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Brown trout phylogenetics: a persistent mirage towards (too) many species

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“... such abundant literature and insightful studies may be little more than a mirage”

J. Lobón-Cerviá (2018)

The mirage surrounding one extensively studied species

Because of its socio-economic importance in fisheries or as highly-prized sport species, the brown trout, *Salmo trutta* (Linnaeus, 1758) has acquired one iconic status. A statement commonly done about this umbrella species affected by habitat degradation is that trout is a highly polytypic Pan-European species and/or species complex naturally distributed over >1 million km², actually ranging from northern continental Europe and Iceland to North Africa, and extending eastward perhaps to northern foothills of Himalaya in Kyrgyzstan and Tajikistan (e.g. Lobón-Cerviá, 2018). The diversity of phenotypes and life histories has motivated the description of numerous taxonomic units, often ranked as species and subspecies. The number of trout within the *S. trutta* complex varies considerably among authors. It spans from 23 (International Union for Conservation of Nature [IUCN], 2020) to generally 30-35 (Kottelat & Freyhof, 2007; Sanz, 2018), and to more than 50 or 60 (Behnke, 1986; Froese & Pauly, 2019; Jonsson & Jonsson, 2011). The delimitation of trout taxa by ichthyologists was certainly first impacted by poor awareness of phenotypic plasticity and speciation as a process, then based on species concepts based on diagnosability, while these taxa are not reproductively isolated and contradict Mayr's (1942) biological species concept. Reliance in “non-adaptive taxonomic types” (Savvaitova, 1995) and in supremacy of one or other species concept could have participated to the belief that one or a huge number of taxa are existing, while obscuring the very information that provide insight in the diversification process (e.g., Willis, 2017). Trout may also have suffered taxonomic inflation (i.e., the recognition of unnecessary taxa; Isaac *et al.*, 2004) in order to support management and conservation decisions.

Authors interested in trout however agree on a point: the complex is complex and the rise of molecular phylogeny during the 80's tried to address this issue to gain

understanding in both the phylogeny of the *Salmo* genus and the phylogeny of the brown trout, thus in their origin, diversification and other aspects of their evolutionary history. The brown trout complex received an interest not found in any other (Pan-)European fish 'species' so far. Indeed, outside population genetics issues that target one single 'species' or – generally speaking – a single operational taxonomic unit (OTU), a literature review estimated the number of original phylogeographic and phylogenetic studies considering at least two OTUs – clades, lineages or (sub)species – to approx. 130 (Fig. 1). Such interest should have led to significant improvements of knowledge, including taxonomic progress.

While a review of knowledge based also on over 100 studies has been already provided by Sanz (2018), we however believe we are facing a mirage of extensive knowledge in trout. If we do not oppose to contents of her work, a difference between Sanz's (2018) review and this opinion piece could be framed in the well-known 'splitter-lumper' dichotomy. Hereby, our goal is to be 'splitters' in order to stress that the genealogical relationships resulting from speciation within the brown trout complex remains unfortunately neglected. Otherwise, works as summarized by Sanz (2018) *rather* depict 'lumping' in which most molecular data produced to help for the delimitation of trout species or another taxonomic entity are aggregated to one existing reference, *S. trutta* sensu stricto (hereafter, StSS) whose origin is offered below. This opinion piece hence suggests to reconsider the outcomes and to come back on some issues that initially motivated trout molecular studies, i.e. providing a sound phylogenetic frame and the evolutionary history of described taxa, before to establish, redefine or improve – or not - operational criteria necessary to species delimitation.

Thirty years of research in few lines: what is really known?

If former allozyme studies anticipated the rise of (mt)DNA studies in trout and identified phylogenetic/phylogeographic signals (reviews in Ferguson, 1989; Guyomard, 1989; García-Marín *et al.*, 1999), works by Bernatchez *et al.* (1992), Bernatchez and Osinov (1995), and Bernatchez (2001) represent the main first attempts to investigate trout diversity

using sequencing molecular tools in a Pan-European perspective. Since these works, main observations might be briefly summarized:

