.5 mM MgCl2, 0.2 mM of each dNTP, 0.4 µM of each primer, and 0.02 U/µL of *Taq* DNA Polymerase (Invitrogen). Reactions were initially denatured at 95 °C for 8 min. Amplifications were carried out in 30 cycles: denaturation at 95 °C for 30 sec, primer annealing at 60 °C for 30 sec, extension at 72 °C for 2 min, and additional extension at 72 °C for 3 min. The products were purified with 50% isopropanol and 0.5 M sodium acetate and sequenced with primers pJETforward and pJETreverse in the Docker laboratory using an Applied Biosystems 3500 Genetic Analyzer (Applied Biosystems Inc.). The internal primer TAP2intrR1 (see Section 3.2.2 and Table 3.3) was designed from the sequences and paired with TAP2a-289F to amplify about 700 bp of partial TAP2 intron for the whole sample set. The PCR condition was the same as that for TAP2a-289F - TAP2b-15R and soxD-F - soxD-R.

The PCR products of TAP2a-289F - TAP2b-15R, TAP2a-289F - TAP2intrR1 and soxD-F - soxD-R were purified with 50% isopropanol and 0.5M sodium acetate, and sequenced in the Docker laboratory as above. TAP2a-289F, TAP2b-15R, TAP2intrR1, soxD-F, and soxD-R were used as sequencing primers. The overlapping sequences generated by TAP2a-289F, TAP2b-15R, and TAP2intrR1 were assembled into

approximately 947 bp (the length of aligned matrix) of TAP2 intron and used for the phylogenetic analyses. The soxD sequences from the two primers (soxD-F sequences were 700-800 bp approximately; soxD-R sequences were approximately 120 bp) did not overlap due to the length of the whole PCR fragment (above 1100 bp). Furthermore, because the soxD-R sequences were of low quality, only approximately 400 bp of the soxD-F sequences were used for the phylogenetic analyses.

In another attempt to generate concentrated PCR products with primer pairs TAP2a-289F - TAP2b-15R and soxD-F - soxD-R, and obtain clear sequences of TAP2 and soxD introns, the Phusion® High-Fidelity DNA Polymerase (New England BioLabs Inc.) was used in PCR. Each 20 μL PCR reaction contained 1X Phusion® HF Buffer (New England BioLabs Inc.) containing 1.5 mM MgCl2, 0.2 mM of each dNTP, 0.5 μM of each primer, and 0.02 U/μL of Phusion® High-Fidelity DNA Polymerase (New England BioLabs Inc.). Reactions were initially denatured at 98 °C for 30 sec. Amplifications were carried out in 30 cycles: denaturation at 98 °C for 5 sec, primer annealing at 67 °C for 20 sec, extension at 72 °C for 2 min, and additional extension at 72 °C for 5 min. Multiple bands were found in the electrophoresis in all samples.

Another effort to improve the quality of the sequences was to do gel extractions to the PCR products with multiple bands instead of the isopropanol precipitation before sequencing with the E.Z.N.A. Gel Extraction Kit (Omega Biotek) following the instructions. However, the sequencing failed because of the low amount of the DNA obtained from the gel extractions. The clean-up of the PCR products prior to the sequencing was also tried with 1.5 U/μL Exonuclease (Exo I, Thermo Scientific) and 0.15 U/μL FastAP™ Thermosensitive Alkaline Phosphatase (Thermo Scientific). However,

the electrophoresis showed brightness around the wells and no bands. The follow-up sequencing also failed.

3.2.4 Phylogenetic analyses

Maximum parsimony analyses and Bayesian analyses were conducted for each TAP2 intron, soxD intron and a combined dataset. The TAP2 intron dataset included 22 sequences from *Lethenteron camtschaticum*, *Lethenteron kessleri*, *Lethenteron appendix*, *Lethenteron reissneri*, *Lethenteron zanandreai*, *Lethenteron ninae*, *Eudontomyzon danfordi*, *Eudontomyzon mariae*, *Lampetra fluviatilis*, *Lampetra lanceolata*, *Lampetra aepyptera*, *Lampetra pacifica*, *Lampetra ayresii*, *Lampetra hubbsi*, and *Petromyzon marinus*. The soxD intron dataset included 16 sequences from *Lethenteron camtschaticum*, *Lethenteron kessleri*, *Lethenteron zanandreai*, *Lethenteron ninae*, *Eudontomyzon danfordi*, *Eudontomyzon mariae*, *Lampetra fluviatilis*, *Lampetra aepyptera*, *Lampetra pacifica*, and *Petromyzon marinus*. Among them, the *Lethenteron camtschaticum* and *Lethenteron kessleri* sequences were from GenBank. The *Petromyzon marinus* TAP2 and soxD introns sequences were from the sea lamprey genome database.

Sequences were aligned in MEGA 5.1 (Tamura et al. 2011) using MUSCLE with the option “align DNA”, and the indels in the TAP2 and soxD intron data matrices were subsequently coded at the end of each sequence using GapCoder (Young and Healy 2003). Gaps in the data matrices were treated as missing data in the phylogenetic analyses. The combined dataset combined 11 TAP2 intron sequences and 11 soxD intron sequences selected from the data matrices. *Lethenteron camtschaticum*, *Lethenteron kessleri*, *Lethenteron zanandreai*, *Lethenteron ninae*, *Eudontomyzon danfordi*, *Eudontomyzon*

mariae, *Lampetra fluviatilis*, *Lampetra aepyptera*, *Lampetra pacifica*, and *Petromyzon marinus* were included. The two gene introns from one each of *Lethenteron zanandreai*, *Lethenteron ninae*, *Eudontomyzon mariae*, *Lampetra aepyptera*, and *Lampetra pacifica* were sequenced from the same individual. In other cases, given the difficulties experienced with amplification and sequencing noted above (Section 3.2.3), two parts were from different individuals of the same species (Table 3.4).

For the TAP2 intron, soxD intron, and the combined datasets, the maximum parsimony trees were inferred using the same method as for the cyt *b* maximum parsimony tree (Section 2.2.3) but using 50% majority rule consensus trees instead of strict consensus trees of maximum parsimony trees (since the strict consensus only keeps the nodes with 100% consensus frequency, too much information will be discarded using strict consensus). *Petromyzon marinus* was used as the outgroup in all three datasets. The consensus frequencies and the bootstrap values were mapped onto the consensus trees.

For Bayesian analyses, MrModelTest 2.3 (Nylander 2004) was used to select the models for the three datasets with the Akaike Information Criterion (AIC). For each dataset, Bayesian analysis was run until the average standard deviation of split frequencies was below 0.01 with every 5,000 generations sampled in MrBayes 3.2.1 (Huelsenbeck and Ronquist 2001; Ronquist and Huelsenbeck 2003). The priors for the phylogenetic model were set as default. Stationarity of negative log-likelihood values was evaluated by plotting these values against generation. The fifty-percent majority rule consensus of posterior distribution of trees was inferred with the burnin period of 25% discarded. *Petromyzon marinus* was used as the outgroup in all analyses.

3.3 Results

3.3.1 TAP2 intron phylogenetic analyses

The length of the aligned partial TAP2 intron sequences was 947 bp including gaps and missing data. The numbers of gaps and missing data were listed in Table 3.5. GapCoder added 21 gap codes to the end of each sequence. Among the 968 characters, 119 were variable, and 33 were parsimony informative. Each independent run of the ratchet discovered 201 trees. Ten runs of the ratchet have discovered 2010 maximum parsimony trees. The length of the trees was 128. The 50% majority rule consensus tree of the 2010 most parsimony trees is shown in Fig 3.2.

Genus *Lethenteron* excluding *Lethenteron ninae* and *Lethenteron zanandreai* was monophyletic. This clade was referred to as Le following Section 2.3.1. Genus *Lampetra* plus *Lethenteron ninae* and *Lethenteron zanandreai* was not monophyletic, even if excluding the *Lampetra aepyptera* with 835/947 missing data. The Pacific *Lampetra* (PLa, following Section 2.3.1) was monophyletic, but Atlantic *Lampetra* (ALa) was not. Even without *Lampetra aepyptera*, the relationship between ALa (see Section 2.3.1) and *Eudontomyzon* was resolved in this tree. The clade combining ALa and *Eudontomyzon* was referred to as Eu-ALa. Clade Le (bootstrap support = 14%; consensus frequency = 54%) included *Lethenteron camtschaticum*, *Lethenteron appendix*, *Lethenteron kessleri* and *Lethenteron reissneri*; clade PLa (bootstrap support = 30%; consensus frequency = 100%) included *Lampetra ayresii*, *Lampetra hubbsi*, and *Lampetra pacifica*; clade Eu-ALa (bootstrap support < 5%; consensus frequency = 100%) included *Eudontomyzon danfordi*, *Eudontomyzon mariae*, *Lampetra fluviatilis*, *Lampetra lanceolata*, *Lethenteron ninae*, and *Lethenteron zanandreai*. Eu-ALa and Le were sister clades. Clade PLa was

sister to Eu-ALa plus Le. *Lampetra aepyptera* was sister to all these three clades.

Within the clade Eu-ALa, *Lethenteron ninae*, *Lampetra lanceolata* and *Lethenteron zanandreai* formed a clade. The bootstrap support for this clade was 24%, and the consensus frequency was 99%. *Lethenteron ninae* from Shakhe River, Russia, was sister to *Lampetra lanceolata* from Ykizdere Brook, Turkey (the only sample of this species), near the type locality, although the consensus frequency was only 80% and the bootstrap support was only 53%. This clade was sister to the *Lethenteron ninae* from Mzymta River, Russia, plus two *Lethenteron zanandreai* with different colorations. The other *Lethenteron zanandreai* (spotted) was sister to them. This clade of *Lethenteron ninae*, *Lampetra lanceolata*, and the relationships among *Lethenteron zanandreai*, *Eudontomyzon danfordi*, *Eudontomyzon mariae*, *Lampetra fluviatilis* from Neva River, Russia, and *Lampetra fluviatilis* from Syas' River, Russia were unresolved. Two *Lampetra fluviatilis* from Syas' River with different colorations formed a clade with the consensus frequency of 100% and the bootstrap support of 85%.

The data matrix including the gap codes for the Bayesian analysis was the same as that for the maximum parsimony analysis. MrModelTest 2.3 suggested GTR model (Tavaré 1986) with a gamma distribution shape parameter ($\Gamma = 0.6535$). The 50% majority rule consensus tree of 900 trees with the posterior probabilities is shown in Fig. 3.3.

In the Bayesian tree, *Lethenteron* excluding *Lethenteron ninae* and *Lethenteron zanandreai* (= the Le group in Fig. 3.2) was not monophyletic. The Eu-ALa and PLa groups (as in Fig. 3.2) were reciprocally monophyletic. The posterior probabilities of Eu-ALa and PLa clades were 0.98 and 0.52, respectively. The relationship between

Eudontomyzon and ALa (excluding *Lampetra aepyptera*) was unresolved. The PLa clade and *Lampetra aepyptera* (missing data = 835/947) from the Atlantic Ocean basin of North America formed a clade with the posterior probability of 0.6. The relationships among Le, PLa plus *Lampetra aepyptera*, and Eu-ALa were unresolved.

Two of three *Lethenteron zanandreai* (both are spotted), both two *Lethenteron ninae* and the only *Lampetra lanceolata* formed a clade with the probability of 0.61, while the relationships of this clade to the remaining one *Lethenteron zanandreai* (monotonous coloration), *Eudontomyzon danfordi*, *Eudontomyzon mariae* and two *Lampetra fluviatilis* clades were unresolved. The two *Lampetra fluviatilis* from Syas' River, Russia, with different colorations formed a clade with the probability of 0.89. The *Lethenteron ninae* from Shakhe River, Russia, and the only *Lampetra lanceolata* formed a clade with the probability of 0.77.

3.3.2 SoxD intron phylogenetic analyses

The length of aligned partial soxD intron sequences was 806 bp. The numbers of gaps and missing data for each sequence are listed in Table 3.6. Seven gap codes were added to the end of each sequence. Among the 813 characters, 38 were variable, and nine were parsimony informative. Each independent run of the ratchet discovered 201 trees. Ten runs of the ratchet have discovered 2010 maximum parsimony trees. The length of the trees was 38. The 50% majority rule consensus tree of the 2010 trees is shown in Fig 3.4.

Lethenteron excluding *Lethenteron ninae* and *Lethenteron zanandreai* formed a clade (Le; bootstrap support = 78%; consensus frequency = 100%). The Atlantic

Lampetra including *Lampetra aepyptera*, *Lethenteron ninae* and *Lethenteron zanandreai* formed a clade with *Eudontomyzon* (Eu-ALa; bootstrap support = 70%; consensus frequency = 100%). The relationship between *Eudontomyzon* and ALa was unresolved. The only Pacific *Lampetra* species for which SoxD intron data were available, *Lampetra pacifica*, was not included in clades Le and Eu-ALa. The relationship among Le, Eu-ALa and *Lampetra pacifica* was unresolved.

In clade Eu-ALa, the two *Lethenteron zanandreai* from the same locality (with different colorations) were monophyletic (bootstrap support = 60%; consensus frequency = 100%), while the two *Lethenteron ninae* from the same locality were not monophyletic. The relationships among two *Lethenteron ninae* and the only one *Lampetra fluviatilis* from the type locality were unresolved, with only 8% bootstrap support and 70% consensus frequency for their clade. These two clades were sister to each other (bootstrap support = 55%; consensus frequency = 100%), and together, they formed a clade with *Lampetra aepyptera*, *Eudontomyzon mariae* from Stryy River, Ukraine, and *Eudontomyzon danfordi* plus *Eudontomyzon mariae* (*Eudontomyzon vladykovi*) from Krka River, Slovenia. *Eudontomyzon mariae* (including *Eudontomyzon vladykovi*) was not monophyletic. The bootstrap support for the clade of *Eudontomyzon danfordi* plus *Eudontomyzon mariae* (*Eudontomyzon vladykovi*) from Krka River, Slovenia, was 64%, and the consensus frequency was 100%.

The data matrix including the gap codes for the Bayesian analysis was the same as that for the maximum parsimony analysis. MrModelTest 2.3 suggested HKY model (Hasegawa et al. 1985) for the soxD dataset. The 50% majority rule consensus tree of 1200 trees with the posterior probabilities is shown in Fig. 3.5.

The topology of the Bayesian tree was the same as the maximum parsimony tree (Fig. 3.4), except that the two *Lethenteron ninae* and one *Lampetra fluviatilis* did not form a clade and the relationships among these three taxa and *Lethenteron zanandreai* were unresolved. The main clades Le (probability = 0.98) and Eu-ALa (0.95) were also discovered in the Bayesian tree.

