On speciation and its reversal in adaptive radiations
The central European whitefish system

Inauguraldissertation

Der Philosophisch-naturwissenschaftlichen Fakultät

Der Universität Bern

Vorgelegt von

Pascal Vonlanthen

von Giffers/Schmitten

Leiter der Arbeit:

Prof. Dr. Ole Seehausen

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Abteilung Aquatische Ökologie

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TABLE OF CONTENTS

Chapter 1

GENERAL INTRODUCTION

Biodiversity, defined as *"the variety of life on Earth at all its levels, from genes to ecosystems, and the ecological and evolutionary processes that sustain it"*, is the basis for human survival and economic well-being (McNeely *et al*., 1990) and provides us with enormous direct and indirect economic benefits, such as food, fiber, and timber (Costanza *et al*., 1997, Singh, 2002). It is responsible for all biogeochemical processes regulating the earth system, such as atmospheric gaseous composition or climate (Loreau *et al*., 2001), and plays an important role in ecosystem functioning and stability (Singh, 2002, Hooper *et al*., 2005). Despite full awareness of it, today we continue to induce massive environmental perturbations on the global biosphere that have initiated the sixth and fastest ever global mass extinction (Myers, 1990, Daily, 1995, Singh, 2002). To understand how changes in the environment impact on biodiversity and in order to develop efficient conservation policies to preserve global diversity, the underlying ecological, genetic and evolutionary processes must be understood (Seehausen, 1999). All the diversity observed today is the result of 3.5 to 4.5 billion years of evolution where speciation on average exceeded extinction. Even though speciation can be rapid on geological time scales (Johnson *et al*., 1996b, Gavrilets, 1999, Seehausen, 2002), it remains very slow when compared to the speed at which diversity is lost today. There is unfortunately no way to get back what has been lost. It is therefore crucial to implement evolutionary concepts to efficiently manage and preserve biodiversity and the processes that generate it (Ashley *et al*., 2003), especially when considering that novel anthropogenic global disturbances like climate change will continue. The past has, however, shown that the incorporation of evolutionary components in conservation and environmental management has been difficult (Ashley *et al*., 2003). The reasons mostly lie in the simplicity of adopting measures related to biodiversity patterns, which are based on apparently rigid taxonomic species definitions, instead of attempting to deal with more complex processes underlying diversity (Mace & Purvis, 2008). Furthermore, conservation policies tend to be directed towards the preservation of the "status quo" instead of incorporating the fact that evolution of biodiversity is a continuous and ongoing process; thus potentially omitting early stages in speciation and processes driving future species diversity and adaptive variation.

This PhD thesis therefore wants to contribute to the understanding of which evolutionary mechanisms are responsible for speciation and how anthropogenic environmental disturbance affects these mechanisms which ultimately leads to diversity loss.

Speciation

Speciation is the evolutionary process leading to the emergence of new reproductively-isolated species. It has gathered considerable attention in the last two decades (Schluter, 2000, Coyne & Orr, 2004, Gavrilets, 2004, Dieckmann *et al*., 2004, Bolnick & Fitzpatrick, 2007) with theoretical publications sometimes outnumbering experimental ones (Kirkpatrick & Ravigne, 2002). It took evolutionary biologists almost 150 years since Darwin's *"On the origin of Species by Means of Natural Selection" (Darwin, 1859)* to accept that the origin of species is to large extents the result of natural selection (Schluter, 2009). The mechanisms leading to speciation are varied and they were usually classified by geographical arrangements of the diverging populations, i.e. focusing on the inhibitory effects of gene flow on the evolution of reproductive isolation (Schluter, 2001). These geographical arrangements range from

allopatric speciation where gene flow is completely absent to sympatric speciation where gene flow is unimpeded (Gavrilets, 2003). It is now accepted that this classification may not be the most informative as most speciation will be parapatric, i.e. with intermediate levels of gene flow compared to speciation in sympatry or allopatry (Gavrilets, 2000). Instead, a classification based on the mechanisms responsible for the evolution of reproductive isolation has recently been applied (Schluter, 2000, Schluter, 2001, Via, 2001, Rundle & Nosil, 2005, Schluter, 2009, Rundell & Price, 2009).

There are many processes that can lead to speciation and they can be grouped into two categories: non-ecological and ecological (Rundle & Nosil, 2005). Non-ecological speciation includes speciation models in which chance events like polyploidy, hybridization, genetic drift, and founder effects play an important role (reviewed in Coyne & Orr, 2004), but does not exclude the fact that natural selection might be occurring during some stages of the process. Selection is however non-ecological and/or is not divergent between environments (Schluter, 2001, Rundle & Nosil, 2005, Schluter, 2009). On the other hand, ecological speciation is defined as the process by which reproductive isolation evolves between populations as a result of ecologically-based divergent selection (Schluter, 2000, Schluter, 2001, Rundle & Nosil, 2005). This thesis will to a large extent deal with processes implicated in ecological speciation and its reversal. I will therefore introduce these two processes in more detail.

Ecological speciation

The concept of ecological speciation date back to the time of development of the "*Biological Species Concept*" (Schluter, 2001). Dobzhansky believed that speciation in *Drosophila* proceeds by evolving suits of physiological adaptations that are successful in their respective environments (Dobzhansky, 1946). Almost simultaneously, Mayr recognized that genetic differences affecting physiological and ecological characters that accumulate between populations might act as isolating mechanisms (Mayr, 1942). In following years, acceptance of ecological speciation was largely based on its inherent appeal and simple plausibility (Schluter, 2001). Experimental evidence, precise models and methods testing for ecological speciation were, however, remarkably absent (Schluter, 2001). It is only in the past two decades, and to some extent due to the emergence of the polymerase chain reaction (PCR) and other molecular techniques in combination with powerful simulations allowing researchers to predict and test many assumptions underlying ecological speciation, that speciation research has seen a revival (Rundle & Nosil, 2005). It lead to significant advances in understanding the different mechanisms implicated, but also revealed that these are very complex, which makes a detailed understanding difficult, even in the best studied model systems (Rundle & Nosil, 2005). Nevertheless, several convincing theoretical studies (Kondrashov & Kondrashov, 1999, Kirkpatrick & Ravigne, 2002, Gavrilets, 2004, Dieckmann *et al.*, 2004) suggest that ecological speciation can occur. This is further supported by experimental (Rice & Hostert, 1993), and empirical studies (reviewed in Schluter, 2000, Rundle & Nosil, 2005, Schluter, 2009) that show that ecological speciation can occur in nature.

Ecological sources of divergent selection

For ecological speciation to occur, an ecological source of divergent selection is needed. For example, divergent selection among populations might be induced in

heterogeneous environments harboring multiple niches. This is consistent with classic models of allopatric speciation (Mayr, 1942, Mayr, 1947), but divergent selection might also arise in the presence of gene flow (Schluter, 2000, Via, 2001, Dieckmann *et al*., 2004, Rundle & Nosil, 2005). In particular the interaction of divergent selection with a reduction in gene flow in heterogeneous environments, possibly coupled with inter specific competition and/or mate choice has been suggested to greatly facilitate speciation when a high number of demes form a cline along ecological gradients (Endler, 1973, May *et al*., 1975, Doebeli & Dieckmann, 2003, Gavrilets, 2004, Leimar *et al*., 2008). Some examples of clinal divergence include the Caribbean lizard *Anolis roquet* (Ogden & Thorpe, 2002), the little Greenbull *Andropadus virens* (Smith *et al*., 2005), and the Periwinkle *Littorina sexatilis* (Butlin *et al*., 2008). Theoretical studies, however, have also suggested that spatial structure is not a prerequisite to speciation and that speciation can theoretically occur in full sympatry. Such models can be grouped into two types: (1) The discrete-habitat models, which assume that individuals belonging to different populations that exploit different ecological niches have the highest fitness in their own niche and mate preferentially within individuals from their own population independent of their birth place (Kondrashov & Kondrashov, 1999, Dieckmann & Doebeli, 1999, Gavrilets *et al*., 2007); and (2) continuous-resource models, which assume a simultaneous evolution of ecological divergence and assortative mating along a continuous resource distribution (Dieckmann & Doebeli, 1999, Doebeli & Dieckmann, 2000, Ito & Dieckmann, 2007). However, both model types have been questioned (Turelli *et al*., 2001, Coyne & Orr, 2004) and empirical evidence has so far not been able to rule out spatial structure.

Divergent selection can arise between populations as a result of their interactions. This typically occurs in sympatry (it could also entail allopatric populations interacting indirectly via a separate mobile species) and is frequency-dependent (Taper & Case, 1992, Schluter, 2000). In nature, it is most commonly observed as a source of divergent selection during ecological character displacement. Evidence for its occurrence in other types of interactions like mutualism, facilitation or apparent competition is scarce (Doebeli & Dieckmann, 2000, Day & Young, 2004).

Finally, sexual selection, because it acts on traits that are directly involved in mate recognition, can also act as an ecological source of divergent selection. This may therefore represent a powerful force in speciation (Panhuis *et al*., 2001). Sexual selection can however be implicated both in ecological and non-ecological speciation (Rundle & Nosil, 2005). It is considered ecological if differences in mate preference evolve ultimately because of divergent selection between environments (Schluter, 2000, Schluter, 2001, Boughman, 2002), otherwise speciation is non-ecological as, for example, in the case for Fisher's runaway and sexual conflict models (Lande, 1981, Chapman *et al*., 2003). Evidence for ecologically-based sexual selection has been found in *Anolis cristatellus* lizards, three-spined sticklebacks (*Gasterosteus aculeatus* spp.), and Lake Victoria cichlids (Boughman, 2001, Leal & Fleishman, 2004, Seehausen *et al*., 2008), but more manipulative laboratory experimental are needed to test the various theoretical predictions of ecological models of sexual selection (Boughman, 2002, Coyne & Orr, 2004).

Mechanisms of reproductive isolation
For ecological speciation to occur, not only ecological divergence has to evolve, but also reproductive isolation between incipient species. Several mechanisms of reproductive isolation are known (Coyne & Orr, 2004), but most are not restricted to ecological modes of speciation. They can be grouped in pre- and post-zygotic

mechanisms. Habitat and temporal isolation of reproducing populations (Johnson *et al.*, 1996a, Dres & Mallet, 2002, Funk *et al.*, 2002). as well as mate choice are the most commonly observed pre-zygotic isolation mechanisms (Rundle & Nosil, 2005). Post-zygotic isolation, on the other hand, can be intrinsic and the result of genetic incompatibilities or it can be extrinsic when hybrid fitness is reduced relative to the individuals of the parental species because of a mismatch of their phenotype compared to the environment (Rice & Hostert, 1993, Rundle & Whitlock, 2001, Coyne & Orr, 2004). Additionally, extrinsic post-zygotic isolation can occur by disruptive sexual selection when hybrids have a reduced mating success and thus decreased reproductive fitness compared to the parental species (Schluter, 2000). Post-zygotic isolation can induce another interesting and potentially important mechanism implicated in speciation called reinforcement. The concept was introduced relatively early and predicts that a strengthening of pre-zygotic isolation is induced by selection against hybridization (Dobzhansky, 1940). Reinforcement can either be extrinsic if hybrid fitness is reduced ecologically compared to parental species (Kirkpatrick, 2001) or intrinsic if hybrid fitness loss is caused by genetic incompatibilities that reduce fertility or survival in early developmental stages (Dobzhansky, 1937, Muller, 1942).

Finally, a genetic mechanism linking the reproductive isolation and ecologically-driven divergent selection is needed. They can be linked in two ways: (1) by genes having a pleiotropic effect on both mechanisms, where reproductive isolation directly evolves due to selection (Rice & Hostert, 1993, Kirkpatrick & Barton, 1997); or (2) by association (linkage disequilibrium) of genes coding for reproductive isolation and ecological adaptation (Kirkpatrick & Barton, 1997).

Adaptive radiation

A particularly fascinating extension of ecological speciation is the adaptive radiation of species. It is "*the evolution of ecological diversity within a rapidly multiplying lineage*" (Schluter, 2000). It occurs when a single ancestor differentiates into an array of species that inhabit different niches and differ in traits used to exploit these niches (Schluter, 2000). It thus does not make any assumptions on the geographical mode of speciation and is characterized by four features that have been employed as criteria in its identification (Schluter, 2000). First, all species of an adaptive radiation share a common ancestor which can be tested by reconstructing multi-locus phylogeneitc trees (Givnish *et al.*, 1997, Schluter, 2000). Second, a significant association between environments and morphological or physiological traits in the species using those environments is expected (phenotype-environment correlation). Third, the utility of those traits in the respective environments has to be demonstrated, for example by conducting reciprocal transplant experiments. This is called trait utility and explains phenotype-environment correlations and connects trait values to a measure of fitness (Arnold, 1983, Schluter, 2000). Finally, rapid speciation is expected. This concept is not very well defined, but expects rapid bursts of speciation that can be identified either by detecting episodes of high branching rates in phylogenies, or periods of speciation significantly exceeding extinction (Schluter, 2000).

Adaptive radiations have gathered a lot of attention as they are considered of foremost importance and are potentially responsible for the generation of much biodiversity (Simpson, 1953, Schluter, 2000). It is well documented for a few classical examples like Darwin finches on the Galapagos islands, *Anolis* lizards in the Caribbean, Hawaiian silverswords, and cichlid fish (Grant, 1986, Losos, 1990, Schluter, 2000, Seehausen, 2006a), and has been suggested in many additional taxa (Schluter, 1996, Taylor, 1999). It has even been demonstrated to occur under

experimental conditions in microbial microcosms (MacLean, 2005). Besides empirical evidence, some theoretical studies trying to understand the different mechanisms and predict circumstances under which adaptive radiation occur has accumulated in recent years (reviewed in Gavrilets & Losos, 2009).

