Speciation in Freshwater Fishes

ABSTRACT

The extraordinary species richness of teleost fishes has attracted much research on mechanisms and modes of speciation. We here review research on speciation in freshwater fish in light of speciation theory, and place this in a context of knowledge about broad-scale diversity patterns in freshwater fishes. We structure our review around the major axes of divergence during speciation in freshwater fishes. These include transitions between marine and freshwater habitats, transitions between lake and stream habitats, and ecological transitions within habitats, as well as allopatric differentiation without distinct niche shifts. Major research directions in the years to come include integrating genomics with evolutionary ecology in studies of freshwater fish speciation, understanding the transition from extrinsic environment-dependent to intrinsic reproductive isolation and its influences on species persistence, and understanding the extrinsic and intrinsic constraints to speciation and how these relate to broad-scale diversification patterns through time.

INTRODUCTION

Ray-finned fishes comprise approximately half of the diversity of all vertebrates, with 28,900 currently described species (FishBase, Froese & Pauly 2014). Remarkably, close to half of these are found strictly in freshwater environments, although freshwaters comprise less than 1% of the earth's surface; in contrast, oceans cover 70% of the earth's surface, but the diversity of ray-finned fishes in the oceans is approximately equal that of freshwaters (Leveque *et al.* 2008; Vega & Wiens 2012). This tremendous diversity of freshwater fishes has incited longstanding interest in understanding the processes contributing to the origination of these species. A Web of Science search with the keywords "Fish" and "speciation" recovers 3263 publications that have been cited a total of 77,133 times. Freshwater fish "species flocks", many of which are celebrated examples of adaptive radiation, have illuminated our understanding of speciation mechanisms and the buildup of species diversity in geographically restricted areas, such as individual lakes. Recurrent speciation in post-glacial north temperate lakes, and also some geologically young tropical lakes, provide replicated examples of the early stages of ecological speciation, and provide an opportunity to study how variation in the environment, history, ecological opportunity, species traits and genomic properties together influence the speciation process (Schluter 1996b; Taylor 1999). Finally, a rich body of phylogeographic and biogeographic studies on a plethora of freshwater fish has illuminated the geographical modes of species diversification in some of the richest biomes on earth (Albert & Reis 2011)**.**

The best-studied cases of speciation in freshwater fishes have been investigated for many years from natural history, ecology, and genetic perspectives, and these data greatly aid our understanding of their evolutionary history (Table S1). New genomic data is now advancing knowledge on several of these systems (Hohenlohe *et al.* 2010; Jones *et al.* 2012; Renaut *et al.* 2012; Gagnaire *et al.* 2013; Schartl *et al.* 2013; Brawand *et al.* submitted), and promises to do so for many others. Integrating across this diversity of taxa, ecosystems and approaches, we can seek to address the characteristics of freshwater fishes and their environments that foster and facilitate the origin of new species. Simultaneously with developments in the ecological genetics of speciation, advances in the attainability of DNA sequence data and of phylogenetic approaches to studying diversification have made possible studies testing hypotheses about diversification in fishes across macroevolutionary timescales with large data sets (Alfaro *et al.* 2009; Rabosky *et al.* 2013).

Freshwater fish speciation research has traditionally been driven from within either one of two strong research traditions: evolutionary ecology and phylogeography, the former emphasizing process, the latter pattern resulting from past processes. Although some of the finest contributions to the field are examples of their successful integration, large parts of the literature in either of these traditions remain disconnected. Not only do the two approaches complement each other importantly, but these two approaches will tend to segregate by mode and mechanism of speciation, and will therefore come to different conclusions about what matters most to the origin of fish species, i.e. ecology and divergent natural and sexual selection (the evolutionary ecology paradigm) or geography and isolation (the phylogeography paradigm). Clearly both are important, but to understand their relative importance and how they interact, the integration of research across these traditions is needed. We attempt to give emphasis to both of these perspectives in this review.

We define speciation as the origin of reproductive barriers that permit the maintenance of genetic and phenotypic distinctiveness among populations in geographical proximity. Reproductive isolation may arise from prezygotic sexual isolation, extrinsic postzygotic isolation that is dependent on genotype–environment interactions, or intrinsic postzygotic isolation that is independent of the external environment. Selection can initiate speciation in situations both with and without gene flow between populations, whereas intrinsic incompatibilities are less likely to accumulate when gene flow is present (Gavrilets 2004). When reproductive isolation is facilitated by or a direct consequence of divergent ecological selection, this is often referred to as ecological speciation (Schluter 2009; but see (Sobel *et al.* 2009)). The “by-product” mechanism is classically considered the best supported: pre- and post-mating isolation arise incidentally as populations accumulate genetic differences favored by different resource environments (Dobzhansky 1937; Mayr 1942). A second route is ecologically-based reinforcement, whereby incomplete postzygotic isolation stemming from adaptation to different resource environments favors the evolution of premating isolation (Dobzhansky 1937).

Speciation often, but certainly not always, involves ecological niche shifts. Niche shifts often happen in periods of geographical isolation, and speciation is sometimes completed by reinforcement in secondary contact (Schluter 2001; Nosil 2012). Freshwater fishes provide compelling examples that the entire process can sometimes happen in the complete absence of a phase of geographical isolation, particularly within lakes (see Box 2**,** Table S1). Speciation in geographical isolation can also happen by the fixation of incompatible mutations during parallel adaptation to similar environments (Schluter 2009), by random genetic drift and its interaction with natural selection in small populations (Gavrilets 2004), through interactions of sexual selection with genetic drift (Lande 1981; Ritchie 2007), and through intragenomic conflict (Presgraves 2010; Crespi & Nosil 2013).

To review the prevalence of these various mechanisms in the diversification of freshwater fishes, we review major recurrent themes in freshwater fish speciation, including review of what the most intensely studied cases of speciation teach us about the mechanisms at work within each of these major themes. We ask about how these mechanisms may relate to broader-scale patterns in the diversity of freshwater fishes. Finally, we review contributions to understanding speciation in general that have been gained through study of freshwater fish speciation.

MAJOR THEMES IN FRESHWATER FISH SPECIATION

Despite the myriad of differences in the details of speciation that quickly become apparent as one reads a broad swath of fish speciation literature, there are a few recurrent themes that capture the major axes of divergence associated with freshwater fish speciation (Figure 1):

1. transitions between saltwater and freshwater environments (and other physio-chemical transitions)
2. transitions between lotic (river) and lentic (lake) environments
3. transitions along major physical habitat axes: water depth, benthic-limnetic, benthic substrates
4. trophic transitions (e.g. intraguild predation and durophagy)
5. geographic speciation without niche shifts

Speciation associated with transitions between saltwater and freshwater environments

Speciation associated with the evolutionary transition from saltwater to freshwater environments includes transitions between marine and freshwater habitats, and also those between saline or sulfidic and fresh inland waters. These are typically steep ecotonal gradients between entirely different ecosystems and physico-chemical environments. Associated selection gradients work on a wide range of phenotypes and functions, from osmoregulation and oxygen metabolism to pathogens, parasites, predation and competition regimes, and often have different nutritional resources. Most fishes are narrowly stenohaline. A recent estimate suggests that only 4% of actinopterygian (ray-finned fish) species are found in both freshwater and saltwater (Vega & Wiens 2012). The generally low species richness of fishes in large brackish seas, a pattern that has persisted over evolutionary timescales (Louisy 2002), bears witness to the ecological constraint that freshwater and marine fish alike face when having to cope with variable salinities. This constraint appears to be most acute at intermediate salinities, and is seemingly not readily overcome by evolution, as testified by the low diversity of most multicellular aquatic organisms in brackish waters (Telesh *et al.* 2013).