3.3.3 Combined data phylogenetic analyses

The combined dataset combined 947 bp aligned TAP2 intron plus 19 gap codes and 806 bp aligned soxD intron plus six gap codes. Among the total 1778 characters, 132 characters were variable, and 21 of them were parsimony informative. Each independent run of the ratchet discovered 201 trees. Ten runs of the ratchet have discovered 2010 maximum parsimony trees. The length of the trees was 138. The 50% majority rule consensus of 2010 trees is shown in Fig 3.6.

The Le group was monophyletic (bootstrap support = 85%; consensus frequency = 100%). *Eudontomyzon* and ALa formed a clade (Eu-ALa; bootstrap support = 72%; consensus frequency =100%) which was sister to *Lampetra aepyptera* with the bootstrap support of 67% and consensus frequency of 100%. Le was sister to the Eu-ALa plus *Lampetra aepyptera* clade with the bootstrap support of 51% and the consensus frequency of 100%. The only PLa species included, *Lampetra pacifica*, was sister to Le plus Eu-ALa and *Lampetra aepyptera*.

In clade Eu-ALa, *Eudontomyzon* was polyphyletic. *Lethenteron ninae* and *Lethenteron zanandreai* formed a clade (bootstrap support = 60%; consensus frequency = 100%). *Lampetra fluviatilis* was sister to this clade. *Lampetra fluviatilis*, *Lethenteron*

ninae and *Lethenteron zanandreai* formed a clade with 68% bootstrap support and 100% consensus frequency.

The data matrix including the gap codes for the Bayesian analysis was the same as that for the maximum parsimony analysis. MrModelTest 2.3 suggested HKY model for the combined dataset. The 50% majority rule consensus tree of 750 trees with the posterior probabilities is shown in Fig. 3.7.

The topology of the combined Bayesian tree was the same as the combined maximum parsimony tree (Fig. 3.6). The probabilities of clade Le and Eu-ALa were 1.00 and 0.98. The probability of clade Eu-ALa plus *Lampetra aepyptera* was 1.00. Le plus Eu-ALa and *Lampetra aepyptera* had the probability of 0.76.

3.4 Discussion

3.4.1 Comparisons among phylogenies using different nuclear datasets

This chapter inferred six trees with three different datasets and two different methods. The phylogenetic relationships among genera recovered using each dataset are different. Relationships among main clades are different between two algorithms using TAP2 intron and are similar using soxD intron and the combined dataset. However, some clades were recovered in all trees, such as Pacific *Lampetra* (PLa, with only one individual of *Lampetra pacifica* included in soxD and combined dataset), and *Eudontomyzon* plus Atlantic *Lampetra* (Eu-ALa, with or without *Lampetra aepyptera*).

The Le group (as in Section 2.4.1, i.e., without *Lethenteron zanandreai* and *Lethenteron ninae*) is monophyletic in all nuclear trees except the TAP2 Bayesian tree. The bootstrap support (BS) and consensus frequency (CF) for the Le clade in the TAP2

maximum parsimony tree are low (BS = 14%; CF = 54%). The supports for the Le clade in the other trees are higher: BS = 78%, CF = 100% for the soxD maximum parsimony tree; posterior probability (P) = 0.98 for the soxD Bayesian tree; BS = 85%, CF = 100 % for the combined maximum parsimony tree; P = 1 for the combined Bayesian tree. Le separates into two clades in the TAP2 trees. *Lethenteron camtschaticum* and *Lethenteron appendix* from the Lake Michigan basin (i.e., from the Great Lakes) are in one clade (BS = 85%; CF = 100%; P = 1) while *Lethenteron kessleri*, *Lethenteron reissneri* and *Lethenteron appendix* from Maine (i.e., in the Atlantic Ocean basin) are in the other clade (BS = 62%; CF = 100%; P = 0.93). These two clades are sister taxa in the TAP2 maximum parsimony tree and their relationship to PLa plus *Lampetra aepyptera* and Eu-ALa) were unresolved in the TAP2 Bayesian tree. Since the single gene tree may not estimate the species tree (Pollard et al. 2006), the lack of monophyly of Le in the TAP2 Bayesian tree may reflect incomplete lineage sorting (i.e., whereby shared ancestral polymorphisms have not progressed to reciprocal monophyly) in the TAP gene rather than the true species tree. Since all other trees support Le as a monophyletic clade, Le is likely monophyletic in the species tree.

Another difference among different datasets is the placement of *Lampetra aepyptera*. In the TAP2 maximum parsimony tree, *Lampetra aepyptera* is sister to Eu-ALa plus Le plus PLa (BS < 5%; CF = 100%). In the TAP2 Bayesian tree *Lampetra aepyptera* is sister to PLa (P = 0.6). In the two soxD trees, *Lampetra aepyptera* is included in Eu-ALa clade, with the relationships to the typical *Eudontomyzon mariae* from Stryy River, Ukraine, *Eudontomyzon danfordi* plus *Eudontomyzon vladykovi* (a synonym of *Eudontomyzon mariae*) from the Krka River drainage, Slovenia, and the ALa group (*Lampetra fluviatilis*,

Lethenteron zanandreai and *Lethenteron ninae*) unresolved. In the combined trees, *Lampetra aepyptera* is sister to Eu-ALa.

Among the three datasets, the combined dataset contains the most variable characters (132), while the TAP2 dataset contains the most parsimony informative characters (33). The soxD dataset contains the least variable characters and parsimony informative characters. Thus the resolution of soxD trees is relatively low, i.e., the relationships among the main clades PLa, Le, Eu-ALa (with *Lampetra aepyptera*) are not resolved. The relationship between *Lampetra aepyptera* and other Eu-ALa species is not clear in the soxD trees, since the information in soxD gene is not sufficient for resolving the branch order among *Lampetra aepyptera* and the other three clades within Eu-ALa. Thus, the results of soxD and the combined dataset are not contradictory. The two TAP2 trees, however, do contradict the other trees and also contradict each other. However, the support for the clade of PLa, Le and Eu-ALa or the probability for that of *Lampetra aepyptera* plus PLa was low. Furthermore, the *Lampetra aepyptera* TAP2 intron sequence had 835 missing data out of 947 total characters. Thus, the placement of *Lampetra aepyptera* in the TAP2 trees was not reliable. *Lampetra aepyptera* may be sister to or belong within Eu-ALa.

3.4.2 Division of genera

Chapter 2 recovered four main clades, Le, PLa, Eu, and ALa, based on cyt *b* gene data. *Okkelbergia* (*Lampetra aepyptera*) may be distinct from these clades or sister to ALa (see Section 2.4.1). Using the nuclear datasets, however, clades Eu and ALa comprised a single clade and are not reciprocally monophyletic. Thus this clade is called

Eu-ALa, and includes *Eudontomyzon danfordi*, *Eudontomyzon mariae* (including *Eudontomyzon vladykovi*), *Lampetra fluviatilis*, *Lampetra lanceolata*, *Lethenteron zanandreai* and *Lethenteron ninae*. Chapter 2 and previous molecular studies (Blank et al. 2008; Lang et al. 2009) suggested Eu and ALa plus *Okkelbergia* as sister taxa. In the nuclear gene trees, the relationships among several taxa in the Eu-ALa clade were unresolved. That means the relationship between Eu and ALa is close and the resolution of the nuclear gene trees may not be high enough to resolve it. In the soxD and the combined trees, ALa is actually monophyletic. Thus, I would not suggest merging *Eudontomyzon* with Atlantic *Lampetra* before nuclear gene trees of higher resolution are inferred.

Pacific *Lampetra* and Atlantic *Lampetra* are distinct from each other in the nuclear gene trees. Although the soxD and combined dataset contain only one individual Pacific *Lampetra* (*Lampetra pacifica*), it is apparent that PLa is not sister to ALa. Thus, the morphological phylogeny by Gill et al. (2003) is not supported by nuclear (this chapter) or mitochondrial (Docker et al. 1999; Blank et al. 2008; Lang et al. 2009; Chapter 2) gene phylogenies. Pacific *Lampetra* should thus be a distinct genus from Atlantic *Lampetra*. *Lampetra ayresii*, *Lampetra hubbsi*, and *Lampetra pacifica* are included in Pacific *Lampetra* based on the nuclear gene sequence data. It is notable that *Lampetra hubbsi* is within the clade of PLa, in agreement with the cyt *b* trees (Docker et al. 1999; Lang et al. 2009; Chapter 2). It was initially described as *Entosphenus hubbsi* (Vladykov and Kott 1976), and its placement in *Lampetra* was only officially recognized by the American Fisheries Society recently (Page et al. 2013). The phylogeny based on nuclear gene sequence provided in this chapter supports this.

Clade Le is recovered in five of the six trees. Although TAP2 trees suggest Le as two groups, *Lethenteron camtschaticum*, *Lethenteron appendix*, *Lethenteron reissneri* and *Lethenteron kessleri* are closely related based on the cyt *b* dataset. The separation of the groups within *Lethenteron* is not supported by other trees. *Lethenteron camtschaticum* and *Lethenteron kessleri* are closely related based on soxD intron (Okada et al. 2010), while they are in separate clades in the TAP2 trees. Interestingly, the Kimura's two-parameter (K2P) distance of the TAP2 intron between these two clades is 2.40%, larger than that between Le and Eu-ALa (2.05%), and similar to that between Le to PLa (2.65%). The TAP2 intron is particularly variable within Le (K2P = 2.02%) compared with the situation within PLa (K2P = 1.46%) or Eu-ALa (K2P = 0.64%). This phenomenon may be related to the diversity of environment in the wide geographic range of the genera. These four species are always in genus or subgenus *Lethenteron* in the morphological taxonomy (e.g. Vladykov and Kott 1979; Potter 1980; Renaud 2011), which is supported by Lang et al. (2009) using cyt *b* data. Thus, the preponderance of evidence suggests that *Lethenteron camtschaticum*, *Lethenteron appendix*, *Lethenteron reissneri* and *Lethenteron kessleri* should be in one genus in spite of the TAP2 Bayesian result and the relatively large genetic distance between the clades. Given the apparent variability in TAP2 sequence, even among closely related taxa (and potentially among individuals), this intron will need to be sequenced in more individuals to determine if the variability has taxonomic value.

It is uncertain whether *Lampetra aepyptera* is in Eu-ALa. Since the *Lampetra aepyptera* in TAP2 trees has too many missing data, the placement suggested by the TAP2 trees are not considered. It may be appropriate to put *Lampetra aepyptera* in

Okkelbergia (Hubbs and Potter 1971; Bailey 1980) rather than put it in ALa, since ALa plus *Lampetra aepyptera* are not always monophyletic, while ALa (excluding *Lampetra aepyptera*) and *Lampetra aepyptera* are reciprocally monophyletic in the soxD and combined trees. Also, in the cyt *b* analysis (Chapter 2), the removal of *Lampetra aepyptera* from ALa would not cause ALa to be not monophyletic. The ancestor of *Lampetra aepyptera* is not known but possibly a *Lampetra fluviatilis*-type one (Docker et al. 1999). More *Lampetra aepyptera* sequences of more genes and different localities will be useful to resolve the status of this non-parasitic species. Using mitochondrial ND3 and control region sequences, Martin and White (2008) showed there to be considerable intraspecific variation within this species; broader geographic coverage of this species is required in the cyt *b* and nuclear gene phylogenies.

3.4.3 Relationships among genera

Chapter 2 discovered that Eu and ALa are most closely related to each other among the four groups, Eu, ALa, PLa and Le. The close relationship is supported by the nuclear gene sequence results. These two groups are in one clade Eu-ALa and are not reciprocally monophyletic. In the soxD and combined trees, ALa (excluding *Lampetra aepyptera*) is monophyletic while Eu is not. In the TAP2 trees, both are not monophyletic. Since the relationships among species in Eu-ALa were unresolved in all the nuclear gene trees, nuclear gene trees with higher resolution may recover Eu and ALa (excluding *Lampetra aepyptera*) as reciprocally monophyletic. Thus, the nuclear gene results do not necessarily contradict the previous mtDNA results (Blank et al. 2008; Lang et al. 2009; Chapter 2). The morphological result that ALa and PLa were sister taxa in one genus

Lampetra (Gill et al. 2003) was not supported using molecular data from, presumably, three independent loci: mtDNA and two nuclear gene introns. Thus, the similarity in the dentition pattern of ALa and PLa (Gill et al. 2003) is not the result of recent divergence from a common ancestor with the same derived dentition pattern (synapomorphy), but rather represents retention of an ancestral dentition pattern (i.e., a symplesiomorphy) or phenotypic convergence (i.e., homoplasy; Patterson 1988; Wake 1991). The genetic basis of the possible convergence in dentition characters is an interesting topic for future studies.

In the combined trees, *Lampetra aepyptera* is sister to Eu-ALa and the Eu-ALa clade is supported with BS = 72%, CF = 100%, and P = 0.98. In the TAP2 maximum parsimony tree, Le is sister to Eu-ALa while *Lampetra aepyptera* with many missing data is distinct from other groups. *Lampetra aepyptera* is in the Eu-ALa clade in the soxD trees. Thus, *Lampetra aepyptera* should be sister to Eu-ALa or in the clade Eu-ALa. The previous mtDNA results (Docker et al. 1999; Blank et al. 2008; Lang et al. 2009; the cyt *b* maximum parsimony tree in Chapter 2) suggest *Lampetra aepyptera* is sister to ALa. Since the placement of *Lampetra aepyptera* is different using different datasets, the relationships among *Lampetra aepyptera*, *Eudontomyzon* and Atlantic *Lampetra* are uncertain. These three taxa are closer to each other than to Le and PLa based on cyt *b* (Blank et al. 2008; Lang et al. 2009; Chapter 2), soxD and the combined dataset.

In the soxD trees and the TAP2 Bayesian tree, the relationships among Eu-ALa, Le, and PLa were not resolved. The combined trees and the TAP2 maximum parsimony tree all recovered Le as sister to Eu-ALa (with or without *Lampetra aepyptera*) while PLa was sister to them, although not well supported. The mtDNA studies reported different

results on the branch order of the Le and PLa groups. Some supported PLa as the outgroup (Docker et al. 1999; Lang et al. 2009) while others supported Le as the outgroup (Blank et al. 2008; Chapter 2). As discussed in Section 2.4.2, neither were well supported. Thus, the relationship between Le and PLa is uncertain since different genes or different phylogenetic methods provide different answers. A dataset combining more unlinked genes widely spread in the whole genome may obtain a well supported relationship between them.

In summary, *Eudontomyzon*, Atlantic *Lampetra* and *Lampetra aepyptera* are a clade based on molecular data of mtDNA and nuclear DNA, while Le and PLa are the outgroups of them. The detailed relationship among Eu, ALa and *Lampetra aepyptera*, and the relationship between Le and PLa, will remain unresolved until a well supported tree is inferred from a dataset combining several unlinked genes.