- (1)** The five original mtDNA StSS lineages reported in Bernatchez *et al.* (1992) and Bernatchez (2001) – namely Atlantic, Danubian, Mediterranean, Adriatic and marmoratus (Fig. 2) – still anchored most studies in the field, but, their respective distribution area became more complex, resulting in the co-occurrence of mtDNA clades in some areas (e.g., Italy, Corsica, Balkans) rather than their allopatric distribution, as initially described (Sanz, 2018 for review);
- (2)** Few other StSS lineages have been added to the original ones in Spain (Duero: Suárez *et al.*, 2001), Turkey (Tigris: Bardakci *et al.*, 2006; Sušnik *et al.*, 2005), Morocco (Dades; Snoj *et al.*, 2011) and Northern Africa (Tougard *et al.*, 2018) (Fig. 2). Sub-lineages related to the Atlantic lineage have been reported in Central Europe (e.g. Cortey *et al.*, 2009);
- (3)** Some species within the complex have lost their initial status and now related to one of the original StSS clade (Kalayci *et al.*, 2018; McKeown *et al.*, 2010; Sanz, 2018; Snoj *et al.*, 2010; Sušnik *et al.*, 2007a; Tougard *et al.*, 2018). High rate of invalid taxa is not surprising in trout (Jonsson & Jonsson, 2011), and in salmonids in general (e.g. Adams & Maitland, 2007). However, the problem goes beyond taxon names and affects the understanding of evolutionary history. For example, Lobón-Cerviá (2018) reports a possible synonymy between *S. macrostigma* and *S. cettii*, when Tougard *et al.* (2018) questioned the reality of *S. macrostigma*. Authors proposed *S. cettii* as belonging to the Adriatic StSS (e.g., Gratton *et al.*, 2014), while Tougard *et al.* (2018) linked *S. macrostigma* to the North African StSS (Fig. 2). One or two species? One or two evolutionary histories? What is/are the realised distribution(s)? Confusion reigns. Furthermore, some OTUs should be hybrids (Razpet *et al.*, 2007; McKeown *et al.*, 2010; Gratton *et al.*, 2014). Otherwise, mtDNA data confirmed the classification of some species outside StSS: *S. obtusirostris* restricted to the western Balkans (Snoj *et al.*, 2002) and *S. ohridanus* endemic to Lake Ohrid (Phillips *et al.*, 2000; Sušnik *et al.*,

2006), with additional support coming from nuclear data (e.g. Pustovrh *et al.*, 2014; Lecaudey *et al.*, 2018). A proposal to erect the marmoratus lineage as a species outside StSS has been made by Pustovrh *et al.* (2014) based on nuclear data, but not thoroughly validated so far.

(4) One origin in the Middle East or/and Mesopotamia is postulated with a probable colonization of northern Europe by the Caspian and the Black Sea, and a colonization of Southern Europe following a Mediterranean route, southern of the Anatolian plateau (Bardakci *et al.*, 2006 and references therein). This point is probable and evidence also comes from comparisons with other species groups (e.g. *Squalius* and *Chondrostoma*; Durand *et al.*, 2002, 2003). The phylogeny presented in Fig. 2 based on StSS mtDNA haplotypes captured little of such information, placing in this case all the western European lineages (Duero, Atlantic and North African) at the root of the mtDNA brown trout phylogeny. It thus emphasizes one Atlantic refuge/Western European for European fish (e.g. Bryja *et al.*, 2010; Culling *et al.*, 2006; Durand *et al.*, 1999) that has been discussed in trout (e.g. Sanz 2018) and Atlantic salmon (*S. salar*; e.g., Finnegan *et al.*, 2013). A refuge is however not an origin. MtDNA taxonomic frames provide interesting but incomplete versions of evolutionary histories (Dellicour & Flot, 2018; Toews & Brelsford, 2012), and no Pan-European nuclear-DNA study is available in trout so far. Sanz (2018) reviewed and proposed mtDNA estimates for divergence time among *S. ohridanus*-*S. obtusirostris* and *S. trutta* that rarely exceed 2 Myr (i.e. Early Pleistocene, concordant with the oldest fossil attributed to *S. trutta*; Vladimirov, 1946). However, recent fossil-calibrated, nuclear estimates provided divergence times around 4-6 Myr between brown trout and *S. ohridanus*-*S. obtusirostris*, and divergence with Atlantic salmon dating back from the Middle Miocene (15-10 Myr) (Crête-Lafrenière *et al.*, 2012; Lecaudey *et al.*, 2018). Unfortunately, for both mtDNA and nuclear estimates, this did not extend to other OTUs than those reported in Fig. 2.