Progress towards completion of ecological speciation
Despite all the advances in understanding mechanisms of speciation, little is known about why ecological speciation goes to completion in some cases and why it fails in others. It is evident that levels of phenotypic divergence and reproductive isolation among species vary on a continuous scale (Endler, 1977, Funk *et al*., 2002, Nosil *et al*., 2009, Seehausen, 2009) and that cases in which speciation is considered incomplete are common. Some examples include *Tinema* walking-stick insects, *Pundamilia* cichlids, whitefish, three-spined stickleback, and Trinidan guppys (Rueffler *et al*., 2006, Crispo *et al*., 2006, Nosil, 2007, Seehausen *et al*., 2008). Several non-ecological factors have been suggested to explain variation in the stage of speciation, including the genetic architecture of traits under selection and mate choice traits, time since start of divergence, and levels of gene flow (Kirkpatrick & Ravigne, 2002, Coyne & Orr, 2004, Gavrilets, 2004, Forister *et al*., 2007, Rasanen & Hendry, 2008). Besides these non-ecological factors, two possible ecological explanations for variation in the completeness of speciation have been suggested (Nosil *et al*., 2009): (1) Variation in the strength of divergent selection acting on a given trait ("stronger selection" hypothesis); and (2) variation in the number of traits that selection acts on ("multifarious selection" hypothesis). The first implies that speciation is more likely to go to completion if selection is stronger on an individual trait, i.e. would be the result of a deeper fitness valley between two adaptive peaks in a single dimension of the adaptive landscape. The second mechanism implies that speciation is more likely to go to completion if selection acts on a larger number of traits associated with multiple divergent niche dimensions. It is based on the principle that different dimensions of divergent adaptation have additive effects on reproductive isolation (Nosil *et al*., 2009). It would therefore be the result of fitness peaks that are separated in multiple adaptive landscapes (Nosil & Harmon, 2009). These two hypotheses however do not mutually exclude each other and, in fact, it is likely that both mechanisms operate simultaneously.

Extinction and hybridisation
The opposing process to speciation is extinction. It is often considered negative because extinctions observed today are very often linked to human induced loss of environmental heterogeneity (Buckley, 1982, Tilman, 1994, Rosenzweig, 1995). Environmental change is however not necessarily human induced and extinctions are a natural part of evolution. Analyses of temporal ranges of fossil data have shown that extinction rates are not constant through time. They rather fluctuate widely with extreme peaks known as mass extinctions (Jablonski, 1991). Five major extinctions have occurred in the past (Myers, 1990), in which generally over 50% of the genera and even more of the species present on earth have disappeared (Jablonski, 1991). The recovery from those extinctions varies, but very often once dominant genera become rare while other diversify very quickly to fill vacant niches (Erwin, 1998). Such faunal replacement have for example been observed for mass extinctions which have apparently mediated faunal replacements, as it was the case in the succession from mammal-like reptiles to dinosaurs after the Permian mass extinction and in the

succession from dinosaurs to mammals at the K-T boundary after the Cretaceous mass extinction (Benton, 1987). Some attributes of ancient mass extinctions tend to be similar (Myers, 1990), like for example that species owing the potential to quickly adapt to changing environments seem to preferentially survive (Jablonski, 1991). This is likely true for the mass extinction occurring today, with the major difference that we currently observe the fastest ever global mass extinction (Myers, 1990, Daily, 1995, Singh, 2002). It does not only differ by its rates of extinction, it is also the first that has been directly induced by a living organism changing the environment. To preserve biodiversity for future generations, the evolutionary, ecological and genetic mechanisms that lead to extinction have to be understood (Seehausen, 1999).

Species can go extinct through two fundamentally different processes. The first and most frequently acknowledged mechanism consists of a sequential extirpation of populations until the entire species is extinct. Alternatively, a loss of genetic distinctiveness through introgressive hybridization can lead to species losses (Rhymer & Simberloff, 1996, Seehausen *et al*., 2007). A special case of the latter is speciation reversal (Seehausen, 2006b), whereby changes in the form or strength of divergent or disruptive selection increase introgressive hybridization among historically sympatric or parapatric species; thus eroding the ecological and genetic differences that distinguish closely related but ecologically divergent species (Seehausen *et al*., 1997, Taylor *et al*., 2006). It occurs when intrinsic hybrid sterility is incomplete which has been shown to be the case in a large fraction of species (Seehausen *et al*., 2007)

A considerable fraction of the biodiversity observed today is the outcome of divergent adaptation to heterogeneous environments (Rundle & Nosil, 2005). The importance of habitat heterogeneity in maintaining species richness is well rooted in community ecology (Tilman, 1994, Rosenzweig, 1995) and island biogeography (MacArthur & Wilson, 1967, Buckley, 1982) and is empirically supported (Rosenzweig, 1995). It is therefore the cornerstone of conservation management (Mora *et al*., 2006) and has been widely recognized. The possibility that introgressive hybridization and speciation reversal drive diversity loss did however gather little attention until recently (Seehausen *et al*., 2007). It is most often considered as a cause of extinction due to species translocation or by the removal of dispersal barriers (Rhymer & Simberloff, 1996, Allendorf *et al*., 2001). The potential of speciation reversal as a consequence of changes in environmental heterogeneity has however rarely been recognized as a major cause of biodiversity erosion by conservation managers. Empirical evidence in the three-spined stickleback species pairs (Taylor *et al*., 2006), African cichlid fish (Seehausen *et al*., 1997, Seehausen *et al*., 2008), North American ciscos (Smith, 1964, Todd & Stedman, 1989), and the North American grey wolf (Lehman *et al*., 1991), however, support the theoretical predictions (Seehausen *et al*., 2007). The large prevalence of young species thereby suggests that speciation reversal by hybridization might be more common than previously thought and that this mechanism should be acknowledged and incorporated in conservation management.

Finally, it is important to note that while speciation reversal by introgressive hybridisation erodes diversity, it does not necessarily represent an evolutionary dead-end. Several studies have theoretically and empirically demonstrated that hybridisation could also act as a catalyst of speciation. It could for example increase the additive genetic variation of a population or accelerate the acquisition of specific adaptive traits that confer to a population the ability to respond faster to selection pressures from the environment (Arnold, 2006). Yet it could also lead to evolutionary novelty by transgressive segregation (Rieseberg *et al*., 1999). However, even though these mechanisms might be very important in the long term evolution of diversity, like in the

propensity to adaptively radiate (Seehausen, 2004), a loss of environmental heterogeneity generally leads to a loss of diversity.

Central European Whitefish as a model system for adaptive radiation research
Several fish taxa from northern temperate aquatic ecosystems have in recent years become model systems for rapid postglacial (Holocene) speciation and adaptive radiation research (Schluter, 1996). Along with others, such as the three-spined stickleback, Pacific salmon, brown trout or Arctic charr, Coregonids also called whitefish (Family of *Salmonidae*), have received considerable attention from evolutionary biologists (Schluter, 1996, Taylor, 1999, Robinson & Schluter, 2000, Bernatchez, 2004, Hudson *et al.*, 2007). They are restricted to the Northern Hemisphere, but are found in North America, Europe and Asia. The subfamily of coregonine fishes (*Coregoninae)* is phenotypically and ecologically the most diverse freshwater fish group in Northern Hemisphere and consists of three genera: the piscivorous *Prosopium*, the predominantly benthic feeding *Stenodus*, and the ecologically highly-variable *Coregonus* (Hudson *et al.,* 2007). The whole taxonomy, even in the best studied systems, is often unclear and controversial. Additional investigations are needed to eventually solve this problem. Many have already attempted this task, diverging considerably in their appraisal of the diversity and the taxonomic classification of species (Fatio, 1890, Wagler, 1937, Steinmann, 1950, Himberg, 1970, Reshetnikov, 1980, Reshetnikov, 1988, Lindsey, 1988, Svärdson, 1998, Kottelat & Freyhof, 2007). Some of these difficulties may stem from the inappropriateness of applying traditional taxonomic models to incipient species. Indeed, whitefish occur in environments that have originated 15'000 years ago after the glaciers retreated. This created novel environments that were invaded by only a small number of species, of which some, like whitefish, have started to speciate. While being a nightmare for taxonomists, this situation offers evolutionary biologists the opportunity to study recent and ongoing processes of ecological speciation and adaptive radiation (Bernatchez, 2004).

Adaptive radiation in coregonids
Relatively little is known about diversity and underlying speciation modes for many of the different coregonine species or genera (Kottelat & Freyhof, 2007, Hudson *et al.*, 2007), but considerable progress has been made especially in the North American and central European lake whitefish species complexes (*C. clupeaformis* and *C. lavaretus*) (Bernatchez, 2004). North American lake whitefish are frequently represented by two ecotypes that probably evolved multiple times in parallel (Landry *et al.*, 2007), a "normal" and a "dwarf" form (Bernatchez, 2004). However, most lakes across their geographical distribution harbour one species, similar to the "normal" form. Higher sympatric diversity has only been found in Spider Lake, with three species historically present, but on of them, the "dwarf" form, disappeared following smelt introduction (Fenderson, 1964, Hudson *et al.*, 2007). On the other hand, European lakes can harbour more species with up to five in central Europe and perhaps up to nine in some Russian lakes (Steinmann, 1950, Douglas & Brunner, 2002, Kottelat & Freyhof, 2007).

In a detailed review, Bernatchez (2004) has summarized the available evidence for adaptive radiation in whitefish. The evidence has however mounted since his work has been published. I will therefore try to complete the list of evidence that has accumulated.

Common ancestry, the first criterion of adaptive radiation, was first analysed from large phylogenies based on RFLP data (Bernatchez *et al.*, 1991). While this analysis showed that the different coregonine genera share a common ancestor, it did not include most of the adaptive radiation which occurred much more recently within the different genera or even subgenera (Hudson *et al.*, 2007). A detailed analysis of the literature and comprehensive phylogenetic analysis of central European whitefish showed a likely monophyly, but observed a significant discordance between phylogenies based on nuclear and mitochondrial markers (Douglas *et al.*, 1999, Hudson *et al.*, 2007, Hudson *et al.*, submitted). Thus it is likely that the central European lakes were invaded by a whitefish hybrid swarm that resulted from hybridization of two formerly isolated refugial clades and that speciation occurred within each lake, or lake system independently (Hudson *et al.*, submitted). Whether the hybrid origin has increased the propensity of whitefish to radiate by increasing functional genetic diversity (Seehausen, 2004) as suggested (Svärdson, 1998) remains to be tested.

Bernatchez (2004) then investigated whether rapid speciation in the deep section of the phylogeny has occurred using a method comparing branching rates of the whitefish phylogeny with a simulated tree to test for the null hypothesis of no bursts in speciation (Wollenberg *et al.*, 1996, Bernatchez, 2004). He found no evidence for distinct episodes or rapid speciation, but found that speciation rates generally exceeded extinction rates within the *Coregonus-Stenodus* clade (Bernatchez, 2004). He however did not include the most recent and diverse radiations, like the central European whitefishes where more than 41 species were recognized in 17 lakes (Steinmann, 1950), which emerged after the last glacial maximum about 15'000 years ago. Thus at least some radiations in whitefish have indeed been rapid.

Phenotype environment correlations in sympatric whitefish species are mostly based on associations that have been observed between the number of gill rakers and stomach contents. Gill rakers are small cartilaginous projections located on the inner ridges of the four gill arches. Functional design considerations predict that densely rakered fish feed more efficiently on zooplankton whereas sparsely rakered fish are more efficient on benthic prey (O'Brien, 1987, MacNeill & Brandt, 1990, Link & Hoff, 1998). While this remains to be experimentally confirmed, it allows searching for these associations between phenotype and diet. The best evidence so far stems from a pond experiment where two northern European whitefish species and artificial hybrids have been raised under standardized conditions (Svärdson, 1979). Stomach analyses have shown that the sparsely rakered species predominantly fed on epibenthic prey while the densely rakered species preferred zooplankton. Interestingly, hybrids were intermediate in their food choice (Svärdson, 1979). Similar patterns have also been observed in field studies both in northern European whitefish (*C. lavaretus spp*) (Svärdson, 1998, Kahilainen *et al.*, 2004, Ostbye *et al.*, 2005, Kahilainen & Ostbye, 2006) and in North American lake whitefish (*C. clupeaformis .spp*) (Bodaly, 1979, Bernatchez *et al.*, 1999, Bernatchez, 2004). Some anecdotal evidence seem to suggest that this also holds for central European whitefish (Steinmann, 1950, Rufli, 1978, Michel, 1996, Mookerji *et al.*, 1998), but quantitative and comparative analyses across lakes are lacking. So far, most studies focused on this phenotypic trait, but some studies indicate that additional dimensions of ecological divergence might be implicated in whitefish speciation. For example, spawning habitat choice by depth seems to be related to body size (Steinmann, 1950) in central European lakes. It also seems that feeding habitat choice is related to morphology (Amundsen, 1988, Rogers *et al.*, 2002) or physiology, which has been observed in the closely related *C. albula* complex (Helland *et al.*, 2007, Ohlberger *et al.*, 2008). Trait utility has so far not been

demonstrated for whitefish. It has been suggested to be likely (Bernatchez, 2004), but experimental testing is still needed.