3.4.4 Placement of species

Lethenteron zanandreai and *Lethenteron ninae* were placed in ALa in Chapter 2. These two species are in the clade Eu-ALa in all the nuclear gene trees, and form a clade with *Lampetra fluviatilis* in the soxD and combined trees. The agreement between mtDNA and nuclear DNA data suggests the origin of these two species being a *Lampetra fluviatilis*-type ancestor. *Lethenteron zanandreai* should be put in Atlantic *Lampetra* rather than *Lethenteron* based on the molecular studies using the cyt *b* gene (Docker et al. 1999; Caputo et al. 2009; Lang et al. 2009; Chapter 2) as well as the nuclear gene (this chapter). The view of some morphological studies (Vladykov 1955; Vladykov and Kott 1979) that put this species in *Lampetra* is supported while the *Lethenteron* origin (Hubbs

and Potter 1971; Potter 1980; Renaud 2011) is not supported. The core issue in morphology is whether *Lethenteron zanandreai* possesses the posterial circumoral teeth (posterials; see Section 1.2; Table 1.2). In the original description, Vladykov (1955) noted no posterials (hence his placement of this species into *Lampetra*), but Renaud (2011) examined two paratypes and discovered that both possessed posterials. Note, however, that Renaud (2011) also observed exolateral teeth (exolaterals) in one of these paratypes, although *Lethenteron* "by definition" should not possess exolaterals; *Lampetra* does not possess exolaterals either (Table 1.2). Renaud (2011) stated that it "would seem therefore advisable to provisionally place this species in *Lethenteron*," but it now appears that dentition is not always a reliable indicator of phylogeny. The characters of posterials and/or exolaterals show intraspecific variation in some species (above), are differently defined by different morphologists (Potter 1980), or have evolved independently in *Lethenteron zanandreai* and other *Lethenteron* species. As with the cyt *b* gene trees, the nuclear sequence data provide no evidence supporting the different coloration patterns in *Lethenteron zanandreai* or *Lampetra fluviatilis* as distinct taxa.

Lethenteron ninae was distinguished from *Eudontomyzon mariae* and *Lampetra lanceolata* and described as a *Lethenteron* species by Naseka et al. (2009). However, its placement in *Lethenteron* is not supported by either the cyt *b* or the nuclear gene data. As with *Lethenteron zanandreai*, therefore, some diagnostic characters distinguishing between *Lethenteron* and *Lampetra* (e.g., the presence or absence of posterials and the pigmentation), should be questioned, since convergence may result in similarities in these characters. Another problem is that the dentition in newly metamorphosed specimens may differ from that in sexually mature specimens (Naseka et al. 2009). Thus the

difference in these diagnostic characters may be subtle in one stage but become obvious in another. The original description of *Lethenteron ninae* is based on newly metamorphosed specimens (Naseka et al. 2009). Morphological studies on spawning specimens of *Lethenteron ninae* may be useful for resolving the placement of this species.

Lethenteron ninae was compared with only one paratype of *Lampetra lanceolata* (Naseka et al. 2009). These two species are not reciprocally monophyletic in the cyt *b* trees (Chapter 2) using *Lampetra lanceolata* collected far from the type locality, as well as in the TAP2 trees using *Lampetra lanceolata* from near the type locality (sequencing for the cyt *b* gene failed in this sample). Since the resolution of the TAP2 trees is not high enough to resolve the species level relationships (none of *Lampetra fluviatilis*, *Lethenteron zanandreai*, *Lethenteron ninae* is monophyletic), I do not suggest treating *Lethenteron ninae* as a synonym of *Lampetra lanceolata* at this point, but suggest that this be further investigated. The close relationship among *Lethenteron ninae*, *Lampetra lanceolata* and *Lethenteron zanandreai* recovered using cyt *b* and nuclear genes should be considered when resolving the placement of *Lethenteron ninae*.

3.4.5 Conclusions

As the first study using nuclear genes to resolve the genetic relationships among *Eudontomyzon*, *Lampetra*, and *Lethenteron*, this chapter resolved a number of questions. Most notably, Pacific *Lampetra* and Atlantic *Lampetra* were shown to be distinct from each other (supporting previous studies using mitochondrial DNA sequence; e.g., Docker et al. 1999; Lang et al. 2009; Chapter 2). They should thus be separated into two genera,

despite their high morphological similarity (Gill et al. 2003). Furthermore, *Lampetra hubbsi*, despite being originally placed in the genus *Entosphenus* based on dentition (Vladykov and Kott 1976), is part of the Pacific *Lampetra* clade, and *Lethenteron zanandreai* and *Lethenteron ninae* are part of the Atlantic *Lampetra* clade. It thus appears that dentition is not always a reliable indicator of phylogeny. Furthermore, Atlantic *Lampetra* and *Lampetra aepyptera* were more closely related to *Eudontomyzon* than to Pacific *Lampetra* and *Lethenteron*. Although placement of *Lampetra aepyptera* was uncertain with the current dataset, this study was consistent with the earlier placement of *Lampetra aepyptera* into a distinct genus (*Okkelbergia*), based on its highly degenerate dentition (Hubbs and Potter 1971).

Some additional interesting questions need more information to resolve, such as the relationships among *Eudontomyzon*, *Lampetra aepyptera*, and Atlantic *Lampetra*, the relationships among *Lethenteron zanandreai*, *Lethenteron ninae*, and *Lampetra lanceolata*, and whether Pacific *Lampetra* or *Lethenteron* is more basal in the species tree. With the recently sequenced *Petromyzon marinus* genome, it is hopeful that other nuclear markers for lampreys will soon be available to address these questions.

3.5 References

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3.6 Tables and Figures

Table 3.1 List of tissue samples for the TAP2 and soxD introns analyses. "(?) " after the species name means the identification of the specimen is uncertain. If not indicated, the specimens are all adults. Samples of *Lampetra fluviatilis* and *Lethenteron zanandreai* include populations with different colorations from the same location; the color features are indicated after the species names for these samples. "(T)" after the country name means the collection locality is the type locality and "(NT)" means near the type locality. The GenBank accession numbers of the cyt *b* sequences from the same tissue samples are after the species names if applicable. All the samples are used in the cyt *b* analyses (see Section 2.2, Table 2.1 and 2.2) except ones with an "[a]" after the species names or the GenBank accession numbers.

Species	Year of collection	Basin	Stream	Country	Latitude	Longitude	Collector(s)	Individuals used for TAP2 intron	Individuals used for soxD intron
Eudontomyzon danfordi	2010	Black Sea basin	Borzhava River, Tissa River drainage, Danube River system	Ukraine	48°29'20.87"N	23°13'13.11"E	Talabishka E.	1	
Eudontomyzon danfordi	2003	Mediterranean Sea basin	Lehotsky Brook at Muránska Dlhá Lúka, Muránska planina National Park	Slovakia	48°47′11″N	20°01′47″E			1
Eudontomyzon mariae	2011	Black Sea basin	Stryy River at Verkne Vysotskoye, Dniester River system	Ukraine	48°57'14.90"N	23° 4'9.55"E	Naseka A. M., Talabishka E.	1	1
*Eudontomyzon vladykovi**	2009	Black Sea basin	Stream, Krka River drainage, Sava River drainage, Danube River system	Slovenia (T)	45°49'4.49"N	15°19'57.01"E	Naseka A. M., Kapla A.		1
Lampetra aepyptera [a] (metamorphosing stage)		Mississippi River basin, Atlantic Ocean basin	Hocking River, Ohio River system	USA (NT)	39°12'7"N	81°46'49"W	White M. M.	1	1

Table 3.1 Continued.

Species	Year of collection	Basin	Stream	Country	Latitude	Longitude	Collector(s)	Individuals used for TAP2 intron	Individuals used for soxD intron
Lampetra ayresii (GU120855 [a])		Pacific Ocean basin	Feather, Sacramento, CA	USA	38°33'24"N	121°21'37"W	Reid S., Goodman D.	1	
Lampetra fluviatilis	2007	Baltic Sea basin	Neva River, from a shop, St. Petersburg	Russia (T)	59°56'58.00"N	30°19'54.42"E	Naseka A. M.	1	1 (Different individuals)
Lampetra fluviatilis (black)	2010	Baltic Sea basin, Ladoga Lake basin	Syas' River at Kolchanovo	Russia	60°1'6.65"N	32°35'2.14"E	Naseka A. M.	1	
Lampetra fluviatilis (grey)	2010	Baltic Sea basin, Ladoga Lake basin	Syas' River at Kolchanovo	Russia	60°1'6.65"N	32°35'2.14"E	Naseka A. M.	1	
Lampetra hubbsi (GU120869)		Pacific Ocean basin	Merced River, Sacramento, CA	USA	37°23'48"N	120°48'10"W	Reid S., Goodman D.	1	
Lampetra lanceolata [a]	2003	Black Sea basin	Ykizdere Brook	Turkey (NT)	40°46'46"N	40°33'29"E		1	
Lampetra pacifica (GU120799, GU120800 [a])		Pacific Ocean basin	N. Fork Reservoir, Clackamas, OR	USA(T)	45°14'36"N	122°16'49"W	Boguski D. A., Reid S.	2	1
Lethenteron appendix		Atlantic Ocean basin	Maine	USA	45°13'15"N	69°18'46"W		1	
Lethenteron appendix	2011	Great Lakes basin, Lake Michigan basin	Pigeon River at Sturgeon Valley Road, Cheboygan, Michigan	USA	45°16'2"N	86°12'18"W		1	
Lethenteron camtschaticum	2011	Pacific Ocean basin	Stream without name, Ussuri Bay, Sea of Japan, Vladivostok area	Russia	43°11'43.93"N	132° 6'47.21"E	Naseka A. M., Shedko M.	1	

Table 3.1 Continued.

Species	Year of collection	Basin	Stream	Country	Latitude	Longitude	Collector(s)	Individuals used for TAP2 intron	Individuals used for soxD intron
Lethenteron kessleri (?)	2010	Arctic Ocean basin, White Sea basin	Stream at Chekshino, Dvinnitsa River system, Sukhona River system, Severnaya Dvina River system, Vologodskaya obl.	Russia	59°39'14.83"N	40°38'8.79"E	Naseka A. M.	1	
Lethenteron ninae	2009	Black Sea basin	Mzymta River, Krasnodarskiy Kray	Russia	43°29'50.94"N	39°59'25.66"E	Mosyagina M. B.	1	
Lethenteron ninae	2009	Black Sea basin	Shakhe River, Krasnodarskiy Kray	Russia (T)	43°48'18.48"N	39°40'45.31"E	Mosyagina M. B.	1	2
Lethenteron reissneri	2006	Pacific Ocean basin	Barh River, Onon, Khentii	Mongolia	48°58'13"N	111°46'54"E	Sabaj M.	1	
Lethenteron zanandreai (spotted)	2011	Adriatic Sea basin	Vipava River at Male Zable	Slovenia	45°52'22.81"N	13°50'53.88"E	Naseka A. M.	2	1
Lethenteron zanandreai (monotonous)	2011	Adriatic Sea basin	Vipava River at Male Zable	Slovenia	45°52'22.81"N	13°50'53.88"E	Naseka A. M.	1	1

**Eudontomyzon vladykovi* were considered a synonym of *Eudontomyzon mariae* in Renaud (2011), since *Eudontomyzon mariae* exhibits a broad geographic distribution with clear disjunctions and wide variation in a number of taxonomic characters, and sufficient adult specimens from across the range has not been collected. Kottelat and Freyhof (2007) elevated *Eudontomyzon vladykovi* as a distinct species. It is distinct from the typical *Eudontomyzon mariae* in the soxD trees, while they are not reciprocally monophyletic in the cyt *b* trees. Since the soxD trees have lower resolution, this thesis treats it as a synonym of *Eudontomyzon mariae* following Renaud (2011).

Table 3.2 List of sequences from GenBank for the soxD intron analyses. All the sequences were sequenced by Okada et al. (2010).

Species	GenBank accession No. of soxD intron sequences	Basin	Stream	Country	Latitude	Longitude
Lethenteron camtschaticum	AB565490 AB565491 AB565492	Pacific Ocean basin	Shiribeshitoshibetsu River, Hokkaido	Japan	42°35'9" N	140°11'22"E
Lethenteron kessleri	AB565493 AB565494	Pacific Ocean basin	Irtysh River, Upper Ob River	Kazakhstan	52°16'53"N	76°55'34"E

Table 3.3 Primers used for the amplification and sequencing of nuclear genes. SoxD-F and soxD-R were modified from the soxD-F and soxD-R primer pair used by Okada et al. (2010). TAP2a-289F and TAP2b-15R were designed from the *Petromyzon marinus* sequence (AY171568, Uinuk-ool et al. 2003) and the mRNA sequences of *Cricetulus griseus* (XM003510258) and *Homo sapiens* (AB045381). 40SrE1-74F and 40SrE2-39R were designed from the *Petromyzon marinus* genome and the mRNA sequences of *Lethenteron camtschaticum* (DC611396) and *Salmo salar* (BT049811). MASPE3-123F and MASPE4-52R were designed from the *Lethenteron camtschaticum* MASP-A gene sequence (AB078894) and the mRNA of *Lethenteron camtschaticum* (AB009075) and *Sus scrofa* (GU810083). TAP2intrR1, TAP2intrR2, TAP2intrF1, TAP2intrF2 and TAP2intrF3 were designed from TAP2 intron sequences sequenced with TAP2a-289F and TAP2b-15R. See Section 3.2.2. AT = annealing temperature.

Primer name	Primer complement	Primer sequence	AT (°C)	Gene of interest	Primer location	Approximate product size (bp)
SoxD-F	SoxD-R	GCGGAAAA TCCTTCAA GCTT	64.2	SoxD intron	Sox D gene: ~ 600 bp upstream of the ~400 bp fragment in Table 3.2 (for *Eudontomyzon* and *Lampetra*) *	~1100 in *Eudontomyzon* and *Lampetra*, > 1500 in *Lethenteron**
SoxD-R	SoxD-F	GCTTGTAC TTGTAGTC GGGATACT TC	63.8	SoxD intron	Sox D gene: ~110 bp downstream of the ~400 bp fragment in Table 3.2* (for *Eudontomyzon* and *Lampetra*) *	
TAP2a-289F	TAP2b-15R	TACGCCAC TGTCAAGT TGCT	63.5	TAP intron 2	TAP exon 2a: 289-308 bp	1200
	TAP2intrR1					700
	TAP2intrR2					700
TAP2b-15R	TAP2a-289F	CACATACA GTGCCCAG AACC	63	TAP intron 2	TAP exon 2b: 15-34 bp	1200
	TAP2intrF1					400
	TAP2intrF2					600
	TAP2intrF3					600
40SrE1-74F	40SrE2-39R	TACAAGAA GGCTCACC TGGG	64.1	40S ribosomal protein subunit 23 intron 1	40S ribosomal protein subunit 23 exon 1: 74-93 bp	multiple bands (the desired product is ~800 bp)
40SrE2-39R	40SrE1-74F	ATGAGCTG TACACGCA CACACT	64.7	40S ribosomal protein subunit 23 intron 1	40S ribosomal protein subunit 23 exon 2: 39-60 bp	
MASPE3-123F	MASPE4-52 R	TCCAACGA CGAGCGCT AC	65.3	MASP-A intron 3	MASP-A exon3: 123-140 bp	multiple bands (the desired product is ~1000 bp)
MASPE4-52R	MASPE3-123 F	GGGGTAGT TGAAGCAG TTGTG	63.4	MASP-A intron 3	MASP-A exon4: 52-72 bp	

Table 3.3 Continued.