(5) Finally, if we emphasize the lack of updated molecular phylogeny encompassing most proposed trout taxa in this piece, no complete morphology-based phylogeny has

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been proposed either. Only partial attempts including few taxa have been produced (e.g., Salmanov & Dorofeyeva, 2001). Correlations between molecular and morphological observations supporting taxonomy resulted in mixed results (see Sanz, 2018; Delling *et al.*, 2020). Further investigations are requested to understand the molecular underpinnings of observed phenotypic and life-history differences that led to trout biocomplexity. To date, only Jacobs *et al.* (2018) really addressed this issue at a genomic scale for the *S. trutta*/*S. ferox* 'species' pair.

The initial frame that focused on StSS is thus valid, now better evaluated, and it has been enriched by additional lineages and better delineation of relationships to few other taxa. It however remains mostly dependent on uniparentally inherited mtDNA data (Fig. 1). Overall, the number of supported taxa and more importantly their relationships, their rate and patterns of diversification, their colonization histories, and their links to phenotypic diversification that support taxonomic descriptions remain obscure. Deep knowledge is a mirage and we may question if scientists built a complex to not face their icon.

Two more frames

If a coherent phylogenetic frame is still missing, two other frames strongly cohabit in trout molecular studies: one concerns species definition, taxonomy and their associated revisions (*supra* and Fig. 2), while a second is sounded in conservation issues – often crucial in trout - and should be 'taxonomy independent' (Splendiani *et al.*, 2019; see also Stanton *et al.*, 2019). These two frames are generally considered fundamental to design efficient biodiversity conservation priorities and management strategies (Allendorf, 2012; Eizaguirre & Baltazar-Soares, 2014), but their uncoupling has been promoted by some agencies (e.g. Haig *et al.*, 2006). Briefly said, operational criteria to species delimitation should not be confused with operational criteria for conservation (e.g. Raposo *et al.*, 2020). In trout, the inappropriateness of considering the "old" zoogeographic frame for conservation and the preservation of its evolutionary diversity has been already discussed (Antunes *et al.*, 2001; Apostolidis *et al.*, 2011; McKeown *et al.*, 2010; Schenekar *et al.*, 2014). Its use remains

unfortunately pregnant while focus on local (meta)populations could be advertised to prioritize issues that are prompted to more efficient management and deal with biological characteristics, ecological and/or evolutionary potentials (Antunes *et al.*, 2001; Splendiani *et al.*, 2019). However, because species is the only recognized unit to frame conservation policies and because rare and endemic species rather than widely distributed ones are favoured, the two frames remain unfortunately lumped in trout by putting the species status or the lineage at the conservation forefront. Numerous trout studies thus focused on events occurring at temporal and/or spatial scales that are poorly relevant to conservation management and quite descriptive at the level of OTUs (e.g. updated distribution of haplotypes pointing new records or levels of diversity that are not related to life history or ecological data).

Other approaches that consider “the best of all worlds” might be substituted (e.g., Mee *et al.*, 2015). Pillars of sands should be avoided, and, by abusively mixing the two frames, the goal to address the big phylogenetic picture, to reduce taxonomic uncertainty and to better understand the processes that shaped the brown trout complex has been progressively lost. This big picture has perhaps become still more difficult to address as new putative trout species have been described over the last fifteen years, notably in Morocco (Delling & Doadrio, 2005; Doadrio *et al.*, 2015) and the Anatolian and Taurus regions, including Tigris and Euphrates drainages (e.g., Turan *et al.*, 2011, 2014, 2017, 2020). These are areas where novel StSS lineages were described (Dades, Tigris, see above), and perhaps close to the putative centre of origin of brown trout for Anatolian trout. We thus do not currently know how these taxa are related, if they clustered within or outside StSS (could be considered as valid taxa or not, *sensu* Galtier, 2019), and which portion of trout evolutionary history they may allow to better understand.

