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Inferring phylogenetic structure, hybridization and divergence times within Salmoninae (Teleostei: Salmonidae) using RAD-sequencing

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ABSTRACT

Phylogenetic studies focusing on Salmonidae have revealed significant obstacles in trying to clarify some interspecific relationships within the Salmoninae subfamily, due to a limited number of markers typed, conflicting phylogenetic signals and ancient hybridization events. To infer reliable phylogenetic relationships, evaluate several putative scenarios of ancient hybridization, and estimate divergence times within Salmoninae, we applied restriction-site associated DNA sequencing (RAD-seq) to 43 samples, including 26 genetic lineages across 21 species, largely representing the subfamily, with an emphasis on the genus *Salvelinus*. We identified 28,402 loci and 28,363 putatively unlinked SNPs, which were used in downstream analyses. Using an iterative k-means partitioned dataset and a Maximum Likelihood approach; we generated a well-supported phylogeny, providing clear answers to several previous phylogenetic uncertainties. We detected several significant introgression signals, presumably ancient, in the genus *Salvelinus*. The most recent common ancestor of Salmonidae dates back to approximately 58.9 MY ago (50.8–64 MY) and the crown age of Salmoninae was estimated to be 37.7 MY (35.2–40.8 MY) using a Bayesian molecular dating analysis with a relaxed molecular clock. The divergence among genera of the subfamily occurred between the late Eocene and middle of the Miocene (≈38–11 MY) such as the divergence between the genus *Oncorhynchus* and *Salvelinus*, which we estimated to 21.2 MY ago (95% HPD: 19.8–23.0 MY), while species diversification took place mainly during the Neogene (≈22–1.5 MY), with more than half of these events occurring in the last 10 MY.

1. Introduction

The Salmonidae family, consisting of salmon, trout, charr, grayling, whitefishes and their relatives, is a very important group of temperate freshwater fishes in terms of both economic and ecological value; combined with their tetraploid ancestry, life-history diversity and rates of diversification, they have attracted considerable interest from the research community. The family includes 11 extant genera divided into three monophyletic subfamilies: Coregoninae, Thymallinae and Salmoninae (Nelson, 2006). Salmoninae, the most speciose subfamily, contains seven genera: *Brachymystax*, *Hucho*, *Oncorhynchus*, *Parahucho*, *Salmo*, *Salvelinus* and *Salvethymus*. Salmonid species offer valuable opportunities to investigate mechanisms of speciation and adaptation within an ecological and evolutionary framework. More specifically, they provide the possibility to study the effect of hybridization and genome duplication on species evolution. Indeed, one of the most remarkable features of salmonid evolutionary history is their autopolyploid origin (Allendorf and Thorgaard, 1984; Svärdson, 1945). They descend from a single tetraploid ancestor resulting from a whole genome duplication event (WGD) known as Ss4R (Lien et al., 2016), which took place around 95MY ago (88 - 103MY) based on the latest estimates (Macqueen and Johnston, 2014). However, since the Ss4R,

Abbreviations: RAD-seq/RAD-sequencing, Restriction-site associated DNA sequencing; SNPs, single nucleotide polymorphisms; MY, million years; RAxML, Randomized Axelerated Maximum Likelihood; BIC, Bayesian Information Criterion; IC, internode certainty; ML, Maximum Likelihood; sd, standard deviation; MCMC, Markov Chain Monte Carlo; HPD, Highest Posterior Density; BS, Bootstrap Support; BI, Bayesian Inference; BER, Bering clade; SIB, Siberian clade; ACD, Acadian clade; ATL, Atlantic clade; ARC, Arctic clade; OKH, Okhotsk Sea clade; NORs, nucleolus organizer regions

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salmonids have been through a process of rediploidization, by means of genomic reorganizations driven by selection, retaining only part of the ancestral tetraploid genome. It is estimated that up to 25% of the salmonid genome went through delayed rediploidization (Robertson et al., 2017) and around 10% still retains residual tetrasomy (Allendorf et al., 2015; Lien et al., 2016). WGD has an essential role in long-term evolutionary success; it is a key mechanism driving the development of new expression patterns and gene functions providing lineage-specific physiological adaptations, such as anadromy, therefore potentially promoting evolutionary diversification and facilitating speciation (Robertson et al., 2017). The partially delayed rediploidization is thought to have slowed down functional divergence, explaining the delay of at least 30MY between the Ss4R and lineage divergence (Macqueen and Johnston, 2014; Robertson et al., 2017).

There have been numerous comprehensive attempts to evaluate phylogenetic relationships among salmonids, using molecular methods (Crespi and Fulton, 2004; Osinov and Lebedev, 2004; Wang et al., 2011; Wilson and Turner, 2009; Yasuike et al., 2010). Shed'ko et al. (2013, 2012) provided extensive taxon coverage but was limited to mtDNA markers, and several other studies have extended this approach with whole mitogenomes (Campbell et al., 2013; Ma et al., 2015; Macqueen and Johnston, 2014; Sahoo et al., 2015). Other comprehensive studies included multiple nuclear and mitochondrial genes, such as Alexandrou et al. (2013) which focused on the dating of anadromy, while incorporating ancestral character simulation; and Crête-Lafrenière et al. (2012) who have so far provided the most extensive taxon coverage. Macqueen and Johnston (2014) were the first to estimate salmonid subfamily relationships using a large dataset of nuclear genes with a strict 1:1 orthology, which provided strong support for a sister relationship between Coregoninae and Thymallinae. The same authors also provided the first direct estimate of the timing of the whole-genome duplication event of salmonids. Collectively, these efforts have provided considerable clarifications on the phylogenetic relationships among salmonid taxa. Nonetheless, in spite of the substantial research contributions directed toward investigating phylogenetic relationships within Salmoninae, some knowledge gaps persist, presumably due to partially incomplete taxon coverage, limited number of markers, conflicting phylogenetic signals of different genomic regions and potentially ancient hybridization events. Additionally, the contrasting rates of rediploidization of different regions of the genome, following the WGD, has only recently been demonstrated (Lien et al., 2016; Robertson et al., 2017), and therefore its impact on phylogenetic signals within salmonids has been largely neglected.

Due to these various factors, some critical points of salmonid phylogeny remain unsettled, such as the exact position of certain species within the phylogenetic tree, as well as the placement of the two monotypic genera: *Parahucho* and *Salvethymus*. For instance, The Sakhalin taimen, *Parahucho perryi*, was formally included in the genus *Hucho*, despite the lack of morphological support for this designation (Sanford, 2000), but multiple molecular studies support the taxon as constituting a separate and monotypic genus (Crespi and Fulton, 2004; Matveev et al., 2007; Oakley and Phillips, 1999; Osinov, 1991), although its phylogenetic position within Salmoninae is still unclear. Within the genus *Salmo*, two taxa have also undergone systematic revision based on genetic information, namely *Salmo ohridanus* (formerly in the monotypic genus *Acantholingua*) and softmouth trout *Salmo obtusirostris* (formerly *Salmothymus*) (Snoj et al., 2002), but not without controversy. Hybridization has played a role in the evolution of softmouth trout (Sušnik et al., 2007), and despite molecular evidence supporting its inclusion in the genus *Salmo* (Snoj et al., 2002; Sušnik et al., 2007), some authors still question whether or not its unique behavior and morphology could underscore a hybridization event with a more distant taxon (Esteve et al., 2014). The genus *Salvelinus* has been shown to comprise multiple taxa with a history of interspecific hybridization (Baxter et al., 1997; Bernatchez et al., 1995; Gross et al., 2004; Radchenko, 2004; Redenbach and Taylor, 2002; Wilson and Bernatchez, 1998; Wilson and Hebert, 1993; Yamamoto et al., 2006). Additionally, the long-finned charr, endemic to the Lake El'gygytgyn in the Russian Far East (Siberia), is characterized by a unique and highly distinct morphology, and was thought to represent an ancestral form of charr, and was therefore placed in a new genus (*Salvethymus*) (Chereshnev and Skopets, 1990). However, subsequent phylogenetic studies placed it clearly within the genus *Salvelinus* and identified it as the sister-group to the *S. alpinus–S. malma* complex (Brunner et al., 2001; Crête-Lafrenière et al., 2012; Osinov et al., 2015; Shed'ko, 2002; Shubina et al., 2013), but this placement has not yet prompted taxonomic change. Thus, there are series of questions and uncertainties concerning the evolution and systematics of salmonids that likely involved various degrees of hybridization or require significantly increased resolution to address and resolve.

Restriction-site associated DNA sequencing (RAD-seq) (Baird et al., 2008; Miller et al., 2007; Rowe et al., 2011) produces large datasets with millions of genome-wide short sequences with deep coverage; and therefore is increasingly used to detect single nucleotide polymorphisms (SNPs) across a large number of loci in phylogenetic studies (Cruaud et al., 2014; Díaz-Arce et al., 2016; Eaton and Ree, 2013; Rubin et al., 2012). RAD-seq largely overcomes the limitation of traditional methods by drastically improving locus sampling across the genome in a single sequencing run, and yielding a much more reliable dataset of sequences and SNPs. This method is promising for systematic studies of closely related taxa, as it also allows the detection of introgression. RAD-seq relies on the retention of enzyme restriction sites across samples in order to obtain homologous sequences. Therefore, when using this method for phylogenetic inference, the age of the family or subfamily of interest is a critical parameter for locus recovery across species, since the number of shared loci is expected to be directly linked to evolutionary rates and divergence, due to a higher number of mutations between more distantly related species. This issue is exacerbated in the case of longer enzyme restriction sites. However, although the number of shared loci in a RAD-seq dataset decreases with the increasing phylogenetic distance between taxa, inadequate or unequal coverage can produce comparable proportions of missing data (Eaton et al., 2017). RAD-sequencing is most useful for resolving shallow phylogenetic questions, but with adequate taxa sampling, good quality DNA samples, increased coverage and accurate sample normalization during library preparation, a sufficient number of orthologous loci can be generated for precise phylogenetic inferences of clades as old as 60 to 80 MY (Cariou et al., 2013; Eaton et al., 2017; Herrera and Shank, 2016; Rubin et al., 2012).

The aim of this study is to investigate and more fully resolve the phylogenetic relationships among salmonid fish species within the Salmoninae subfamily, with a focus on the genus *Salvelinus*; as well as detect putative ancient hybridization events. We focus on clarifying some of the remaining uncertainties and controversial points of Salmoninae systematics using a RAD-seq dataset, including the main representatives of the subfamily, to produce a reliable phylogenetic hypothesis. Additionally, we estimate the divergence time between the different clades and genera.

2. Material & methods

2.1. Taxon sampling

This dataset includes representatives of the 7 genera of the Salmoninae subfamily and a subset of 21 species among 122 extant species of Salmoninae (98 species, > 80%, belong to the combined genera *Salvelinus* and *Salmo*) (Froese and Pauly, 2017; "GBIF: The Global Biodiversity Information Facility," 2016; Kottelat and Freyhof, 2007); however, the exact number of extant species remains a topic of debate. More precisely, the dataset consist of 43 individuals: one *Brachymystax* species, one *Hucho* species, five *Oncorhynchus* species, five *Salmo* species, seven *Salvelinus* species, one *Thymallus* species and two

Table 1
Sample names, common names and sampling locations of the individuals used in this study. *(a)*, *(b)* and *(c)* are used to differentiate distinct individuals of the same species.