Large brackish seas then provide an interesting paradox: these are large and highly productive ecosystems, yet very poor in species. In principle, such systems would provide ideal conditions for ecological speciation in response to release from interspecific competition in the few taxa that can adapt to the physiological challenges (Yoder *et al.* 2010). In support of this prediction, there are a few striking examples of fish radiations in brackish seas. These include a monophyletic adaptive radiation of 24 endemic goby species in the brackish inland seas of eastern Europe (Neilson & Stepien 2009a), and recent radiations of herrings of the genera *Alosa* and *Clupeonella*, with respectively 6 and 3 endemic species in the Caspian Sea and the Black Sea (Faria *et al.* 2006).

Despite physiological constraints, a substantial number of freshwater fish species have their direct sister species in the sea. Entire freshwater fish faunas of small and large oceanic islands in both hemispheres, such as New Zealand and Iceland, are derived from marine ancestors, as are substantial fractions of the freshwater fish faunas of Madagascar and Australia (Leveque *et al.* 2008). Fishes are quite unique among animal groups in their frequent transitions between these environments at evolutionary time scales (Figure 2); most other aquatic animal groups are restricted to either the ocean or to freshwaters, or have highly unbalanced diversity across them (Grosberg *et al.* 2012). The evolutionary transition from marine to freshwater environments may often happen via the evolution of anadromy, the lifestyle by which populations that otherwise live in the ocean migrate into freshwaters to reproduce. The larvae remain in freshwater for a variable duration of their ontogeny before they return to the ocean. Once the ability to reproduce in freshwaters is established, evolving a resident freshwater lifestyle may be relatively easy.

Examples of repeated parallel evolution of the transition from anadromy to freshwater resident lifestyles come from Atlantic trout and sockeye salmon. Frequently this transition is associated with ecological speciation in sockeye (Taylor 1999), although the situation is often unclear in Atlantic trout (Klemetsen *et al.* 2003). Transitions can also lack an anadromous stage. Classical work on this type of divergence has been done in the threespined stickleback (*Gasterosteus aculeatus* species complex), which has evolved freshwater forms from marine ancestors many times independently (e.g. Jones *et al.* 2012). Other investigated cases include ninespined stickleback (Ishikawa *et al.* 2013), freshwater gobies derived from the brackish water radiation of the Black Sea (Neilson & Stepien 2009b), silversides (Gosline 1948; Bloom *et al.* 2013), and killifish (Kozak *et al.* 2014). Recent genomic work on stickleback and killifish suggest that a large number of genes experience divergent selection during such transitions (Jones *et al.* 2012; Kozak *et al.* 2014).

Several other strong gradients in physiological environments, comparable to those between saltwater and freshwater, are associated with speciation in fish. The best-studied cases are those in livebearers of the genera *Poecilia* and *Gambusia* inhabiting sulfidic versus freshwater streams and caves in Mexico and the Carribean. Multiple cases of early and advanced incipient speciation and some of older speciation events have been demonstrated in both genera (Tobler *et al.* 2011; Palacios *et al.* 2013; Riesch *et al.* 2014), associated with strong but asymmetric divergent selection between and adaptation to sulfidic and hypoxic waters.

In some cases of marine-freshwater speciation in threespine stickleback strong behavioural assortative mating has evolved, apparently as a by-product of divergence in body size (McKinnon *et al.* 2004). In other cases that have been investigated, despite evidence for genomically widespread divergent selection, the transition between saltwater and freshwater is not associated with evolution of strong reproductive isolation (Jones *et al.* 2006). In the River Tyne, a strongly bimodal distribution of genotypes and phenotypes of riverine versus marine stickleback exist, and a significant heterozygote deficit and cytonuclear disequilibrium in juveniles all confirm that barriers to gene flow exist. However, the frequency of juvenile hybrids within the sympatry zone and the absence of behavioural assortative mating indicate incomplete prezygotic isolation (Jones *et al.* 2008). Incomplete and asymmetric behavioural isolation have also been found among the Mexican mollies from sulfidic and freshwater springs, however, the complementary effects of natural selection and sexual selection maintain the genetic distinctiveness of the species in parapatry (Plath *et al.* 2010). In River Tyne stickleback, only a small fraction of the marine population breeds sympatrically with the freshwater population (Jones *et al.* 2006), leaving little scope for reinforcement selection. Because of the limited opportunity for reinforcement but strong and multifarious divergent selection associated with it, the saltwater to freshwater transition is an interesting model for studying by-product speciation.

After emergence from the ocean new freshwater lineages of fish have often diversified extensively into new species in freshwater environments. Geologically recent examples include the ariid catfish of Australia (Betancur-R *et al.* 2012), the galaxiids of New Zealand (Waters & Wallis 2001), radiations of silversides in central and north America (Bloom *et al.* 2013) and SE Asia (Herder *et al.* 2006), and many postglacial radiations of freshwater fish in the northern hemisphere, such as Icelandic arctic char (Gislason *et al.* 1999) and the stickleback species and ecotype pairs of the Canadian west coast (McKinnon & Rundle 2002; Deagle *et al.* 2013). In this sense, many cases of successful colonization of freshwater can be considered evolutionary key innovations that have opened up new adaptive zones for the colonizing lineages (Simpson 1953). Additionally, the diversification of fish in freshwater may also have been an important source of new lineages that returned to the sea and diversified there (Vega & Wiens 2012).

Speciation associated with transitions between discrete chemico-physical conditions in streams is a relatively understudied theme in freshwater fish speciation, but the widespread existence of chemico-physically discrete and distinct habitats with different fish assemblages, such as the white water/black water/clear water divide in the Amazon, taken together with recent work on ecological speciation between such habitats (Cooke *et al.* 2012), suggest that the importance of such speciation to the origins of fish diversity may have been underestimated (Cooke *et al.* 2012). Characiform fish are one of the most species-rich families of freshwater fish (Figure 2) with more than 850 species in the Amazon basin, occupying a large diversity of riverine habitats (Albert & Reis 2011). Their diversification has classically been explained by allopatric speciation due to river vicariance. However, recent work suggests that parapatric ecological speciation may have been more important in this group than previously acknowledged. Studying the widespread characin *Triportheus albus*, (Cooke *et al.* 2012) found that divergent selection between visual environments and population genetic structure present at the interface of water types appeared more important to generating diversity than spatial structure and biogeographic history. Similarly, work on another characin, *Astyanax*, revealed evidence for repeated parallel ecological speciation in parapatry between different visual environments in caves and surface waters in Mexico (Strecker *et al.* 2012).