Primer name	Primer complement	Primer sequence	AT (°C)	Gene of interest	Primer location	Approximate product size (bp)
TAP2intrR1	TAP2a-289F	GAGTACTC TGCTCTAA TTCCAAGC G	65.2	TAP intron 2	Intron 2 of TAP: ~706-730 bp	700
TAP2intrR2	TAP2a-289F	CCTGCCAG ACACTTAT TTGGTG	65.4	TAP intron 2	Intron 2 of TAP: ~746-767 bp	700
TAP2intrF1	TAP2b-15R	GCTCATTC CATTAGTG TCTATCTT GC	64.8	TAP intron 2	Intron 2 of TAP: ~552-577 bp	400
TAP2intrF2	TAP2b-15R	GCCATAAA TAGCATTG TCAGAGTG	63.4	TAP intron 2	Intron 2 of TAP: ~271-294 bp	600
TAP2intrF3	TAP2b-15R	GAGAGAGA CGCGTGAT TAATAACT TAG	63.2	TAP intron 2	Intron 2 of TAP: ~245-271 bp	600

*The relative positions of the primers to the target fragment (Table 3.2) were estimated using sequences from *Lampetra* and *Eudontomyzon*. The lengths of PCR products were estimated using electrophoresis (Fig. 3.1). Apparently the relative positions of primers in *Lethenteron* are different. Since no *Lethenteron camtschaticum* or *Lethenteron kessleri* was successfully sequenced, only the relative positions in *Lampetra* and *Eudontomyzon* were shown.

Table 3.4 Samples for the combined dataset of TAP2 and soxD introns. Samples of *Lethenteron zanandreai* include populations with different colorations from the same location; the color features are indicated after the species names for these samples. "(T)" after the country name means the collection locality is the type locality and "(NT)" means near the type locality. "*" means different individuals for TAP2 and soxD introns. "a" means the individuals for TAP2 and soxD introns are from different localities. The soxD intron sequences of *Lethenteron camtschaticum* and *Lethenteron kessleri* are from GenBank; GenBank accession numbers are given after the country names. See Smith et al. (2013) for more information on the *Petromyzon marinus* genome. See Table 3.1 and 3.2 for more information about other specimens.

Species	Basin	Stream	Country
Eudontomyzon danfordi *[a]	Black Sea basin	Borzhava River, Tissa River drainage, Danube River system	Ukraine (TAP2)
	Mediterranean Sea basin	Lehotsky Brook at Muránska Dlhá Lúka, Muránska planina National Park	Slovakia (soxD)
Eudontomyzon mariae	Black Sea basin	Stryy River at Verkne Vysotskoye, Dniester River system	Ukraine
Lampetra aepyptera	Atlantic Ocean basin	Hocking River, Ohio River system	USA (NT)
Lampetra fluviatilis *	Baltic Sea basin	Neva River, from a shop, St. Petersburg	Russia (T)
Lampetra pacifica	Pacific Ocean basin	N. Fork Reservoir, Clackamas, OR	USA(T)
Lethenteron camtschaticum *[a]	Pacific Ocean basin	Stream without name, Ussuri Bay, Sea of Japan, Vladivostok area	Russia (TAP2)
	Pacific Ocean basin	Shiribeshitoshibetsu River, Hokkaido	Japan (soxD, AB565490)
Lethenteron kessleri *[a]	Arctic Ocean basin, White Sea basin	Stream at Chekshino, Dvinnitsa River system, Sukhona River system, Severnaya Dvina River system, Vologodskaya obl.	Russia (TAP2)
	Pacific Ocean basin	Irtysh River, Upper Ob River	Kazakhstan (soxD, AB565493)
Lethenteron ninae	Black Sea basin	Shakhe River, Krasnodarskiy Kray	Russia (T)
Lethenteron zanandreai A (spotted)	Adriatic Sea basin	Vipava River at Male Zable	Slovenia
Lethenteron zanandreai B * (TAP2: spotted; soxD: monotonous)	Adriatic Sea basin	Vipava River at Male Zable	Slovenia
Petromyzon marinus	Great Lakes basin	Great Lakes	USA

Table 3.5 Length without gaps, numbers of gaps and missing data of TAP2 intron dataset. The color feature of *Lampetra fluviatilis* and *Lethenteron zanandreai* are after the species names.

Species	Collection locality	Length without gaps (bp)	Gap (bp)	Missing data (bp)
Eudontomyzon danfordi	Borzhava River, Black Sea basin, Ukraine	875	7	65
Eudontomyzon mariae	Stryy River, Black Sea basin, Ukraine	876	11	60
Lampetra aepyptera	Hocking River, Atlantic Ocean basin, USA	107	5	835
Lampetra ayresii	Feather River, CA, Pacific Ocean basin, USA	568	2	377
Lampetra fluviatilis	Neva River, Baltic Sea basin, St. Petersburg, Russia	858	7	82
Lampetra fluviatilis (black)	Syas' River, Baltic Sea basin, Kolchanovo, Russia	810	6	131
Lampetra fluviatilis (grey)	Syas' River, Baltic Sea basin, Kolchanovo, Russia	810	6	131
Lampetra hubbsi	Merced River, Pacific Ocean basin, USA	509	5	433
Lampetra lanceolata	Ykizdere Brook, Black Sea basin, Turkey	518	5	424
Lampetra pacifica	Clackamas River, Pacific Ocean basin, USA	607	5	335
Lampetra pacifica	Clackamas River, Pacific Ocean basin, USA	376	4	567
Lethenteron appendix	Pigeon River, Lake Michigan basin, USA	795	9	143
Lethenteron appendix	Maine, Atlantic Ocean basin, USA	932	12	3
Lethenteron camtschaticum	Vladivostok area, Ussuri Bay, Sea of Japan, Russia	808	7	132
Lethenteron kessleri	Severnaya Dvina River system, White Sea basin, Russia	920	13	14
Lethenteron ninae	Shakhe River, Black Sea basin, Russia	792	7	148
Lethenteron ninae	Mzymta River, Black Sea basin, Russia	527	4	416
Lethenteron reissneri	Barh River, Onon, Khentii, Mongolia	439	2	506
Lethenteron zanandreai (monotonous)	Vipava River, Adriatic Sea basin, Slovenia	166	5	776
Lethenteron zanandreai (spotted)	Vipava River, Adriatic Sea basin, Slovenia	874	8	65
Lethenteron zanandreai (spotted)	Vipava River, Adriatic Sea basin, Slovenia	559	10	378
Petromyzon marinus	Great Lakes, USA	930	17	0

Table 3.6 Length without gaps, numbers of gaps and missing data of soxD intron dataset. The color feature of *Lampetra fluviatilis* and the GenBank accession Numbers of *Lethenteron camtschaticum* and *Lethenteron kessleri* are after the species names.

Species	Collection locality	Length without gaps (bp)	Gap (bp)	Missing data (bp)
Eudontomyzon danfordi	Lehotsky Brook, Mediterranean Sea basin, Slovakia	193	426	187
Eudontomyzon mariae	Stryy River, Black Sea basin, Ukraine	188	426	192
Eudontomyzon vladykovi	Krka River drainage, Black Sea basin, Slovenia	195	426	185
Lampetra aepyptera	Hocking River, Mississippi River basin, Atlantic Ocean basin, USA	189	426	191
Lampetra fluviatilis	Neva River, Baltic Sea basin, St. Petersburg, Russia	378	428	0
Lampetra pacifica	Clackamas River, Pacific Ocean basin, USA	103	0	703
Lethenteron camtschaticum (AB565490)	Shiribeshitoshibetsu River, Pacific Ocean basin, Japan	400	406	0
Lethenteron camtschaticum (AB565491)	Shiribeshitoshibetsu River, Pacific Ocean basin, Japan	399	407	0
Lethenteron camtschaticum (AB565492)	Shiribeshitoshibetsu River, Pacific Ocean basin, Japan	400	406	0
Lethenteron kessleri (AB565493)	Irtysh River, Arctic Ocean basin, Kazakhstan	398	408	0
Lethenteron kessleri (AB565494)	Irtysh River, Arctic Ocean basin, Kazakhstan	400	406	0
Lethenteron ninae	Shakhe River, Black Sea basin, Russia	194	426	186
Lethenteron ninae	Shakhe River, Black Sea basin, Russia	194	426	186
Lethenteron zanandreai (monotonous)	Vipava River, Adriatic Sea basin, Slovenia	189	426	191
Lethenteron zanandreai (spotted)	Vipava River, Adriatic Sea basin, Slovenia	189	426	191
Petromyzon marinus	Great Lakes, USA	800	6	0

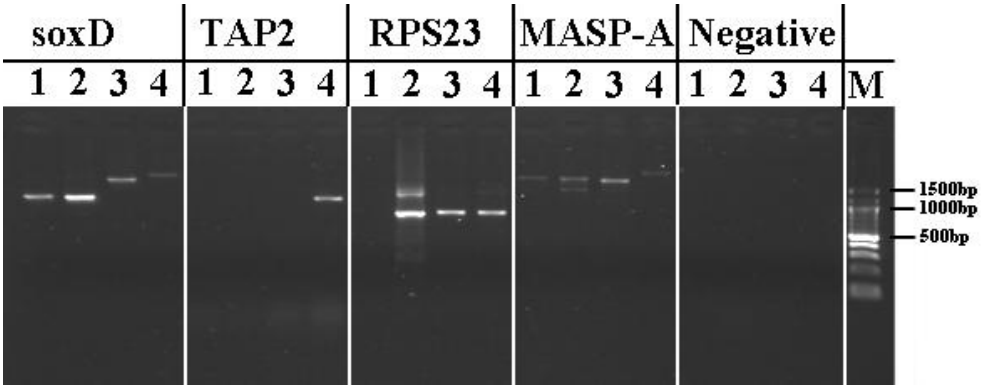

Fig. 3.1 Electrophoresis image for the PCR products with primer pairs for soxD, TAP2, RPS23, MASP-A introns. Numbers under the gene names refer to different DNA templates: 1 is *Eudontomyzon danfordi*, 2 is *Lampetra fluviatilis*; 3 is *Lethenteron camtschaticum*; 4 is *Lethenteron kessleri*. Primers for soxD are soxD-F and soxD-R; primers for TAP2 are TAP2a-289F and TAP2b-15R; primers for RPS23 are 40SrE1-74F and 40SrE2-39R; primers for MASP-A are MASPE3-123F and MASPE4-52R (Table 3.3). Water was used instead of DNA templates for the negative control. Negative 1 is for soxD; 2 is for TAP2; 3 is for RPS23; 4 is for MASP-A. M refers to the marker, 100 bp ladder (New England Biolabs Inc.). The 500 bp, 1000 bp and 1500 bp bands are marked on the right of the ladder. Each 30 μL PCR reaction contained 1X PCR buffer (Invitrogen) (20 mM Tris-HCl pH 8.4; 50 mM KCl), 2.5 mM MgCl2, 0.2 mM of each dNTP, 0.4 μM of each primer, and 0.02 U/μL of Go*Taq*® DNA polymerase (Promega). Reactions were initially denatured at 96 °C for 3 min. Amplifications were carried out in 30 cycles: denaturation at 96 °C for 30 sec, primer annealing at 60 °C, 58 °C, and 55 °C for 30 sec for 10 cycles each, extension at 72 °C for 2 min, and additional extension at 72 °C for 5 min.

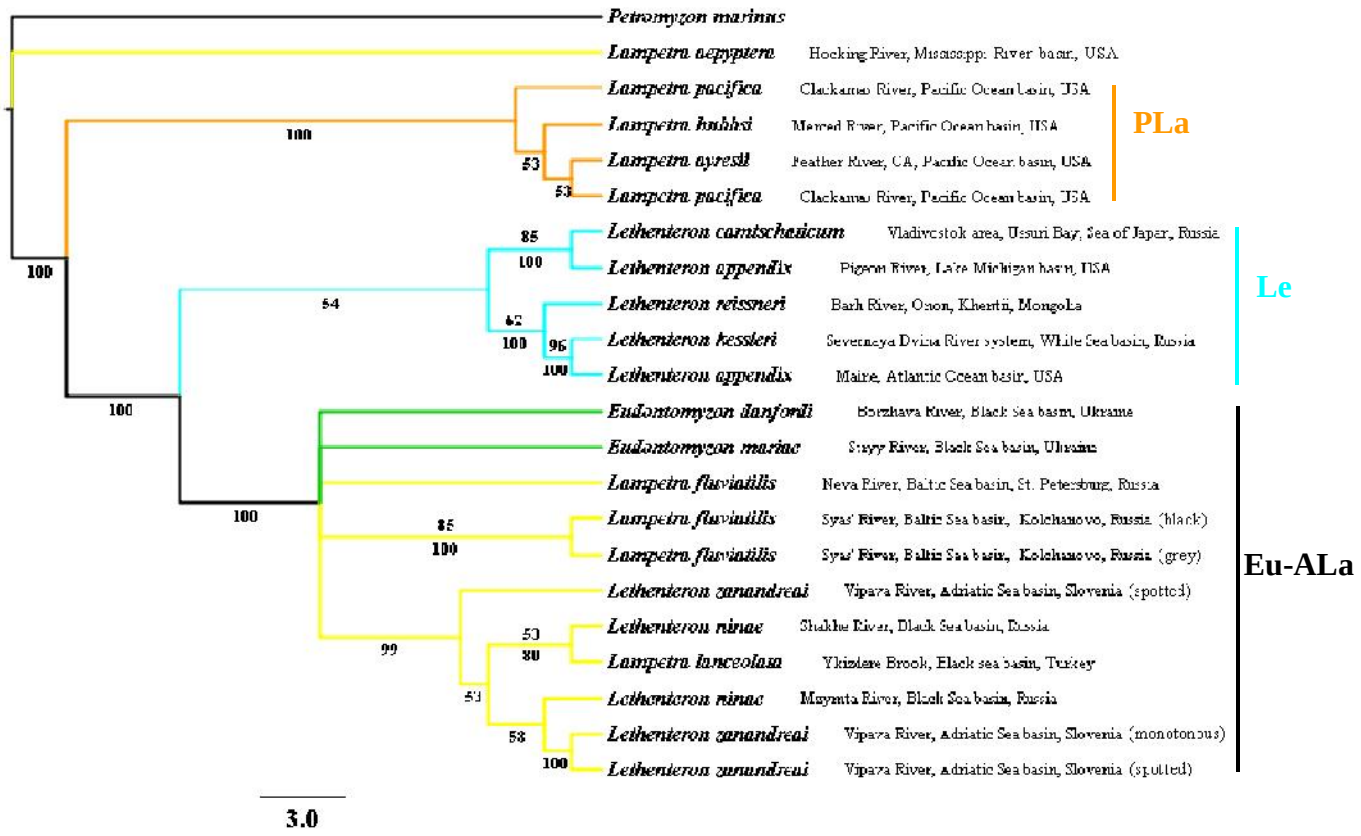

Fig. 3.2 The 50% majority rule consensus of 2010 maximum parsimony trees (length = 128) resulting from heuristic searches using the Parsimony Ratchet for the TAP2 intron dataset. Bootstrap support values greater than 50 are above the branches. Consensus frequencies are under the branches. The scale refers to the number of substitutions. The branches are colored as in the cyt *b* maximum parsimony tree (see Section 2.3.1, Fig. 2.1): *Lethenteron* in blue, Pacific *Lampetra* in orange, *Eudontomyzon* in green, and Atlantic *Lampetra* plus *Lethenteron zanandreai* and *Lethenteron ninae* in yellow. Three main clades are marked out on the right of the species names and localities: PLa, Le, and Eu-ALa.