Samples names	Common name	Country	Location
Thymallus thymallus	European Grayling	Germany	Kösseine (Elbe), Fichtelgebirge, Bavaria
Hucho hucho (a)	Huchen/Danube salmon	Germany	Inn (Wasserburg), Bavaria
Hucho hucho (b)	Huchen/Danube salmon	Germany	Inn (Wasserburg), Bavaria
Brachymystax lenok blunt snout	Blunt-snouted lenok	Russia	Aldan River (Lena), Sakha (Yakutia) Republic
Brachymystax lenok sharp snout	Sharp-snouted lenok	Russia	Indigirka River, Sakha (Yakutia) Republic
Parahucho perryi (a)	Japanese huchen/Sakhalin taimen	Russia	Dagi River, Sakhalin
Parahucho perryi (b)	Japanese huchen/Sakhalin taimen	Russia	Sokol'nikovka River, Sakhalin
Oncorhynchus mykiss (a)	Rainbow trout	Germany	Danube (introduced)
Oncorhynchus mykiss (b)	Rainbow trout	Russia	Kamchatka River
Oncorhynchus gorbuscha	Pink salmon/Humpback salmon	Russia	Reidovaya River, Iturup Island
Oncorhynchus keta (a)	Chum salmon/Dog salmon	Russia	Lagynoe Lake, Iturup Island
Oncorhynchus keta (b)	Chum salmon/Dog salmon	Russia	Lagynoe Lake, Iturup Island
Oncorhynchus masou (c)	Masu salmon/Cherry salmon	Russia	River Tigil, Kamchatka
Oncorhynchus masou (a)	Masu salmon/Cherry salmon	Russia	River Tigil, Kamchatka
Oncorhynchus masou (b)	Masu salmon/Cherry salmon	Russia	River Naiba, Sakhalin
Oncorhynchus nerka	Sockeye salmon/Red salmon	?	North Pacific (bought in supermarket)
Salvelinus namaycush (a)	Lake trout	Canada	Tagish Lake, Yukon
Salvelinus namaycush (b)	Lake trout	Canada	Muncho Lake, Liard River, British Columbia
Salvelinus fontinalis (a)	Brook trout/Brook charr	Canada	Mistassini lake, Quebec
Salvelinus fontinalis (b)	Brook trout/Brook charr	Canada	Tessier Lake, Quebec
Salvelinus leucomaenis (a)	Whitespotted charr	Russia	Dagi River, Sakhalin
Salvelinus leucomaenis (b)	Whitespotted charr	Russia	Yama River, Magadan Oblast
Salvelinus leucomaenis (c)	Whitespotted charr	Russia	Yama River, Magadan Oblast
Salvelinus levanidovi (a)	Levanidov's charr	Russia	Yama River, Magadan Oblast
Salvelinus levanidovi (b)	Levanidov's charr	Russia	Yama River, Magadan Oblast
Salvelinus alpinus (SIB)	Arctic Charr	Russia	Ylyy lake, Suntar-Indigirka
Salvelinus alpinus (ACD)	Arctic Charr	Canada	Paul Lake, Gaspésie, Quebec
Salvelinus alpinus (ARC)	Arctic Charr	Canada	Resolute Lake, Nunavut
Salvelinus alpinus (ATL)	Arctic Charr	Germany	Königssee, Bavaria
Salvelinus malma (BER)	Dolly varden	Russia	Yama River, Magadan Oblast
Salvelinus malma (OKH) (a)	Dolly varden	Russia	Tym River, Sakhalin
Salvelinus malma (OKH) (b)	Dolly varden	Russia	Sopochnoe Lake, Iturup Island
Salvethymus svetovidovi	Long-finned charr	Russia	El'gygytgyn Lake, Chukotka Autonomous Okrug
Salvelinus confluentus (a)	Bull trout	Canada	Fitzsimmons Creek, South-West British Colombia
Salvelinus confluentus (b)	Bull trout	Canada	Lower Fraser River, South-West British Colombia
Salvelinus confluentus (c)	Bull trout	Canada	Pine and Burnt Rivers, Central interior British Colombia
Salmo trutta (a)	Brown trout/Sea trout	Germany	Iller (Danube), Bavaria
Salmo trutta (b)	Brown trout/Sea trout	Germany	Iller (Danube), Bavaria
Salmo salar	Atlantic salmon	Germany	Haspertalsperre, Sauerland
Salmo marmoratus	Marbled trout	Slovenia	Trebuscica
Salmo obtusirostris	Adriatic trout/Softmouth trout	Bosnia Herz.	Neretva, Eastern part of the Adriatic basin
Salmo ohridanus	Ohrid trout/Belvica	Macedonia	Lake Ohrid

BER = Bering Clade.
SIB = East Siberian Clade.
ACD = Acadia Clade.
ARC = Arctic Clade.
ATL = Atlantic Clade.
OKH = Okhotsk Sea Clade.

species from monotypic genera: *Parahucho perryi* and *Salvethymus svetovidovi* (Table 1). Clades represented in this dataset within the genus *Salvelinus* refer to genetic lineages previously identified and defined based on mitochondrial DNA (Brunner et al., 2001; Malyarchuk, 2002).

2.2. RAD-sequencing and raw data analysis

Genomic DNA was extracted from fin clips of 43 specimens using a Qiagen DNeasy Blood & Tissue kit, and digested with the *SbfI* restriction enzyme. Library preparation followed the protocol of Baird et al., (2008). The library preparation and RAD-sequencing were both performed by Eurofins Genomics. The samples were labeled using specific individual barcodes differing by at least two nucleotides to avoid incorrect individual assignment of reads due to potential sequencing error. The 43 samples of this study were run multiplexed on one lane of an Illumina 1.8+ HiSeq2000 sequencer to generate single-end reads of 100 bp.

The raw sequenced reads were filtered using the software pipeline *pyRAD* v.2.7 (Eaton, 2014), designed specifically for *de novo* assembly of RAD-seq data meant for phylogenetic downstream analysis. The software pipeline is well suited to deal with variation across species and higher-level clades since it applies clustering and alignment methods handling high levels of divergence while accounting for indel variation. Reads that could not be reliably attributed to one of the barcodes used in this study, as well as reads of poor overall quality (Phred score < 20), were removed from the analysis. The quality of the retained reads was controlled using the FastQC bioinformatic tool to determine if any trimming was necessary due to lower quality toward the end of the reads (Phred score < 20). In subsequent steps of the pyRAD analysis, only reads with coverage > 5 were retained. Reads were clustered using a 90% similarity threshold, following the pipeline recommendations (Eaton, 2014), to cluster putatively orthologous loci both within and across samples. Loci with sequence data for < 18 individuals were excluded from the clustering, to include a maximum number of loci for the focal genus of our study (*Salvelinus*), while limiting the total amount of missing data, and to avoid potential strong bias due to overpruning of

loci with only little representation across taxa (Jiang et al., 2014).

2.3. Phylogenetic analysis

Analyzing large concatenated datasets, including thousands of loci, can cause the data analysis to be computationally intractable or lead to significant biased estimates and systematic errors, which can result in strong support for erroneous phylogenetic tree topologies (Lemmon and Lemmon, 2013). As partitioning is necessary to account for the heterogeneity in evolutionary rates, the best-fit partition scheme for the dataset was inferred using iterative k-means (Frandsen et al., 2015), which clusters individual sites in different subsets, based on their estimated evolutionary rate calculated using the Tree Independent Generation of Evolutionary Rates program (fast_TIGER) (Frandsen, 2014). This approach splits the concatenated alignment into subsets of sites with similar evolutionary rates, while avoiding over-parameterization. This algorithm and the fast_TIGER program are implemented in the python-based software PartitionFinder (Frandsen et al., 2015; Lanfear et al., 2014, 2012) and offers the major advantage of not requiring any prior pre-partitioning assumptions. The estimation of the best-fit partitioning scheme is directly computed from the data, more accurately accounting for complex patterns of nucleotide rate heterogeneity (Cummins and McInerney, 2011; Moran et al., 2015). Unlike most alternatives, this approach does not present a starting tree bias and the partitioning optimization is phylogeny-independent. This method has also been shown to lead to better fit partitioning schemes of evolutionary models on real data, compared to alternative partitioning approaches; it is the most computationally efficient on data matrices with thousands of loci and can account for potential reticulations in the data (Frandsen et al., 2015). PartitionFinder was also used to evaluate the best-fit nucleotide substitution models of molecular evolution for each partition using the Bayesian Information Criterion (BIC score) (Abdo et al., 2005; Minin et al., 2003).

For maximum likelihood inference, we used RAxML (Randomized Axelerated Maximum Likelihood), v. 8.1.17 (Stamatakis, 2014), which allows parallel processing and can handle partitioned datasets with large amounts of missing data. Our phylogenetic inferences were calculated using the best-fit partition scheme estimated by iterative k-means, and the general time-reversible nucleotide substitution model (GTRGAMMA). Node support of the best ML tree topology was assessed in RAxML with bootstrap replicates through the automatic bootstopping method and using internode certainty (IC). The IC allows detection of potential incongruencies (Salichos et al., 2014; Salichos and Rokas, 2013) by giving an estimation of the support of each node based on its frequency in a set of trees. An IC equal to 0 represents equal support for the two most prevalent conflicting bipartitions, while an IC of one represents the absence of conflict. The resulting tree, with node support, was visualized using Dendroscope (Huson et al., 2007).

Additionally, we conducted a Bayesian phylogenetic inference on the partitioned dataset using the software MrBayes v3.2.6 (Ronquist & Huelsenbeck 2003; Ronquist et al. 2011, 2012). Two independent runs were performed using the GTR + G evolutionary model and random starting trees. Each one was run for five million generations, with four Markov chains under default heating settings, with sampling every 1000 generations. Default priors were used in all analyses. The software Tracer v1.6 (Rambaut et al., 2014) was used to evaluate parameters convergence. The trees and posterior probabilities were summarized in MrBayes, after the removal of a 25% burn in. The resulting trees and posterior probabilities were visualized using FigTree v1.4 (Rambaut, 2012).

2.4. Neighbor-net analysis

A Neighbor-Net analysis was performed using SplitsTree4 (Huson and Bryant, 2014, 2006), which provides greater resolution for large datasets (Bryant and Moulton, 2004). The software uses molecular sequence data to generate an unrooted network, representing the evolutionary relationships (Bryant and Moulton, 2004, 2002). Networks can represent phylogenetic relationships in a more accurate way than trees, as they can also account for complex evolutionary processes such as hybridization, duplication events and gene recombination. This method is particularly suitable when there is evidence of hybridization events between some species in the dataset. For this analysis, 28,363 putatively unlinked SNPs (instead of the whole concatenated alignment) were used to perform the Neighbor-Net analysis to overcome the computational limitations of SplitsTree4 in handling very large datasets.

2.5. Taxonomic jackknife

The taxonomic jackknife method was applied to test the effect of taxon sampling on the topology and branch support. This measures the tree robustness and overall data consistency, by assessing the stability of the clades, branching topology and bootstrap support when removing a specific taxon. Phylogenetic relations are first estimated using the entire set of taxa; analyses are then repeated by pruning each taxon of interest from the dataset, one at a time. Changes in the tree topology and/or support values can indicate hybrid taxa or “rogue taxa”. This approach can therefore help detect hybridization signals in multilocus phylogenetic trees (Seehausen, 2004). Since Neighbor-Joining method produced the same topology as obtained using RAxML and MrBayes, while being much faster to compute, we implemented the taxonomic jackknife by producing multiple Neighbor-Joining (Saitou and Nei, 1987) phylogenetic trees, with the R packages APE (Paradis et al., 2004) and phangorn (Schliep, 2011) (R software 3.0.1, The R Foundation, 2013). The outgroup species was *Thymallus thymallus*, and node support was estimated with 500 bootstrap replicates. The final trees, with bootstrap values, were visualized in Dendroscope (Huson et al., 2007).