Evidence for divergent natural selection
The next step consists of providing evidence for the role of divergent natural selection. The notion that divergent natural selection plays a role in whitefish speciation is not new - it has already been suggested more than 50 years ago (Steinmann, 1950) and different mechanisms have been suggested more than 20 years ago (Smith & Todd, 1984). They suggested three selection mechanisms to be important: (i) because lakes are characterized by steep ecological gradients that may submit whitefish to divergent selection pressures along these gradients, also known as clinal speciation; (ii) that inter-specific competition for food might lead to divergence of populations; and (iii) that the use of distinct resources might trigger temporal and spatial spawning isolation. Some evidence for the third hypothesis emerged from indirect tests for selection inferred from Q_{ST}-F_{ST} comparisons (Bernatchez, 2004, Ostbye *et al.*, 2005). However, such analyses and resulting conclusions have been heavily questioned (Pujol *et al.*, 2008). Evidence for interspecific resource competition resulted from the acquisition of observational evidence for ecological character displacement (Bernatchez, 2004). Theory predicts that phenotypic differences between evolutionary lineages are greater when these occur in sympatry than in allopatry (Schluter, 2001). In whitefish, the best evidence stems from observations of shifts in gill raker number of North American lake whitefish found in sympatry (Lindsey, 1981). Whitefish fulfil four of the six criteria needed to satisfy the theory of character displacement (Robinson & Wilson, 1994, Bernatchez, 2004): (1) phenotype differentiation in gill rakers has a genetic basis; (2) chance as an explanation for this pattern can be ruled out; (3) observed differences represent evolutionary shifts and not lineage sorting; (4) The shifts in resource use match the phenotypic changes observed. Some additional indirect evidence stems from a recent study of North American whitefish showing that genetic differentiation, and thus reproductive isolation, is strongest in lakes where habitat availability is reduced due to oxygen depletion that leads to an increase in competition (Landry *et al.*, 2007). Additional investigations are therefore clearly necessary, but these observations strongly suggest that competitive interactions related to feeding ecology might be implicated in whitefish speciation. Finally, reproductive isolation has to develop as a consequence of divergent selection. Evidence is still scarce and comes mainly from comparison between estimations of gene flow based on neutral genetic loci as a surrogate of reproductive isolation and phenotypic differentiation (Bernatchez, 2004). A negative correlation between gene flow and divergence in phenotypic traits that are subject to divergent selection is expected and has indeed been observed in gill rakers for North American lake whitefish species pairs (Lu & Bernatchez, 1999).

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Chapter 2

SUMMARY OF CHAPTERS

Chapters 3 and 4: Speciation along environmental gradients in central European whitefish.
Despite recent theoretical advances, the relative importance of spatial and ecological factors as well as their interaction in adaptive radiation is not well understood. One of the reasons might be that few studies have sampled ecological species pairs or multi-species complexes in a taxonomically, ecologically, and spatially unbiased fashion at the appropriate scale to directly compare these factors.

In the first two chapters I thus investigate the effects of divergence in depth, time and geographical distance between spawning sites on the distribution of microsatellite allele frequencies and ecologically relevant traits in whitefish species flocks of lakes Neuchâtel and Lucerne, Switzerland, central Europe. The whitefish of these lakes are part of a larger postglacial adaptive radiation that occurred in parallel in multiple lakes after the glaciers retreated about 15'000 years ago. In Lake Neuchâtel, two species have previously been described: a large sized, shallow and winter spawning species (*C. palea*); and a small sized, deep and winter spawning species (*C. candidus*). Lake Lucerne was thought to harbor three distinct species: a large sized, shallow and winter spawning species (*C. sp. "Bodenbalchen"*); a small sized, deep and winter spawning species (*C. zugensis*); and an intermediately sized, summer and deep spawning species (*C. nobilis*). In both lakes, however, fishermen and old literature suggest that an intermediately sized species spawning in winter at intermediate depths exists. To investigate how the whitefish diversity is structured along ecological gradients, and to determine the mechanisms that might be implicated in generating these species gradients, we conducted taxonomically unbiased quantitative sampling along the spawning depth in both lakes.

In Lake Neuchâtel, genetic variation in multiple microsatellite loci and several phenotypic traits are structured continuously without apparent phenotypic and genetic discontinuities along the spawning depth gradient. Both the genetic and phenotypic diversity were structured exclusively by water depth at spawning site, but not by spawning time nor by geographical distance, or any two-way interactions between variables. These results suggest a dominant role for isolation-by-adaptation along the water depth gradient in whitefish speciation. In agreement with theory suggesting that populations can differentiate along ecological gradients in narrow parapatry despite gene flow (Endler, 1973, Doebeli & Dieckmann, 2003, Leimar *et al.*, 2008), adaptive differentiation along the water depth gradient is likely maintained by divergent selection, as indicated by very large P_{ST} values obtained for gill raker counts and other morphometric variables related to feeding ecology. That the phenotypic cline has a parallel in a genotypic cline suggests that gene flow restriction in Lake Neuchâtel whitefish is a by-product of divergent selection between different spawning depths.

Similarly in Lake Lucerne, the quantitative sampling reveals that winter spawning whitefish are phenotypically and genetically structured in a cline-like pattern along the water depth gradient on their spawning grounds. However, body size was not normally distributed within depths. A clear tri-modal distribution of body size within age-class was observed that cannot be explained by gill net selectivity, suggesting demographic admixture of differently sized species within depths. The three distinct size clusters were confirmed by population genetic analyses where the intermediate size class was composed of genetically intermediate fish. Furthermore, the genetic variation among all individuals but also within two of the three size classes was strongly correlated with body size, suggesting isolation-by-adaptation in body size (Nosil *et al.*, 2008). The occurrence of a strong relationship between neutral allele

frequencies and size, together with admixture of different size classes within an age class on the spawning site further suggest that assortative mating based either on size, or on a trait correlated with size, might be implicated in the process of speciation. At least two different scenarios could have lead to the pattern observed in Lake Lucerne: clinal speciation along an environmental gradient (Endler, 1973, Doebeli & Dieckmann, 2003, Gavrilets, 2004) or hybrid speciation (Arnold, 2006, Mallet, 2007).

The Neuchâtel and Lucerne systems therefore show some similarities. In both lakes, whitefish diversity is distributed along and structured a by spawning depth gradient. Some of the mechanisms of reproductive isolation might therefore be quite similar, like isolation by depth and, potentially, size-based assortative mating. The genetic and phenotypic differentiation along these gradients, however, differs markedly between the lakes. While Lake Neuchâtel whitefish are structured continuously without apparent phenotypic and genetic discontinuities, discrete clusters can be identified phenotypically and genetically in Lake Lucerne. These different stages in the progress towards speciation observed in the two lakes could have two reasons: (1) either the environmental conditions and thus the adaptive landscapes differ among the two lakes, giving Lake Lucerne whitefish a greater opportunity for speciation (speciation scenario), or (2) the recent eutrophication, that was more pronounced in Lake Neuchâtel, could have lead to an increase in gene flow between two formerly isolated species (speciation reversal scenario; see chapter 6).

Chapter 5: Can variation in the multifariousness of ecological divergence explain the progress towards speciation in whitefish radiations?

The results obtained in chapters 3 and 4 show that different degrees of reproductive isolation and phenotypic divergence can be observed in central European whitefish. This has also been shown in other Alpine lakes, like Lakes Biel, Thun or Brienz (Bittner, 2009). Two possible ecological explanations for variation in the completeness of speciation have been suggested (Nosil *et al.*, 2009): variation in the strength of divergent selection acting on a given trait (the "stronger selection" hypothesis) and variation in the number of traits that selection acts on (the "multifarious selection" hypothesis). In this chapter I study the different stages of speciation in four Alpine lakes (Lake Lucerne, Lake Neuchâtel, Lake Thun and Lake Walen) and test whether variation in the number of divergent ecological traits explains the variation in stages of speciation. The following four traits were included: (1) Spawning time, which is directly related to the timing of fry emergence and might thus influence survival (Cushing, 1990, Sogard, 1997, Svensson & Sinervo, 2000, Woods *et al.*, 2009); (2) Spawning depth, which is related to different physiological or behavioural adaptations to spawn in different water depths (Weaver *et al.*, 1996, Rogers *et al.*, 2002, Woods *et al.*, 2009); (3) Gill raker numbers, which are likely related to feeding ecology (Svärdson, 1979, Bernatchez, 2004, Kahilainen & Ostbye, 2006); (4) Growth rate, which could reflect physiological adaptations to different feeding and predation regimes (Rogers *et al.*, 2002, Helland *et al.*, 2007, Ohlberger *et al.*, 2008).

The analyses were performed using microsatellite allele frequencies at ten loci as well as phenotypic and ecological data from 1570 whitefish sampled in the four lakes. The results first confirm the monophyletic status of whitefish in each lake, supporting a sympatric origin of whitefish species within each lake. The population genetic analyses further revealed highly variable degrees of genetic differentiation between species, confirming the occurrence of different stages of speciation within lakes, but also in different lakes. Using partial Mantel correlations between genetic and ecological distances among individuals, and thus removing confounding effects of

non-independence of the different traits, we show that up to three different ecological traits significantly explain genetic differentiation within lakes. I find that while the same traits are important in most lakes, the relative importance of the different traits differs among lakes, implying that whitefish populations in different lakes either experienced different adaptive landscapes, or evolved differently in response to similar adaptive landscapes. Furthermore, we find a positive relationship between the Levene's Index (a measure of variance that has been suggested for evaluating the multifariousness of selection) and the stage of speciation. This suggests that divergent selection acts on multiple ecological traits. The analysis does however not allow distinguishing between multifarious selection and stronger selection in a given speciation event and it does also not allow to distinguish between either of the above and selection acting on different traits in different speciation events. Altogether, the strong relationships between ecological traits and genetic differentiation suggest that any change in the environment that relaxes divergent selection along either one of the important ecological dimensions will weaken reproductive isolation and genetic differentiation among species.

Chapter 6: Anthropogenic eutrophication drives large scale extinction by speciation reversal in adaptive radiations

Because gene flow in young radiations is typically not yet completely inhibited by intrinsic post-zygotic isolation (Seehausen *et al*., 1997, Seehausen *et al*., 2008), environmental changes reducing the available ecological or reproductive niches of different species will likely relax the selective forces that drive ecological speciation and/or maintain ecological and genetic differentiation. As shown in the previous chapters, sympatric whitefish species are divergent in at least four ecological traits (spawning time, spawning depth, body size and gill raker counts). This suggests that changes in the environment that relax divergent selection in any of these traits could lead to an increase in gene flow among species. During the latter half of the last century, anthropogenic phosphate enrichment increased primary production that lead to increased microbial decomposition rates and thus greater oxygen demand at the water-sediment interface. This lead to the loss of deep waters as spawning habitats and likely influenced the reproductive success of whitefish (Müller, 1992). The degree of eutrophication however varied among lakes.

In this chapter I explore how whitefish diversity has been affected by this eutrophication phase. I used species richness and the within-lake range in gill raker number as a measure of functional diversity to compare historical and present day diversity. Additionally, molecular population genetic data based on 10 microsatellite loci was used to estimate the number of genetically differentiated species and the extent of genetic differentiation among them in seven lakes. The results show that historical diversity was strongly related to maximum lake depth, confirming that environmental heterogeneity in water depth plays an important role in whitefish speciation. Interestingly, the best explanatory variable for contemporary diversity was the minimum oxygen concentration at the lake bottom measured during the last ten years. Oxygen rich lakes today harbour a greater diversity than oxygen poor lakes. Furthermore, the degree of genetic differentiation among whitefish species from seven lakes was negatively related to the maximum extent of organic pollution observed during the most eutrophic phase. To test whether reproductive success is related to the extent of pollution, we analysed egg survival in 12 lakes. The results show that the survival is very strongly negatively correlated to the extent of pollution. The comparison of historical and contemporary diversity shows that about 33% of the

initial 41 species and 16.7% of the functional diversity has been lost during the last century. Furthermore, the genetic differentiation among the remaining species is 59% lower in more impacted lakes, suggesting that gene flow among species has increased with increasing pollution. Taking together loss in species richness and the weakening of genetic differentiation among the remaining species, at least 55% of the whitefish diversity in the alpine radiations has been lost in less than 100 years.

All this strongly suggests that eutrophication drives the erosion of species distinctiveness and associated functional phenotypic diversity by increasing gene flow among species that previously partitioned a wider range of reproductive niches, consistent with the speciation reversal scenario. These alterations of the conditions which drive and maintain species richness in whitefish adaptive radiations have resulted in a rapid and massive loss of whitefish diversity. Together with evidence from similar study systems, the case of central European whitefish suggests that species richness in lake fish radiations might be rapidly lost globally due to anthropogenic activities that disrupt the evolutionary processes that drive adaptive radiation.

SYNTHESIS

The understanding of ecological speciation by natural selection has gone a long way since Darwin suggested 150 years ago that natural selection is important in speciation (Darwin, 1859). It has in recent years not only been demonstrated that it occurs, but also that ecological speciation might be more common than previously thought (Rundle & Nosil, 2005, Schluter, 2009, Gavrilets & Losos, 2009). It has however also become evident that the underlying processes are very complex, that several different mechanisms (see Introduction) can lead to ecological speciation and that little is so far known about their relative importance (Rundle & Nosil, 2005, Gavrilets & Losos, 2009). The number of model systems is rapidly increasing since the interest in ecological speciation has been renewed. This is very much needed as many case studies across different taxonomic groups are necessary to draw general conclusions about the different mechanisms implicated in ecological speciation (Gavrilets & Losos, 2009).