Fig. 3.3 The 50% majority rule consensus of 900 Bayesian trees using TAP2 intron dataset. The posterior probabilities are above the branches. The scale refers to the number of substitutions per site. The branches are colored as in the cyt *b* Bayesian tree (see Section 2.3.2, Fig. 2.2): *Lethenteron* in blue, Pacific *Lampetra* in orange, *Eudontomyzon* in green, and Atlantic *Lampetra* plus *Lethenteron zanandreai* and *Lethenteron ninae* in yellow. Two clades are marked out on the right of the species names and localities: PLa and Eu-ALa.

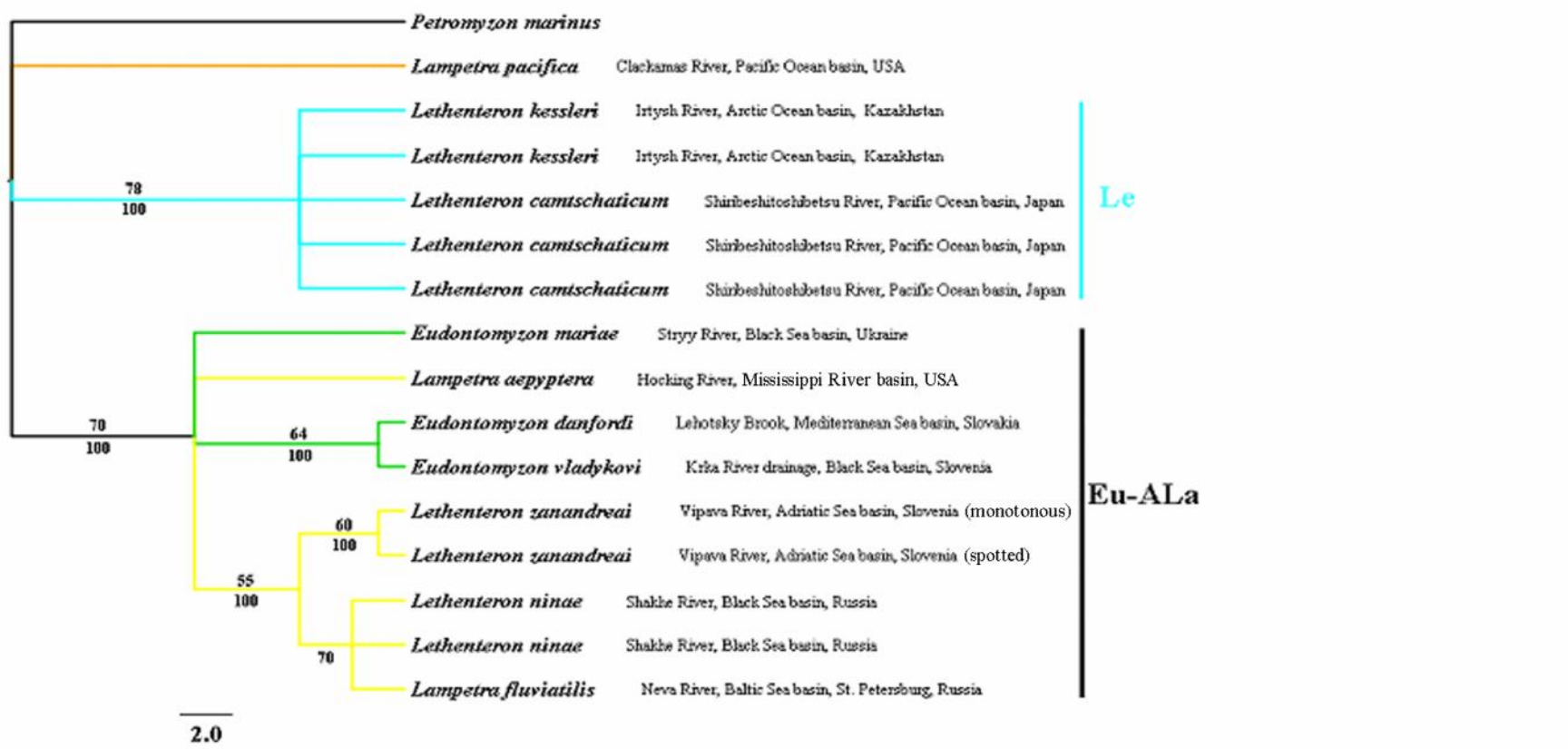

Fig. 3.4 The 50% majority rule consensus of 2010 maximum parsimony trees (length = 38) resulting from heuristic searches using the Parsimony Ratchet for the soxD intron dataset. Bootstrap support values greater than 50 are above the branches. Consensus frequencies are under the branches. The scale refers to the number of substitutions. The branches are colored as in the cyt *b* maximum parsimony tree (see Section 2.3.1, Fig. 2.1): *Lethenteron* in blue, Pacific *Lampetra* in orange, *Eudontomyzon* in green, and Atlantic *Lampetra* plus *Lethenteron zanandreai* and *Lethenteron ninae* in yellow. Two main clades are marked out on the right of the species names and localities: Le and Eu-ALa.

Fig. 3.5 The 50% majority rule consensus of 1200 Bayesian trees using soxD intron dataset. The posterior probabilities are above the branches. The scale refers to the number of substitutions per site. The branches are colored as in the cyt *b* Bayesian tree (see Section 2.3.2, Fig. 2.2): *Lethenteron* in blue, Pacific *Lampetra* in orange, *Eudontomyzon* in green, and Atlantic *Lampetra* plus *Lethenteron zanandreai* and *Lethenteron ninae* in yellow. Two clades are marked out on the right of the species names and localities: Le and Eu-ALa.

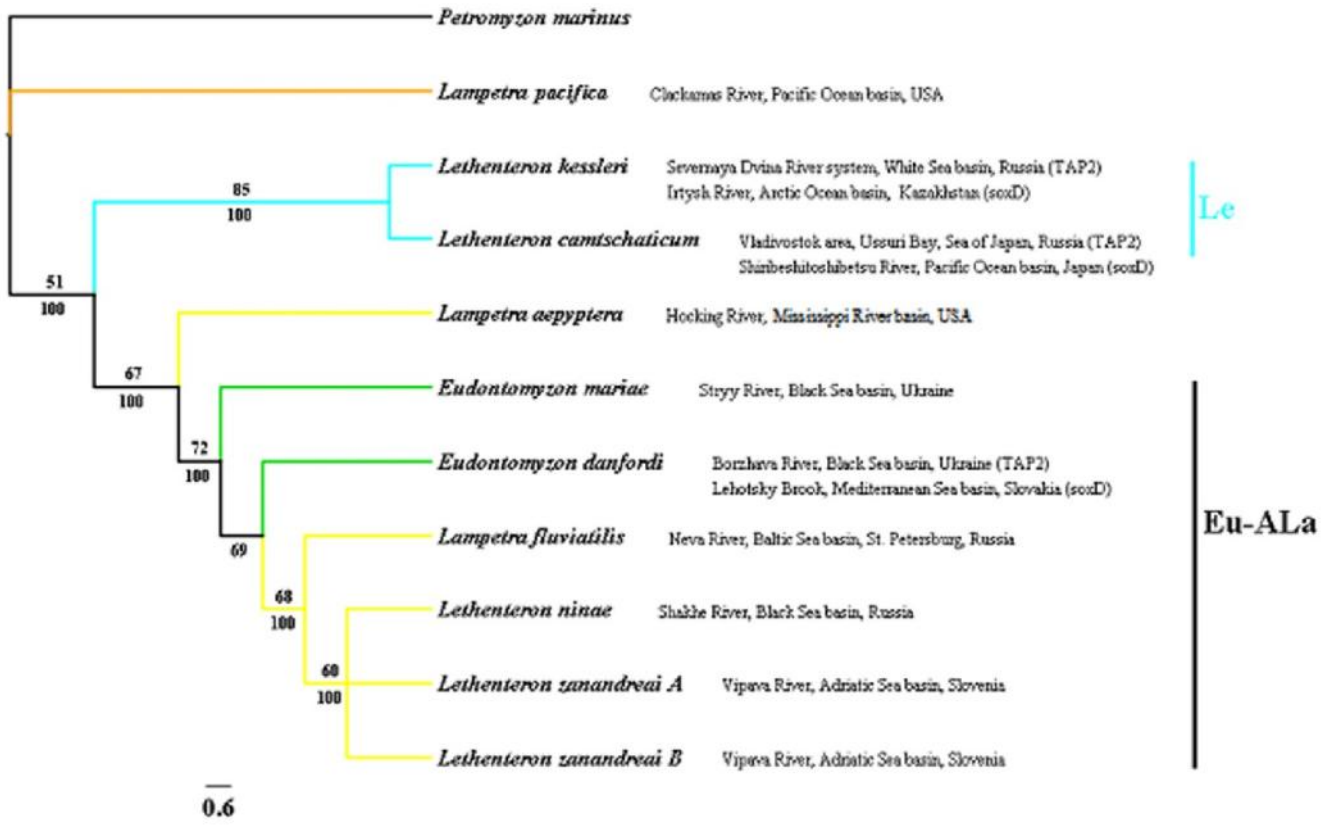

Fig. 3.6 The 50% majority rule consensus of 2010 maximum parsimony trees (length = 138) resulting from heuristic searches using the Parsimony Ratchet for the combined dataset. Bootstrap support values greater than 50 are above the branches. Consensus frequencies are under the branches. The scale refers to the number of substitutions. The branches are colored as in the cyt *b* maximum parsimony tree (see Section 2.3.1, Fig. 2.1): *Lethenteron* in blue, Pacific *Lampetra* in orange, *Eudontomyzon* in green, and Atlantic *Lampetra* plus *Lethenteron zanandreai* and *Lethenteron ninae* in yellow. Two main clades are marked out on the right of the species names and localities: Le and Eu-ALa.

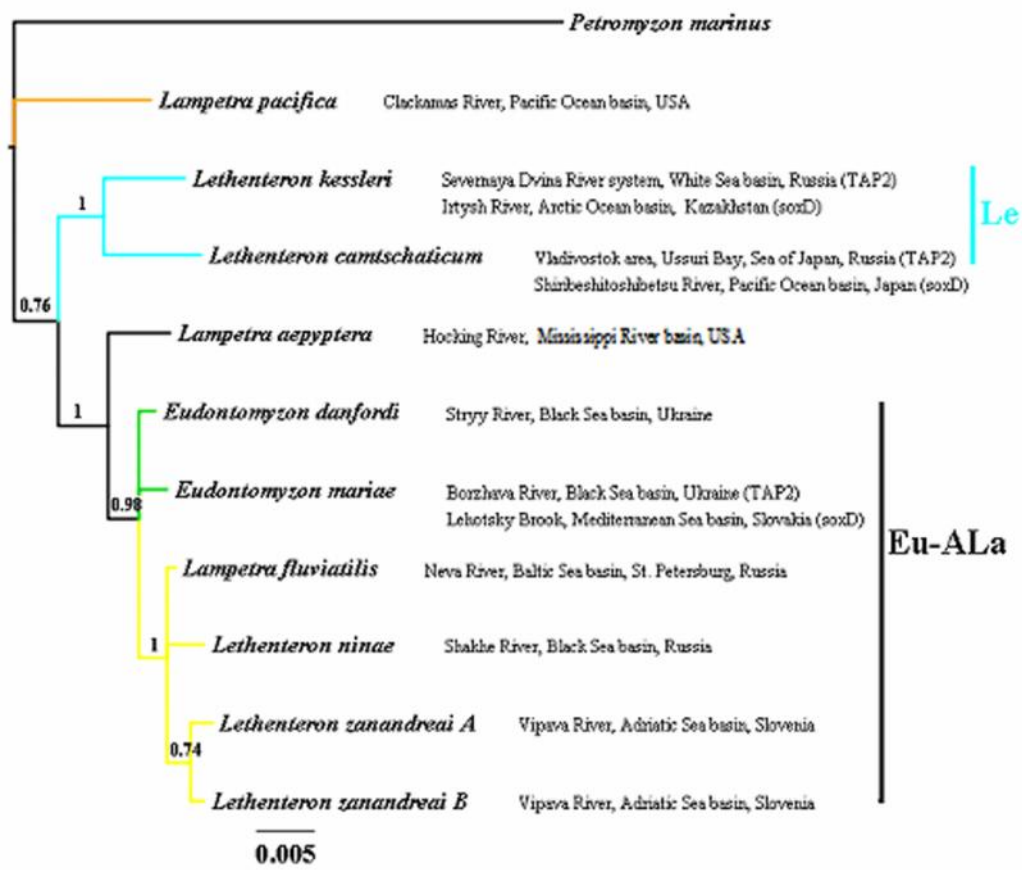

Fig. 3.7 The 50% majority rule consensus of 750 Bayesian trees using combined dataset. The posterior probabilities are above the branches. The scale refers to the number of substitutions per site. The branches are colored as in the cyt *b* Bayesian tree (see Section 2.3.2, Fig. 2.2): *Lethenteron* in blue, Pacific *Lampetra* in orange, *Eudontomyzon* in green, and Atlantic *Lampetra* plus *Lethenteron zanandreai* and *Lethenteron ninae* in yellow. Two clades are marked out on the right of the species names and localities: Le and Eu-ALa.