2.6. Detection and estimation of introgression events

To test for past introgression events and gene flow, we used the D-statistic test (Durand et al., 2011; Green et al., 2010; Patterson et al., 2012) as implemented in the pyRAD v 2.7 software pipeline (Eaton, 2014; Eaton and Ree, 2013), and based on the topology recovered from Maximum Likelihood (ML) searches in RAxML. Applied to a four-taxa topology, including three sister taxa and one outgroup, the D-statistic test can reliably detect asymmetry in allele pattern frequencies, which are inconsistent with the topology. Although this test has been mainly used to detect inter-population hybridization, recent studies have shown that it is also suitable to detect introgression on genome-wide data between more distantly related taxa (Eaton and Ree, 2013; Escudero et al., 2014). In a (((P1,P2),P3),O) topology, the D-statistic test analyzes the common loci to detect incongruent apomorphic characters, which only occur in both P3 and P1 or both P3 and P2. The test reveals a positive hybridization signal when the number of alleles only shared by P3 and P1 is significantly different from the number of alleles shared only by P3 and P2, indicating an exchange of alleles through introgression. Indeed, a similar number of inconsistent allele patterns in both pairs of taxa are expected to be the result of stochastic lineage sorting without gene flow. For these tests, heterozygous sites were excluded, following a conservative method (Eaton and Ree, 2013). For each test, the standard deviation of the D-statistic was calculated with 1000 bootstrap replicates. Statistical significance was determined by converting the obtained Z-scores, into a two-tailed p-value using the R software 3.0.2 (R Core Team, 2015) with the alpha level adjusted to 0.01 using the Holm-Bonferroni correction for multiple tests (Holm, 1979). The D-statistic test is implemented to detect significant signals of hybridization, but does not estimate the proportion of introgressed loci. Therefore, when a significant hybridization signal was detected based on the D-statistic, the proportion of genetic introgression involved was

Table 2
Raw number of reads obtained for each sample, number of aligned clusters from the pyRAD analysis with a minimum of five reads per cluster, number of consensus loci after filtering for paralogs, and number of loci in the final dataset including a minimum of 18 taxa.

Taxon	Raw reads ($\times 10^6$)	Clusters at 90%[a]	Mean depth	Consensus loci[b]	Number of loci in final data set[c]
Thymallus thymallus (a)	2.08	52,575	29.97	48,904	6193
Thymallus thymallus (b)	1.85	54,653	24.10	50,071	6193
Hucho hucho (a)	9.38	79,496	24.62	66,931	16,133
Hucho hucho (b)	6.11	73,149	20.51	62,077	16,059
Brachymystax lenok blunt snout	1.24	53,350	12.85	48,902	13,863
Brachymystax lenok sharp snout	1.19	53,138	12.92	48,702	13,746
Parahucho perryi (a)	2.30	60,242	22.45	55,152	20,678
Parahucho perryi (b)	4.64	66,238	32.03	59,795	21,101
Oncorhynchus mykiss (a)	2.62	63,957	23.85	58,278	18,032
Oncorhynchus mykiss (b)	0.53	28,348	5.21	25,084	7702
Oncorhynchus gorbuscha	4.35	66,682	37.95	61,536	17,798
Oncorhynchus keta (a)	1.93	60,760	19.72	55,722	17,484
Oncorhynchus keta (b)	2.71	66,163	20.76	58,973	17,632
Oncorhynchus masou (a)	4.07	68,975	34.47	62,330	18,182
Oncorhynchus masou (b)	0.29	16,683	4.62	14,760	4579
Oncorhynchus masou (c)	3.55	68,161	28.71	61,692	18,141
Salvelinus namaycush (a)	4.01	63,892	30.87	57,387	25,140
Salvelinus namaycush (b)	1.43	55,299	14.81	50,600	23,784
Salvelinus fontinalis (a)	1.78	56,637	20.01	52,071	22,965
Salvelinus fontinalis (b)	1.88	57,386	20.52	53,081	23,493
Salvelinus leucomaenis (a)	5.13	66,239	44.23	60,002	25,392
Salvelinus leucomaenis (b)	2.11	58,284	22.67	53,363	24,484
Salvelinus leucomaenis (c)	6.17	66,446	49.52	60,048	25,297
Salvelinus levanidovi (a)	1.34	53,459	14.68	49,068	23,413
Salvelinus levanidovi (b)	5.43	64,680	41.89	58,715	25,399
Salvelinus malma (BER)	2.33	60,067	24.42	54,398	26,069
Salvelinus malma (OKH) (a)	3.36	63,949	31.82	57,586	26,451
Salvelinus malma (OKH) (b)	12.03	74,798	62.34	66,308	26,636
Salvelinus alpinus (SIB)	1.53	54,413	17.13	50,054	25,027
Salvelinus alpinus (ACD)	3.28	60,319	32.00	55,267	26,260
Salvelinus alpinus (ARC)	1.05	48,525	9.81	44,206	21,411
Salvelinus alpinus (ATL)	2.36	58,828	23.93	54,084	26,139
Salvethymus svetovidovi	1.33	51,701	14.85	47,774	24,025
Salvelinus confluentus	1.44	55,701	15.51	51,096	24,530
Salmo trutta (a)	1.51	57,127	15.41	52,067	19,091
Salmo trutta (b)	3.07	63,943	29.33	57,943	20,515
Salmo salar	2.78	61,846	27.64	56,653	20,023
Salmo marmoratus	1.05	42,428	10.44	37,838	13,232
Salmo obtusirostris	0.35	20,660	4.89	18,083	6383
Salmo ohridanus	1.09	52,329	11.50	47,314	17,448

[a] Clusters with minimum coverage of 5 reads.
[b] Consensus loci which passed filtering for paralogs.
[c] Minimum taxa in a final locus = 18.

estimated using the *f*-estimator (Durand et al., 2011; Green et al., 2010; Martin et al., 2015; Reich et al., 2010). The *f*-estimator provides the opportunity to compare, for the two taxa, the observed difference in number of incongruent allele patterns to what would be expected in the case of a complete introgression event with homogenization of allele frequencies. To check if the percentage of introgression calculated by the *f*-estimator was consistent with the D-statistic results, we calculated this percentage for a subset of the non-significant D-statistic results.

2.7. Divergence time estimation

A molecular dating analysis was performed on the partitioned dataset in BEAST 2.3.1 (Bouckaert et al., 2014; Drummond et al., 2006; Heled and Drummond, 2012), using a Bayesian relaxed molecular clock with uncorrelated lognormal rate heterogeneity, to allow for variable evolutionary rates between lineages, and a Yule speciation tree prior (Gernhard, 2008; Yule, 1925), as the focus was on the divergence time at the inter-specific level. This analysis included 26 individuals representing clearly separated genetic lineages or different species. For divergence time estimation, the partitioned dataset was used as input, and the best ML tree topology inferred by RAxML analysis was used as a starting tree. The BEAST analysis was conducted using linked trees, linked clock models and unlinked substitution-rates, under the general time-reversible nucleotide substitution model (GTR + G) for each partition. To reduce the risk of incorrect molecular dating due to unreliable fossil dating, only four reliable fossil records, with their best or most conservative age estimate (i.e. minimum estimate), were used to calibrate the divergence time estimation. Each fossil used in this analysis was used as a minimum time constraint for the node being calibrated. †*Eosalmo driftwoodensis* is the oldest known fossil of Salmonidae (Wilson, 1977; Wilson and Li, 1999), which was found in Driftwood Canyon (British Columbia) from which the sediments have been dated to early Eocene (Ypresian), more precisely estimated to be 51.8 MY (± 0.3 MY) (Greenwood et al., 2005). This extinct species is considered to be a stem lineage to Salmoninae (Stearley and Smith, 1993; Wilson and Li, 1999; Wilson and Williams, 1992); and therefore, 50 MY was used as a conservative minimum boundary for the Salmonidae family, as done previously in some studies (Crête-Lafrenière et al., 2012; Macqueen and Johnston, 2014) to calibrate the most recent common ancestor of Salmonidae (Ln offset: 50, mean: 10, sd: 1). †*Salvelinus larsoni* is dated to the middle of the Miocene (Kimmel, 1975; Smith et al., 1982; Stearley and Smith, 1993), and is more specifically estimated to be 11 MY old (Power, 2002). Therefore, 11 MY was used as a minimum time constraint for the stem node of the genus *Salvelinus* (Ln offset: 11, mean: 23, sd: 1). †*Oncorhynchus rastrosus* (Berggren et al., 1985; Koch et al., 1992; Smith et al., 1982) constitutes the oldest

representative of the genus *Oncorhynchus* (Barnes, 1976) and is dated to the Late Miocene, 11.5 MY (± 0.5 MY) (Eiting and Smith, 2007). Thus, 11 MY was used to constrain the minimum age of the crown node of (*Oncorhynchus masou*, (*Oncorhynchus keta*, *Oncorhynchus gorbuscha*)) as previously done (Crête-Lafrenière et al., 2012) (Ln offset: 11, mean: 15, sd: 1). †*Oncorhynchus ketopsis* is dated to the late Miocene, between 6 and 8 MY (Eiting and Smith, 2007; Stearley and Smith, 1993), therefore 6 MY was used as a minimum divergence time between *O. keta* and *O. gorbuscha* based on the relationships to extant taxa inferred from the description of the fossils (Eiting and Smith, 2007) (Ln offset: 6, mean: 8, sd: 1). The input file for BEAST 2, with all the parameters and priors, was set up using BEAUti 2.3.1 (Bouckaert et al., 2014). All parameters were estimated using the Bayesian method based the Markov Chain Monte Carlo (MCMC) algorithm. The molecular dating analysis was run for a total of 90 million generations, sampled every 3000th generation. Tracer v1.6 (Rambaut et al., 2014) was used to explore the output of the BEAST analysis, in order to check for adequate effective sample size (> 200) and to determine the burn-in percentage. A 25% burn-in was applied in TreeAnnotator v2.1.2 (Rambaut and Drummond, 2014), and the posterior sample estimates of the trees from the BEAST analysis were summarized and combined to produce a consensus maximum clade credibility tree. Finally, FigTree v1.4 (Rambaut, 2012) was used to display the best molecular phylogeny and visualize the 95% Highest Posterior Density (HPD) for each node.

3. Results

3.1. RAD-sequencing and raw data analysis

The Illumina RAD-sequencing produced on average 2.96×10^6 reads per sample with an average of 23.9× coverage. Following quality filtering and assignment of the reads to each individual, the retained reads of thirty individuals had an optimal Phred score (> 28) for all bases, and thirteen samples had lower Phred scores toward the end of the reads (> 20). After removing the barcodes, the reads were trimmed to 92 bp. Using the pyRAD software pipeline with a 90% similarity and a minimum coverage of 5, the final dataset of 28,402 loci, 373,331 SNPs including 28,363 putatively unlinked SNPs and 258,849 parsimony informative sites (Table 2), created a concatenated matrix of 2.59×10^6 aligned nucleotides. Three samples, one individual of *Oncorhynchus nerka* and two individuals of *Salvelinus confluentus*, were filtered out during the pyRAD analysis due to a very low number of RAD tags and mean coverage. Missing data in the overall final matrix were partly due to divergent evolution of some restriction sites in certain taxa, especially in the outgroup taxon, as well as variable quality of template DNA (Table 2).

3.2. Phylogenetic analysis

For the partitioning of the final dataset from the pyRAD analysis, the iterative k-means algorithm, based on the best BIC score, clustered the individual sites of the alignment into 28 subsets. The best-fit nucleotide substitution model of molecular evolution was the GRT + Γ (general time-reversible substitution and gamma distributed rate variation across sites).

The Maximum Likelihood searches in RAxML and the Bayesian Inference from MrBayes produced strikingly similar and well-resolved phylogenetic trees with BS, IC and posterior density values (Fig. 1 and Appendix A). The relative tree certainty was estimated in RAxML to be 0.97. Only 0.1% of the sites were completely undetermined, while the overall percentage of missing data in the whole RAD-Seq dataset is 35.5%.