Speciation associated with transitions between lotic and lentic ecosystems

The transition from life in streams to life in lakes is another major theme in freshwater fish speciation. While perhaps not affecting the physiology of fish as strongly as the transitions between salt and freshwater, living in streams and living in lakes requires many contrasting adaptations. Many stream fish cannot reproduce in lentic environments because they require the high oxygen tensions of running waters to hatch their eggs. On the other hand, life in permanent running water requires adaptations to swimming against the currents or avoiding them by becoming strictly benthic with reduced swimbladders and/or structural modifications of the mouth and pelvic fins. Pathogens, parasites, predators, food and light also differ. Geographically adjacent river and lake fish assemblages are generally very different in species composition. Yet, there is a relative paucity of speciation studies across the stream-lake habitat gradient. Threespine stickleback are one of the best studied model systems for this transition (Berner *et al.*

2009a; Deagle *et al.* 2012b; Kaeuffer *et al.* 2012; Lucek *et al.* 2013; Lucek *et al.* in revision). Other studied examples include lacustrine ecotypes of kokanee salmon, spawning either on wave-washed lake shores or migrating into inlet streams (Taylor *et al.* 1997; Hendry *et al.* 2000), and European trout that have in a few instances evolved fully lacustrine endemic species from more widely distributed stream-dwelling species (Giuffra *et al.* 1996). There are several examples of rapid incipient speciation by this mode of origin, specifically in Pacific salmon and threespine stickleback (Hendry *et al.* 2000; Lucek *et al.* 2012).

Work on the divergence between lake and stream populations of threespine stickleback started in the 1970s with the discovery of phenotypically highly divergent yet broadly parapatric populations (Moodie 1972a; Reimchen *et al.* 1985). Sources of divergent selection that have been invoked include predation regimes (Moodie 1972b; Reimchen 1988), dietary resources (Lavin & McPhail 1986; Berner *et al.* 2008), ambient light (Reimchen 1989) and parasite communities (Eizaguirre *et al.* 2012); all differ strongly between streams and lakes. Despite these contrasting ecological environments, reproductive isolation is often lacking or incomplete (Hendry *et al.* 2013). Significant isolation by adaptation has been inferred from genetic data in several instances including very young and older population pairs, together making for some of the best-sampled continuums of the speciation process (Thompson *et al.* 1997; Berner *et al.* 2009b; Lucek *et al.* 2012). Among the most striking instances are the cases of Mayer and Drizzle Lake in the Haida Gwaii archipelago (British Columbia) (Reimchen *et al.* 1985; Thompson *et al.* 1997), where black giant stickleback have evolved in parallel from smaller stream stickleback. These black giant stickleback are sufficiently strongly isolated to be recovered as reciprocally monophyletic with respect to parapatric stream populations in recent studies based on genome-wide SNPs (Deagle *et al.* 2012b; Deagle *et al.* 2013), suggesting that reproductive isolation is maintained in geographical sympatry and ecological parapatry. Although early work suggested behavioural reproductive isolation has evolved between these forms (Reimchen *et al.* 1985), no indications of behavioural isolation were found despite strongly differentiated phenotypes in the only other case where this has been directly investigated, lake and stream stickleback from Misty Lake on Vancouver Island (Rasanen et al. 2013). This shows that strong reproductive isolation in stickleback does not always evolve simply as a by-product of phenotypic adaptation, and also that strong phenotypic differentiation can sometimes be maintained in the absence of sexual isolation, perhaps because of strong divergent natural selection and reduction of gene flow via limitations to dispersal (Hendry *et al.* 2002).

Whether differentiated lake-stream stickleback ecotype pairs represent cases of parapatric speciation from a single colonization or from secondary contact after repeated colonization from the sea is not always clear. Phylogeographic work on the Misty Lake pair suggests secondary contact between two very divergent mitochondrial lineages (Thompson *et al.* 1997). Recent work using genome-wide markers demonstrated the segregation of distinct mitochondrial lineages also in the Haida Gwaii radiation, but the haplotypes were not associated with habitats or ecotypes, suggesting either parapatric speciation from an admixed seeding population, or secondary contact with maintenance of habitat-specific phenotypes in the face of extensive and long-term homogenizing gene flow at neutral loci (Deagle *et al.* 2013).

How representative are these studies of stickleback for the origin of new fish species in the evolutionary shifts between lotic and lentic environments? The importance of this lotic-lentic transition is easy to see if one considers the many large lacustrine adaptive radiations, including the African cichlid radiations, the sculpins of Lake Baikal, the barbs of Lake Tana and the radiations of whitefish in large lakes across the northern hemisphere (Echelle & Kornfield 1984). All of these radiations have arisen from widespread river-dwelling specialists. In this sense, this transition can be considered another repeatedly evolved evolutionary key innovation in freshwater fish that opened up new adaptive zones for the lineages involved.

Nevertheless, it seems that the lotic/lentic transition itself does often not lead to strong reproductive isolation. Recent work on the genetics of several species-rich lake fish radiations has uncovered surprisingly weak reproductive isolation between members of endemic lake radiations and widespread parapatric riverine species (Schwarzer *et al.* 2008; Stelkens & Seehausen 2009; Loh *et al.* 2013). Fully sympatric sister species within the lake radiations are often more strongly isolated. This implies that either the rates of evolution of reproductive isolation are higher during within-lake speciation than during stream-lake speciation, or that strong sexual isolation fails to evolve in the latter, perhaps due to the absence of effective reinforcement selection in parapatry (Stelkens & Seehausen 2009). In addition, some freshwater fish groups have achieved the stream-lake transition through phenotypic plasticity without speciation, such as the widespread life history polymorphism between resident stream trout and migratory lake trout (Klemetsen *et al.* 2003)**.** In this case, reproductive sympatry and sneaking male mating strategies may impede by-product speciation**.** Studying cases where speciation has not occurred may inform us about the conditions facilitating speciation as much as studying cases where reproductive isolation has evolved.

Speciation along habitat gradients within lentic or within lotic ecosystems

Research on species flocks of fishes in large lakes has considerably influenced the development of speciation theory, specifically the debate about sympatric speciation, ever since the modern synthesis (Mayr 1942; 1984). A strong proponent of allopatric speciation, Mayr felt compelled to devote a section of the chapter “nongeographical speciation” in his 1942 book to lacustrine fish species flocks. Arguing that the accumulation of many closely related species in these lakes was due to multiple independent colonizations from rivers, he rejected the theories of Herre (1933) and Woltereck (1931) who had suggested species flocks originated through ecological specialization in response to ecological opportunity (Mayr 1942). Later in life, faced with overwhelming evidence, (Mayr 1984) changed his view and accepted intralacustrine speciation for the most species-rich flocks, specifically the cichlid fish of the African Great Lakes. Research on speciation in lakes has contributed importantly to developments in speciation research ever since (Schluter 1996a, 2000; Coyne & Orr 2004; Gavrilets & Losos 2009; Nosil 2012), and much of the recent literature on speciation in lakes echoes some of the original ideas of Herre and Woltereck (Schliewen *et al.* 1994; Schluter 1996b; Gislason *et al.* 1999; Streelman & Danley 2003; Bernatchez 2004; Kocher 2004; Seehausen 2006). In Bolnick and Fitzpatrick’s (Bolnick & Fitzpatrick 2007a) summary of putative cases of sympatric speciation, 5 out of the 12 cases of sympatric speciation that they classified as “generally accepted” involved speciation of fish in lakes: four cases of cichlid fish and one of Arctic charr. The only other taxon for which sympatric speciation was as frequently invoked was phytophagous insects. Lake fish also featured prominently among their category of “possible cases of sympatric speciation”. Several more cases can now be added to this list (Table S1). This begs the question of if there is something fundamentally different about fish in lakes that diminishes the constraining effects of gene flow on the speciation process (Felsenstein 1981) (see Box 2). We address this question by reviewing the major recurrent themes in speciation of fish within lakes.