Whitefish as a model organism for ecological speciation along environmental gradients

Despite considerable advances in studying whitefish adaptive radiation (Bernatchez, 2004), very little is actually known about how the diversity is related to environmental variables, about what mechanisms drive the emergence of reproductive isolation, and even about the degree of reproductive isolation observed among sympatric species. The central European system is particularly interesting as it harbours multiple lakes in very close geographic proximity with whitefish diversity varying between one and five species in each lake (Chapter 6). After the glaciers retreated about 15'0000 years ago, these lakes were colonised by a whitefish hybrid swarm (Hudson *et al.*, submitted) from which adaptive radiations emerged within each lake (Douglas *et al.*, 1999, Hudson *et al.*, submitted, Chapter 5). In this thesis I use quantitative sampling of whitefish along environmental gradients, population genetic and morphometric tools to study how whitefish diversity is arranged in the environment. I investigate mechanisms of reproductive isolation and how the environment influences genetic differentiation and species diversity.

In the first two chapters, I explore the effects of divergence in depth, time and geographical distance between spawning sites on gene flow and genetic differentiation of whitefish in lakes Neuchâtel and Lucerne, Switzerland, central Europe. I discovered parallel clines of allele frequencies in multiple microsatellite loci and in several phenotypic traits along the spawning depth gradient in Lake Neuchâtel, likely maintained by divergent selection (Chapter 3). Theoretical studies have demonstrated that the establishment of stable clines along spatial environmental gradients is a potential outcome of local adaptation when inter-specific competition among different demes along this depth gradient is absent and mating is random (Doebeli & Dieckmann, 2003, Leimar *et al.*, 2008). Similarly in Lake Lucerne, winter spawning whitefish are phenotypically and genetically structured in a cline-like fashion along the spawning water depth gradient. However the gradient here consists of three distinct phenotypic and genetic clusters that are distributed along the depth gradient and which are to some extent demographically admixed within depths (Chapter 4). The same models as above predict such an outcome when inter-specific competition leads to negative frequency dependent selection and/or when mate choice is non-random with regard to phenotype (Doebeli & Dieckmann, 2003, Leimar *et al.*, 2008). The overlapping spawning depth ranges in the three Lake Lucerne whitefish species

together with population genetic data indeed suggest that mate-choice by body size, or a trait correlated to body size, might be implicated. Whitefish thus represent an excellent model system to test theoretical predictions of clinal speciation theory.

The above models assume that population divergence is driven by divergent adaptation and competition for resources (Doebeli & Dieckmann, 2003, Leimar *et al.*, 2008). However, the clines that we observed occur along spawning depth gradients. While this could be the result of the evolution of reduced dispersal (Heinz *et al.*, 2009) and thus foster reproductive isolation, it does not explain how suites of phenotypic traits, associated with specific feeding ecologies (Bernatchez, 2004, Kahilainen & Ostbye, 2006), are related to adaptation or preferences for particular spawning depths. Two general patterns have emerged, first large whitefish tend to spawn at shallower depths than small ones, and spawning during lake stratification is confined to depths below the thermo-cline where the water is cool enough to allow for successful egg development. Variation in spawning depth might thus represent physiological or behavioural adaptations to different water depths. In North American lakes, the dwarf whitefish ecotype has been found to use shallower feeding niches than the large ecotype. In central European lakes, small type whitefish either use deeper epibenthic habitats or performe dial migrations in the pelagic (Eckmann, 1991, Kahilainen *et al.*, 2004, Knudsen & Gjelland, 2004). Physiology does therefore not seem to consistently cause the same constraint to foraging and spawning at different depths. It has also been shown that predators (brown trout, burbot, arctic charr, pike) fed preferentially on small sized whitefish (Kahilainen & Lehtonen, 2003). The use of different spawning depths and dial migrations could therefore also reflect behavioural adaptation to predator avoidance.

Whitefish are not only reproductively isolated by depth, but also by time and possibly mate choice (Chapters 4+5). The most obvious consequence of variation in spawning times is variation in the timing of fry emergence. It might thus be the consequence of inter-specific food competition in larval stages where zooplankton nauplia are the predominant food item. This would imply that peaks of high zooplankton nauplia concentration in the lakes should match hatching times of larvae of the different species. Evidence for this is still lacking. On the other hand, predation and over-wintering mortality could also play a role (Cushing, 1990, Sogard, 1997, Svensson & Sinervo, 2000, Woods *et al.*, 2009).

To get more insight into the processes of clinal speciation in whitefish, reciprocal transplant experiments are needed to test whether clinal divergence is associated with different feeding efficiencies on different food items. Then the ecological divergence needs to be linked to the mechanisms of reproductive isolation. This involves a better understanding of the physiological and behavioural mechanisms that may constrain it. Furthermore, mate choice experiments are needed to determine whether mate choice plays a role in speciation. If it does, it has to be determined whether divergent selection directly acts on the mate recognition cues (by-product speciation) or whether adaptive speciation might be implicated (Schluter, 2000, Rundle & Nosil, 2005). The results obtained in chapter 3 seem to suggest that some kind of assortative mating by size or a trait correlated with size might be implicated. Lake Thun, however, harbours two species of similar size which spawn at similar depth and at similar time, suggesting that some additional mechanism other than size-assortative mating is implicated in reproductive isolation. It has been suggested that breeding tubercules in whitefish could be a trait signalling for good genes, but experimental work did not reveal any conclusive evidence for this hypothesis (Wedekind *et al.*, 2008). Finally, the major difficulty in establishing whitefish as model system in speciation research likely lies in the difficulty associated with doing good experimental

studies. Whitefish have a long generation time (3 years) and fish grow to large sizes. Experimental work therefore requires substantial time and space.

Stages in the process of speciation

It is common to observe cases in nature where divergent selection generates only incomplete reproductive isolation between populations, resulting in weak or no differentiation at neutral genetic loci (Rueffler *et al.*, 2006, Nosil, 2007, Seehausen *et al.*, 2008). It, however, often remains unclear why speciation goes to completion in some cases, and why it does not in most others (Nosil *et al.*, 2009). Several different stages of speciation have been observed and studied in a few species complexes like walking stick insects, East African cichlids and three spined sticklebacks (Hendry *et al.*, 2002, Seehausen *et al.*, 2008, Nosil & Sandoval, 2008, Nosil *et al.*, 2009). The results of chapters 3-5 show that different degrees of reproductive isolation and thus different stages of speciation into phenotypically very similar pairs of species also occur in central European whitefish. Taxa with replicated adaptive radiations with parallel evolution and similar demographic histories, as in the case of whitefish, represent powerful model systems for exploring the causes of variation in the progress towards speciation (Seehausen, 2009).

Besides several non-ecological explanations like the genetic architecture of traits under selection, mate choice traits, and time since divergence or gene flow, two potential ecological explanations for variation in the completeness of speciation have been suggested (Nosil *et al.*, 2009): (1) Variation in the strength of divergent selection acting on a given trait ("stronger selection" hypothesis), i.e. that speciation is more likely to go to completion if selection is stronger on an individual trait; (2) variation in the number of traits that selection acts on ("multifarious selection" hypothesis), i.e. that speciation is more likely to go to completion if selection acts on a larger number of traits. Very few tests of these alternatives exist (Nosil *et al.*, 2009). The strongest support in favour of multifarious selection probably comes from experimental work on *Drosophila* where it has been demonstrated that divergent selection on multiple traits lead to reproductive isolation whereas only incomplete reproductive isolation resulted from divergent selection on a single trait (Rice & Hostert, 1993). There are almost no tests from natural systems, mainly because the generation of the selection estimates are difficult (Nosil *et al.*, 2009).

In chapter 5 I investigate four ecological traits that are likely important in whitefish speciation: spawning depth, spawning time, body size at a given age, and gill raker numbers. While controlling for confounding effects of non-independence among the different traits, I show that divergence in multiple traits best explains genetic differentiation among individuals. I also demonstrate that selection on multiple ecological traits is implicated in speciation. However, it was not possible to discriminate between multifarious and stronger selection. Furthermore, trait divergence as a surrogate for divergent selection has some limitations. First it represents only indirect evidence for multifarious selection (Nosil *et al.*, 2009). Second, it assumes that the classes of taxon pairs being compared do not differ strongly in the genetic variance-covariance (G) matrix (Nosil *et al.*, 2009). Third, using phenotypic traits suffers the same caveat as Q_{ST}-F_{ST} comparisons, i.e. one cannot differ between environmental effects and additive genetic divergence (Whitlock, 2008).

My results also show that divergence among whitefish species is very tightly linked to ecological parameters of lakes. It includes adaptations to different feeding niches, to different spawning depth and times, and inter-specific competition for resources may also be important. As a consequence, any change in the environment

that relaxes selection in any of these important dimensions will weaken reproductive isolation and genetic differentiation among species.

Further investigations are thus needed to evaluate the roles of the multifariousness of selection, and the strength of selection in whitefish speciation. Towards this aim, experimental estimates of divergent selection on adaptive traits, are necessary to directly test these non-exclusive hypotheses. This task will however not be easy as selection strength is difficult to estimate accurately. Furthermore, before selection can be tested, we need to better understand why whitefish are divergent along these four axes of divergence we observed. I here suggest a few, in my opinion, interesting experimental and environmental survey approaches to address some of these questions. (1) Spawning time: It would be interesting to find out how the adaptation to different spawning times evolved. One approach, could be to use hatchling survival as a fitness measure. This depends on food availability and predation intensity. Several laboratory experiments and environmental surveys are needed to estimate such a fitness landscape: (a) survey of zooplankton species diversity, biomass, and densities in the lake water column, measured semi-continuously during a full annual cycle; (b) feeding efficiency experiments with whitefish larvae of multiple species, using nauplii of the various zooplankton species found within their lake of origin; (c) experimental determination of critical zooplankton nauplii densities that allow survival and optimal growth of the different species; (d) survey of the habitat use of whitefish larvae of different species in multiple lakes and over a whole year period; (e) determination of the prey preference and year-round habitat use of potential predators. The results from these experiments could then be used to generate a fitness landscape. (2) Gill rakers: here, testing feeding efficiency of different species on different food types under experimental conditions should be performed. This is the first step needed to test the main prediction of ecological speciation in whitefish, that resources and adaptations to different resources drive ecological speciation. (3) Spawning depth: It is difficult to develop hypotheses about why different whitefish species should spawn in different depths, and it would be difficult to experimentally manipulate this environmental dimension to simulate natural conditions in divergent depth niches: Two scenarios seem plausible: (a) it could be that different species are behaviourally or physiologically constrained to spawn in a certain depth range; (b) it could be that predation is not equally distributed in the different spawning depths. A survey of predation risks in different spawning depth, combined with an estimation of egg survival under different light, pressure and temperature conditions might give first insights into these questions. (4) Body size: this could be related to a trade off between predator avoidance against the energy costs of swimming. Experimental evaluation of the swimming energetics when searching for different food types that the different whitefish species feed on, similar to what has been carried out in North American (*C. clupeaformis*) whitefish, however taking into count the effects of intra- and inter-specific differences in growth/size., could be performed.

Linking evolution to conservation

While we are only starting to understand how species arise, we are globally losing diversity at a rate that earth has never faced before (Myers, 1990, Daily, 1995, Singh, 2002). A considerable fraction of the world's biodiversity is the result of recent ecological divergence in heterogeneous environments (Rundle & Nosil, 2005), and whitefish are a prime example of this phenomenon. The processes by which these young species can be lost and under which circumstances it occurs, however, has

gathered little attention (Seehausen *et al.*, 2007). In young species, intrinsic postzygotic isolation is generally absent (Seehausen *et al.*, 1997, Seehausen *et al.*, 2008). A loss of environmental heterogeneity and associated selection can thus trigger gene flow between previously reproductively isolated species, leading to introgressive hybridization, and potentially speciation reversal (Seehausen *et al.*, 2007). The importance of habitat heterogeneity in maintaining ecological species richness has been widely recognized (Buckley, 1982, Tilman, 1994, Rosenzweig, 1995). The incorporation of evolutionary theory in conservation and environmental planning has, however, been difficult (Ashley *et al.*, 2003), even though genetic diversity has been recognized to be an important part of conservation since the Biodiversity Treaty of Rio de Janeiro in 1992.

In chapter 6 I study how environmental heterogeneity links to whitefish species richness in different central European lakes. I further explore diversity losses that occurred during the last century as well as the underlying causal mechanisms of these diversity losses. The results indicate that historical whitefish diversity was significantly related to lake depth, where deep lakes harbored more species than shallow lakes. This is consistent with the results obtained in chapters 3-5, which show that depth is a key environmental component in structuring whitefish diversity. However, I also show that about 55% of the historical diversity has been lost in less than 100 years by speciation reversal. The main driving force was eutrophication by phosphorous enrichment that caused oxygen depletion on the sediment surface. This has lead to a loss of deep water spawning habitats in some lakes, with associated loss of reproductive isolation among species. Consistent with this scenario, the present day diversity is best explained by the minimum oxygen concentration measured close to the sediments in the recent past.