The trees reveal three major clades within Salmoninae, with *Brachymystax/Hucho* clade splitting off basal, while the *Parahucho/Salmo* clade is a sister-group to the *Salvelinus/Oncorhynchus* lineages. *Salvethymus* grouped within the genus *Salvelinus*, which is consistent with the findings of previous studies (Crête-Lafrenière et al., 2012; Osinov et al., 2015; Shed'ko et al., 2013; Shubina et al., 2013). This monotypic genus appears to be the sister taxon of the *S. alpinus–S. malma* complex, and is located within what used to be considered a single taxon: *S. alpinus/S. malma/S. confluentus* (McPhail, 1961; Taylor, 2016). *Salvelinus* and *Oncorhynchus* are supported as sister genera in our results, which supports previous Salmonidae phylogenetic studies (Fig. 5) (Alexandrou et al., 2013; Crespi and Fulton, 2004; Crête-Lafrenière et al., 2012; Koop et al., 2008; Ma et al., 2015; Macqueen and Johnston, 2014; Shed'ko et al., 2013; Wang et al., 2011; Wilson and Turner, 2009; Yasuike et al., 2010). The well-supported clade of *Salvelinus leucomaenis* and *S. levanidovi* appears as a sister-group to the remaining members of the genus *Salvelinus*. Our results also show *Salvelinus namaycush* as the closest species to *S. fontinalis,* as shown in some previous analyses (Crespi and Fulton, 2004; Crête-Lafrenière et al., 2012). Within the genus *Oncorhynchus*, among the taxa included in our dataset, *O. mykiss* is the sister-group to a clade composed of the remaining *Oncorhynchus*, with *O. gorbuscha* and *O. keta* clustering together and appearing as a sister clade to *O. masou*. In the genus *Salmo*, the taxon sampling is limited to five species and *S. salar* is the sister-group to all remaining *Salmo* taxa in our analysis. *S. marmoratus* and *S. trutta* appear as sister taxa, and the exact position of *S. obtusirostris* and *S. ohridanus* shows low BS support, low posterior probability and very low IC score (Figs. 1 and 2), which may be due to a much lower number of reads for *S. obtusirostris,* leading to a large amount of missing data for this species in the final dataset. Finally, our results show that *Parahucho* is the sister-group to *Salmo*, which has only been observed in few studies so far (Fig. 5) (Alexandrou et al., 2013; Crespi and Fulton, 2004; Oakley and Phillips, 1999).

3.3. Neighbor-net analysis

The Neighbor-Net analysis produced a network with well resolved phylogenetic relationships, and only very few conflicting signals of unresolved relationships likely resulting from ancient hybridization between some species (Fig. 2a), especially within the genus *Salvelinus*, such as between *S. namaycush* and *S. fontinalis*, and within the *S. alpinus–S. malma* complex (Fig. 2b), but also between *O. keta* and *O. gorbuscha* (Fig. 2a). The phylogenetic inference of the relationships between species is predominantly tree-like and highly consistent both with the ML phylogenetic tree and with the Bayesian Inference from MrBayes.

3.4. Taxonomic jackknife

The multiple Neighbor-Joining trees, estimated using the taxonomic jackknife, show an overall robustness and topological stability (Fig. 3a & 3b). Variations in bootstrap values reveal a few instabilities, most likely due to hybridization events between certain taxa. The BS supports estimated using R are slightly different from those inferred by RAxML (Fig. 3a A and Fig. 1); indeed two lower BS values appear in the genus *Salmo*. The pruning of *S. marmoratus* (Fig. 3a B) changes the position of *S. ohridanus* with a very low BS support, while the pruning of *S. ohridanus* (Fig. 3a C) only affects the BS support. The pruning of *S. obtusirostris* (Fig. 3a D) does not affect the topology but the BS supports reach 100 for all nodes potentially indicating hybridization involving this taxon but also the possible effect of missing data. Within *Salvelinus*, the removal of *S. leucomaenis* (Fig. 3b E) does not change the topology but a significant decrease in BS support occurs at the node separating (*S. confluentus*, *Sv. svetovidovi*, *S. alpinus–S. malma* complex) and (*S. levanidovi*, *S. fontinalis*, *S. namaycush*), revealing some instability likely induced by ancient hybridization in the genus. The pruning of several other taxa (Fig. 3b) within *Salvelinus* did not show any effect on either topology or node support.

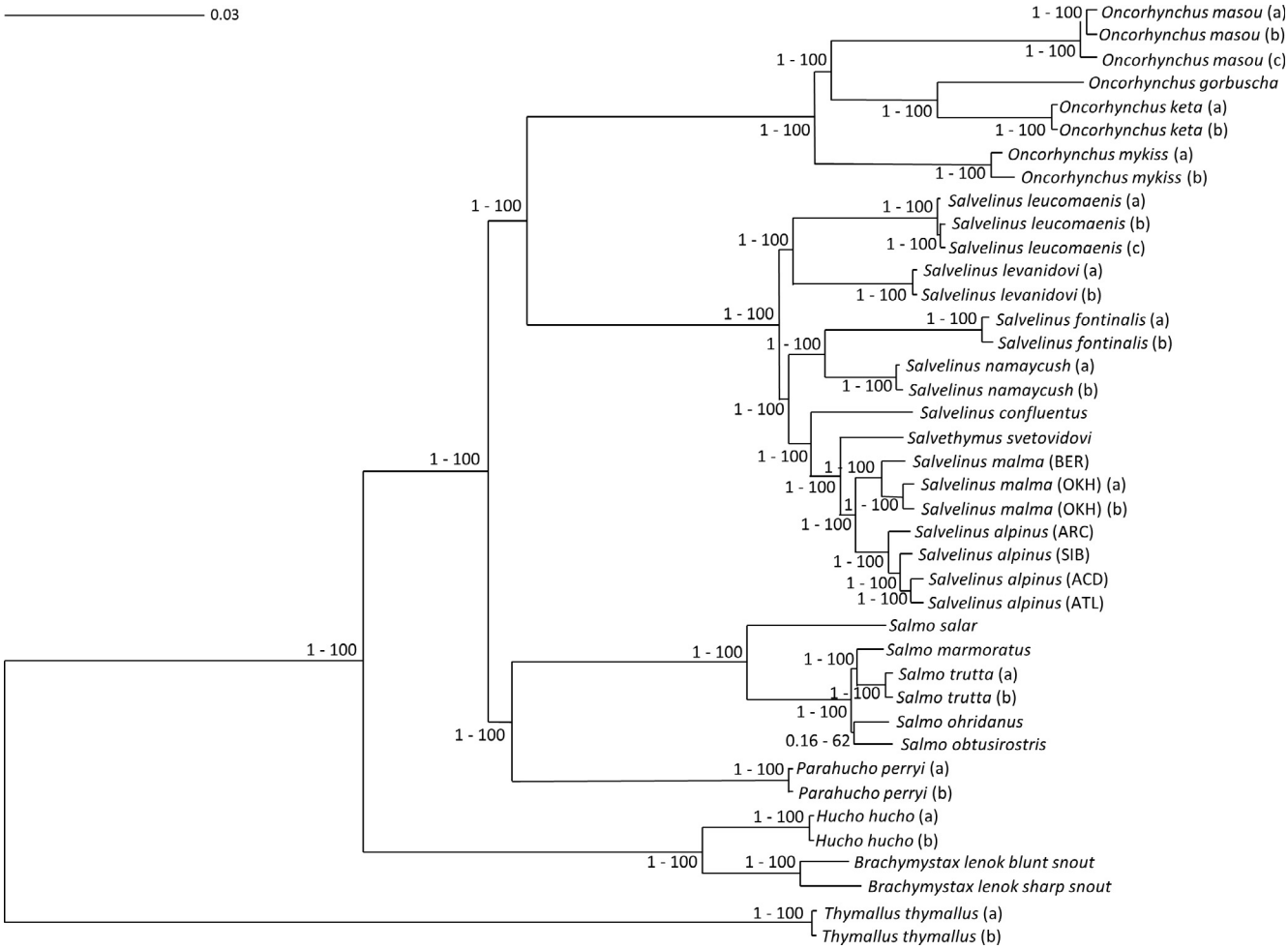

Fig. 1. Maximum-likelihood (ML) phylogenetic tree of 40 salmonid taxa from RAxML analysis, based on the best partition scheme of the dataset from PartitionFinder. On the node labels, the first number represents the Internode Certainty (IC scores) and the second number represents the bootstrap support value (BS) of each node. The scale bar represents the nucleotide substitutions per site.

3.5. Detection and estimation of introgression events

Based on a number of hypotheses and the position of particular taxa thought to have undergone reticulate evolution, with a focus on the genera *Salvelinus* and *Salmo*, we tested 64 four-taxa combinations for introgression using the D-statistic test. Some of these tests merely involved a different individual for a taxon with replicates in the dataset. In total, nine four-taxa combinations were statistically significant (Table 3). The results from the D-statistic tests revealed several signals of introgression events in the genus *Salvelinus*. For instance, *Sv. svetovidovi* shows hybridization signals with *S. levanidovi* (2.56%) and *S. namaycush* (2.19%). *S. confluentus* also shows evidence of introgression with *S. namaycush* (2.48%), while *S. namaycush* also reveals potential ancient hybridization with *S. leucomaenis*. Additionally, significant signals of introgression were detected with the D-statistic test between *S. malma* of the Bering clade (BER) and *S. alpinus* of the Siberian (SIB), Acadian (ACD) and Atlantic (ATL) and Arctic (ARC) clades. Finally, in the *Salmo* genus, only one pair of taxa exhibits introgression signal, *S. marmoratus* and *S. obtusirostris*.

Estimated percentage of introgression, calculated using *f*-estimator, for taxon pairs revealing significant D-statistic signals, ranged from 1.66%, between *S. namaycush* and *S. leucomaenis*, up to 4.24%, between *Salmo marmoratus* and *Salmo obtusirostris*. Higher percentage of introgression could be, at least partially, associated with hybridization that is more recent. Values of the *f*-estimator calculated for a subset of the non-significant D-statistic results resulted in lower introgression estimates, ranging from 0 to 1.61% (mean: 0.49, sd: 0.41).

3.6. Divergence time estimation

The tree topology recovered from the molecular dating analysis, based on the 28,402 putative orthologous loci across 21 salmonid species (Fig. 4), was identical to those recovered from the Maximum Likelihood analysis and Bayesian Inference (Figs. 1 and 2). The node clustering *Salmo ohridanus* and *S. obtusirostris* once again showed much lower support with a posterior probability of 0.77, while posterior probability was equal to 1 for all the other nodes in the tree.

The age of the most recent common ancestor of Salmonidae, at the divergence point between Salmoninae and Thymallinae, was estimated by the BEAST analysis (Fig. 4) to be 58.9 MY, with the 95% Highest Posterior Density (HPD) ranging from 50.8 to 64.0 MY. The crown age of Salmoninae subfamily was predicted to be around 37.7 MY (95% HPD: 35.2–40.8 MY). The divergence separating *Salmo/Parahucho* and *Oncorhynchus/Salvelinus* took place about 29.8 MY ago (95% HPD: 27.6–33.2 MY), while the divergence between the genus *Oncorhynchus* and *Salvelinus* was estimated to have occurred 21.2 MY ago (95% HPD: 19.8–23.0 MY). The crown age of the genus *Salvelinus* was predicted to be 15.1 MY (14.1–16.4 MY), slightly older than the crown age of the genus *Salmo* estimated to be 13.8 MY (13.3–14.8 MY). *Salvelinus confluentus* arose around 4.6 MY ago (4.1–5.9 MY), while *Salvethymus svetovidovi* emerged approximately 3.2 MY ago (2.6 – 3.6 MY), which is consistent with the estimated age of the lake El'gygytgyn of 3.58 MY