Three major physical niche axes characterize lake ecosystems and make them distinct from most other ecosystems. All three have to do with the physical characteristics of water, and offer a diversity of ecological opportunity for fishes. The first is **water depth**. A large number of physical and biotic variables change with water depth, including light, temperature and oxygen, and associated pathogens, parasites, food and predators, all of which are known to affect fish populations in important ways. Populations that colonize broad ranges of depth habitat experience strong divergent selection (Seehausen & Magalhaes 2010; Ingram 2011; Ohlberger *et al.* 2013). Mayr was among the first who recognized the importance of this third dimension of lakes (Mayr 1984). The best terrestrial comparison to water depth gradients is elevation gradients, and indeed some of the best examples of parapatric ecological speciation in terrestrial animals can be found here, including birds (Mayr 1942) and butterflies (Gompert *et al.* 2006). However, the spatial scale of divergence is often very different in fish speciation, in some cases involving depth differences as little as 10m and without any habitat discontinuity (Seehausen et al. 2008). Although no studies have directly compared divergent selection and evolutionary response along altitudinal versus water depth gradients, one plausible reason for this difference in the spatial scale of divergence is that water is a denser medium than air; changes of pressure and light with underwater depth happen at smaller spatial scales than equivalent changes in air, and changes in temperature can be large and abrupt around the thermocline, especially in temperate lakes (Ohlberger *et al.* 2008).

Divergence in depth utilization is one of the first axes of divergence in many cases of freshwater fish speciation, and non-geographical speciation in fishes is often more common than geographical speciation in water bodies with sufficient and temporally stable variation in habitable water depth. Geographically sympatric incipient and sibling species of lake fish often utilize depth differently (whitefish (Bernatchez 2004; Vonlanthen *et al.* 2009; Ingram *et al.* 2012), vendace (Ohlberger *et al.* 2013), pigmy whitefish (Gowell *et al.* 2012), charr (Knudsen *et al.* 2006), stickleback in British Columbia (Bentzen *et al.* 1984; Boughman 2001)) and Lake Thingvallavatn (Olafsdottir *et al.* 2007), cichlids in Lake Victoria (Seehausen *et al.* 2008b; Seehausen & Magalhaes 2010), Lake Malawi (Genner & Turner 2012), and Nicaraguan crater lakes (Elmer *et al.* 2009), sailfin silversides in lakes on Sulawesi (Herder *et al.* 2008). Exceptions include some incipient cichlid species pairs in Cameroonian crater lakes (Martin 2012) and pupfish (Martin & Feinstein 2014).

For divergent selection to lead to speciation when there is gene flow, ecological differentiation must become coupled to reproductive isolation, and this coupling needs to persist against the effects of recombination (Felsenstein 1981). Theory suggests that this may be most likely through one-allele mechanisms and “magic traits” (Felsenstein 1981; Kirkpatrick & Ravigne 2002; Gavrilets 2004). Divergent selection along water depth gradients may be a powerful mechanism to generate initial coupling between adaptation and reproductive isolation when populations both feed and breed at different depths (Seehausen & Magalhaes 2010), a mechanism that has been referred to as an “automatic magic trait” (Servedio *et al.* 2011). There is indeed evidence for breeding at slightly different water depth in many of the above cases, but there are exceptions (Bernatchez 2004; Martin 2012). Unfortunately, detailed microhabitat and depth utilization data are not available for most lacustrine species pairs, such that it is at present difficult to quantify the prevalence of reproductive isolation as a by-product of microhabitat adaptation. Additional evidence for the importance of the depth-related by-product mechanism comes from work demonstrating that habitable lake depth is a strong predictor not just of species richness but of the strength of species genetic differentiation (Vonlanthen *et al.* 2012b), and from studies showing a positive correlation between spatial segregation along the water depth axis and the extent of genetic differentiation (Seehausen & Magalhaes 2010; Ingram *et al.* 2012).

The second major physical niche axis in lakes is the **benthic-limnetic axis**, where fish are either lake-bottom associated or living in the open-water environment (Schluter 1996b; Taylor 1999). Although the higher density of water makes evolutionary transitions from benthic to pelagic less constraining than the non-aquatic equivalent, the transition from land to air, it is nevertheless associated with strong biomechanical performance trade-offs in locomotion and feeding. Living in the open water of the pelagic zone requires sustained swimming whereas living in the structured benthic zone requires maneuverability and burst swimming. Feeding in the open water requires ram or suction feeding with a wide reach to ingest items in the water column, whereas feeding from the bottom requires forceful suction feeding or increased bite force to dislodge items from the sediment. The constructional trade-offs between adaptation to these alternative feeding styles are quite well understood (Barel 1983; Wainwright 2007; Holzman *et al.* 2012), and there is evidence that speciation can be associated with disruptive selection on the force-reach trade-off in suction feeding (Schluter 1995; McGee & Wainwright 2013). There is evidence that anatomical properties that allow for behavioural flexibility in some taxa facilitate evolvability (Alfaro *et al.* 2005; Holzman *et al.* 2011; Wainwright *et al.* 2012). In addition, the gill rakers, a prey retention apparatus, are under divergent selection between primarily plankton feeding limnetic niches and benthic niches, mediated by trade-offs between being able to retain small food items and to release inedible sediment particles (Bentzen & McPhail 1984; Roesch *et al.* 2013).

Speciation associated with the benthic-limnetic transition has been demonstrated for whitefish in lakes in North America, Switzerland, Scandinavia, pigmy whitefish in Alaska, Arctic charr in Iceland and Scotland, threespine stickleback in Canada, trout in Ireland, and kokanee salmon in Russia (Figure 1, Table S1). Although less prominent, examples also exist from lakes at lower latitudes. Some of these, such as pupfish in Lake Titicaca, are depauperate in other fish species and in that way comparable to postglacial lakes, but others are more species-rich. These latter include Lakes Biwa and Tana with benthic-limnetic species pairs of barbs, some of the cichlid species pairs in crater lakes of Nicaragua, a cichlid pair in Cameroonian Lake Ejagham, and a small number of species pairs in the large cichlid radiation of Lake Malawi (Figure 1, Table S1). Phylogenetic patterns in several older lacustrine fish radiations are consistent with the concept that divergence along this axis has led to lasting lineage differentiation beyond young species pairs. The phylogenetically partially resolved radiations of cichlids of Lakes Malawi (Shaw *et al.* 2000) and Barombi Mbo (Schliewen & Klee 2004), sculpins of Lake Baikal (Kontula *et al.* 2003) and pupfish of Lake Titicaca (Garrigos *et al.* 2013) all have clades with specialized pelagic and benthic feeders.

As predicted by the structural features of aquatic ecosystems, speciation associated with benthic-limnetic divergence has primarily been documented in lakes, and not many cases in river fish are known. However, in stream-dwelling North American cyprinids, a shift from a purely benthic to a pelagic lifestyle and morphology is associated with a diversification rate increase

(Hollingsworth *et al.* 2013). The evolution of a pelagic life style in cases like this qualifies an evolutionary key innovation, bearing witness to the discrete nature of the benthic-limnetic niche contrast and associated adaptations.