The future

The next step in trout study is to handle the full complexity of phylogenetic relationships to learn how trout diversity arises and changes. Picking up only previously described ‘species’

one by one for further molecular comparison with StSS has become a dead-end. After decades of studies using maternally inherited mtDNA markers or - more generally - reduced marker sets, “going large” with next generation sequencing (NGS) technologies that provide access to thousands of nuclear loci (e.g. single nucleotide polymorphisms [SNPs]) and genomic features (e.g., Wellenreuther *et al.*, 2019) to delineate the underlying genealogy is undoubtedly the key in trout. Panels of genome-wide distributed SNP markers have proved suitable for phylogenomic analysis (Leaché & Oaks, 2017) and updated the understanding about the patterns and the mechanisms of lineage diversification, together with species delimitation (e.g., Chan *et al.*, 2020; Díaz-Arce *et al.*, 2016; Hughes *et al.*, 2020; Leaché *et al.*, 2014; Song *et al.*, 2017). This includes fish species complex in sticklebacks (Guo *et al.*, 2019), roaches (Baumsteiger *et al.*, 2017), cichlids (e.g. Willis, 2017); suckers (Bangs *et al.*, 2020), topminnows (Duvernell *et al.*, 2019), and coregonids (*Coregonus ardeti* species flock; Ackiss *et al.*, 2020). Based on several thousands of polymorphisms, Copus *et al.* (2018) proposed to reduce the *Gila robusta* complex to a single species. NGS data also provided with better assessment of origin, colonization routes, demographic histories including admixture and introgression, and/or identification of stage of divergence during the speciation process in diverse fish species (e.g., Jeffries *et al.*, 2016; Fang *et al.*, 2018; Lucek *et al.*, 2018; Ravinet *et al.*, 2018; Rougemont & Bernatchez, 2018). For example, the pattern of reticulate evolution between *S. obtusirostris* and the Adriatic clade of *S. trutta* described by Sušnik *et al.* (2007b) should receive in depth evaluation. Trout, however, covers perhaps more putative OTUs than any other examples, and OTU-rich taxa appeared yet especially challenging to investigate these issues (e.g. Dincă *et al.*, 2019; Chambers & Hillis, 2020). Actually, it is recognized that a limited number of sampled genomes per OTU may provide access to substantial information, including “evolutionary process connectivity” (i.e. how contemporary observed connectivity and diversity reflect long term selective/adaptive and demographic history, including patterns of genetic admixture and speciation; Gagnaire, 2020). The ever-decreasing cost to access genome data would probably no longer constrains this issue.

The release of important genomic resources such as the Atlantic salmon (Lien *et al.*, 2016; GenBank: GCA_000233375.4) and brown trout (GenBank: GCA_901001165.1) genomes provide substrates to progress in trout phylogeny. SNPs are accessible in *S. trutta*, but mostly used however in a population genomics framework (e.g., Andersson *et al.*, 2017; Drywa *et al.*, 2013; Leitwein *et al.*, 2016; Saint-Pé *et al.*, 2019). Jacobs *et al.* (2018) used SNPs to document the divergence in the *S. trutta/S. ferax* 'species' pair occurring in Loch Maree catchment (Scotland), but this did not represent a phylogenetic study. Only Lecaudey *et al.* (2018) used SNPs in a phylogenetic context, but they were interested in higher phylogenetic relationships than the ones sustaining the brown trout complex.

The production of a more complete phylogeny including many taxa seem possible, but challenges have to be overcome. This includes the relatively young history of divergence for some OTUs, taxon sampling, choosing marker types, or identification of orthologous/paralogous genes (single-copy vs gene duplicates) in trout. As paralogs impede investigations in salmonids (Lecaudey *et al.*, 2018), databases (Pasquier *et al.*, 2016; Samy *et al.*, 2017) and tools providing guidelines for *de novo* or graph-based inference methods for orthologous gene identification have been developed (Kapli *et al.*, 2020; Smith & Hahn, 2020). Many of them seem however already identified owing to transcriptome assembly in the brown trout (Carruthers *et al.*, 2018) and consolidate resources to address this challenging key task. While the number of markers cannot be considered sufficient to accurate phylogeny estimation (Pyron, 2015) and their nature may lead to conflicting results (e.g. Reddy *et al.*, 2017), results by Collins and Hrbek (2018) suggested good performance of SNP data coming from restriction-site-associated DNA (RAD) analysis to address relatively recent and shallow phylogenies. However, relevance of models behind RAD-based and other SNP analysis remains to be considered further (Bravo *et al.*, 2019). Among methods, the multispecies coalescent (MSC) emerged to tackle complex phylogenies in the age of increasing access to genomic data (e.g., Rannala & Yang, 2017). MSC is a statistical framework based on coalescent theory that explicitly