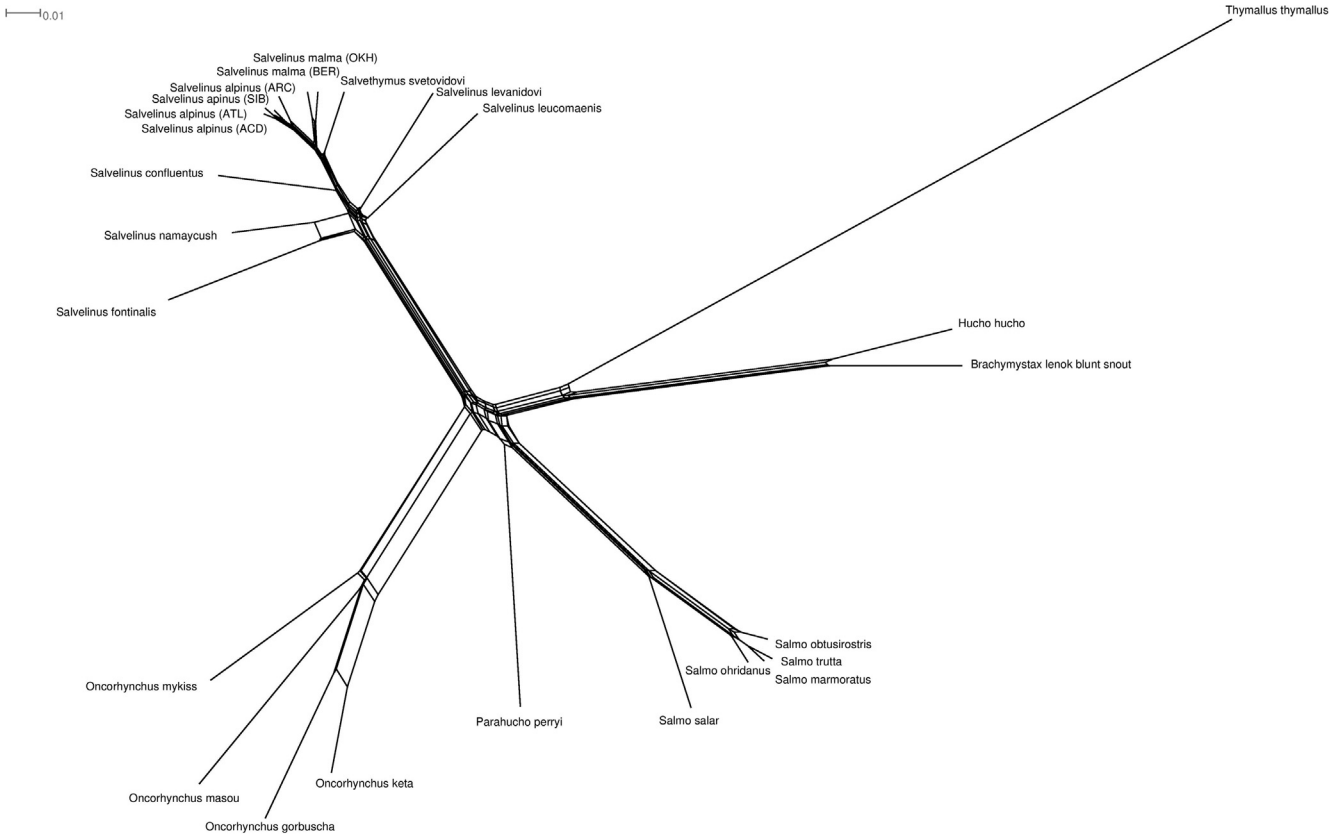

Fig. 2a. Split graph of the Neighbor-Net phylogenetic network analysis of 40 salmonid taxa, generated using SplitsTree4. The scale bar represents the nucleotide substitutions per site.

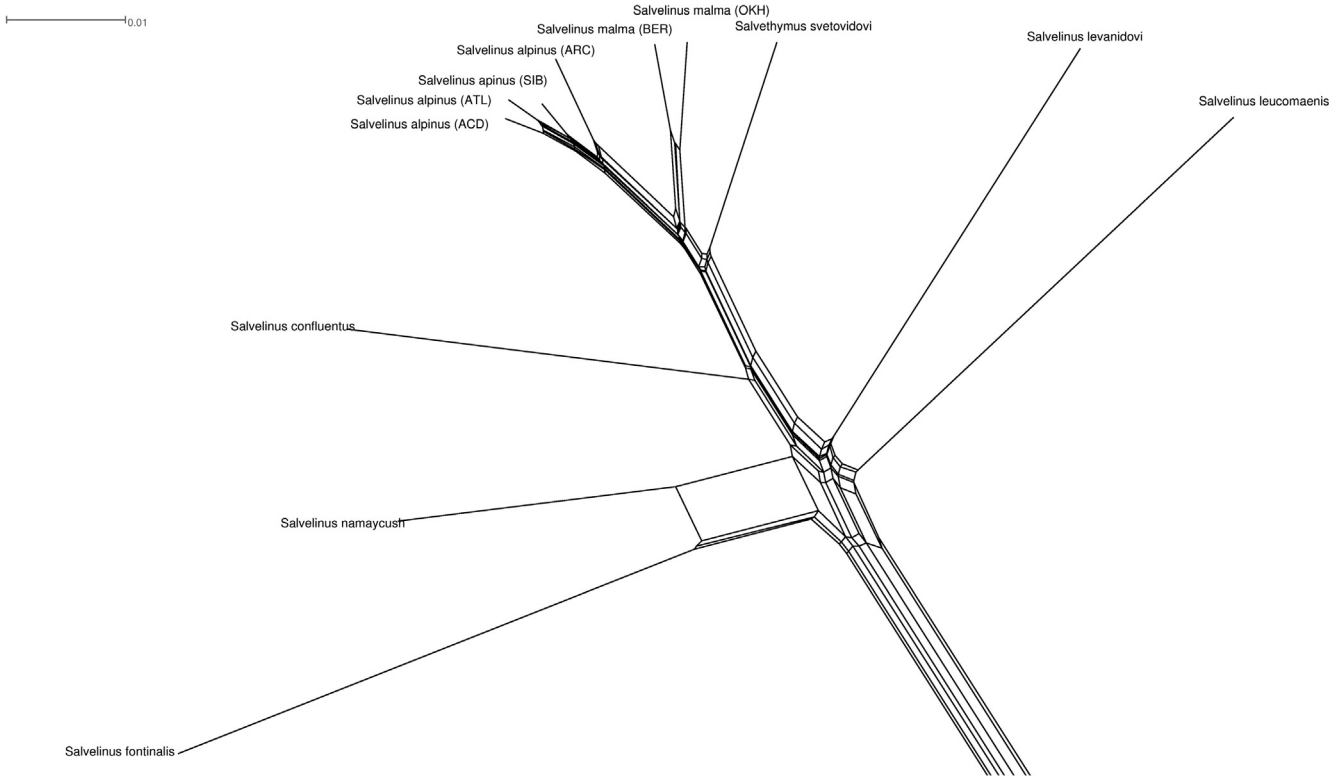

Fig. 2b. Zoom in of the genus *Salvelinus* in the Neighbor-Net phylogenetic network generated using SplitsTree4. The scale bar represents the nucleotide substitutions per site.

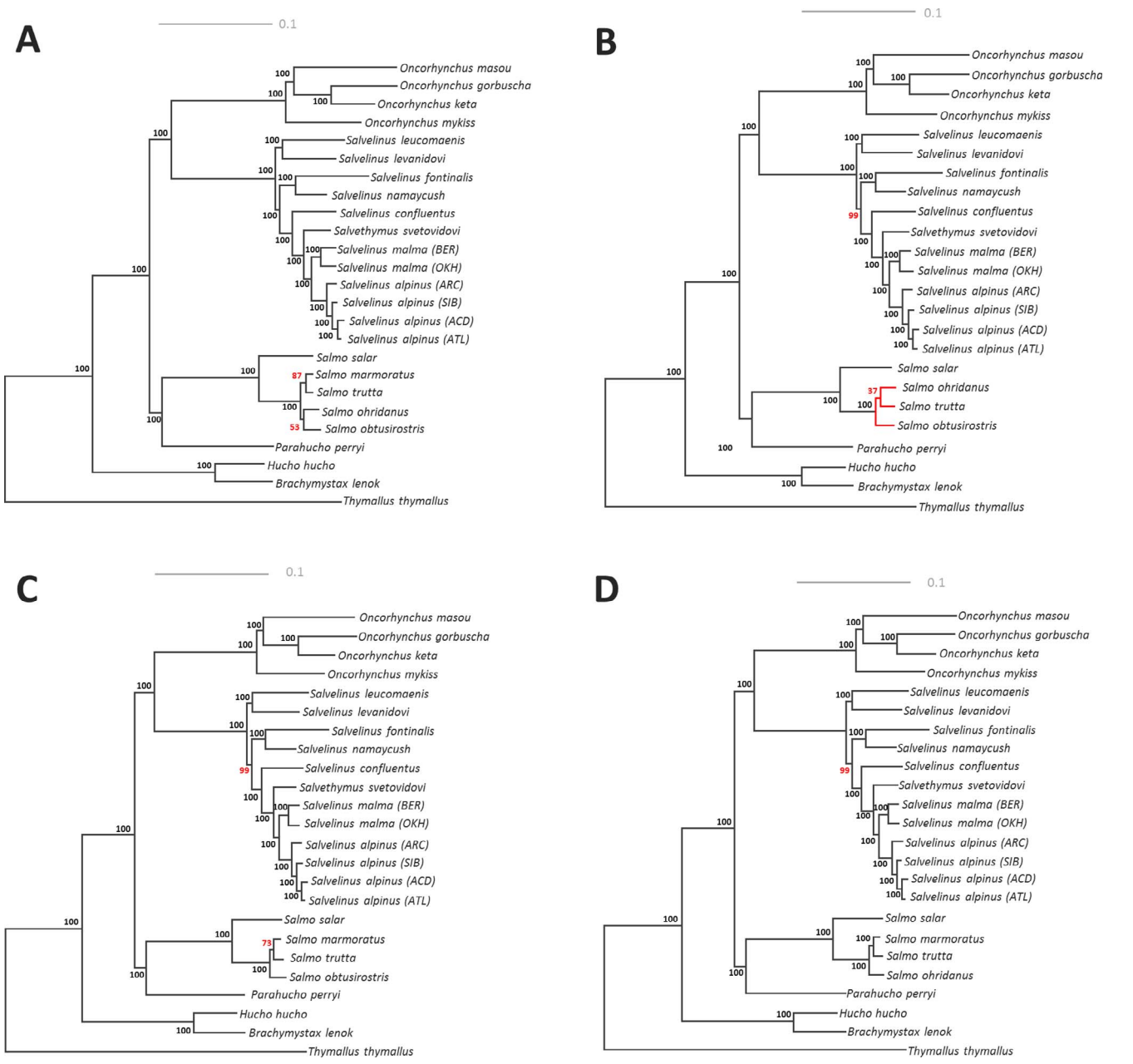

Fig. 3a. Neighbor-Joining (NJ) phylogenetic trees of all taxa and *Salmo* taxa using the taxonomic jackknife method in R. The node labels represent the bootstrap values (BS) of each node; the ones in red correspond to BS values lower than 100. (A) All taxa, (B) pruning of *Salmo marmoratus*, (C) pruning of *Salmo ohridanus*, (D) pruning of *Salmo obtusirostris*. Branches affected by the pruning are marked in red. The scale bar represents the nucleotide substitutions per site. (For interpretation of color in this figure legend, the reader is referred to the web version of this article.)

(± 0.04 Ma) (Layer, 2000) where this species is endemic. Except for *Salvethymus*, the most recent genera split within Salmoninae took place around 11.5 MY ago (8.9–14.6 MY) between *Hucho* and *Brachymystax*. Overall, the divergence between genera of the Salmoninae subfamily occurred between the late Eocene and middle of the Miocene (≈38 to 11 MY), while the species diversification took place mainly during the Neogene (≈22 to 1.5 MY). In fact, all the extant taxa in our dataset emerged within the last 22 MY, with more than half of them in the last 10 MY.