These results demonstrate that divergent selection in more heterogeneous environments is likely to explain the higher diversity observed in some central European lakes. It also shows that the loss of this environmental heterogeneity by pollution relaxed divergent selection and lead to introgressive hybridization resulting in diversity loss. The mechanisms implicated in diversification and diversity loss are thus very similar. Strikingly, this diversity loss has occurred in one of the commercially most important group of fish species in the central European freshwater fishery. In this system, different species are often not recognized as distinct management and conservation units. Indeed, even now that a fairly detailed taxonomic coverage has been published (Kottelat & Freyhof, 2007), the Swiss government has decided to not treat each species separately in a new red list for endangered freshwater fish species, and grouped them all in *Coregonus lavaretus spp.,* rendering conservation and management at the appropriate level impossible. Whitefish are not the only example of large scale diversity loss by speciation reversal. This phenomenon has also been demonstrated in east African cichlid fish and three spined sticklebacks (Seehausen *et al.*, 1997, Taylor *et al.*, 2006, Seehausen *et al.*, 2008). In these examples from other fish systems, the processes are surprisingly similar: eutrophication in the case of cichlid fish and the introduction of a crayfish species in stickleback lakes have lead to changes in the adaptive landscape perceived by stickleback and cichlid species that lead to an increase in gene flow between recently diverged fish species in both examples.

Because a large fraction of the world's biodiversity is of recent origin, diversity loss by speciation reversal might be much more common and more relevant than previously thought. The evolutionary ecology of speciation reversal should thus be fully integrated into conservation biology (Seehausen *et al.*, 2007). Furthermore the theoretical framework linking hybridization and environmental heterogeneity (Buerkle

et al., 2003, Seehausen *et al.*, 2007) should be refined. Indeed, predicting the effects of environmental change on dynamics of biodiversity is probably one of the most pressing issues in ecology, evolutionary biology and conservation (Sala *et al.*, 2000, Thomas *et al.*, 2004, Seehausen *et al.*, 2007). Additionally, the ecological value of these locally adapted species and their fitness advantages in comparison to less adapted populations, should be demonstrated to strengthen the necessity to fully integrate evolutionary processes into conservation biology.

To develop and better understand the importance of environmental heterogeneity in whitefish speciation and its reversal, historical time series of morphological and genetic data should be compared to contemporary data. Furthermore, more detailed genetic, morphometric and environmental investigations of present day diversity should be undertaken in a higher number of lakes. This is needed to revise the current taxonomy to provide managers, ecologists and evolutionary biologists with reliable species identities and diversity numbers. At the same time, these studies would strengthen our understanding of how whitefish diversity is linked to the environment. Finally, resource profiles for the different lakes should be linked to experimental assessment of feeding efficiencies on different naturally occurring food items to eventually compare predictive adaptive landscapes with the biodiversity observed. This is the ultimate goal and would be a powerful tool to predict how whitefish diversity will react to different environmental changes, but would also represent a major step forward in the understanding of the mechanisms of ecological speciation in whitefish adaptive radiations.

Final conclusions

Whitefish have many attributes that makes them a fascinating and valuable model organism for speciation research. It is however evident that much basic information about the diversity present and the mechanisms implicated in the evolution of this diversity is still lacking. A better and more detailed documentation of current diversity is very much needed, in the same way as experimental approaches, to test the very many mechanisms and predictions resulting from ecological speciation theory. This would not only add to the understanding of ecological speciation, but also help to predict how changes in the environment impact on biodiversity and thus helps to determine efficient conservation and management strategies.

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Chapter 3

Divergence along a steep ecological gradient in lake whitefish (*Coregonus* sp.)

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Running title: Divergence along a steep ecological gradient in whitefish

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ABSTRACT

To understand mechanisms structuring diversity in young adaptive radiations, quantitative and unbiased information about genetic and phenotypic diversity is much needed. Here we present the first in-depth investigation of whitefish diversity in a Swiss lake, with continuous spawning habitat sampling both in time and space. Our results show a clear cline like pattern in genetics and morphology of populations sampled along an ecological depth gradient in Lake Neuchâtel. Divergent natural selection appears to be involved in shaping this cline given that trait specific P_{ST} values are significantly higher than F_{ST} values when comparing populations caught at different depths. These differences also tend to increase with increasing differences in depth, indicating adaptive divergence along a depth gradient, which persists despite considerable gene flow between adjacent demes. It however remains unclear, whether the observed pattern is a result of currently stable selection-gene flow balance, incipient speciation, or reverse speciation due to anthropogenic habitat alteration causing two formerly divergent species to collapse into a single gene pool.

INTRODUCTION

Adaptive radiations often occur with little apparent opportunity for geographical isolation of diverging populations (Schluter, 2000). A large number of studies have addressed the genetic differences between phenotypes in adaptive radiation but they mostly focus on phenotypically defined "ecotypes", and often demonstrate that "ecotypes" are genetically differentiated despite geographical proximity (McKinnon & Rundle, 2002; Bernatchez, 2004; Kocher, 2004). Such work has been used to invoke sympatric speciation. However, in many classical radiations, such as the cichlid fish of the African Great Lakes, populations are structured both ecologically and geographically, raising the possibility that genetic divergence, even when currently maintained by ecological gradients, may have arisen during periods of geographical isolation in the past (Sturmbauer *et al.*, 2003; Koblmüller *et al.*, 2007). The prevalence of ecological speciation and adaptive radiation varies considerably among groups of organisms, and the reasons for this remain largely unknown (Kassen *et al.*, 2004; Seehausen, 2006b). Two competing hypotheses are (1) lineage-specific propensity for populations to become spatially isolated, i.e. related to stenotopy and dispersal ability; and (2) lineage-specific propensity to undergo evolutionary divergence despite gene flow, i.e. related to the genetic architecture of adaptive traits. However, few studies have sampled ecological species pairs or multi-species complexes in a taxonomically, ecologically, and spatially unbiased fashion at the appropriate scale. Despite recent theoretical advances, the relative importance of spatial and ecological factors, and their interaction in adaptive radiation, are not well understood (Dieckmann *et al.*, 2004; Gavrilets, 2004; Gavrilets & Vose, 2005). As a consequence, comparative studies of rapidly speciating and non-speciating taxa, much needed to identify the causes of variation in speciation rates between lineages, suffer from lack of data for informative comparisons.

Coregonids (whitefish and relatives), along with other salmoniformes and sticklebacks (*Gasterosteus aculeatus*), have become a model system for rapid postglacial (Holocene) speciation and adaptive radiation research in the Northern hemisphere (Douglas *et al.*, 1999; Bernatchez, 2004; Kahilainen & Ostbye, 2006; Hudson *et al.*, 2007; Landry *et al.*, 2007). They have colonized areas of formerly glaciated regions that became ice free only 15'000-20'000 years ago (Steinmann, 1950;

Taylor, 1999). Coexistence of multiple species in many lakes (Steinmann, 1950; Behnke, 1972; Reshetnikov, 1988; Pigeon *et al.*, 1997; Svärdson, 1998; Hudson *et al.*, 2007) and apparent parallel evolution (Pigeon *et al.*, 1997; Ostbye *et al.*, 2006; Landry *et al.*, 2007) suggest that many independent adaptive radiations have occurred (Bernatchez, 2004). Divergent ecological selection on feeding, involving specialization for benthic *versus* pelagic food, has frequently been proposed as a driving mechanism for these radiations (Bernatchez, 2004; Kahilainen *et al.*, 2004; Kahilainen & Ostbye, 2006; Landry *et al.*, 2007). In particular, gillraker number and density, which often differ between sympatric species, is a key morphological trait purportedly evolving under divergent selection between feeding regimes (Schluter, 1996; Bernatchez, 2004; Ostbye *et al.*, 2005). Functional design considerations predict that densely rakered fish feed more efficiently on zooplankton whereas sparsely rakered fish are more efficient on benthic prey (O'Brien, 1987; MacNeill & Brandt, 1990; Link & Hoff, 1998). It has also been demonstrated that gillraker number and density are highly heritable in whitefish (Bernatchez, 2004). While coexistence between several genetically differentiated species has been shown in many lakes, gene flow among sympatric species seems common (Todd & Stedman, 1989; Lu *et al.*, 2001). Despite the promise of the Coregonid system, little is known about the relevance of within-lake geographical, temporal and habitat isolation for these species flocks. A single study has simultaneously addressed geographical and habitat isolation within one lake, but not temporal isolation (Ostbye *et al.*, 2005). Classical studies have addressed temporal isolation, but did not utilise genetic data (Smith & Todd, 1984).

Up to six whitefish species are known to coexist in larger lakes (20-600 km²) at the northern edge of the Swiss Alps. They differ in growth rate, spawning time, spawning habitat, colour, morphology and gillraker number and density (Wagler, 1937; Steinmann, 1950; Rufli, 1978; Kottelat & Freyhof, 2007). Available genetic data generally agrees with taxonomic classifications confirming that ecotypes are typically genetically differentiated species (Douglas *et al.*, 1999; Douglas & Brunner, 2002; Douglas *et al.*, 2005). Anecdotal evidence from fishermen and old literature (Dottrens & Quartier, 1949; Bargetzi, 1960), however, suggests the existence of intermediate phenotypes found in intermediate spawning habitats which remained uncharacterized by taxonomists (Kottelat & Freyhof, 2007) and unstudied by population geneticists (Douglas *et al.*, 1999; Douglas & Brunner, 2002; Douglas *et al.*, 2005). To understand the mechanisms of speciation and maintenance of sympatric whitefish species, it is important that the entire range of available geographical space, habitat and spawning seasons within a lake is considered and that the sampling is done using taxonomically unbiased methods.

We applied a taxonomically unbiased quantitative sampling to Lake Neuchâtel where two to three sympatric species have been described in most of the literature; a deep and late spawning Bondelle (*Coregonus candidus*), and a shallow early spawning Palée (*Coregonus palea*). A third (taxonomically undescribed) species spawning at intermediate depths and at intermediate times has also been reported, although less frequently (Dottrens & Quartier, 1949; Bargetzi, 1960). The typical spawning depths and times of the two common species are supposedly well separated. We present the first genetic, morphological and ecological analysis of whitefish diversity sampled along an environmental and temporal gradient in spawning, alongside with samples from populations from several geographically distant spawning sites within the lake. We assess the relative importance of divergent selection along gradients of habitat, time and geographical distance, and evaluate the potential scenarios leading to the observed patterns in diversity.

MATERIAL AND METHODS

Sampling

The following sampling design for Lake Neuchâtel whitefish was used to collect in a taxonomically unbiased fashion covering the range of spawning depths (depth transect), the range of spawning times and the maximum possible range of geographical distances. Shallow littoral sites correspond to known spawning locations of the large Palée, while deep ones correspond to those of the small Bondelle. All fish except the river sample (electro-fishing) were caught with gill nets. Fishing along the depth transect was done on four separate days across the spawning period between the 7th of December 2005 and the 12th of January 2006. Fish were caught in shallow (2 & 10m), medium (30m) and deep (60m) areas, chosen in the closest possible geographic proximity to one another (Fig. 1a). For each sampling day, and at all depths, nets with an area of 200m^2 and composed of panels with mesh sizes of 34mm, 40mm and 45mm were set over night for approximately 15 hrs (Fig. 1b). Different mesh sizes were used to cover the entire size spectrum of spawning fish. Additional samples, geographically separate from the depth transect, were collected from a shallow Palée reference site (Shallow 1) with a mesh size of 45mm and from two deep Bondelle reference sites (95m & Deep1) with a mesh size of 34mm (Fig. 1a). All fish were mature or very close to maturation (maturation degree 5 and 6 (Smolina, 1920)) and were measured, weighed, sexed, and a standardized digital image was taken. A piece of muscle tissue was preserved in absolute ethanol for DNA analysis and scales were collected from all fish for age and growth determination.
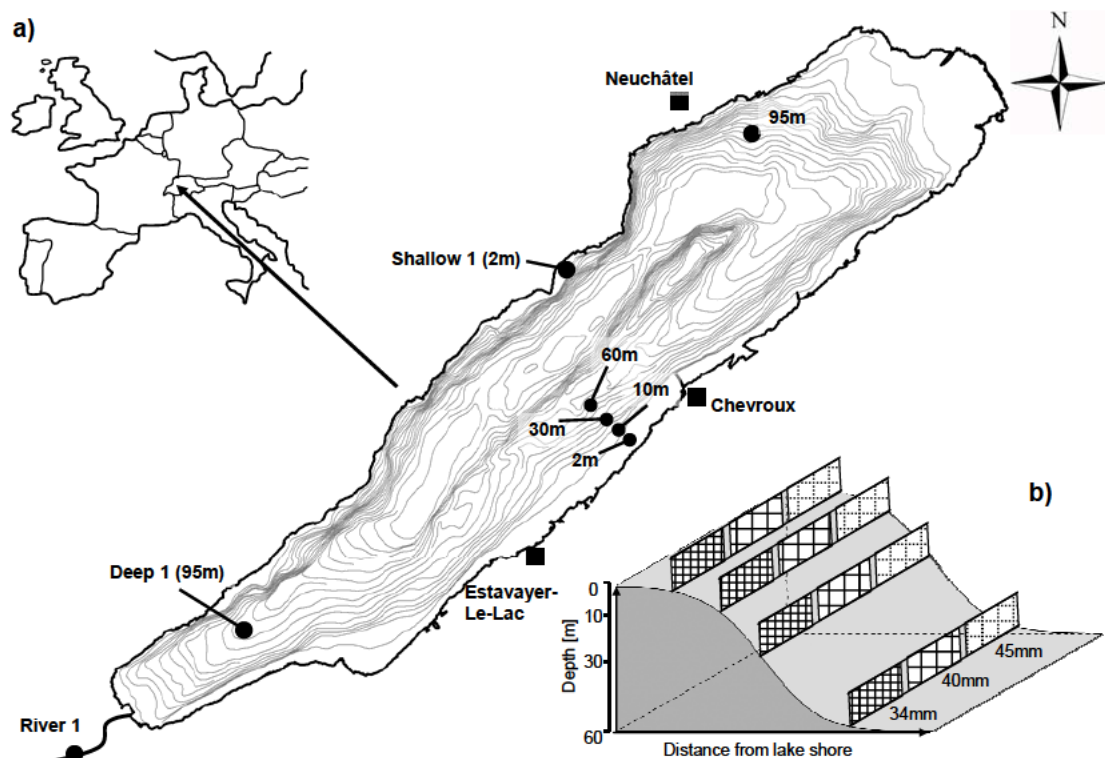

Figure 1. Map of Lake Neuchâtel, Switzerland, with sampling sites and details (a). The sampling sites 2m, 10m, 30m, and 60m are at different water depths along a straight depth transect. The relative spacing between and the three different mesh sizes (34mm, 40mm and 45mm) used at those four sites are shown in (b). Each of these four sites was sampled four times covering the entire spawning period. Three further samples were taken two at 95m deep sites, (95m and Deep 1) and once at shallow site (Shallow 1, approximate depth 2m). Three additional samples were available for genetic analyses (River 1, Shallow 2, Deep 2). The River 1 sample was taken from the Orbe river which flows into the lake. The exact locations and water depths of the Shallow 2 and Deep 2 sites are unknown.