A third major physical niche axis, transitions between **soft and hard benthic substrates**, has received less attention in the speciation literature, although the trade-offs associated with the associated transition in feeding specialization on loose versus attached prey have been extensively studied. Due to the high density and viscosity of water, a large portion of benthic nutritional resources, including both free-living and attached to organic debris, is not or only loosely attached to the substrate. Firmly attached biocover growing on rocks is also a rich dietary resource in the benthic habitat. Opportunities to feed on loose versus attached benthic resources mediate a strong tradeoff between suction feeding and biting because these feeding styles place conflicting demands on the cranial musculo-skeletal system (Barel 1983; Konow *et al.* 2008). Specialized bite feeding has repeatedly evolved in percomorph fish lineages, most famously among coral reef dwelling marine fish, but also in cichlids and in several groups of cyprinids among the freshwater fish.

In cichlids, ecological speciation within the benthic niche in lakes has been shown to sometimes be associated primarily with shifts between feeding on attached versus loose prey. Examples include sympatric incipient species in the Lake Victoria genus *Neochromis* (Magalhaes *et al.* 2012), cichlids in the genus *Alcolapia* from Lake Natron (Seegers *et al.* 1999), and the hybrid speciation event that generated a specialized sponge eater *Pungu maclareni* in Lake Barombi Mbo (Schliewen & Klee 2004). Similar transitions between living and feeding on soft sediment versus rocky bottom have occurred in cyprinids in Lake Tana (de Graaf *et al.* 2008) and Lake Biwa (Komiya *et al.* 2011) but are not associated with the evolution of a strong biter morphology. Additionally, the trade-off between adapting to loose and attached resources in the benthic zone may contribute to the high diversity of littoral freshwater fish communites compared to those of the pelagic in large lakes worldwide (Vadeboncoeur *et al.* 2011).

Evidence for speciation along the water depth axis and the benthic-limnetic axis is available for many different groups of fish. Importantly, both of these modes of speciation may come with some “automatic” coupling of adaptation and reproductive isolation because the transitions between these fairly discrete niches tend to be associated with some spatial segregation. However, these mechanisms alone will rarely allow the evolution of species flocks with more than three or four species (Taylor 1999; Knudsen *et al.* 2006; Vonlanthen *et al.* 2012b), particularly when reproductive isolation emerges mainly as the by-product of spawning site segregation. For building larger sympatric species numbers, more subtle ecological differentiation seems to be required, as well as additional mechanisms of reproductive isolation. Larger intralacustrine species flocks often reveal additional trophic radiations through the evolution of adaptations to feeding on hard-shelled molluscs (durophagy) and intraguild predation (piscivory) (Figure 1). In many of the groups that have made even larger species flocks, including cichlids, pupfish, and sailfin silversides, behavioural mate choice is highly developed (Chunco *et al.* 2007; Young *et al.* 2010; Kodric-Brown & West 2014), and many of these taxa display sexual dimorphism in colour (Wagner *et al.* 2012b) . We know from theory that the interaction of sexual and natural selection can be a particularly powerful mechanism for generating new species in the absence of geographical isolation (Kirkpatrick & Ravigne 2002; van Doorn *et al.* 2009). Several empirical examples from lacustrine fish suggest that intraspecific sexual selection facilitates the evolution of sexual isolation between populations that experience divergent ecological selection, and may allow speciation with much more subtle ecological differences among sympatric species than mechanisms relying on divergent natural selection alone (Boughman 2002; Maan & Seehausen 2011). Sensory drive, resulting from fine scale adaptations in the sensory systems to environmental variation in signal transmission and detection, may be one mechanism that is particularly powerful in generating or facilitating reproductive isolation (Boughman 2001; Seehausen et al. 2008b).

All of these mechanisms of speciation appear to be rare in rivers compared to lakes, but some speciation associated with the evolution of intraguild predation and durophagy has happened, apparently sympatrically, in large rivers (Figure 1; (Burress *et al.* 2013)).

Geographical speciation without distinct niche shifts

Classical allopatric speciation without niche shifts in rivers frequently occurs through vicariance events driven by changes in the geohydrology of freshwater drainage systems. There are several mechanisms for speciation in this context. Genetic drift in stable populations (Wright 1940) and drift associated with founder events (Mayr 1954), as well as the interaction of the latter with selection for new constellations of co-adapted gene complexes (Templeton 1981; Carson & Templeton 1984) can generate reproductive incompatibility, such as through the accumulation of Dobzhansky-Muller incompatibilities (DMIs; see (Presgraves 2010) for review). DMIs can also arise through the fixation of alternative advantageous genes in allopatric populations that adapt to similar selection pressures (Muller 1940), referred to as mutation order speciation (Schluter 2009). Additionally, the interaction of drift with sexual selection can generate sexual isolation (Fischer 1930; (Lande 1981). Studies of freshwater fish provide support for many of these mechanisms. Finally, although rarely explicitly discussed in speciation literature, many species have simply accumulated sufficient phenotypic and/or DNA sequence differences for populations to be assigned species status by taxonomists. Such species boundaries likely often do reflect reproductive isolating barriers, but in many cases such barriers would never be “tested” in sympatry.

Conspecific populations of fish isolated by drainage system vicariance diverge via several routes. In some cases, geographical isolation persists long enough for the accumulation of intrinsic incompatibilities, inhibiting the production of fertile or viable offspring upon secondary contact that would eventually occur via river capture or drainage reshuffling (Albert & Reis 2011). Alternatively, and arguably much more often, populations will come into contact before they have acquired complete intrinsic reproductive isolation. This is perhaps particularly the case for freshwater fishes, as studies of the rate at which intrinsic incompatibilities arise in freshwater fishes have shown hybrid viability and fertility despite millions of years of divergence time (Mendelson 2003; Bolnick & Near 2005; Stelkens *et al.* 2010). In absence of strong intrinsic incompatibilities, populations can either remain reproductively isolated through differences in behavior or habitat occupation, or hybridize. In the latter case, populations may merge into a hybrid swarm, reinforcement selection may lead to the evolution of behavioural isolation, or ecological character displacement may cause behavioural isolation as a by-product, as may be the case in the benthic-limnetic stickleback species pairs (Rundle *et al.* 2000).

Allopatric speciation via the mechanisms described above is probably the mode of speciation by which the majority of riverine fish species has arisen. But there is a remarkable paucity of studies that test such speciation and the evidence to date is mostly based on phylogeographic data (but see (April *et al.* 2013)). Many groups of species-rich teleosts show very strict allospecies distribution patterns where sister taxa occupy exclusive geographical ranges and sympatric species are phylogenetically relatively distant (Near & Benard 2004; Dias *et al.* 2013). Such patterns point to two important predictions: that the evolution of reproductive isolation as a consequence of evolution in geographical isolation is slow, and that reinforcement upon secondary contact is rare. This is corroborated by many examples of hybridization in contact zones (April *et al.* 2013; Bossu & Near 2013) and few examples for reinforcement. Geographical speciation in river fish is most often associated with isolation of different drainage systems, but can also happen by persistent isolation of smaller tributaries of the same larger river by intervening habitat that is inhabitable for stream fish. That main channels of large rivers can act as a formidable barrier to genetic exchange among populations of stream fish has been demonstrated in the Amazon (Ready *et al.* 2006) and the Congo (Schwarzer *et al.* 2012), but also in the diverse riverine fauna of eastern North America (Harrington *et al.* 2013).