4. Discussion

Although phylogenetic relationships of salmonids, based on a RAD-sequencing dataset, were previously inferred by Gonen et al. (2015), the analysis included only 5 salmonids species and 3050 loci. Therefore, we present the first phylogeny of salmonid fishes based on a large RAD-sequencing dataset, with an extensive taxon sampling of the family. With a focus on the subfamily Salmoninae and extensive taxon coverage of the genus *Salvelinus*, the topology recovered, based on > 28,000 loci, is well resolved and highly supported across all applied methods, thus providing some clear answers to a few phylogenetic uncertainties highlighted by the conflicting results from previous studies (Fig. 5). For instance, *Salvelinus* and *Oncorhynchus* appear as sister genera, which is very well supported in our data and there is multiple independent evidence supporting this relationship such as higher shared synteny, morphology, biogeography, and ecology (Crespi and Fulton, 2004). The estimated divergence of 21.2 MY (HDP: 19.8–23.0 MY) (Fig. 4) between the two genera is similar to the estimated divergence time in two separate studies: 23.5 MY (Macqueen and Johnston, 2014) and 20 MY (Shed'ko et al., 2013) (Fig. 5). Another example is the position of the

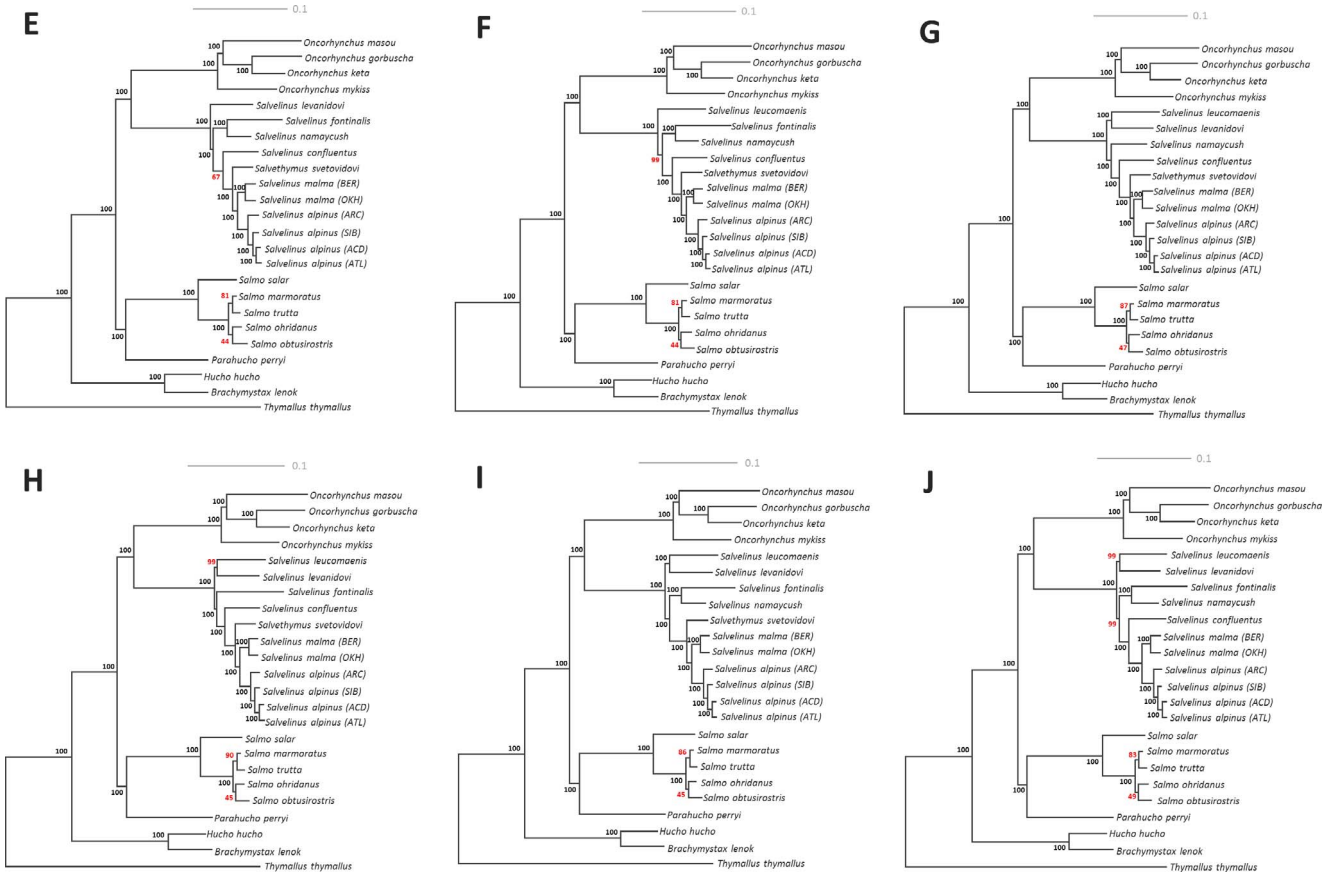

Fig. 3b. Neighbor-Joining (NJ) phylogenetic trees of *Salvelinus* taxa using the taxonomic jackknife method in R. The node labels represent the bootstrap values of each node; the ones in red correspond to BS values lower than 100. (E) Pruning of *Salvelinus leucomaenis*, (F) pruning of *Salvelinus levanidovi*, (G) pruning of *Salvelinus fontinalis*, (H) pruning of *Salvelinus namaycush*, (I) pruning of *Salvelinus confluentus*, (J) pruning of *Salvethymus svetovidovi*. The scale bar represents the nucleotide substitutions per site. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

monotypic genus *Parahucho* as a sister genus to *Salmo* with a mean divergence time of 21.9 MY. This placement has also appeared in Crespi and Fulton (2004), as well as Alexandrou et al. (2013), but in contrast to a number of other studies that either grouped *Parahucho* with *Salvelinus* or simply as the sister-group to the *Oncorhynchus/Salvelinus* clade, or to the *Oncorhynchus/Salmo* clade (Campbell et al., 2013; Crête-Lafrenière et al., 2012; Ma et al., 2015; Shed'ko et al., 2013) (Fig. 5). Our results also support the placement of *Salvethymus,* from Lake El'gygytgyn, within the genus *Salvelinus,* here as sister-group to the *S. alpinus–S. malma* clade, supporting conclusions that its morphological distinctiveness might be based on paedomorphic characters (Alekseyev, 2000; Osinov et al., 2015) rather than being a primitive form of *Salvelinus* as initially described (Chereshnev and Skopets, 1990). Therefore, based on molecular evidence, *Salvethymus svetovidovi* should be included within the genus *Salvelinus*.

Some concerns could be raised regarding the impact of the WGD on our phylogenetic inferences, more specifically the effect of differential rates of rediploidization across the genome (Robertson et al., 2017). In regions characterized by extremely delayed rediploidization, known as 'Lineage-specific ohnolog resolution' regions (LORe), species divergence occurred before the divergence of ohnologs, which leads to the absence of true orthology across species, potentially affecting phylogenetic signals (Robertson et al., 2017). We evaluated postliminary the potential impact on our dataset by mapping the loci included in our final dataset for *Salmo salar* to the corresponding reference genome (ICSASG_v2) using Bowtie2 v2.2.9 (Langmead and Salzberg, 2012). Subsequently, we identified which of these loci were located within the LORe regions using the coordinates retrieved from the supplementary materials in Lien et al. (2016) and Robertson et al. (2017). We found a relatively negligible percentage (4.6%) of our loci located within LORe regions of the Atlantic salmon genome, and thus we expect a small effect on our phylogenetic inferences considering the size of the dataset.

Table 3
Summary table of the significant four-taxa D-statistic tests results and proportion of introgression calculated using *f*-estimator. Blue indicates the pairs of taxa showing signals of hybridization.

P1	P2	P3	O	D	sd(D)	Z	p-value	BABA	ABBA	Common loci	Introgression
Salmo trutta	*Salmo marmoratus*	*Salmo obtusirostris*	*Salmo salar*	0.425	0.110	3.86	$1.13x10^{-4}$	21	52	3535	**4.24 %**
Salvelinus fontinalis	*Salvelinus namaycush*	*Salvelinus leucomaenis*	*Oncorhynchus mykiss*	0.172	0.041	4.14	$3.47x10^{-5}$	258	365	12959	**1.66 %**
Salvelinus namaycush	*Salvelinus fontinalis*	*Salvelinus confluentus*	*Oncorhynchus mykiss*	-0.201	0.041	4.96	$7.05x10^{-7}$	382	254	12032	**2.48 %**
Salvelinus leucomaenis	*Salvelinus levanidovi*	*Salvethymus svetovidovi*	*Oncorhynchus mykiss*	0.104	0.031	3.35	$8.08x10^{-4}$	567	699	12060	**2.56 %**
Salvelinus fontinalis	*Salvelinus namaycush*	*Salvethymus svetovidovi*	*Oncorhynchus mykiss*	0.167	0.043	3.90	$9.62x10^{-5}$	250	350	11829	**2.19 %**
Salvelinus malma (OKH)	*Salvelinus malma (BER)*	*Salvelinus alpinus (SIB)*	*Oncorhynchus mykiss*	0.135	0.051	2.65	0.0080	170	228	14720	**2.38 %**
Salvelinus malma (OKH)	*Salvelinus malma (BER)*	*Salvelinus alpinus (ACD)*	*Oncorhynchus mykiss*	0.178	0.050	3.55	$3.85x10^{-4}$	213	305	15331	**3.14 %**
Salvelinus malma (OKH)	*Salvelinus malma (BER)*	*Salvelinus alpinus (ARC)*	*Oncorhynchus mykiss*	0.249	0.062	4.05	$5.12x10^{-5}$	134	223	12402	**4.22 %**
Salvelinus malma (OKH)	*Salvelinus malma (BER)*	*Salvelinus alpinus (ATL)*	*Oncorhynchus mykiss*	0.149	0.053	2.79	0.0053	203	274	15289	**2.62 %**

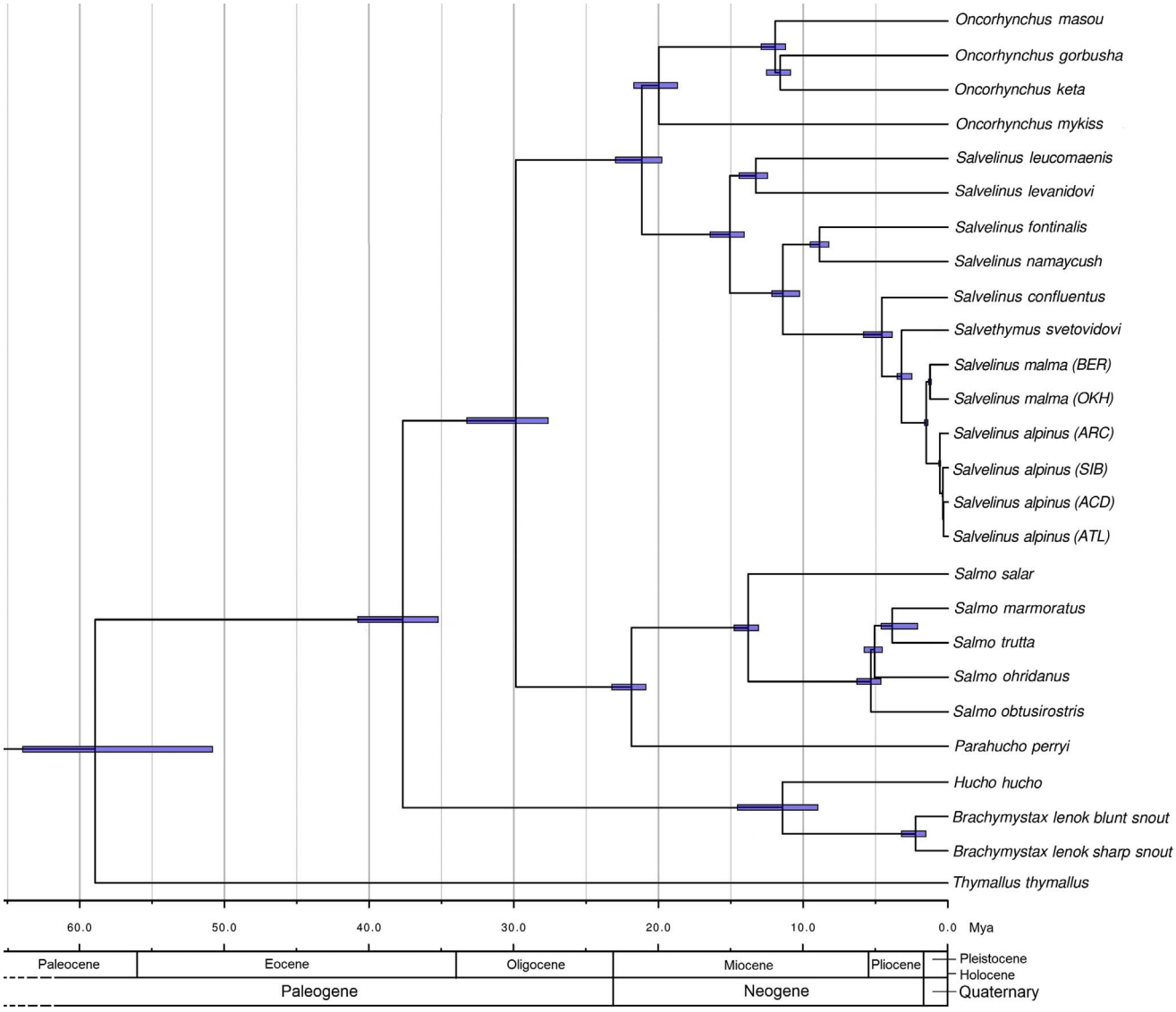

Fig. 4. Fossil-calibrated phylogeny generated using BEAST 2. The horizontal blue bars on the nodes represent 95% highest posterior density. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Nonetheless, future NGS studies addressing salmonid phylogenetics should carefully consider performing appropriate preliminary steps to filter out the loci located in LORe regions in order to avoid any potential bias, although at this time these regions have only been described and clearly defined in the Atlantic salmon genome, making it challenging to completely remove all such regions across many salmonid species.