Table 1. This table summarizes the number of whitefish sampled and data extracted from those samples. For each sample we report the population codes used (Code), the sampling date or period in case of multiple samplings (Date), the net mesh size (Mesh size) and the depth of capture (Depth). Numbers of individuals used for; microsatellite genotyping (MG), gill raker analyses (GR), geometric morphometric analyses (GM), linear morphometric analyses (TM) and growth analyses (GW). Results for; observed (H_O) and expected (H_E) heterozygosities, the deviations from HWE for each population over all loci after sequencial Bonferroni correction (HWE), the allelic richness (A_R), inbreeding coefficients (F_{IS}) with significance level, and the number of deviations from linkage equilibrium at the five and one percent significance level (N_{LD}). Three populations (Deep 2, Shallow 2 and River 1) were sampled earlier and only microsatellite genotypes collected at nine instead of 14 loci were used (results based on nine loci are labelled °). The Population coded as Simshallow 1, Simdeep 1 and Simadmix 1 represent the average results for ten simulated populations for each code, based on the parental populations sampled at the water depth extremes (2m, shallow1 and 95m, deep1). The results from these groups are labelled with * and are not included in the Total/overall summary.

Code	Date	Mesh size (mm)	Depth (m)	MG (n)	GR (n)	GM (n)	TM (n)	GW (n)	H_O	H_E	HWE	A_R	F_{IS}	N_{LD} P < 0.05	N_{LD} P < 0.01
2 m	7.12 2005–12.01.2006	34/40/45	2	24	24	23	24	24	0.59	0.61	0	5.93	0.031 n.s.	2	1
10 m	7.12 2005–12.01.2006	34/40/45	10	47	47	47	38	47	0.66	0.63	0	6.05	-0.053 n.s.	5	3
30 m	7.12 2005–12.01.2006	34/40/45	30	135	135	133	96	133	0.63	0.64	0	6.51	0.011 n.s.	8	3
60 m	7.12 2005–12.01.2006	34/40/45	60	52	46	39	39	51	0.63	0.61	0	6.21	-0.039 n.s.	11	4
95 m	19.01.2006	34	95	29	30	30	30	29	0.63	0.63	0	6.65	-0.004 n.s.	3	2
Deep 1	19.01.2006	34	95	30	30	30	30	30	0.64	0.62	0	6.27	-0.031 n.s.	6	1
Deep 2	January 2003	34	–	20	–	–	–	–	0.51*	0.55*	0*	4.44*	0.066 n.s.*	2*	1*
Shallow 1	09.12.2004	45	2	21	21	21	20	–	0.61	0.59	0	5.93	-0.026 n.s.	5	0
Shallow 2	December 2002	45	–	36	–	–	–	–	0.56*	0.54*	0*	3.92*	-0.042 n.s.*	4*	2*
River 1	December 2002	–	–	29	–	–	–	–	0.51*	0.51*	0*	4.01*	0.003 n.s.*	4*	2*
Simshallow 1	–	–	–	50	–	–	–	–	0.64†	0.62†	0	7.53†	-0.027 n.s.†	5.3†	1.1†
Simdeep 1	–	–	–	50	–	–	–	–	0.64†	0.62†	0	8.29†	-0.031 n.s.†	6.7†	1.6†
Simadmix1	–	–	–	50	–	–	–	–	0.63†	0.62†	0	8.23†	-0.009 n.s.†	5.5†	1.2†
Total/overall				423	333	323	277	314	0.60	0.59	0	5.59		50 (6.7%)	19 (2.6%)

A minimum of ten fish from each sampling site, sampling date, mesh size and sampling depth were preserved in 4% formalin for linear morphometric analyses. In 2001-2002, tissue samples but no whole fish or photos were obtained from fishermen from two additional shallow sites, in the Orbe river (River 1) and the lake (Shallow 2) and one deep water site (Deep 2). The exact sampling locations for the Shallow 2 and Deep 2 sample are not known. The sampling scheme and sample sizes per site are summarized in table 1.

DNA extraction and microsatellite amplification

Total DNA was extracted using a QIAGEN BioSprint 96 extraction robot with corresponding standard extraction method. All specimens were genotyped at 14 microsatellite markers, except River 1, Shallow 2 and Deep 2 where only nine markers were used (Patton *et al.*, 1997; Turgeon *et al.*, 1999; Rogers *et al.*, 2004). Forward primers of the following loci were labelled with fluorescent dyes: Cocl-Lav4, Cocl-Lav-49, Cocl-Lav61, and Cocl-Lav224 with Cy5; Cocl-Lav6, Cocl-Lav10, Cocl-Lav45, Cocl-Lav68, Cocl-Lav23, BWF1, and BWF2 with Cy5.5; Cocl-Lav18, SsBgIIIM.26, and Cisco-157 with Cy7. Because of overlapping allele size ranges of many markers, three different loci sets were generated for multiplexing. The first set included Cocl-Lav-49, Cocl-Lav61, Cocl-Lav6, Cocl-Lav68, Cocl-Lav10, and Cisco-157. The second set included Cocl-Lav4, Cocl-Lav224, BWF2, Cocl-Lav45 and Cocl-Lav18 (Bittner *et al.*, unpubl.). The third set included Cocl-Lav23, BWF1 and SsBgIIIM.26. Multiplex PCR amplification was performed using QIAGEN® (Basel, Switzerland) Multiplex PCR kit according to the manufacturer's protocols. PCR were carried out in 10μl reactions volumes containing 5μl QIAGEN Multiplex PCR Master mix, 3μl ddH$_2$O, 1μl DNA (20ng/μl) and 1μl primer mix (2pmol/μl each primer). The thermocycler profile began with an initial denaturation step at 95°C for 15min, followed by 30 cycles of 30 sec at 94°C, 90sec at 57°C, 90 sec at 72°C and ended with a final extension of 10 min at 72°C. Fragments from each PCR product was resolved on an automated capillary loading DNA sequencer (CEQ 8000, Beckmann and Coulter, Fullerton, CA, USA) following manufacturer protocols and guidelines and scored using the provided fragment analysis software (Beckman Coulter, Fullerton, CA, USA). All allele designations were verified by eye.

Fish morphology

Digital pictures were taken from all sampled specimens and used for a landmark-based analysis of body shape variation (Zelditch *et al.*, 2004). Fourteen homologous landmarks, selected based on standard landmark descriptions (Bookstein, 1991; Zelditch *et al.*, 2004), were identified on *Coregonus* images (Fig. 2a) and were set using the TPSDIG program (Rohlf, 2006). Landmark configurations for all specimens were translated to a common position, scaled to unit centroid size and corrected for orientation using the Generalized Least Squares Procrustes Superimposition Analysis (GPA;(Rohlf, 1999)). GPA generates a consensus configuration to which the landmark configurations of each individual fish are compared. Deviations from the consensus for each individual are assessed using the thin-plate spline method, which determines orthogonal interpolation functions, or shape axes called principal warps, describing the major axes of shape change (Zelditch *et al.*, 2004; Rohlf, 2006). The bending energies calculated along each principal warp required to fit the landmark configuration of each fish to that of the consensus configuration generates a set of partial warps, or shape variables, for each fish usable in standard multivariate statistical analyses (Rohlf, 1999). The GPA, thin-plate spline and generation of partial warps were performed with TPSRelw (http://life.bio.sunysb.edu/morph/).

Seventeen linear morphometric distances were measured using digital callipers (Fig. 2b-d). Gill raker numbers were counted on the first gill arch from the left side of each fish. The age and the growth rate of each fish was estimated by scalimetry where fish size per year is back-calculated using the distance between the scale centre and each annular ring. The relationship between length of all fish at the age (L_i) and scale size was estimated by a linear regression using the following equation (Caranhac, 1999):

$$L_i = -\frac{a}{b} + \left(L_c + \frac{a}{b}\right) * \frac{R_i}{R_c}$$

where a and b are the intercept and the slope of the regression, R_c is the scale radius and R_i the distance from the scale centre to the year ring, L_c is the total fish length (Smale & Taylor, 1987; Caranhac, 1999).
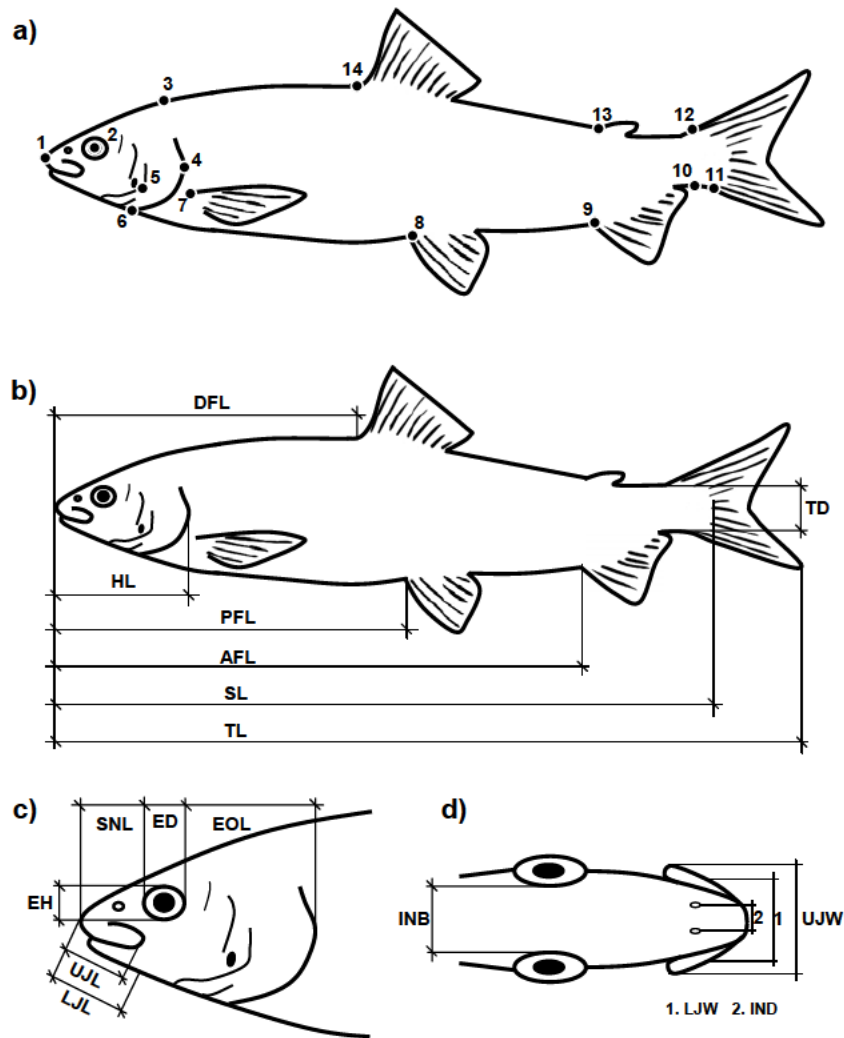

Figure 2. The 14 landmarks used for the geometric morphometric analysis of shape (a). The linear morphometric distances (b-d): Total length (TL), standard length (SL), dorsal fin length (DFL), pelvic fin length (PFL), anal fin length (AFL), tail depth (TD), head length (HL), eye diameter (ED), snout length (SNL), eye-operculum length (EOL), eye height (EH), lower jaw length (LJL), upper jaw length (UJL), intra orbital width (INB), intra nostril distance (IND), lower jaw width (LJW), upper jaw width (UJW).

Data analysis

Sources of genetic population structure

Three potential sources of "population" structure were recorded for each fish: capture date (spawning date), mesh size used, and capture depth (spawning depth) (Table 2). A hierarchical analysis of molecular variance (AMOVA; Excoffier *et al.*, 1992) was performed to determine which factor accounted for the largest source of genetic variation between samples. The populations used for further investigations were then defined by the best structuring factor.

Table 2. AMOVA results for the alternative structuring of the sampled whitefish. Shown are the global F_{ST} value and the corresponding P-value. Because only depth significantly structured our samples, the populations will be grouped by depth for further analyses.