accounts for gene tree discordance due to incomplete lineage sorting and was initially devoted to identify 'species' as entities between which genetic exchanges have been negligible. It may now address more complex demographic modelling during speciation (e.g., Hey *et al.*, 2018; Long & Kubatko, 2018), have been extended to phylogenetic network as the multispecies network coalescent (e.g., Wen *et al.*, 2016; Zhang *et al.*, 2018), and recognized as "broadly" efficient (Hillis, 2019; Kapli *et al.*, 2020; Rannala & Yang, 2017). Guidelines and tools regarding coalescent-based species delimitations and further testing procedures are regularly enriched (e.g., Hey and Pinho, 2012; Carstens *et al.*, 2013; Flouri *et al.*, 2020; Jones, 2017; Kapli *et al.*, 2020; Liu *et al.*, 2019; Luo *et al.*, 2018). In fish, ancestral geographic distribution or estimation of divergence times among closely related lineages benefited of such new statistical modelling approaches in sticklebacks (Fang *et al.*, 2020). In cichlids, Olave and Meyer (2020) illustrated capacities to deal with recent radiation events that might also be present in some lacustrine trout (e.g., *S. ischchan* in Lake Sevan, Armenia, in which four sympatric morphs were recorded; Dadikyan, 1986).

Despite access to statistical procedures and genomic resources, species are hypotheses and not inherently subjectives, because of artificial cut-offs in the process of divergence (e.g. Hey, 2001; De Queiroz, 2007; Pante *et al.*, 2015). MSC – or any other method (e.g. approximate Bayesian computations) - is then not a magic bullet and improving trout phylogeny that describe a speciation process by analyzing genome-wide data sets is not sufficient to establish criteria for taxon delimitation (e.g. Chambers & Hillis, 2020; Stanton *et al.*, 2019; Sukumaran & Knowles, 2017; Willis, 2017). Result accuracy and criteria for taxon delimitation have to be considered in light of complementary knowledge coming from different perspectives, including phenotypic and ecological information (Barley *et al.*, 2018; Campillo *et al.*, 2020; Hillis, 2019; Solís-Lemus *et al.*, 2015; Sukumaran & Knowles, 2017), as well as geographical/distributional consideration (e.g., De Queiroz, 2007; Hillis, 2019, 2020). In the roundtail chub (*G. robusta*) complex, Copus *et al.* (2018) showed that morphological variation reflected phenotypic plasticity and that only molecular variation could offer consistent material for taxa delimitation. Willis (2017) also discussed

the real necessity of species delimitation in peacock cichlids (*Cichla pinima* species complex), because their evolutionary history could not be unambiguously rendered as discrete units. Concurrently, Kautt *et al.* (2018) showed that Midas cichlid species complex from Nicaraguan crater's lakes can be described as independent evolutionary lineages that established differences in morphology, ecological settings and distribution, suggesting the likely existence of criteria for taxa/species delimitation in less than 2000 generations.

'Going large' offers to explore many facets of trout speciation, strengthen the interplay between systematics, phylogeography and speciation genomics (Galtier, 2019), and operate to renew links with conservation issues (Coates *et al.*, 2018; Stanton *et al.*, 2019; Raposo *et al.*, 2020). It may allow for the definition of shared operational criteria for trout (sub-)species delimitations and to set aside a current situation where many trout taxa – while reproductively non-isolated - have been described as tips of one unknown phylogeny. Carstens *et al.* (2013) and others proposed to be conservative when delimiting species and the current situation militates for recognizing only few trout species. Actually only *S. ohridanus* and *S. obtusirostris* seem to have received (relatively) sufficient molecular support as independent taxa. Much work has still to be done and trout from the Anatolian and Taurus regions are certainly the most interesting taxa that might contribute to elucidate the trout phylogenetic puzzle.