Our age estimation of the most recent common ancestor of Salmonidae is 58.9 MY (50.8–64 MY), which is highly consistent with the 59.1 MY (58.1–63.2 MY) estimated by Crête-Lafrenière et al. (2012), but also very close to the age estimated in some other studies (Campbell et al., 2013; Ma et al., 2015; Macqueen and Johnston, 2014). Overall, most of the divergence times we estimated between genera are very similar to those estimated in several recent studies that include molecular dating (Campbell et al., 2013; Crête-Lafrenière et al., 2012; Ma et al., 2015; Macqueen and Johnston, 2014) (Fig. 5). There are however, some significant contrasts with divergence times shown in Alexandrou et al. (2013) and Shed'ko et al. (2013), which have respectively the oldest and youngest estimates compared to similar studies (See comparison in Fig. 5). These differences in divergence time estimates are mainly explained by the use of different calibration points at critical nodes. However, the topology between genera inferred in Alexandrou et al. (2013) is the most consistent with ours, especially concerning the branching of *Parahucho perryi* (Fig. 5).

Despite the stability of our topology, multiple statistically significant signals of hybridization were detected within the genus *Salvelinus* and *Salmo*, all of which reveal comparatively low levels of introgression (1.66–4.24%). These estimates are very similar to the introgression levels inferred between *Homo sapiens* and Neanderthals, which were between 1 and 4%, predicted to have occurred 50,000 to 80,000 years ago (Durand et al., 2011; Green et al., 2010; Reich et al., 2010). Therefore, these proportions could indicate ancient hybridization events, but could also potentially reflect low levels of modern introgression, at least for marbled and softmouth trout, as ongoing hybridization does occur in these species (e.g. Sušnik Bajec et al., 2015), and this taxon pair represents the only clade in our analysis lacking 100% node support regardless of the analytical method applied.

Hybridization between two species requires at least partial overlapping distribution, at one point in time, as well as sharing some life history traits pertaining to reproduction. However, even when these conditions are combined, the sympatry of closely related species does not necessarily lead to hybridization due to various pre- or post-zygotic isolating mechanisms. Hybridization is a particularly common process in fishes (Allendorf and Waples, 1996; Bernatchez et al., 1995; Scribner et al., 2000), and is quite prevalent in salmonid species, mainly due to

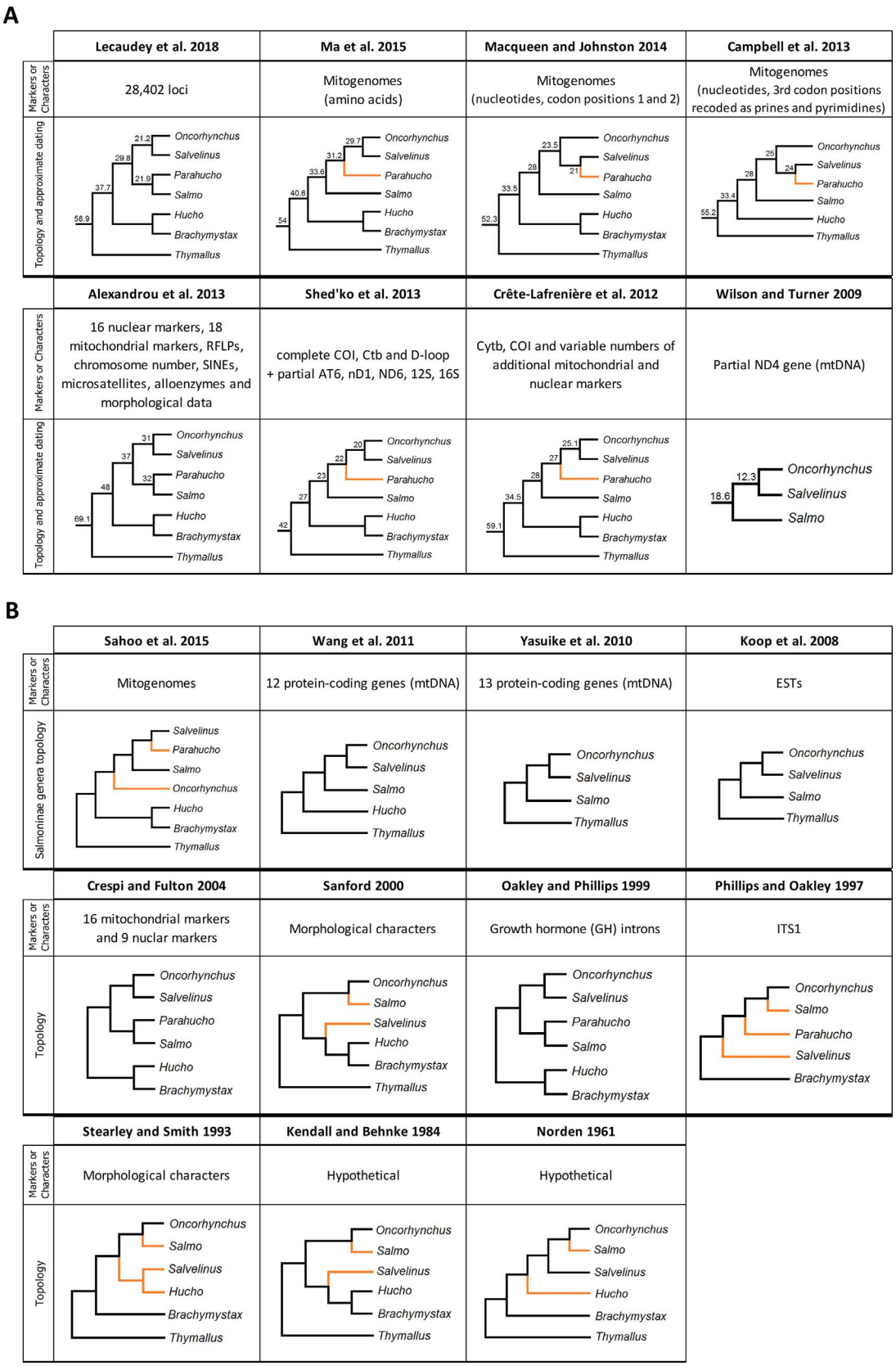

Fig. 5. A: Summary figure of genera topology and approximate node dating within Salmoninae, based on 8 studies (Alexandrou et al., 2013; Campbell et al., 2013; Crête-Lafrenière et al., 2012; Ma et al.,2015; Macqueen and Johnston, 2014; Shed'ko et al., 2013; Wilson and Turner, 2009). B: Summary figure of genera topology within Salmoninae, based on 11 studies (Crespi and Fulton, 2004; Kendall and Behnke, 1984; Koop et al., 2008; Norden, 1961; Oakley and Phillips, 1999; Phillips and Oakley, 1997; Sahoo et al., 2015; Sanford, 2000; Stearley and Smith, 1993; Wang et al., 2011; Yasuike et al., 2010). The topology differences, in comparison to the one found in this study, are marked in orange. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

low post-zygotic barriers (Taylor, 2004). This phenomenon occurs mainly among closely related species when secondary contact occurs and reproductive isolation is not complete. There are numerous documented hybridization and introgression events within the genus *Salvelinus* occurring across millions of years between multiple pairs of species, and at different geographical scales (Baxter et al., 1997; DeHaan et al., 2009; Kanda et al., 2002; Redenbach and Taylor, 2002; Taylor et al., 2001). To understand more clearly the results from the D-statistic tests, the significant hybridization signals should be placed within phylogenetic and phylogeographic contexts.

For *Salvelinus* species, several introgression signals were detected, for instance between *S. malma* and *S. alpinus*, which recently diverged from each other, around 1.5 MY ago (Fig. 4), and have current distributions that partially overlap (Appendix B) (Taylor, 2016). Our results show more specifically signals of hybridization between *S. malma* from the Bering clade and *S. alpinus*. A previous study has shown *S. alpinus* individuals with introgressed haplotypes from the Bering clade of *S. malma* along eastern Siberian coasts where they are parapatric (Alekseyev et al., 2009). This study shows evidence of shared haplotypes between the two species from Arctic Canada, and similarly, shared haplotypes were also found in Alaska (Taylor et al., 2008; Taylor and May-McNally, 2015). In both studies, the observed introgression is expected to be the result of historical hybridization between the species and thus is concordant with our results. A recent study also revealed low levels of hybridization (< 1%), kept low due to ecological segregation, between *S. alpinus* and *S. malma* in Alaska, where they occur in sympatry (May-McNally et al., 2015). Introgression between the two species had also been shown in earlier studies (Brunner et al., 2001; Hamada et al., 1998). This hybridization and respective diversity has even created debate concerning their status as separate species (Brunner et al., 2001; McPhail, 1961; Taylor et al., 2008) but more recent studies show clear evidence that they are indeed distinct species (Moore et al., 2015; Taylor et al., 2008). Additionally, post-glacial hybridization between different glacial lineages of *S. alpinus*, which survived in separate refugia, has also been recently demonstrated (Moore et al., 2015).

We found, for the first time, signals of introgression between *S. namaycush* and *S. confluentus*, who diverged around 11.4 MY ago (Fig. 4) and have native ranges that partially overlap in the western part of North America (Appendix B), implying that ancient hybridization between these two species is plausible. Another instance of hybridization signal in our data is between S. *namaycuch* and *S. leucomaenis*, for which the common ancestor can be traced back to around 15.1 MY (Fig. 4). The native location of *S. leucomaenis* is the Sea of Japan, the Sea of Okhotsk and the Russian coast of the Bering Sea, while *S. namaycush* is native to North America (Appendix B), but the distribution range of these two species, being geographically proximate in the Bering Sea area, could have been parapatric in the past and therefore compatible with an ancient hybridization event, which is also supported by the relatively low introgression proportion we detected. The endemic species to Lake El'gygytgyn, *Sv. svetovidovi*, exhibits introgression signals with *S. namaycush*, as well as with *S. levanidovi,* in our results. In both cases, the pairs of species are allopatric (Appendix B), and hybridization is difficult to explain based on current geographic distributions; therefore, the introgression we detect between these pairs of species could in reality stem from an unknown closely related species or specific lineage, potentially extinct, not included in our dataset, as this is a known issue with D-statistic test (Durand et al., 2011; Eaton and Ree, 2013). When the real taxon or lineage involved in the hybridization is not sampled, a significant introgression signal can potentially be detected between the real allele receiver and the most closely related taxon or lineage to the real allele donor in the dataset, due to their shared ancestry (Durand et al., 2011; Eaton et al., 2015; Eaton and Ree, 2013). It can also be challenging to distinguish between separate introgression events when one species is involved in hybridization events with several species. Additionally, hybridization between certain pairs of species can result in asymmetrical genetic introgression and/or bias sex ratio, which can potentially hinder its detection. Finally, signals of hybridization are expected to be diluted over time by the accumulation of mutations and genetic drift occurring since the hybridization events, and percentage of ancient introgression is underestimated to some extant due to the fact that back mutations are not accounted for by the D-statistic test or *f*-estimator.