Chapter 4 General Discussion

In this thesis, the relationships among the lamprey genera *Eudontomyzon* Regan 1911, *Lampetra* Bonnaterre 1788, and *Lethenteron* Creaser and Hubbs 1922 and the relationships within genus *Lethenteron* were studied. The mitochondrial DNA (mtDNA) was used for resolving the relationships among genera and within the genus *Lethenteron*, and the nuclear DNA was used for resolving the generic level relationships. The complete mitochondrial cytochrome *b* (cyt *b*) gene (1191 bp) was sequenced in a total of 72 individuals from 11 species, from multiple localities for each species where samples were available. In particular, for the genus *Lethenteron*, where differences among species were expected to be subtle (and where intraspecific variation could be as great as or greater than interspecific variation), most main basins in the ranges of the species were sampled. A haplotype network was inferred for the Arctic lamprey *Lethenteron camtschaticum* (Tilesius 1811) and its closely related satellite species. A total of 20 haplotypes were discovered in the five species, more than previously reported (Yamazaki et al. 2006; Lang et al. 2009), and the haplotype frequency distribution in species and geographic regions was analyzed.

Previous studies on generic relationships or placement of species in lampreys (Docker et al. 1999; Blank et al. 2008; Caputo et al. 2009; Lang et al. 2009) all used mtDNA. In this thesis, nuclear DNA was used for the generic relationships and

placement of species in *Eudontomyzon*, *Lampetra*, and *Lethenteron* for the first time. Several questions were resolved using introns of two nuclear genes, the transporter associated with antigen processing (TAP) gene and the SRY-related high mobility group box D (soxD) gene. For example, nuclear DNA sequence data supported the separation of Pacific *Lampetra* and Atlantic *Lampetra*, the status of the least brook lamprey *Lampetra aepyptera* (Abbott 1860), the placement of the Lombardy brook lamprey *Lethenteron zanandreai* (Vladykov 1955), the Western Transcaucasian brook lamprey *Lethenteron ninae* Naseka, Tuniyev, and Renaud 2009 and the Kern brook lamprey *Lampetra hubbsi* (Vladykov and Kott 1976). The relationships discovered using multiple loci are more likely the relationships in the species tree than those inferred using only mtDNA data. The discoveries of this thesis are discussed in the forthcoming sections.

4.1 Comparison between cyt *b* and nuclear phylogenies

Based on the knowledge of the mitochondrial genome in several lamprey species (sea lamprey *Petromyzon marinus* L., Lee and Kocher 1995; European river lamprey *Lampetra fluviatilis* (L.), Delarbre et al. 2000; *Lethenteron camtschaticum*, Hwang et al. 2013a; Far Eastern brook lamprey *Lethenteron reissneri* (Dybowski 1869), Hwang et al. 2013b), many primers have been designed to amplify and sequence mitochondrial genes in lampreys. Mitochondrial genes, especially cyt *b* gene, have been widely used in phylogenetic studies on lampreys (e.g., Docker et al. 1999; Yamazaki et al. 2006;

Espanhol et al. 2007; Blank et al. 2008; Caputo et al. 2009; Lang et al. 2009; Boguski et al. 2012; Docker et al. 2012). The nuclear genome is available in only one lamprey species, *Petromyzon marinus* (Smith et al. 2013), and few nuclear genes have been sequenced to resolve the phylogenetic relationships among lampreys (internal transcribed spacer region 1 of the rRNA gene (ITS1) and the ninth intron of TAP gene (TAP9) in *Lampetra*, Boguski 2009; the second intron of TAP gene (TAP2) in *Eudontomyzon*, *Lampetra* and *Lethenteron*, and soxD gene intron in *Eudontomyzon* and *Lampetra*, Chapter 3). Lamprey nuclear genes have been used in deep-level phylogenies of vertebrates, that is, among lampreys, hagfishes, and jawed vertebrates (rRNA genes, Stock and Whitt 1992; Mallatt and Sullivan 1998; protein-coding genes, Kuraku et al. 1999), but these genes provide insufficient resolution for more recent divergences. Okada et al. (2010) sequenced the soxD gene intron in *Lethenteron camtschaticum* and the Siberian lamprey *Lethenteron kessleri* (Anikin 1905), but only in an attempt to find diagnostic differences between them. Different from previous lamprey nuclear DNA studies, in Chapter 3, gene introns were used to resolve the phylogeny among genera. However, compared with mtDNA, it is relatively difficult to obtain clear nuclear DNA sequences, particularly those that show sufficient variation for resolution of relationships among species and genera. Some nuclear genes (e.g., the 18S rRNA gene, Stock and Whitt 1992) may have multiple copies (making amplification and direct sequencing difficult), whereas single-copy nuclear genes often have low yields and thus generate

comparatively poor sequences (see Section 3.2). Thus, fewer species and individuals are included in the nuclear phylogeny than in the cyt *b* phylogeny; for example, sequencing attempts failed in *Lethenteron* sp. N and the Korean lamprey *Eudontomyzon morii* (Berg 1931), and tissue samples were not available for *Lethenteron* sp. S (whereas cyt *b* sequence data was available on the GenBank).

Due to the higher substitution rate of mitochondrial genes (Brown et al. 1979; Brown et al. 1982; Pesole et al. 1999), the cyt *b* phylogeny has higher resolution than the nuclear phylogeny. For instance, groups Eu (*Eudontomyzon* without *Eudontomyzon morii*) and ALa (Atlantic *Lampetra* including *Lethenteron ninae* and *Lethenteron zanandreai* without *Lampetra aepyptera*) are reciprocally monophyletic in the cyt *b* trees, while the relationship between them is not resolved in the nuclear DNA trees. The relationships among more taxa were unresolved in the nuclear consensus trees than in the cyt *b* consensus trees, which means more uncertain relationships. A similar case occurred in Boguski's (2009) study: well supported clades in mitochondrial phylogenies received lower support or collapsed in nuclear DNA phylogenies. To recover some poorly supported clades in the nuclear gene trees, this study used 50% majority rule consensus instead of strict consensus for the nuclear genes. Even so, the resolution of the nuclear trees is lower than the cyt *b* trees.

Although with low resolution, the nuclear gene trees still provide some evidence for the genus division and species placement. It is agreed in all trees that ALa and Eu are

most closely related among ALa, PLa (Pacific *Lampetra*), Le (*Lethenteron* plus *Eudontomyzon morii* without *Lethenteron* sp. S, *Lethenteron ninae* and *Lethenteron zanandreai*) and Eu. Thus the division of the genus *Lampetra* (as defined in Renaud 2011) into two parts, Atlantic and Pacific *Lampetra*, are supported by both mitochondrial and nuclear phylogenies (Docker et al. 1999; Blank et al. 2008; Lang et al. 2009; this thesis). Another similarity in mitochondrial and nuclear phylogenies is that *Lethenteron zanandreai* and *Lethenteron ninae* are in Atlantic *Lampetra* rather than *Lethenteron*. These issues will be discussed in the next section.

The difference occurs in the status of *Lampetra aepyptera* among different genes. It is sister to Atlantic *Lampetra* or *Eudontomyzon* (unresolved in the Bayesian tree) in the cyt *b* trees, and is sister to *Eudontomyzon* plus Atlantic *Lampetra* in the combined nuclear trees. However, since many characters are missing in the *Lampetra aepyptera* TAP2 sequence, the TAP2 and combined nuclear trees may not be reliable. The soxD trees do not contradict the cyt *b* trees.

Another difference in the topology of trees was that PLa was sister to all other taxa in the nuclear gene trees while *Lethenteron* was sister to all other ingroup taxa in the cyt *b* trees. Neither topology was well supported. The relationship between PLa and Le also varied in previous mitochondrial studies (Docker et al. 1999; Blank et al. 2008; Lang et al. 2009). Thus this difference may not be caused by lineage sorting, but may be caused by the lack of substitutions or indels in selected genes that diagnostically distinguish Le

or PLa from all others. More genes from the whole genome are needed to resolve the phylogenetic status of PLa and Le groups.

In summary, the nuclear gene trees contain fewer species and individuals and are of lower resolution than the cyt *b* trees in this study, but phylogenetic analyses with these independent datasets are generally consistent. The differences in relationships among genera/groups therefore are not likely the result of lineage sorting. There is uncertainty on the placement of PLa and Le since both are poorly supported as the sister taxa to all other ingroups, but both nuclear genes and cyt *b* gene recover similar relationships among *Eudontomyzon*, *Lethenteron*, Pacific and Atlantic *Lampetra*. See Table 4.1 for a summary of the results for different datasets concerning questions about genera division and species placement.

4.2 Suggestions on taxonomy of lampreys

4.2.1 Genus Lampetra *may be divided*

It was suggested by several previous molecular studies using mitochondrial genes (Docker et al. 1999; Blank et al. 2008; Lang et al. 2009) that *Lampetra* [as in Renaud (2011), but including *Lampetra hubbsi* which was later put in *Lampetra* by Page et al. (2013) based on the mtDNA phylogeny by Docker et al. (1999) and Lang et al. (2009)] was not monophyletic. Although the morphological studies and recent taxonomic lists do not separate them (Vladykov and Kott 1979b; Potter 1980; Gill et al. 2003; Nelson 2006;

Renaud 2011), the phylogenies generated in this study using nuclear genes and cyt *b* gene all support the view that *Lampetra* is not monophyletic. *Lampetra* from the Atlantic drainages and *Lampetra* from the Pacific drainage form distinct clades, and the two clades are not sister to each other. Also, the placement of *Lampetra hubbsi* in Pacific *Lampetra* is supported in both cyt *b* and TAP2 trees (the soxD trees and the combined trees do not include this species).

Pacific *Lampetra* and Atlantic *Lampetra* are very similar in morphology. The river lamprey *Lampetra ayresii* (Günther 1870) and *Lampetra fluviatilis* were initially considered identical and treated as the same species; likewise for the western brook lamprey *Lampetra richardsoni* Vladykov and Follett 1965 and the European brook lamprey *Lampetra planeri* Bloch 1784 (see Section 2.1.1). The phylogenies using mtDNA (Docker et al. 1999; Lang et al. 2009) were not adopted by the recent taxonomic list (Renaud 2011) but, with the supporting results of nuclear gene phylogenetic analysis (Chapter 3), the different results of morphological and molecular studies are not likely due to the incomplete lineage sorting in the molecular studies. Rather, they appear to be due to morphological similarity in genetically distinct genera. Morphology only studies phenotypic characters, without concerning the genetic basis of them. Mutations in different genes could result in the same morphological character state (Steiner et al. 2009). Thus, morphology would sometimes be misleading. The phenotypic convergence in Atlantic and Pacific *Lampetra* is presumably the adaptation to a certain environment.

A possible explanation is that the parasitic species (*Lampetra ayresii* and *Lampetra fluviatilis*) have developed the same dentition pattern independently in adaptation to similar hosts (lamprey dentition is related to their diet, Potter and Hilliard 1987; Renaud et al. 2009) or have both retained the ancestral pattern of dentition (again due to constraints related to feeding), while non-parasitic (satellite) species diverged from the similar stems and kept the same dentition pattern.

The recent taxonomic list (Renaud 2011) put seven species in *Lampetra*: three from the Atlantic drainage of Eurasia (*Lampetra fluviatilis*, Turkish brook lamprey *Lampetra lanceolata* Kux and Steiner 1972 and *Lampetra planeri*), one from the Atlantic drainage of North America (*Lampetra aepyptera*), and three from the Pacific drainage of North America (*Lampetra ayresii*, *Lampetra richardsoni* and Pacific brook lamprey *Lampetra pacifica* Vladykov 1973). Results from this thesis indicated that *Lethenteron zanandreai* and *Lethenteron ninae* should be included in the Eurasian *Lampetra* clade, and further supported placement of *Lampetra hubbsi*, which was *Entosphenus hubbsi* in the original description (Vladykov and Kott 1976a), in the Pacific *Lampetra* clade (see Section 4.2.3). Based on phylogenetic analyses of both mitochondrial and nuclear gene sequences, therefore, it appears that *Lampetra* may be separated into at least two genera, one including four species from the Pacific drainage and the other including five Eurasian species from the Atlantic drainage. Considering the low support values obtained in all previous molecular studies (Docker et al. 1999; Blank et al. 2008; Lang et al. 2009) for

the clade of *Lampetra aepyptera* plus Eurasian Atlantic *Lampetra*, and the results of this study, it may be more appropriate to put *Lampetra aepyptera* in another genus *Okkelbergia* rather than to put it in the genus of Eurasian Atlantic *Lampetra*. Hubbs and Potter (1971) suggested separating *Lampetra aepyptera* from genus *Lampetra* as genus *Okkelbergia*, since the generic diagnostic dentition characters are too poorly developed in this species to assign it to *Lampetra* or any other genus. Vladykov and Kott (1976b) disagreed with them and pointed out that poorly developed dentition is associated with the non-parasitic life history type rather than a generic diagnostic character, and that Hubbs and Potter (1971) might have used a newly metamorphosed sample with the teeth not yet fully cornified. Although Renaud (2011) did not adopt the genus *Okkelbergia*, the author pointed out that *Lampetra aepyptera* is the only *Lampetra* species possessing both exolaterals and posterials while other *Lampetra* species do not possess both.

Thus, the results of this thesis support dividing *Lampetra* into Eurasian Atlantic *Lampetra*, Pacific *Lampetra* and *Okkelbergia*. This suggestion is based on data from three genes (cyt *b*, TAP2 intron and soxD intron) used in this study, and needs more evidence from both morphological and molecular studies. Especially for *Okkelbergia*, a larger sample set covering its geographic range is desirable; considerable intraspecific variation in the mitochondrial genome has been reported (Martin and White 2008).

4.2.2 Relationships among genera

According to the morphological phylogeny by Gill et al. (2003) using only parasitic species, *Eudontomyzon* and *Lampetra* are sister taxa while *Lethenteron* is sister to the clade of their two. This study found *Eudontomyzon* and Atlantic *Lampetra* are sister taxa, while Pacific *Lampetra* is distinct from them. Although Pacific *Lampetra* is similar to Atlantic *Lampetra* morphologically (Gill et al. 2003), differences are discovered in both mitochondrial (Docker et al. 1999; Blank et al. 2008; Lang et al. 2009; Chapter 2) and nuclear genes (Chapter 3). Except for the status of Pacific *Lampetra*, previous molecular studies and this study support the morphological phylogeny (Gill et al. 2003). Atlantic *Lampetra* and *Eudontomyzon* are sister taxa based on mitochondrial and nuclear genes (Blank et al. 2008; Lang et al. 2009; this thesis).