Finally, because of the availability of OTUs present only as museum specimens (e.g. *S. pallaryi*), the study of ancient or degraded DNA has also to be more deeply considered in trout. Few studies considered museum specimens, only using mtDNA (Splendiani *et al.* 2016, 2017; Levin *et al.*, 2018; Tougard *et al.*, 2018). Study of nuclear ancient DNA is challenging, but may improve knowledge (Chassaing *et al.*, 2016; Lopez *et al.*, 2020).

In the meantime

If the bar is too high before to gather extensive genome-wide data, a pragmatic approach remains the study of complete mitogenomes. Trout mitogenomes are recent and now retrieved from both contemporary and archived samples (e.g., Nedoluzhko *et al.* 2018;

Sahoo *et al.*, 2016; Tougard *et al.*, 2018; first *S. salar* mitogenome by Hurst *et al.*, 1999). While mtDNA is uniparentally inherited, produces over-pronounced geographic structuring and is a notable source of mito-nuclear discordance (i.e. produces very partial pictures of connectivity and gene flow among OTUs; Petit & Excoffier, 2009; Toews & Brelsford, 2012), phylogenies using complete mitogenome remain largely indicative of the speciation process and paleogeographic events (Ben Chehida *et al.*, 2020; Jacobsen *et al.*, 2012; Wang *et al.*, 2019; see also Collins & Hrbek, 2018)). Mitogenomes enabled support to complex situations in which specific status was not previously assessed and/or ambiguous (e.g. Beheregaray *et al.*, 2017; Buckley *et al.*, 2018), providing also support for a more integrative and corroborative taxonomy in which morphology and molecular species delineation have been shown to match (Pedraza-Marrón *et al.*, 2019) or not (Vernygora *et al.*, 2018).

In the meantime, we may also ask ourselves if progress in molecular trout studies do not also depend on a large cooperative research network that may gather samples from many OTUs, from many selected locations (and museums) and further ecological information to push trout research to new areas. If local research groups exist (e.g., Balkan Trout Restoration Group: <http://www.balkan-trout.com/research.htm>), transnational studies remain few, mostly binational, and large-scale international programs regarding trout date back to two decades ago (TroutConcert; Laikre, 1999). New collaborations are certainly necessary to reach novel achievements and make the mirage of trout molecular studies disappear to both splitters and lumpers.

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Captions of the Figures

Fig. 1: Evolution of the number of publications over the 1989-2020 period dealing with trout systematics found in public reference databases (Web of Sciences, Google Scholar, PubMed) in June 2020 using “Salmo”, “phylogeny”, or “systematics” as queries, then curated (e.g. reviews reporting data from former studies were discarded to avoid to count a study twice). A reference was considered relevant when, at least, two *Salmo* taxonomic species or *S. trutta* evolutionary lineages were included in the studied dataset, and phylogenetic relationships were represented by a tree or a network. The full list of references ($N = 128$) used in this study is available upon request to the contact author. MtDNA: PCR-based sequence of mitochondrial DNA genes; nucDNA: PCR-based sequence of nuclear genes and microsatellites; NGS: data obtained through next-generation sequencing technologies, including nuclear SNPs and mitogenomes. Studies strictly based on allozymic data were not considered.

Fig. 2: Simplified Bayesian phylogenetic tree reconstructed from a concatenated dataset including the complete mitochondrial control region (1019 base pairs) and cytochrome *b* (1140 base pairs) genes. Numbers at nodes are for posterior probabilities (≥ 0.80) and bootstrap percentages ($\geq 50\%$), while “-” are for nodes weakly supported in either the Bayesian or maximum likelihood analyses. Lineage and species names are indicated on the right. Outside the Atlantic salmon taken as outgroup, note that only four taxa are considered in this tree (*S. trutta*, *S. ohridanus*, *S. obtisrostris* and *S. marmoratus* as the marble trout lineage is often considered a separate OTU [e.g., Pustovrh *et al.*, 2014]), meaning this phylogeny includes only 17.4% (4 out of 23) species recognized by IUCN. The horizontal bar under the phylogenetic tree indicates the number of nucleotide substitutions per site. For details about sampling and phylogenetic analyses, see Tougard *et al.* (2018).