Sv. svetovidovi interestingly possesses a much lower number of chromosomes than the average observed among *Salvelinus* as a result of multiple Robertsonian translocations (Frolov, 1997, 1993; Oleinik et al., 2015; Ráb and Phillips, 2001; Sutherland et al., 2016). These major chromosomal rearrangements are likely the main reason for its morphologically aberrant characters, associated with a primitive or paedomorphic phenotype among *Salvelinus*. Furthermore, most of the primitive features of *Salvelinus* are inherent in the karyotypes of *Sv. svetovidovi*, *S. namaycush*, *S. fontinalis* and *S. levanidovi* (Frolov, 1997), such as the presence of multiple nucleolus organizer regions (NORs) (Frolov, 2001, 1997, 1995). *Sv. svetovidovi* presents a peculiar and unique mosaic of plesiomorphic and apomorphic characters of the genus *Salvelinus* (Chereshnev et al., 2002; Oleinik et al., 2015). Therefore, the Robertsonian translocations and subsequent rearrangements that have occurred in the genome of the ancestor that gave rise to *Sv. svetovidovi,* can provide an alternative explanation for the detection by the D-statistic test of asymmetry in allele pattern frequencies, inconsistent with the topology, in pairs involving *Sv. svetovidovi*. Lastly, the literature on *Salvelinus* also provides evidence for ancient hybridizations not detected in our study, most likely because each species in our dataset is represented by only a few individuals that do not cover the current distribution. Hybridization has been shown for instance between *S. alpinus* and *S. fontinalis* (Bernatchez et al., 1995; Glémet et al., 1998; Hammar et al., 1991), between *S. malma* and *S. confluentus* (Baxter et al., 1997; McPhail and Taylor, 1995; Redenbach and Taylor, 2002; Taylor and May-McNally, 2015), between *S. fontinalis* and *S. confluentus* (Kanda et al., 2002), or even between *S. alpinus* and *S. namaycush* (Wilson and Bernatchez, 1998). However, in some of these studies, introgression was detected using mtDNA, whereby evidence of nuclear introgression could disappear over time via several generations of paternal back-crossing.

Our phylogenetic results suggest that the discordance between some of the previous studies (Fig. 5) is likely due to insufficient resolution as a result of the limited number of markers and/or conflicting phylogenetic signals between different parts of the genome, for instance due to the contrasting rates of rediploidization (Robertson et al., 2017), or as a result of incongruences between different types of characters used for inferences. Using the RAD-sequencing approach considerably increases the number of loci and provides genome-wide characters leading to a more reliable representation of the evolutionary relationships within the Salmonidae family. Considering the age of the Salmonidae family, our study includes one of the oldest clades among vertebrates empirically investigated so far using RAD-sequencing. The successful application of RAD-seq on such divergent taxa to address and resolve phylogenetic questions shows the usefulness of this NGS method to study large-scale phylogenetic relationships.

The findings of this study present a significant improvement and a valuable contribution to the systematics of Salmoninae. Our results shed light on some of the previously recalcitrant phylogenetic relationships. Consequently, our analyses more fully resolve the phylogenetic relationships among salmonid fish species on some long-standing controversial points and provide more reliable divergence time estimates. For a greater understanding of the evolutionary history of Salmoninae, it would be valuable to increase the taxon sampling with systematic replicates for each species, ideally including representatives of each of the main genetic lineages or putative subspecies/distinct phylogeographic groups (e.g., coastal and interior lineage of bull trout (Taylor et al., 1999), northern and southern Asian and North American Dolly Varden (Taylor and May-McNally, 2015; Yamamoto et al., 2014).

In future investigations, the focus provided in this study for the genus *Salvelinus,* should also be given to the genera *Salmo* and *Thymallus*. For *Salmo*, there are still considerable uncertainties concerning the evolutionary history of a number of prominent taxa, such as *Salmo marmoratus* (marble trout) and *S. obtusirostris* (softmouth trout), as well as *S. carpio* (carpione) (see Gratton et al., 2014), and other larger-growth phenotypes throughout the range of the *Salmo trutta* species complex, all of which may have been involved in significant events of hybridization. The genus *Thymallus* requires comprehensive molecular investigation in both eastern and central Asia. In eastern Asia, due to its relatively high species diversity, and in central Asia, due to a rather cryptic association between current taxonomy and phenotypic diversity. For all salmonids, more extensive genome-wide studies on specific groups revealing significant radiations, such as *Salvelinus*, *Salmo* and *Coregonus*, should provide very useful insights, on both the mechanisms of evolutionary radiations and the distinctiveness of specific taxa, needed to promote and carry out efficient management and conservation measures.

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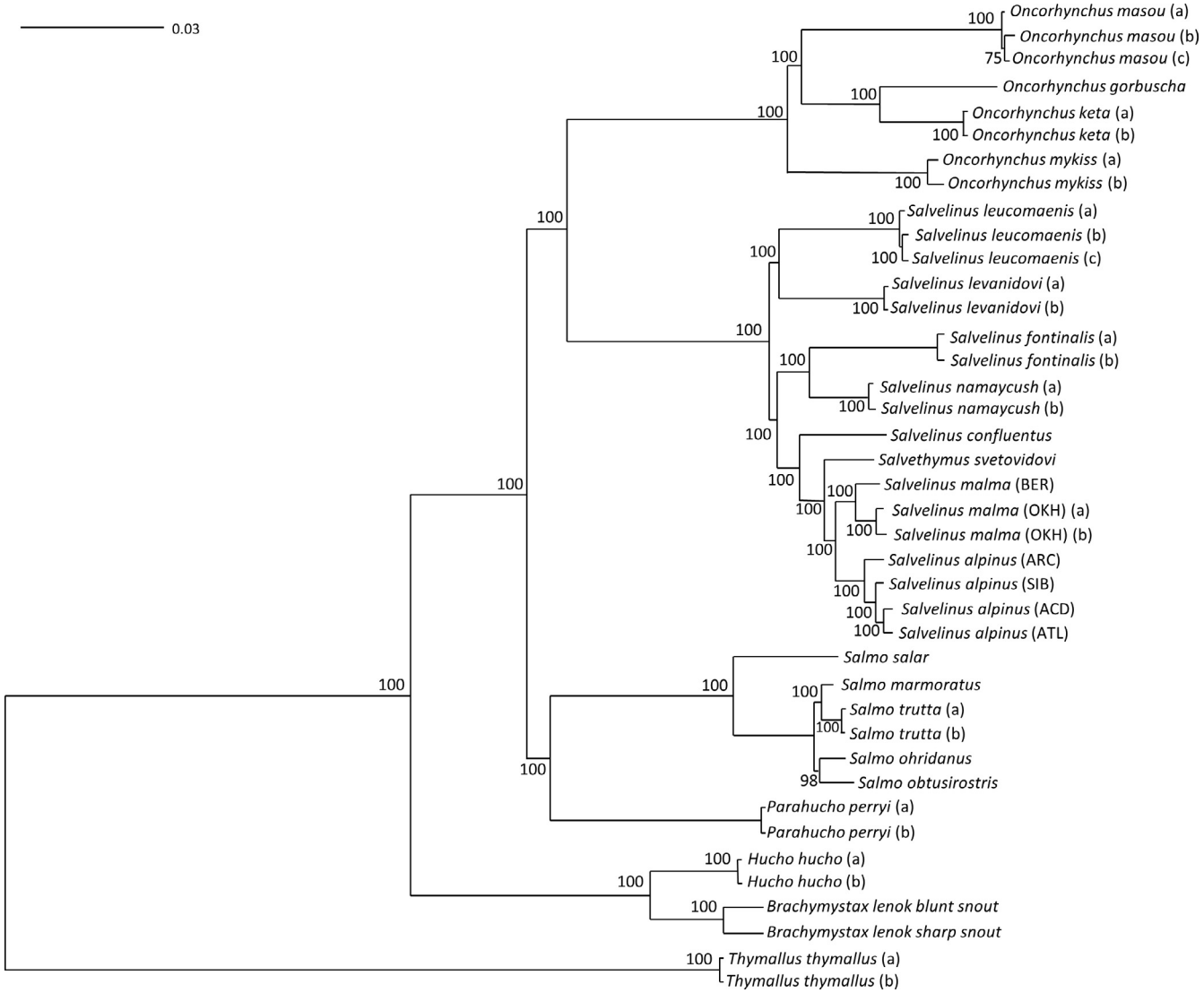
Appendix A

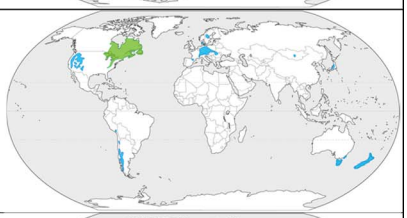
Bayesian Inference (BI) phylogenetic tree of 40 salmonid taxa from MrBayes analysis. The node labels represent the posterior probabilities, converted in percentages, for each node. The scale bar represents the nucleotide substitutions per site.



Appendix B

Table with the known distribution range for each *Salvelinus* species included in this study. For each species, the native range is represented in

green and the naturalized range is represented in blue. (Chereshnev et al., 1989; Chereshnev and Skopets, 1990; Linnaeus, 1758; Mitchill, 1814; Pallas, 1814; Suckley, 1859; Walbaum, 1792).

Species/Complex	Description	Common Name	Distribution	Distribution Map
Salvelinus malma complex	(Walbaum, 1792)	Dolly Varden	Arctic, NW-NE Pacific, drainages from Alaska to Washington; South Korea to Bering Sea, Kamchatka Peninsula into northern Japan (Hokkaido). Russia: from the Kolyma River to Sakhalin and the southern Kuril Islands	
Salvelinus alpinus complex	(Linnaeus, 1758)	Arctic charr	Europe: Northern Atlantic, Norway, southern Sweden, eastern Finland, Iceland, Greenland coasts, Northern UK & west Ireland. North America: Hudson Bay, Quebec, Maine & New Hampshire. Northern Russia & Siberia, Arctic coast to Chukotka, Kamchatka and Transbaikalia	
Salvelinus namaycush	(Walbaum, 1792)	Lake trout	North America: from northern Canada, Alaska, to New England, Great Lakes in Canada & USA. Introduced in Europe and certain parts of Asia	
Salvelinus fontinalis	(Mitchill, 1814)	Brook charr	North America: Canada from Newfoundland to west Hudson Bay; Great Lakes & Mississippi River to Minnesota & northern Georgia. Introduced in west part of North America and in South America. Also introduced in temperate regions of other continents	
Salvelinus leucomaenis	(Pallas, 1814)	Whitespotted charr	Hokkaido Japan, NE Korea & Russia: Sakhalin, Kuril Islands & Kamchatka. Bering and Okhotsk Sea	
Salvelinus levanidovi	(Chereshnev, Skopets & Gudkov, 1989)	Levanidov's charr	Yama River and neighbouring rivers, river mouths and Marine areas in the northern Sea of Okhotsk (Magadan Oblast)	
Salvethymus svetovidovi	(Chereshnev & Skopets, 1990)	Long-finned charr	Endemic to the Lake El'gygytgyn in Chukchee Peninsula, Russia	
Salvelinus confluentus	(Suckley, 1859)	Bull trout	Confined to NW North America; upper Missouri River, Athabasca and Saskatchewan River drainages; southern Yukon and Northwest Territories south to Columbia River drainage including Nevada; extirpated from the McCloud River in northern California	

Appendix C. Supplementary material

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.ympev.2018.02.022.

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