Geographic speciation in freshwater fishes can also occur in large lakes, particularly in taxa with limited dispersal. In cichlids of the African great lakes, evidence for geographic speciation comes both from genetic studies showing very fine-scale spatial genetic structure (Danley & Kocher 2001; Wagner & McCune 2009), and from the allopatric distributions of many closely related taxa (Seehausen *et al.* 1999; Genner *et al.* 2004). Allopatric sister taxa differ frequently in color but rarely in any other obvious phenotypic or ecological traits, suggesting a role of divergent sexual selection in divergence of populations (Allender *et al.* 2003; Knight & Turner 2004; Kocher 2004; Salzburger 2009; Mattersdorfer *et al.* 2012). That such taxa at least sometimes remain distinct in secondary contact has been demonstrated in unplanned translocation experiments (Young *et al.* 2009). However, in other cases such allopatric incipient species may fuse again upon secondary contact (Egger *et al.* 2012).

LINKING DIVERSITY AND SPECIATION

Hutchinson famously asked, “Why are there so many kinds of animals?” (Hutchinson 1959), and this seems a particularly apt question for the tremendous diversity of ray-finned fishes, a group which contains approximately half of known vertebrate species. However, as the sections of the paper above elaborate, there is a great variety of mechanisms and circumstances by which speciation in fishes can occur. Rates of speciation measured for some freshwater fish are the highest of any known vertebrates (McCune 1997). In Lake Victoria cichlids, speciation rates calculated from calibrated molecular phylogenies range from ~5-7 species per lineage per million years, meaning that with unconstrained speciation over time, there would exist hundreds of thousands of species after only 2 million years. The more appropriate question, given these observations, may be why there are so few kinds of freshwater fishes (e.g. Felsenstein 1981).

We know the answer to this question, at least in broad strokes: Speciation rates vary among lineages; the number of species at any given time is influenced not just by speciation but also extinction; there are genetic constraints to the origin of species (e.g. Felsenstein 1981); and there are limits to the number of species that can coexist without competitive exclusion. The real unknown is the extent to which these different kinds of limits influence realized patterns of diversity, and whether and how variation in their importance across clades impacts the relative diversity of these groups. One pointed question directly related to the focus of this review, speciation, is to what extent variation in the mechanisms of and constraints on the speciation process impact the relative diversity of clades. Of the four factors listed above, only two of them relate to the process of speciation, and it is thus conceivable that factors influencing the speciation process have little impact on patterns of species richness among clades. Although no consensus exists on the answers to these questions, addressing them requires not only the study of speciation, but understanding of broader-scale diversity and its drivers and limits.

Variation in speciation and extinction rates

Freshwater fishes have long been a focus in discussions of rapid speciation, due the endemic diversity present in young lakes (McCune 1997; McCune & Lovejoy 1998). As new genetic data enable increasingly well-resolved trees and large-scale phylogenetic analyses, our understanding of the details of evolutionary relationships among fish groups and their dynamics of diversification are increasing rapidly (e.g. Alfaro *et al.* 2009; Near *et al.* 2013; Rabosky *et al.* 2013). Rabosky et al (2013) provide evidence for correlated changes in body size evolution and speciation rate across actinopterygian (ray-finned) fishes, and in doing so provide new perspective on the degree of speciation rate variation across lineages in this group (see Figure 2). Among the families with the fastest inferred speciation rates are some of the taxa best known from case studies of rapid speciation, including cichlids and salmonids, and also taxa known for adaptive radiation like pupfishes, telmatherinids, and cottids (Figure 1, Table S1). However, other taxa that are well-known from studies of speciation, such as stickleback, do not have high rates of speciation, inferred in this phylogenetic context. Yet other taxa have very high species richness, but not exceptionally high speciation rates, such as cyprinids and characids. Such discrepancies between speciation rates known from individual cases of speciation, rates measured in a phylogenetic context, and species diversity may arise for many reasons, but at a basic level these observations underscore the point that even if speciation is rapid, this does not necessarily generate species-rich groups that will persist over macroevolutionary timescales.

Factors influencing the persistence of species may be particularly important to consider when studying broad-scale patterns of diversity in freshwater fishes, given that freshwater habitats are often ephemeral. Although many of the world's "ancient lakes" host species-rich adaptive radiations of fish and other taxa, most of the world's lakes are geologically young. Many north temperate lakes, frequently studied in the context of the evolution of salmonid species pairs, were ice-covered in the last glacial maximum. Even at tropical latitudes, global climate changes over glacial cycles mean that only the deepest of lakes persist over millions of years. For lake-specialized taxa the ephemerality of lakes will most often result in extinction over macroevolutionary timescales. The beautifully preserved semionotid fossil fishes of the Newark Supergroup show such a dynamic repeatedly over ~20,000 year timescales, with endemic species flocks arising, going extinct upon the drying up of the lake, and arising anew, with differences in variety and form, upon the refilling of the lake (McCune 1987). Even without the disappearance of lakes, environmental changes that destabilize lake conditions, like eutrophication, can cause the collapse of differentiated species that have diverged along ecological gradients within lakes (Behm *et al.* 2010; Vonlanthen *et al.* 2012a). Thus, although worldwide there are likely many thousands of cases of intralacustrine divergence and speciation, the vast majority of diversity in these environments will, over macroevolutionary timescales, be ephemeral. When viewing freshwater fish diversity at long timescales, we are thus perhaps left with more questions than we have answers: What is it that allows some lineages to persist through time and to grow in diversity, despite environmental fluctuations, while others repeatedly evolve into species pairs but not to accumulate diversity over macroevolutionary timescales? What impact does the ephemerality of freshwater environments have on the evolution of novel traits, phenotypes, and on evolvability itself? It is possible that cycles of species radiation and species extinction through the breakdown of reproductive isolation in lakes that are ephemeral at geological timescales, leaves populations with high diversity that are able to diversify quickly again in subsequent lakes.

Given that the ephemerality of freshwater environments has dramatic consequences for speciation and the persistence of diversity in fishes through time, and marine habitats are subject to entirely different scales and kinds of environmental change, marine versus freshwater fish groups may have very different histories of diversification. In the oceans, habitats are perhaps less ephemeral, population sizes may be generally larger, and dispersal is generally less constrained than in freshwater systems, making for interesting contrasts of speciation processes in freshwater versus marine environments (Puebla 2009). How is it that freshwater environments are home to half of actinopterygian diversity when these environments are so much less extensive than marine environments? Vega and Wiens (2012) addressed this question by calculating and comparing the net diversification rates from clades including predominantly marine and freshwater fishes to ask if there were differences in net diversification rate or clade age by major habitat across the actinopterygian tree. They found no consistent differences in rate or clade age across 22 major actinopterygian clades, and interpret their finding of roughly equal diversity in marine and freshwater environments as an effect of ancient marine extinctions leading to a relative paucity of species in the ocean given available “space”, and to the effect of barriers to dispersal in freshwater environments elevating species richness in these environments. Other hypotheses could also explain these results (e.g. Grosberg *et al.* 2012), and these patterns should be investigated with diversification models that can contrast lineage accumulation patterns through time in marine and freshwater clades.