Grouping	df	Global F_{ST}	P-value
Date	3	0.00297	0.824
Depth	2	0.00803	<0.001
Mesh size	2	0.00126	0.985
Date and Depth	10	0.00808	0.981
Date and Mesh size	11	0.00156	1
Depth and Mesh size	8	0.00494	0.999

Genetic diversity within populations

In order to determine the ability to detect demographic population admixture with the 14 microsatellite loci used in this study, we simulated 10 admixed, 10 shallow and 10 deep populations. For these simulations, we used the allele frequencies measured in populations sampled at the extreme ends of the spawning depth distribution (2m and 95m), and applied a resampling procedure implemented in POPTOOLS for MICROSOFT EXCEL 2002 (http://www.cse.csiro.au/poptools/).

Expected (H_E) and observed (H_O) heterozygosity, were calculated using Arlequin 3.1 (Excoffier *et al.*, 2005). Deviations from Hardy-Weinberg equilibrium (HWE) were tested with exact tests (Guo & Thompson, 1992) for each locus and population using GENEPOP 3.4 (Raymond & Rousset, 1995) with 1'000'000 steps in the Markov chain and 5'000 dememorization steps. Inbreeding coefficients (F_{IS}; Weir & Cockerham, 1984) were calculated at each locus within each population and over all loci for all populations and tested for significant deviation from zero using FSTAT version 2.9.3 (Goudet, 2001). Significance levels of both HWE and F_{IS} were corrected for multiple comparisons using sequential Bonferroni corrections (Rice, 1989). FSTAT was also used to calculate an unbiased measure of the number of alleles for each population (allelic Richness (A_R)) and the average number of alleles (A_N) per population. Deviations from linkage equilibrium between all pairs of loci for each population were tested using ARLEQUIN 3.1 (Excoffier *et al.*, 2005). Differences in linkage disequilibrium among population groups were tested using Student's T-tests.

Genetic population structure among populations

The genetic structure among populations was investigated by performing pairwise computations of single locus and multilocus F_{ST} values with corresponding 95 % confidence intervals estimated with 20'000 bootstrap replicates (Weir & Cockerham, 1984) using ARLEQUIN 3.1(Excoffier *et al.*, 2005). Isolation by distance (IBD calculated using $F_{ST}/(1-F_{ST})$) and by depth (called IBZ hereafter) as well as partial correlations, were assessed using Mantel tests (Mantel, 1967; Smouse *et al.*, 1986;

Rousset, 1997) as implemented in ARLEQUIN 3.1 (Excoffier *et al.*, 2005). To visualize population differentiation and similarity, a factorial correspondence analysis (FCA) over populations but based on individual allele frequencies was performed using GENETIX version 4.04 (Belkhir *et al.*, 1996-2002). Finally, the migration rate (m_{Beerli}) among all populations was estimated using a maximum-likelihood framework based on coalescent theory as implemented in the program MIGRATE (Beerli & Felsenstein, 2001). The estimations were performed three times to control for consistency in the results.

Morphology

Univariate residual methods were used to adjust each linear morphological distance for size heterogeneity among individuals (Fleming & Gross, 1994). Each linear morphological distance was regressed against standard length (SL) and standardized residuals were used as variables in subsequent analyses. Because this method potentially also removes information that is not due to allometry, but looks like allometry when populations have non-overlapping or weakly overlapping size distributions (Klingenberg, 1996), we additionally calculated ratios between trait size and total body length. These ratios were then compared between the small subset of fish of similar size caught at depths of 2m and of 30m using Mann-Whitney U-tests and Bonferroni corrected (Rice, 1989). These groups were chosen because they were the most distinct depth groups with sufficient size overlap to allow statistical testing.

Partial warps from the geometric morphometric analyses were reduced to principal components (relative warps). A main effect ANOVA was used to calculate which factor (spawning date, mesh size, spawning depth) explained a significant proportion of the variance in relative warps similar to the AMOVA used for genetics above. Based on those results (see results section), canonical variates analysis (CVA) on the matrix of all relative warps was used to quantify differences among populations from different spawning depths (Rüber & Adams, 2001; Klingenberg *et al.*, 2003; Zelditch *et al.*, 2004; Roy *et al.*, 2007). To test if observed clinal relationships between shape and spawning site water depth are statistically significant, second degree polynomial or linear regression terms were fitted to the relationships between every morphological variable and spawning site depth using JMP 4.0. Since growth patterns are likely influenced by feeding ecology and because feeding is thought to be related to the gill raker morphology (O'Brien, 1987; MacNeill & Brandt, 1990; Link & Hoff, 1998), correlations between growth patterns and gill raker counts were calculated within depth classes. In a scenario of hybridisation, one would expect to find an increase in variance of genetically based morphological traits in hybrids compared to their parental populations (Seehausen 2004). In order to test whether morphological variance was enhanced in populations from intermediate depth, we tested for homogeneity of variance (or homoscedasticity) along the depth gradient in all morphometric variables using Levenes statistic.

Comparison of morphology and genetic data

Several approaches are available for testing if phenotype-environment associations are evolving under divergent natural selection, including reciprocal transplant experiments, direct measurements of selection, and testing predictions of optimal phenotype distributions from estimated adaptive landscapes, i.e. based on resource distributions (Schluter, 2000). Empirical support, however, is often difficult to obtain with any of these methods, mainly due to experimental constraints (Rueffler *et al.*, 2006). Alternatively, inferences may be made from comparisons between population differentiation in phenotypes and neutral genetic loci.

The Q_{ST}/F_{ST} method (Spitze, 1993) has been frequently applied for this purpose (Podolsky & Holtsford, 1995; Lynch *et al.*, 1999). It is based on the principle that divergence in quantitative traits evolving neutrally and having a quasi-pure additive genetic basis should show a divergence similar to that of allele frequencies at neutral loci (Wright, 1951). If these assumptions are met, Q_{ST} values that significantly exceed F_{ST} values are consistent with divergent selection on those traits, whereas significantly smaller values are consistent with purifying selection (Merilä & Crnokrak, 2001; McKay & Latta, 2002). Traditionally, Q_{ST} is quantified using additive components of genetic variance, requiring prior knowledge of genetic variation in quantitative traits. Such information is however not available for most wild populations. Several authors have suggested using phenotypic variance in wild populations as a surrogate for additive genetic variance in phenotypic traits (Merilä, 1997; Bernatchez, 2004; Ostbye *et al.*, 2005). Here, we calculated such P_{ST} values for gill raker number, back-calculated size at age three years (hereafter size), upper jaw length, and the scores on the first axis of one PCA each for morphometric distances and geometric morphometrics. Fully appreciative of the potential pitfalls of this approach (Goudet & Buchi, 2006; Whitlock, 2008), we merely use it to guide our search for traits that may be involved in divergent adaptation and those that are unlikely to be. Furthermore, both growth and gill raker numbers are known to be highly heritable in Salmonid fishes in general and in whitefish in particular (Nilsson, 1992; Gjedrem, 2000; Bernatchez, 2004), which at least for these traits, supports the use of the Q_{ST} analyses.

A Bayesian likelihood approach using the lme4 package implemented in R was applied to estimate P_{ST} values (Palo *et al.*, 2003) and confidence intervals were estimated by an MCMC simulation approach with 10'000 replicates. Pairwise P_{ST} values were considered significantly different from pairwise F_{ST} values, only when the 95% confidence intervals of both were non-overlapping. In order to test for associations between phenotypes, microsatellite genotypes and environments, Mantel correlations and partial Mantel correlations were calculated between P_{ST} values and spawning-depth differences, distances between sampling locations, and linearly corrected F_{ST} ($F_{ST}/(1-F_{ST})$) in ARLEQUIN version 3.11 (Excoffier *et al.*, 2005). Genotype-to-phenotype association were tested with correlations calculated between the individual scores on the first CVA of linear and geometric morphometric data, and the individual scores on the first FCA scores of microsatellite data, using all individuals with known spawning depths. Finally, to test for linkage disequilibrium between morphological traits and microsatellite loci, linear regressions were fitted for informative univariate morphological variables against PCA scores of informative microsatellite loci, where informative means morphological variables or microsatellite loci showing significant differentiation among populations with different spawning depths.

RESULTS

Sources of genetic population structure

Of the three structuring variables; depth, time and mesh size, only spawning depth explained significant molecular variance between groupings (populations) with a significant global F_{ST} of 0.008 (Table 2). None of the other variables seemed to have any consistent effect (all $p>0.8$). Further subdividing samples by the combination of spawning date and spawning depth yielded a similar but non-significant global F_{ST} (Table 2). The drop in the significance level between these two analyses can be explained by the drop in sample sizes if the samples are subdivided into a large number

of groups. Structuring by mesh size, spawning date or any combination of these with each other and with depth did not reveal any significant structuring. Subsequent genetic analyses were thus performed with populations structured by spawning depth, and not by time or mesh size.

Genetic diversity within populations

No significant deviations from HWE and no significant F_{IS}-values were observed at any of the microsatellite loci or in any of the populations after sequential Bonferroni correction (Table 1). The tests for deviations from linkage equilibrium were significant in 50 out of 745 at $p<0.05$ and in 19 out of 745 comparisons at $p<0.01$. The number of deviations (N_{LD}) was slightly but significantly higher in the 30m and 60m populations than in shallow and deep populations (two samples t-test, equal variances, 8df, t=-4.6, $p<0.01$; t=-2.8, $p<0.05$ for N_{LD} defined by $p<0.05$ and <0.01 respectively), whereas they did not differ between shallow and deep water populations (6df, t=2.92, $p=0.78$). We tested the consistency of this pattern by simulating 10 pure shallow, 10 pure deep and 10 admixed populations between these two extremes. The results did not show any significant signs for increased observed heterozygosities (H_O), significant deviations from HWE or significant negative F_{IS} values (Table 1). Similarly, the number of deviations from linkage equilibrium was not higher in simulated admixed populations compared to simulated shallow and deep populations (Table 1). Therefore, and also because the significant linkage tests involved different pairs of loci in different populations, we concluded that they were unlikely to be due to physical linkage between particular loci. Instead, we are likely looking at effects of type I errors and/or random genetic drift in finite populations (Ohtha, 1982). This conclusion is consistent with a recent genetic linkage map for North American whitefish (*Coregonus clupeaformis*) which included seven of the loci used in this study (Rogers *et al.*, 2007). The 14 loci showed a wide range of variability, with three to 35 alleles observed across all populations and 176 alleles over all loci and all populations. Within populations, the mean number of alleles per locus ranged from 4.33 to 10.28, H_E ranged from 0.52 to 0.64, and A_R ranged from 3.92 to 8.06 (Table 1). Thus, the genetic diversity observed in the marker used in this study corresponds to common observations in microstallite markers.

Genetic population structure among populations

We found low overall genetic differentiation among populations, with pairwise F_{ST} values ranging between 0 and 0.070 (Table 3). The highest pairwise differences were found between shallow and deep spawning populations (F_{ST} between 0.037 and 0.070). No significant differences among geographically distant spawning sites could be detected within any given spawning depth, not even between geographically very distant populations such as "shallow 1" and "2m" or "deep1" and "95m" (Fig.1, Table 3). Significant genetic differences were neither observed between spawning times on any given spawning ground nor within any given spawning depth. Congruent with this, neither isolation by distance (IBD) nor isolation by time (IBT) were observed among spawning populations (Mantel correlation between geographical distance and linearized F_{ST}: r=0.161; $p=0.26$), whereas isolation by depth (IBZ) was strong and significant.

Table 3. Pairwise F_{ST} values between Lake Neuchâtel whitefish populations based on 14 microsatellite loci (comparisons based on nine loci are labelled °) with significance level (below diagonal) and the corresponding 95% confidence intervals above the diagonal. A key to sample designation is given in Table 1

	2 m	10 m	30 m	60 m	95 m	Deep 1	Deep 2†	Shallow 1	Shallow 2†	River 1†
2 m	–	-0.003 to 0.010	0.004 to 0.030	0.015 to 0.045	0.018 to 0.065	0.020 to 0.055	0.013 to 0.090	-0.009 to 0.007	-0.012 to 0.0002	-0.009 to 0.002
10 m	0.004 n.s.	–	-0.003 to 0.014	0.002 to 0.022	0.004 to 0.038	0.006 to 0.029	0.001 to 0.064	-0.002 to 0.018	-0.007 to 0.014	-0.004 to 0.023
30 m	0.015**	0.004 n.s.	–	0.001 to 0.009	-0.002 to 0.010	0.005 to 0.019	-0.004 to 0.010	0.007 to 0.054	0.004 to 0.037	0.007 to 0.057
60 m	0.029***	0.010*	0.005 n.s.	–	-0.004 to 0.008	0.003 to 0.022	-0.003 to 0.016	0.010 to 0.061	0.010 to 0.052	0.015 to 0.064
95 m	0.040***	0.019***	0.004 n.s.	0.002 n.s.	–	-0.003 to 0.015	-0.014 to 0.003	0.020 to 0.094	0.020 to 0.084	0.023 to 0.105
Deep 1	0.037***	0.017***	0.012**	0.012*	0.005 n.s.	–	-0.013 to 0.003	0.021 to 0.072	0.024 to 0.081	0.024 to 0.090
Deep 2†	0.047***	0.029***	0.003 n.s.	0.007 n.s.	-0.006 n.s.	-0.005 n.s.	–	0.023 to 0.132	0.018 to 0.085	0.015 to 0.106
Shallow 1	-0.002 n.s.	0.008 n.s.	0.027***	0.034***	0.054***	0.045***	0.070***	–	-0.003 to 0.021	-0.009 to 0.003
Shallow 2†	-0.006 n.s.	0.002 n.s.	0.017***	0.03***	0.050***	0.052***	0.048***	0.009 n.s.	–	-0.077 to 0.024
River 1†	-0.004 n.s.	0.008 n.s.	0.027***	0.039***	0.062***	0.057***	0.053***	0.005 n.s.	-0.006 n.s.	–

The genetic differentiation between populations increased with increasing difference in water depth between spawning locations (Mantel correlation: r=0.736; *p*<0.01). A partial Mantel correlation using geographical distance and the residuals from the Mantel correlation between linearized F_{ST} and spawning depth revealed no significant complementary effect of geographic distance either.