Vladykov and Kott (1979b) put *Eudontomyzon*, *Lampetra* and *Lethenteron* in subfamily Lampetrinae. Bailey (1980) treated *Eudontomyzon*, *Lampetra*, *Lethenteron*, *Entosphenus*, *Tetrapleurodon* and *Okkelbergia* as subgenera under genera *Lampetra*. Potter (1980) put subgenera *Lampetra* (including *Lampetra aepyptera*), *Lethenteron* and *Entosphenus* under the genus *Lampetra*. Based on the morphological phylogenetic study by Gill et al. (2003), Nelson (2006) put *Eudontomyzon*, *Lampetra*, *Lethenteron*, *Entosphenus*, *Tetrapleurodon* and *Caspiomyzon* under the subfamily Lampetrinae. This study suggests that Atlantic *Lampetra* and *Eudontomyzon* are more closely related to each other than to *Lethenteron* or Pacific *Lampetra*. Thus if *Lampetra* and *Lethenteron*

are lumped together in a subfamily or as subgenera under the same genus, *Eudontomyzon* should be included in their group. The genus *Lampetra* defined by Potter (1980) is not supported by other morphological studies and molecular studies including this thesis. The subfamily Lampetrinae by Vladykov and Kott (1979b) was monophyletic (with some movement of species among genera, similarly hereinafter) in Lang el al. (2009) using cyt *b* gene, and the genus *Lampetra* by Bailey (1980) is supported in the maximum parsimony tree but not the Bayesian tree, while the subfamily Lampetrinae by Nelson (2006) was not supported in either trees. Based on Lang et al. (2009), *Lethenteron*, *Eudontomyzon*, Atlantic and Pacific *Lampetra* could be one subfamily (Lampetrinae in Vladykov and Kott 1979b), and *Entosphenus* and *Tetrapleurodon* are another subfamily (Entospheninae in Vladykov and Kott 1979b), while *Caspiomyzon* is distinct from them, clustered with *Petromyzon* and *Ichthyomyzon* (the Bayesian tree) or being sister to all other genera in family Petromyzontidae (the maximum parsimony tree). Lang et al. (2009) did not resolve the division of subfamilies within Petromyzontidae since the information in cyt *b* gene is not sufficient. A phylogenetic study including all genera in family Petromyzontidae using nuclear genes is needed. Morphological phylogeny with parasitic and non-parasitic lampreys included may also be useful.

4.2.3 Relict species in Atlantic and Pacific Lampetra

This thesis supports previous molecular results suggesting that *Lethenteron*

zanandreai and *Entosphenus hubbsi* belong to the Atlantic and Pacific *Lampetra* clades, respectively (e.g., Docker et al. 1999; Caputo et al. 2009; Lang et al. 2009)—but is the first to use both mitochondrial and nuclear genes, and is the first to indicate that *Lethenteron ninae* belongs to the Atlantic *Lampetra* clade. Although all the *Lethenteron zanandreai* samples in this thesis are from the same locality, *Lethenteron ninae* individuals from the type locality and several other localities were included. As discussed in Section 4.3.2, molecular phylogenetic analyses have been particularly useful in resolving the phylogenetic placement of these non-parasitic "relict" lampreys that, due to poorly developed or variable dentition characters, have been not been unambiguously assigned to a certain genus (Docker 2009; see Section 4.3.2).

It was controversial whether *Lethenteron zanandreai* possesses posterials (Vladykov 1955; Hubbs and Potter 1971; Tutman et al. 2009; Renaud 2011; see Section 1.3.5). Thus, some authors believed this species was *Lethenteron* (Hubbs and Potter 1971; Potter 1980; Renaud 2011), others believed it was *Lampetra* (Vladykov 1955; Vladykov and Kott 1979b). These morphological studies may have different understandings on the recognition of posterials. Interestingly, even if the presence of posterials were reported, the teeth in the only row of posterials in *Lethenteron zanandreai* are less than other *Lethenteron* species except *Lethenteron ninae*. Vladykov and Kott (1979a) considered *Lethenteron zanandreai* as the descendant of the parasitic Eurasia Atlantic *Lampetra* species *Lampetra fluviatilis*. The initial placement of *Lethenteron zanandreai* in

Lampetra by Vladykov (1955) is supported by molecular studies.

Lethenteron ninae was described from the Black Sea basin by Naseka et al. (2009). The comparison was made among *Lethenteron ninae* and *Lampetra lanceolata* and the Ukrainian brook lamprey *Eudontomyzon mariae* (Berg 1931). However, *Lethenteron ninae* and *Lethenteron zanandreai* are geographically closer to Eurasian Atlantic *Lampetra* than to any other *Lethenteron* species; this is consistent with the results of the molecular results suggesting that *Lethenteron zanandreai* and *Lethenteron ninae* both belong to Atlantic *Lampetra*. Given that this study tested relationships using three unlinked genes, I feel this is sufficient evidence for placing both species in Atlantic *Lampetra*.

Lampetra hubbsi differs from other *Lampetra* species by its transverse lingual lamina and endolaterals, which resemble the *Entosphenus* species (Vladykov and Kott 1976a). However, the transverse lingual lamina is poorly developed and the endolateral count is occasionally variable (being like that in *Lampetra*) (Renaud 2011). *Lampetra hubbsi* was recently put in *Lampetra* by Page et al. (2013) based on the mtDNA phylogeny by Docker et al. (1999) and Lang et al. (2009); nuclear DNA sequence data now confirm this placement (specifically, in Pacific *Lampetra*).

4.3 Relationships among stem and satellite species

With the above reassignment of *Lethenteron ninae* and *Lethenteron zanandreai* to

the Eurasian *Lampetra* clade, the majority of *Lethenteron* species represent closely related stem and satellite species. Are the satellite species separate species from the stem, or are they non-parasitic forms or freshwater forms of the same species? Docker (2009) pointed out that different answers may apply to different cases. This thesis likewise suggests that the degree of similarity differs among different satellite and stem species. Vladykov and Kott (1979a) illustrated this variable degree of relatedness between different satellite and stem with lines of different lengths connecting them. The following sections will discuss three types of relationships among satellite species and the stem based on variable degrees of relatedness.

4.3.1 Closely related satellite species

Some satellite species are directly sister to the stem and, as is being increasingly shown, are often not reciprocally monophyletic (e.g., Espanhol et al. 2007; Boguski et al. 2012; Docker et al. 2012). These satellite species are closely related to the stem and are called closely related satellite species in this thesis (see Chapter 2). *Lethenteron camtschaticum* has four closely related satellite species: Alaskan brook lamprey *Lethenteron alaskense* Vladykov and Kott 1978, American brook lamprey *Lethenteron appendix* (DeKay 1842), *Lethenteron kessleri* and *Lethenteron reissneri* (*s.s.*). In this thesis, these five species are not reciprocally monophyletic in the cyt *b* gene trees. Similarly, *Eudontomyzon mariae* [including the synonyms *Eudontomyzon vladykovi*

(Oliva and Zanandrea 1959) and *Eudontomyzon stankokaramani* (Karaman 1974)] is a closely related satellite species of the Carpathian lamprey *Eudontomyzon danfordi* Regan 1911, *Lampetra planeri* for *Lampetra fluviatilis*, and *Lampetra richardsoni* for *Lampetra ayresii*. *Eudontomyzon danfordi* and *Eudontomyzon mariae* are not reciprocally monophyletic in the cyt *b* trees. *Lampetra fluviatilis* and *Lampetra planeri* are also not reciprocally monophyletic. *Lampetra ayresii* and *Lampetra richardsoni* are reciprocally monophyletic (Chapter 2). However, *Lampetra ayresii* and *Lampetra richardsoni* were recovered in one well supported clade and not reciprocally monophyletic using cyt *b* gene in a previous study using more individuals by Boguski et al. (2012). In this thesis, the genetic distances (Kimura's two-parameter distance, K2P) between *Lethenteron camtschaticum* and its closely related species, between *Lampetra fluviatilis* and *Lampetra planeri*, and between *Lampetra ayresii* and *Lampetra richardsoni* are small (< 2%). The genetic distance between *Eudontomyzon danfordi* and *Eudontomyzon mariae* are relatively large (>2% for the typical *Eudontomyzon mariae* plus *Eudontomyzon vladykovi* and *Eudontomyzon stankokaramani* or for each of them).

The nuclear genes have lower resolution and the datasets include fewer species. The genetic distance of TAP2 intron between *Eudontomyzon danfordi* and *Eudontomyzon mariae* is zero, while that between *Lethenteron camtschaticum* and *Lethenteron kessleri* (2.27%) or *Lethenteron reissneri* (3.27%) are very large (the K2P between *Lethenteron camtschaticum* and the outgroup, *Petromyzon marinus*, is 5.50%). However, only a single

individual for each these species were included. The K2P between *Lethenteron camtschaticum* and *Lethenteron appendix* (two individuals from different localities) is 1.07%. In the TAP2 tree, *Lethenteron camtschaticum*, *Lethenteron appendix*, *Lethenteron kessleri*, and *Lethenteron reissneri* separate into two clades. However, the two clades are not diagnosable. Other closely related stem and satellite species are not included in the TAP2 tree. In the soxD dataset, *Eudontomyzon danfordi* and the typical *Eudontomyzon mariae* (single individual) have relatively large genetic distance (1.62%; the K2P between *Eudontomyzon danfordi* and the outgroup, *Petromyzon marinus*, is 9.50%), while *Eudontomyzon danfordi* and *Eudontomyzon vladykovi*, *Lethenteron camtschaticum* and *Lethenteron kessleri* have a distance of zero. Since only a single individual is included for each of *Eudontomyzon danfordi* and the typical *Eudontomyzon mariae*, it is not known whether each species would be reciprocally monophyletic when multiple individuals are included. The relationships at the species level are not clear in the nuclear trees.

Does it mean that the closely related species and the stem are the same phylogenetic species where they are not reciprocally monophyletic or the genetic distances are less than 2% (Avise and Walker 1999)? Although phylogenetic species were defined to be diagnosable and reciprocally monophyletic (Mayden 1997), the gene tree may not reflect the species tree (Pamilo and Nei 1988; Pollard et al. 2006). Thus, those species, which were not reciprocally monophyletic in the cyt *b* tree, could be reciprocally monophyletic

using more informative data from the whole genome. One example is in the case of *Lampetra fluviatilis* and *Lampetra planeri* from the Sorraia River: they are not reciprocally monophyletic using mitochondrial genes (Espanhol et al. 2007), but are reciprocally monophyletic using the SNPs from the whole genome obtained from restriction site-associated DNA sequencing (RADseq) (Mateus et al. 2013b). Since the latter study included only one locality, there is still no conclusion on whether *Lampetra fluviatilis* and *Lampetra planeri* range-wide would be reciprocally monophyletic.

The genetic distances of cyt *b* gene are more than 2% between 98% vertebrate sister species (Avise and Walker 1999). Fewer differences in cyt *b* gene between stem and satellite species may reflect a shorter divergence time or ongoing gene flow (Docker 2009). For those stem and satellite species with a genetic distance less than 2%, it is possible that the speciation occurred recently and the divergence of cyt *b* gene has not accumulated to 2% genetic distance. The genetic distance could give us a clue about between-taxa relationships. However, the species delimitation could not be based solely on genetic distance data.

Thus, without testing more loci from the whole genome, it is uncertain whether the closely related satellite species, which are not monophyletic in gene trees, are the same species as the stem. No genetic marker has been found to distinguish between *Lethenteron alaskense* and *Lethenteron camtschaticum*. Yamazaki and Goto (1998) recognized a fixed allele of malate dehydrogenase 3 (MDH3) that diagnostically

distinguished *Lethenteron kessleri* from *Lethenteron camtschaticum*. However, Yamazaki et al. (2006) discovered that *Lethenteron reissneri* (*s.s.*) possesses the same allele as *Lethenteron kessleri* and suggested both should be the same species under the name *Lethenteron reissneri*, although they are morphologically distinguishable (Iwata et al. 1985). However, the sampling of *Lethenteron reissneri* (*s.s.*) in Yamazaki et al. (2006) was limited to two localities near each other; it is not known whether the allele difference is diagnostic between species range-wide. This thesis has not found any other genetic marker distinguishing among *Lethenteron kessleri*, *Lethenteron reissneri* and *Lethenteron camtschaticum*. More studies using several unlinked genes are useful for answering the question whether *Lethenteron camtschaticum* and its closely related species are the same species. Allozyme allele analyses, microsatellite, and restriction fragment length polymorphism (RFLP) could provide evidences on hybridization or reproductive isolation, which would also help answer the question.

4.3.2 Divergent satellite species – relict species

There are some non-parasitic species for which the stem cannot be unambiguously identified based on the morphological data. Compared with closely related species discussed in the last section, these satellite species are relatively divergent from the stem. Thus they were often called relict species (Hubbs and Potter 1971; Docker et al. 1999; Docker 2009). For example, *Lethenteron* sp. N is a divergent satellite species of

Lethenteron camtschaticum. Based on the cyt *b* trees, *Lethenteron zanandreai*, *Lethenteron ninae* and *Lampetra lanceolata* are divergent satellite species of *Lampetra fluviatilis*, and *Lampetra pacifica* and *Lampetra hubbsi* are divergent satellite species of *Lampetra ayresii*. These satellite species and their stem (plus closely related satellite species) are reciprocally monophyletic, and are thus "good" phylogenetic species. In the nuclear gene trees, the resolution is not high enough, and the closely related satellite species (e.g., *Lampetra planeri*) are sometimes not included, making the divergent satellite species directly sister to the stem. Thus the discussion of divergent satellite species is based on the cyt *b* gene. The genetic distances between divergent satellite species and the stems are relatively large (most K2P > 3%, except for between *Lethenteron zanandreai* and *Lampetra fluviatilis*, K2P ranges from 2.76% to 3.14%).

Interestingly, the phylogenetic placement of these divergent satellite species based on molecular data is often different from the placement based on morphological characters. Some relict species were taken as satellite species of other stems. For example, *Lethenteron zanandreai* (Hubbs and Potter 1971; Potter 1980; Renaud 2011) and *Lethenteron ninae* (Naseka et al. 2009) were considered derived from *Lethenteron camtschaticum*. Similarly, *Lampetra hubbsi* was originally described as *Entosphenus* species (see Section 4.2.3), but molecular studies (Docker et al. 1999; Lang et al. 2009; this thesis) clearly placed it within the Pacific *Lampetra* clade. In other cases, molecular data has been useful in identifying divergent or relict lineages in morphologically

conserved species. For example, *Lethenteron* sp. N was treated as the same species as *Lethenteron reissneri*, which is a closely related satellite species of *Lethenteron camtschaticum*. However, both mitochondrial (Yamazaki et al. 2003; Yamazaki et al. 2006; Lang et al. 2009; Chapter 2) and nuclear genomes (Yamazaki and Goto 1996; Yamazaki and Goto 2000) show them to be reciprocally monophyletic and genetically distinct, although *Lethenteron* sp. N has not been formally described as a separate species (Renaud 2011). Due to similar morphology, *Lampetra pacifica* was once synonymized with *Lampetra richardsoni*, but Reid et al. (2011) separated them based on subtle morphological and distinct molecular differences, which is supported by Boguski et al. (2012) and Chapter 2 using cyt *b* gene. Boguski et al. (2012) discovered four unrecognized divergent satellite species (*Lampetra* spp.) of *Lampetra ayresii* using cyt *b* gene. Mateus et al. (2013a) described three cryptic species which were previously treated as *Lampetra planeri* due to the lack of diagnostic morphological characters. Mateus et al. (2011) recovered these cryptic species and *Lampetra fluviatilis* plus *Lampetra planeri* were reciprocally monophyletic using mtDNA. Thus they are also divergent satellite species of *Lampetra fluviatilis*. Their relationships to *Lethenteron ninae*, *Lethenteron zanandreai* and *Lampetra lanceolata* are to be studied.