SYNTHESIS

We started this review by asking how research on speciation has helped understand the exceptional species richness of freshwater fish and how studying this exceptionally diverse clade has contributed to our understanding of speciation. Many different modes and mechanisms of speciation have been demonstrated in freshwater fish. Yet, some of these are supported by more cases than others, and although the rich literature on fish speciation remains incompletely integrated, some conclusions can be drawn.

First, freshwater fish often speciate at small spatial scales, perhaps because the steep environmental gradients in the dense medium of water cause steep selection gradients that affect both resource utilization and reproductive isolation at the same time. Examples from many different fish groups suggest speciation can sometimes proceed in the absence of any geographical isolation. This allows fish to respond to ecological opportunity via ecological speciation and adaptive radiation, and very different research approaches converge on the conclusion that such responses can be exceptionally fast in freshwater fishes (McCune 1997; Hendry *et al.* 2000; Rabosky *et al.* 2013): ecological speciation can happen within just hundreds of generations and sometimes perhaps even faster. At the same time it is clear that the evolution of intrinsic postzygotic isolation among fish species is slow and generally takes on the order of millions of years (April *et al.* 2013).

Our review of the literature from work on phylogeographic patterns and on evolutionary mechanisms suggests that the slow and steady generation of new species through allopatric speciation, rapid speciation by sexual selection in allopatry, and rapid ecological speciation, often with gene flow, all contribute importantly to freshwater fish diversification. Which of these contributes most is at present difficult to say. Sympatric and parapatric speciation with gene flow is of considerable interest to evolutionary biologists and our review confirms earlier suggestions that it is more common in freshwater fish than in most other animal taxa (Bolnick & Fitzpatrick 2007a) . Many of the cases that we reviewed fulfill criteria of by-product speciation, where at least partial reproductive isolation is a by-product of divergent ecological adaptation, often because adaptation to different habitats and microhabitats tends to be associated with reduced encounter rates during reproduction. In many cases, parapatric and sympatric incipient and sister species of fish are also behaviourally isolated by mate choice, but while this is widespread in some groups, such as cichlid fish, its prevalence in other groups such as salmonids and cyprinids is unclear. There is convincing evidence that behavioural assortative mating can evolve by reinforcement or reinforcement-like mechanisms (Rundle *et al.* 2000), but it is also clear that this does not always happen. Behavioural isolation is often lacking or incomplete in situations where sister taxa are parapatric with little overlap, but seems more often complete among sympatric taxa. This is support for a role of reinforcement-like mechanisms. It is also clear though that parapatric sister taxa can often retain considerable phenotypic and genetic differentiation without strong sexual isolation.

We have outlined several recurrent themes in terms of modes and mechanisms of speciation in freshwater fish. It is important to realize that these are not exclusive. The data in Figure 1 and Table S1 reveal that some major clades are known for speciation by several of these modes, whereas others are known for just one or two of them. Stickleback and whitefish for instance speciate by shifts between major habitats but also by shifts along water depth gradients and along the benthic-limnetic axis within lakes. Cichlids are known to speciate by habitat shifts within lakes, but also by sexual selection and by several kinds of trophic transitions within habitats. Herrings are at the other extreme: they do speciate by shifts between the sea and freshwater, but are not known for marked further radiation within freshwaters. It is likely that such differences between taxa explain some of the variation in species richness among clades.

Differences in evolvability are likely to contribute to among-clade differences in speciation rate and species richness.. Many of the ecological transitions associated with speciation in freshwater fish require taxa to overcome adaptive constraints. Complexity in the functional basis of performance, referred to as many-to-one mapping of form to function (Alfaro *et al.* 2005), and modularity (Galis & Metz 1998), have for instance been proposed as mechanisms that reduce trade-offs in the feeding performance of fish. Variability among clades in complexity and modularity of performance underlying traits may help to explain variability in diversification.

Both of these mechanisms have been suggested to contribute to evolvability in cichlids (Galis & Metz 1998; Liem & Summers 2000; Parnell *et al.* 2008). Variability in coping with extreme physical conditions and with interspecific competition are likely additional traits that help predict speciation. Interestingly, these might be expected to be negatively correlated through life history tradeoffs (Stearns 1977). The data tabulated in Figure 1 and Table S1 indeed suggest that the clades that radiate in extreme environments (poeciliids, pupfish and other killifish) are not known to radiate in "benign" environments. It is possible that release from interspecific competition in extreme environments contributes to this pattern. Similarly, stickleback are only known to speciate in fairly small lakes that have few other fish species. The presence of resource competitors such as sculpin and rainbow trout appears to significantly hinder the formation of species pairs (Vamosi 2003). Likewise, species pairs in Arctic charr appear to be more common in lakes with few other fish species (Griffiths 1994). Whitefish, in contrast, have radiated repeatedly even in the presence of many other fish species, but they do not occupy small or extreme environments. Cichlids finally have radiated in very many very species rich lakes, but some cichlids have also become extremophiles and speciated in alkaline lakes where no other fish survives (Seegers *et al.* 1999). Such variation among fish taxa may well to some extent explain spatial and phylogenetic variation in speciation, but formal tests of these ideas have yet to be developed.

Hypotheses about the influence of traits on patterns in fish diversification are now possible to test in a rigorous phylogenetic comparative context, as new genetic data enable increasingly large and well-resolved trees and as analytical methods develop and expand. These include tests of the influence of traits on species diversification (Santini *et al.* 2009; Wainwright *et al.* 2012), and tests of broad-scale patterns in diversification (Rabosky *et al.* 2013). In tropical cichlids, recent analyses revealed that the best predictors of when adaptive radiation occurs in a set of numerous lakes across Africa include both ecological factors (lake depth, net solar radiation) and lineage specific traits (the presence of sexual dichromatism) (Wagner *et al.* 2012a). Studies like this underscore the importance of considering both intrinsic and extrinsic factors together when assessing the suite of factors influencing speciation.

Many studies of species flocks of in fish have recently uncovered signals of an admixed history of the founding populations, and several lacustrine adaptive radiations seem to have evolved from hybrid swarms. There is also increasing evidence that many species flocks have collapsed again through interspecific hybridization. Future studies should therefore address the role of interspecific hybridization and admixture between genetically distinct lineages both in the evolution of evolvability and reproductive isolation.

Lakes are home to some of the most celebrated and species-rich adaptive radiations, and also to dozens of species pairs, most often in post-glacial lakes (Taylor 1999). Many more populations exhibit bimodality in phenotype and ecology, and freshwater fishes are among the classic cases of persistent resource polymorphism (Robinson & Wilson 1994). These replicated situations represent important opportunities to understand the circumstances in which reproductive isolation evolves, and when it does not (Vamosi 2003; Wagner *et al.* 2012a), and detailed comparative studies of many more of these systems are warranted. Finally, future research should seek a fuller integration of the process-oriented evolutionary ecology paradigm and the pattern-oriented phylogeography paradigm in freshwater fish speciation research. Process-oriented research has recently begun to uncover unexpected speciation by natural and sexual selection in Amazonian characids (Cooke *et al.* 2012; Kolm *et al.* 2012), long thought to have diversified solely through geographical isolation. In addition, increasingly well-resolved phylogenomic approaches in ecological speciation research have uncovered surprising signatures of ancient differentiation of divergent alleles between very young stickleback species pairs (Jones *et al.* 2012). These classes of approaches will undoubtedly uncover many more surprising facets of the interactions between ecology, geography and historical contingency in freshwater fish speciation in the years to come.

FIGURES AND TABLES.

Figure 1. **Major themes in freshwater fish speciation.** For each theme we provide a non-exhaustive list of taxa that have been investigated, an number indicating the number of replicate cases for which published data exist, and key references provided in electronic supplement. See also Table S1. Taxa in bold correspond to those depicted in the boxes.

Figure 2. **Speciation rates and species richness varies dramatically across the phylogeny of Actinopterygian fishes**. The phylogeny depicts relationships among families with greater than 10 species, and terminal branches are scaled to speciation rates inferred in Rabosky et al. (2013). Circles on the tips of the tree are proportional to species richness. Red circles represent marine families; blue circles are families where the FishBase (Froese & Pauly 2010) entry for at least one species contained the word "freshwater". We highlight several families that are classic systems of speciation and/or species diversity research: a) Gasterosteidae (stickleback), b) Cichlidae, c) Cottidae (sculpins), d) Salmonidae, e) Cyprinodontidae (pupfishes), f) Telmatherinidae (silversides), g) Mormyridae (African weakly electric fish), h) Cyprinidae (minnows). Tree redrawn from Rabosky et al. (2013).

Table S1. Case studies of the major themes of freshwater fish speciation/expanded info Figure 1.

Box 1. THE EXTENDED SPECIATION CONTINUUM

We define speciation as the origin of reproductive barriers that permit the maintenance of genetic and phenotypic distinctiveness among populations in geographical proximity. One of the major challenges in speciation research lies in clearly distinguishing between events during speciation that are important for understanding the speciation process, and divergence that happens after reproduction isolation has evolved. Once populations are reproductively isolated, diverging populations will accrue differences due to drift, selection and adaptation that have little to do with the process of speciation, and inferring mechanisms of speciation from such differences can confound cause and consequence of speciation. The best assurance against this is to study speciation at its earliest stages. This approach, however, may lead researchers towards studying cases of population divergence that will never result in speciation (Nosil *et al.* 2009). In this context it is of considerable importance to distinguish between divergence of allopatric populations simply by drift or local adaptation, and incipient speciation. Much of the contemporary speciation research program rests on an assumption that speciation proceeds as a continuum from an incipient stage to complete reproductive isolation. However, many or even most cases of early-stage speciation will not produce species that ultimately persist (Seehausen *et al.* 2008a), and many species will be short lived, such that many cases of speciation may not leave a lasting signature over macroevolutionary timescales (Rosenblum *et al.* 2012).

Addressing these issues in understanding the origins of species and linking these origins to larger-scale diversity patterns requires studying the speciation process from early deviations from panmixia to complete reproductive isolation, and further to the assembly of communities and regional biota. By studying replicated cases of speciation in the same species complexes, and doing so in taxa that are part of phylogenetically well-characterized clades, we can emulate such an approach. Taking this approach, ideal speciation model systems should have replicated pairs of species and incipient species at varying stages of divergence, and also be embedded within clades of species varying in divergence time. Freshwater fish are exceptionally rich in such species complexes. In addition, the long history of detailed research in natural history and ecology of several species-rich freshwater fish groups has become an invaluable asset in developing these systems into speciation research models.

Box 2: SYMPATRIC SPECIATION

Why is there a seemingly more frequent occurrence of sympatric speciation in fishes compared to most other taxa? Three nonexclusive possibilities may contribute to this pattern.

First, it could be that the frequency of sympatric divergence in fishes is not greater than for other taxa, but simply that small lakes are environments in which allopatric scenarios can more readily be ruled out than most other environments. One commonality between fish and phytophagous insects is their occurrence in areas with well-circumscribed boundaries. All well-supported examples of sympatric speciation in fishes are taxa living in small freshwater lakes, whereas all cases of putative sympatric speciation in phytophagous insects are species that live and breed on specific host plants. If allopatric speciation is taken as the null hypothesis, geographically restricted areas where it is possible to rule out the possibility of geographic speciation within the habitat, or secondary colonization of the habitat, are those most likely to stand up to scrutiny.

Second, it could be that freshwater fish differ from many other taxa in their dispersal and movement patterns in such a way that facilitates speciation at small geographic scales. Many freshwater fishes return to spawn in their natal spawning grounds, and/or have specialized preferences in spawning habitat and spawning time (Figure 1, Table S1). Such behaviors facilitate divergence at small spatial scales if they are heritable and there is variance among populations, and can lead to the coupling of traits under ecological selection to facilitate assertive mating (Adams *et al.* 2006).

Third, it could be that freshwater environments actually do foster divergence at small spatial scales more so than other environments. Strong gradients for selection are necessary prerequisites to sympatric speciation (Bolnick & Fitzpatrick 2007b), and whether or not speciation is truly sympatric, such gradients are expected to facilitate divergence in the face of gene flow if selection is strong. Lakes have strong ecological gradients in resources (e.g. food type and availability in littoral-pelagic and shallow-deep water axes) and in environmental characteristics (e.g. light gradient with depth). Coupled with mechanisms facilitating assortative mating, such gradients may readily create environments strongly facilitative of divergence at small spatial scales (Boughman 2001, 2002; Maan *et al.* 2006). Likewise, gradients in habitat between lake and riverine environments, and between freshwater and marine environments, are strong, and many cases of species divergence occur along such gradients (see Main Text, Figure 1**).**

Box 3: GENOMICS OF SPECIES DIVERGENCE

Genetic data have in the past decades dramatically increased our understanding of freshwater fish speciation by allowing us to rigorously investigate the evolutionary relationships among species, the phylogeographic context of speciation, and the population genetic processes at play in empirical case studies of speciation. The recent surge in the availability of genomic data is advancing studies in the field of speciation at a rapid pace. Annotated fish genomes are available from an increasing number of species across the teleost tree (Volff 2005), including the recent sequencing of several species particularly important to studies in freshwater fish speciation (Jones *et al.* 2012; Schartl *et al.* 2013; Brawand *et al.* submitted). Genome reduction techniques combined with next-generation sequencing (NGS) are rapidly expanding the extent of the genome which can be readily examined in studies of incipient and recent speciation, and allow unprecedented insight into the process of divergence even in the absence of reference genomes (Hohenlohe *et al.* 2010; Deagle *et al.* 2012a; Renaut *et al.* 2012; Wagner *et al.* 2013; Martin 2014).

This deluge of genomic data will allow for major inroads into understanding the genetic basis for speciation, a field still in its fledgling stages (Rogers *et al.* 2013). One major challenge in studying the genetic basis of speciation is differentiating changes that occurred after the speciation process from those crucial to the initial splitting of populations (see Box 1). Addressing this challenge requires study of multiple populations and species, from small deviations from panmixia up through fully differentiated species, but special emphasis should be placed on sampling incipient species at the very early stages of divergence because genomic changes can accumulate quickly after the origin of reproductive isolation, and obscure the signal of the initial genetic changes driving the split (Seehausen *et al.* 2014).

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