4.3.3 Highly divergent "satellite species"

Some "satellite species" are actually not genetically and morphologically similar to

the stem. They are not even in the genus of the stem or their placements in the genus are uncertain. In this discussion, they are considered as highly divergent satellite species. *Lethenteron* sp. S is an example of highly divergent satellite species. Like *Lethenteron* sp. N, *Lethenteron* sp. S is under *Lethenteron reissneri* in the recent taxonomic list (Renaud 2011), which means it is also considered derived from *Lethenteron camtschaticum*. Although morphologically similar, *Lethenteron* sp. S is highly divergent from *Lethenteron camtschaticum* based on cyt *b* gene (Lang et al. 2009; Chapter 2). It is sister to all genera considered in this study (Le, PLa, ALa and Eu; see Fig. 2.1 and 2.2). In the study including more lamprey genera and species by Lang et al. (2009), this species is actually not in the clade of any known genus. Thus, it may not be appropriate to treat it as a satellite species of *Lethenteron camtschaticum*. If every non-parasitic lamprey is derived from a parasitic one (Hubbs and Trautman 1937; Zanandrea 1959; Potter 1980), the parasitic ancestor of *Lethenteron* sp. S should be the common ancestor of *Lethenteron*, *Lampetra*, *Eudontomyzon*, and *Entosphenus* based on Lang et al. (2009). This would suggest that the non-parasitism of *Lethenteron* sp. S arose at an early time. Another possibility is that the true stem of *Lethenteron* sp. S, which diverged from the common ancestor of *Lethenteron*, *Lampetra*, *Eudontomyzon*, and *Entosphenus* early, died out before being discovered. This true stem might resemble *Lethenteron camtschaticum* morphologically, which should be the result of phenotypic convergence.

Lampetra aepyptera was once considered a satellite species of *Lampetra ayresii*

(Vladykov and Kott 1979a). Other authors suggested that its stem is *Lethenteron camtschaticum* (Bailey 1980) or *Lampetra fluviatilis* (Docker et al. 1999). The genetic distance (cyt *b*) to *Lampetra fluviatilis* from *Lampetra aepyptera* is not appreciably larger than that from *Lethenteron zanandreai*, *Lethenteron ninae* or *Lampetra lanceolata* (see Table 2.6). However, unlike the other three satellite species, which are geographically closer to *Lampetra fluviatilis* than to all other parasitic lampreys, the distance from *Lampetra aepyptera* to *Eudontomyzon danfordi* is similar to that to *Lampetra fluviatilis*. The relationships among *Lampetra aepyptera*, ALa and Eu are uncertain and it may be most appropriate to put it in *Okkelbergia* as the only species in this genus (see Section 4.2.1).

4.4 Limitations of this study

To study the relationships among *Lethenteron*, *Eudontomyzon* and *Lampetra*, it is ideal to include all species of these genera, or at least most species in all the mitochondrial and nuclear DNA datasets. However, since some samples were unavailable for this study, and many sequencing efforts failed in the nuclear genes, there are several species missing, especially in the nuclear datasets. The Epirus brook lamprey *Eudontomyzon graecus* Renaud and Economidis 2010, of which the tissue samples and sequences were unavailable, is not included in this thesis. For *Lethenteron* sp. S, no tissue sample was available and the cyt *b* sequence (Okada et al. 2010) is from the

GenBank. Tissue samples of adult *Eudontomyzon morii*, *Lampetra planeri*, *Lampetra richardsoni*, *Lethenteron alaskense* and *Lethenteron* sp. N were available, but attempts to amplify the target nuclear genes with polymerase chain reaction (PCR) failed. In the soxD dataset, *Lethenteron* excluding *Lethenteron zanandreai* and *Lethenteron ninae* were not sequenced and only *Lethenteron camtschaticum* and *Lethenteron kessleri* sequences were retrieved from GenBank. Thus, the placement of *Eudontomyzon morii*, *Lampetra planeri*, *Lampetra richardsoni*, *Lethenteron alaskense*, *Lethenteron* sp. N and *Lethenteron* sp. S are only based on cyt *b* gene data. DNA sequence data from nuclear genes is needed for the placement of *Eudontomyzon morii*, *Lethenteron* sp. N and *Lethenteron* sp. S. In this study, 24 cyt *b* sequences are from GenBank. The identification of these samples directly follows the references (Lee and Kocher 1995; Delarbre et al. 2000; Lang et al. 2009; Okada et al. 2010; Boguski et al. 2012). The species identification and gene sequences were not personally verified. Identification of *Eudontomyzon morii*, which was based on a metamorphosing specimen, has been questioned (Lang et al. 2009; see Section 2.4.3.2).

For TAP2 and soxD sequences, many characters are missing in several sequences (see Table 3.5 and 3.6). This is due to the quality of the sequences. For TAP2 intron, some sequences are better at the 5' end while some are better at the 3' end. The fragment that is clear in all sequences is short. Thus some sequences have missing data at the 5' end and some have missing data at the 3' end. For the soxD gene, the target fragment

(Okada et al. 2010) is actually closer to the soxD-R primer. However, the soxD-R sequences are unclear. Thus, soxD-F sequences were used. Several sequences have missing data at 3' end. This missing data decreased the number of variable and parsimony informative characters. The resolution and the accuracy of the trees will increase with the missing parts sequenced.

In the cyt *b* dataset, multiple individuals, preferably from different localities, were used for each species when available. However, for the nuclear datasets, most species only have one individual successfully sequenced and used for the analyses. Multiple individuals for one species may significantly decrease the effect of individual mutations. Especially for species like *Lampetra aepyptera*, of which the placement is controversial, the results using only one individual could be biased due to the individual mutation.

With the cyt *b*, TAP2 and soxD datasets, there are still unresolved relationships among species and genera. For example, the relationship between *Lethenteron ninae* and *Lampetra lanceolata* needs further study. The question about the ancestor of *Lampetra aepyptera* has no certain answer. Because the two nuclear genes provide less information than the mitochondrial genes, more nuclear genes are preferred if possible.

The basic methods used for this study are DNA sequencing and phylogenetic analyses (maximum parsimony and Bayesian analysis). Relationships among some closely related species (e.g., *Lethenteron camtschaticum* and its closely related satellite species) remain uncertain, even though a median-joining network was inferred to resolve

the population-level relationships. Other methods such as microsatellite, RFLP and RADseq may be useful for resolving the relationships among closely related taxa. These methods may be tried in future studies.

4.5 Future studies

This thesis discussed the relationships among genera *Lethenteron*, *Lampetra* and *Eudontomyzon* and within genus *Lethenteron*. Several questions are still open. The relationships among *Lethenteron camtschaticum* and its closely related satellite species are still uncertain. The relationships among *Lethenteron*, Pacific *Lampetra*, *Eudontomyzon* and Atlantic *Lampetra* are also uncertain. The placements of *Lampetra aepyptera*, *Lethenteron* sp. N, *Lethenteron* sp. S and *Eudontomyzon morii* are to be resolved with other genes (especially from the nuclear genome).

To resolve the relationships among *Lethenteron camtschaticum* and its closely related satellite species, other methods may be useful. Methods like microsatellite and RFLP may provide more information about the population connectivity and hybridizations between species. Yamazaki et al. (2011) used microsatellite analysis for the estimation of population variation, structure, connectivity, and divergence time among different life history forms of *Lethenteron camtschaticum*. Docker et al. (2012) found that the silver lamprey *Ichthyomyzon unicuspis* Hubbs and Trautman 1937 and the northern brook lamprey *Ichthyomyzon fossor* Reighard and Cummins 1916 were not

reciprocally monophyletic, and were indistinguishable using the allele frequency of RFLP in mtDNA and nuclear microsatellite analysis. The microsatellite loci used by Yamazaki et al. (2011) could be used for *Lethenteron alaskense*, *Lethenteron reissneri* and *Lethenteron kessleri*. These species are suspected to be the same species as *Lethenteron camtschaticum*, and hybridizations may occur among populations of these species (between the satellite and the stem or between two satellite species). RFLP assays could be developed for mtDNA, for which numerous sequences of *Lethenteron* are available in GenBank. Even if diagnostic differences would not be found among them, significant differences in the allele frequency would indicate barriers to gene flow. RADseq was used by Mateus et al. (2013b) to distinguish *Lampetra fluviatilis* and *Lampetra planeri* from the same locality, which are not reciprocally monophyletic using mitochondrial genes (Espanhol et al. 2007). A similar method may apply to *Lethenteron camtschaticum*, *Lethenteron alaskense*, *Lethenteron kessleri*, *Lethenteron reissneri* and *Lethenteron appendix*, using a sample set covering most river systems within their ranges.

The soxD intron sequenced by Okada et al. (2010) is not complete. Different product sizes were detected by electrophoresis (see Fig. 3.1), but the indels causing such difference were not included in the published sequences. This thesis failed to sequence these *Lethenteron* samples with different sizes. It may be worth trying to sequence this intron in *Lethenteron* in future studies.

To resolve the placement of genus *Lethenteron*, Pacific *Lampetra*, and *Okkelbergia* (*Lampetra aepyptera*), more nuclear genes are to be explored. The *Petromyzon marinus* genome (Smith et al. 2013) provides information on numerous genes of lampreys. More lamprey nuclear gene or mRNA sequences are available in GenBank. Primers can be designed based on these data.

Many questions are to be studied in lamprey phylogeny. Future studies using more samples from more localities with various technologies may provide new vision on the relationships among genera and species. These studies would help resolve the taxonomy of lampreys, and help the conservation of lamprey species and their host fishes.

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4.7 Tables and Figures

Table 4.1 Summary of the results of the phylogenetic analysis using cyt *b*, TAP2, soxD, and the combined dataset and suggestions for taxonomy by this thesis. K2P means Kimura's two-parameter distance.

	Dataset				
Phylogenetic questions	**cyt *b***	**TAP2**	**soxD**	**Combined**	**Suggestions for taxonomy**
Is Pacific *Lampetra* a distinct genus from Atlantic *Lampetra*?	Yes: genera are reciprocally monophyletic and differ by 7.89–8.63% K2P	Yes: Pacific *Lampetra* and Atlantic *Lampetra* plus *Eudontomyzon* are reciprocally monophyletic	Yes: genera are reciprocally monophyletic	Yes: genera are reciprocally monophyletic	Put Pacific *Lampetra* in a new genus and only Atlantic *Lampetra* in *Lampetra*.
Is *Lampetra aepyptera* distinct from Atlantic *Lampetra*?	Poorly supported sister taxa	Yes: *Lampetra aepyptera* (with 835/947 missing data) is sister to Pacific *Lampetra* or is the outgroup	Unresolved	Yes: *Lampetra aepyptera* is sister to Atlantic *Lampetra* plus *Eudontomyzon* while Atlantic *Lampetra* is monophyletic	Put *Lampetra aepyptera* in *Okkelbergia*, a genus containing only this species.

Table 4.1 Continued.

	Dataset				
Phylogenetic questions	**cyt *b***	**TAP2**	**soxD**	**Combined**	**Suggestions for taxonomy**
Are *Lethenteron camtschaticum septentrionalis* and *Lethenteron camtschaticum camtschaticum* distinct subspecies*?*	No: subspecies not reciprocally monophyletic and differ by only 0–0.27% K2P	Not included	Not included	Not included	Treat *Lethenteron camtschaticum septentrionalis* as a synonym of *Lethenteron camtschaticum.*
Are *Lethenteron camtschaticum*, *Lethenteron alaskense*, *Lethenteron appendix*, *Lethenteron kessleri*, *Lethenteron reissneri* (*s.s.*) each phylogenetic species?	No: these taxa are not reciprocally monophyletic and differ by only 0–1.09% K2P	*Lethenteron alaskense* not included; *Lethenteron appendix* polyphyletic; the others only single individual.	*Lethenteron camtschaticum* and *Lethenteron kessleri* not reciprocally monophyletic; the others not included.	Only single individual of *Lethenteron camtschaticum* and *Lethenteron kessleri* included.	These five species are one by the phylogenetic species concept. Considering the differences between *Lethenteron camtschaticum* and *Lethenteron reissneri* plus *Lethenteron kessleri* in the one allozyme allele locus (Yamazaki et al. 2006), and the lack of sufficient nuclear DNA information in *Lethenteron alaskense*, *Lethenteron appendix* keep them as separate species for now.
Is *Lethenteron zanandreai* in *Lethenteron* or Atlantic *Lampetra*?	Atlantic *Lampetra*	Atlantic *Lampetra*, with *Eudontomyzon* and *Lampetra aepyptera*	Atlantic *Lampetra*	Atlantic *Lampetra*	Change *Lethenteron zanandreai* to *Lampetra zanandreai.*

Table 4.1 Continued.

	Dataset				
Phylogenetic questions	**cyt *b***	**TAP2**	**soxD**	**Combined**	**Suggestions for taxonomy**
Is *Lethenteron ninae* in *Lethenteron* or Atlantic *Lampetra*?	Atlantic *Lampetra*	Atlantic *Lampetra*, with *Eudontomyzon* and *Lampetra aepyptera*	Atlantic *Lampetra*	Atlantic *Lampetra*	Change *Lethenteron ninae* to *Lampetra ninae*.
Is *Lethenteron* sp. N and *Lethenteron* sp. S distinct from each other?	Yes: they differ by 12.31% K2P and are placed in different clades	Not included	Not included	Not included	Descriptions for these two new species are needed.
Is *Eudontomyzon morii* in *Eudontomyzon* or *Lethenteron*?	*Lethenteron*	Not included	Not included	Not included	Since the only sample is a metamorphosing individual with uncertain identification (sequence from Lang et al. 2009), this sample could belong to a new species rather than *Eudontomyzon morii*.
Is *Lampetra hubbsi* in Pacific *Lampetra*?	Yes	Yes	Not included	Not included	Put it in the genus of Pacific *Lampetra*
Are *Eudontomyzon vladykovi* and *Eudontomyzon mariae* phylogenetic species?	They are in the same clade and not reciprocally monophyletic.	*Eudontomyzon vladykovi* not included	One individual for each included, distinct from each other.	*Eudontomyzon vladykovi* not included	Since the mitochondrial and nuclear results are different, this thesis has no conclusion for this question.

Table 4.1 Continued.

	Dataset				
Phylogenetic questions	**cyt *b***	**TAP2**	**soxD**	**Combined**	**Suggestions for taxonomy**
Is *Eudontomyzon stankokaramani* distinct from *Eudontomyzon mariae*?	Yes: they are reciprocally monophyletic and differ by 2.49-3.05% K2P	Not included	Not included	Not included	Since only one *Eudontomyzon stankokaramani* sequence from Lang et al. (2009) is included, this thesis has no conclusion for this question.