A Locus by locus inspection of the distribution of genetic variance showed that significant population structure was largely attributable to five of the 14 loci used (Cocl-Lav-61, Cocl-Lav-6, Cocl-Lav-68, Cocl-Lav-10 and Cocl-Lav-23; Global F_{ST} range: 0.014-0.060; *p*<0.05). Mantel regressions of linearized F_{ST} against depth were significant for each of the five loci separately (Cocl-Lav-61, r=0.56, *p*<0.05; Cocl-Lav-5, r=0.68, *p*<0.05; Cocl-Lav-68, r=0.64, *p*<0.05; Cocl-Lav-10, r=0.60, *p*<0.05; Cocl-Lav-23, r=0.44, *p*<0.05). Hence, we observed a single lake-wide genetic cline between spawning sites arranged along water depth. This can be visualized by plotting the population mean scores on the first FCA axis based on individual allele frequencies calculated over all populations against water depth (Fig. 3a). Finally, the estimation of migration rates show that most migration occurs from the 30m (intermediate depth) population into the neighbouring ones (Table 4).

Table 4. Estimation of migration rates (m$_{Beerli}$) between whitefish populations sampled at different depth. Given in columns are the migration rates from populations A into populations B (e.g. A 10m vs. B 2m is the migration rate from the 10m population into the 2m population, A2 vs. B10 is the migration rate from the 2m population into the 10m population and so on). A key to sample designation is given in Table 1.

	A 2m	A 10m	**A 30m**	A 60m	A 95m	A Deep1	A Shallow1
B 2m	-	5.83	**11.75**	3.04	3.32	3.09	2.57
B 10m	4.41	-	**19.22**	3.87	2.08	3.99	2.06
B 30m	3.49	6.43	-	5.12	3.43	3.96	2.83
B 60m	4.16	6.38	**21.26**	-	4.13	3.30	2.14
B 95m	3.13	5.13	**14.98**	4.23	-	5.04	2.30
B Deep1	2.92	3.62	**19.64**	9.54	5.28	-	3.64
B Shallow1	3.13	2.76	**9.19**	2.14	2.08	2.35	-

Morphology

We found clines by spawning depth in size (Fig. 3b) and in gill raker counts (Fig. 3c). From shallow to deep waters, size decreases and number of gill rakers increases. Both phenotypic clines are steep in shallow water and flatten towards greater depths. Second order polynomial fits were highly significant for both traits (Figure 3b & c). Correlations between growth and gill raker counts were non-significant within spawning sites of the same depths, except for fish caught at 30m where a significant negative correlation was detected (larger fish have fewer rakers, r=0.25, *p*<0.01; data not shown). This implies some admixture at this intermediate depth between fish originating from shallow and deep water. While spawning depth explained a significant fraction of variance in size at the age of three years (Main effect ANOVA, 2df, F=48.67, *p*<0.001), neither spawning date nor mesh size did. Spawning depth and mesh size, however, both explained significant amounts of variance in the number of gill rakers (Main effect ANOVA spawning depth: 2df, F=15.66, *p*<0.001; mesh size: 2df, F=11.06, *p*<0.001). In contrast, neither significant differentiation, nor significant clinal trends in PCA scores were observed in linear or geometric morphometrics. CVA scores, however, did show clinal trends similar to those seen for the gill raker and growth data (Fig. 3d & e). Second degree polynomial regression terms provided

significant fits to the CVA scores from shape analysis ($r^2=0.632$; $p<0.001$) and a weak but significant linear regression did fit for linear distances ($r^2=0.178$; $p<0.001$).

When analysing ratios of morphometric distances to standard length, distinct and significant clinal patterns emerged in seven of 13 traits (results not shown). The absence of a cline using PC scores based on size corrected residuals, but the presence in ratios, suggests that the apparent clinal pattern in external morphology is largely due to allometric size effects.
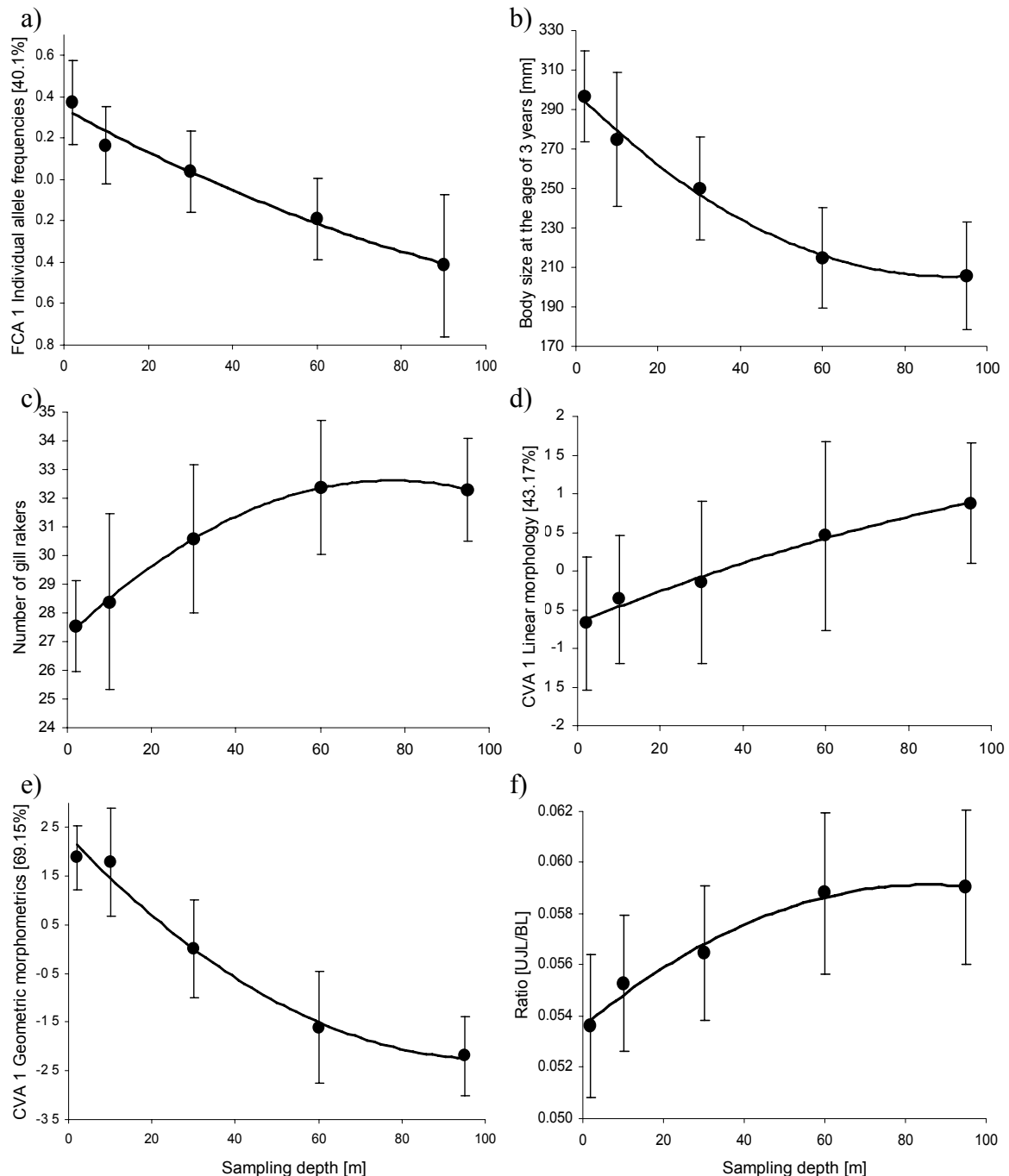
Figure 3. The eco-morphological and genetic clines by water depth on spawning sites: the first factorial correspondence axis (a) of individual allele frequencies at 14 microsatellite loci, estimated body length at the age of three years (size) (b), the number of gill rakers (c), the scores of the first canonical variate analyses based on linear morphometric distances (d) and on geometric morphometrics (e), the upper jaw length (UJL) ratio (f). Error bars are standard deviations, solid lines show the polynomial second degree fitting. The amount of variation explained by the raw data (r^2) and its significance were calculated on individual data and are reported in each panel.

However, if allometric size effects accounted for all morphological variation, fish of similar size caught at different spawning site depths should be morphologically indistinguishable. Contrary to this prediction, two size ratios (12% of measures taken), namely upper jaw length (UJL, $p<0.05$) and pelvic fin length (PFL, $p<0.05$) differed significantly between similarly sized fish from 2 and 30m water depth. These differences, however, were no longer significant after Bonferroni correction. Interestingly, UJL showed a cline along spawning site depth very similar to that demonstrated for gill rakers and size (Fig. 3f) while PFL did not show any clinal trend (results not shown).

Hence size, gill raker number and UJL, showed distinct parallel clines along spawning site depths, while several other morphological traits showed strong allometric covariation with size along depth.Finally, tests for deviation from homogeneity of variance between the spawning site depths were only significant for gill raker counts (Levenes statistic=4.487; 4df1, 282df2, $p<0.01$) where variances were elevated in intermediate water depths (Fig. 3c). Similar, but nonsignificant trends were observed in the first CVA axes of linear and geometric morphometrics.

Comparison of morphological and genetic data

Mantel and partial Mantel correlations between P_{ST} calculated from gillraker number, size and UJL and the pairwise spawning site depth differential were highly positive and significant, whereas correlations with geographic distance were not significant (Table 5). Neither P_{ST} based on PCA scores calculated from linear morphology, nor those calculated from geometric morphometrics correlated with spawning site depth differential or geographical distances (Table 5). Controlling for spawning site depth differential, partial Mantel correlations between P_{ST} and geographical distance were non-significant (Table 5). On the other hand, controlling for geographical distance, P_{ST} for gill raker number, size and UJL were highly positive and significantly correlated with spawning site depth differential (Table 5).

P_{ST} values for gill rakers, size, and UJL were significantly larger than microsatellite-based F_{ST} values, especially when the spawning site depth differential exceeded 30m (Fig. 4a,b & e). However, no significant differences were observed between F_{ST} and P_{ST} based on PC1 scores of either linear morphology or geometric morphometrics (Fig. 4c & d). Mantel correlations between P_{ST} and linearized F_{ST} were significantly positive for gill rakers and size after sequential Bonferroni correction and for UJL before but not after correction (Table 5).

Table 5. Mantel correlations between P_{ST} values of morphological traits and $F_{ST}/(1-F_{ST})$ and partial Mantel correlations between P_{ST} values and depth of capture or distance between sampling locations. Populations included in this analysis are: 2m, 10m, 30m, 60m, 95m, deep 1 and shallow 1. N Populations represents the number of populations included in each analysis. N Comparisons is the number of pairwise comparison in the Mantel correlation. "Depth" is the depth difference and "Distance" is the geographic distance between two populations. "Residuals Depth" is the partial Mantel correlation between residuals from the mantel correlation between water depth difference and $F_{ST}/(1-F_{ST})$ against geographical distance and reciprocally, "Residual Distance" is the partial

Trait	N	N	Depth		Distance		Residuals Depth		Residuals Distance		$F_{ST}/(1-F_{ST})$	
	Populations	Comparisons	r	P- value	r	P-value	r	P-value	r	P- value	r	P-value
Gill rakers	7	21	**0.781**	**0.012***	-0.054	0.707	-0.498	1.000	**0.840**	**0.008***	**0.754**	**0.003***
Size at 3 years	6	15	**0 822**	**0.006***	-0.095	0.636	-0.490	0.980	**0.867**	**0.008***	**0.734**	**0.001***
PC 1 Geo. morphometrics	7	21	-0.081	0.506	0.258	0.171	0.299	0.068	-0.175	0.764	-0.072	0.481
PC 1 morphology	7	21	0.253	0.140	0.396	0.104	0.346	0.136	0.149	0.223	0.125	0.263
Upper jaw length ratio	7	21	**0 873**	**0.005***	0.361	0.047	0.196	0.203	**0.858**	**0.007***	0.534	0.030

* Significant after sequential Bonferroni correction.

We found no significant correlations between P_{ST} and linearized F_{ST} based on either linear or geometric morphometrics. When comparing the individual scores on the first CVA axis for linear and geometric morphometrics to the individual scores on the first FCA axis of microsatellite allele frequencies, strong genotype-to-phenotype correlations emerged (Fig. 5; geometric morphometrics: r=0.608, $p<0.001$; linear morphometrics: r=-0.329; $p<0.001$). Finally, linear regressions between informative morphological traits (gill raker number, size and UJL) and the five informative microsatellite loci were significant if performed over all samples. However, within depth groups, after sequential Bonferroni correction, none were significant (Table 6), suggesting that the five microsatellite loci are not physically linked to phenotypic traits under selection.
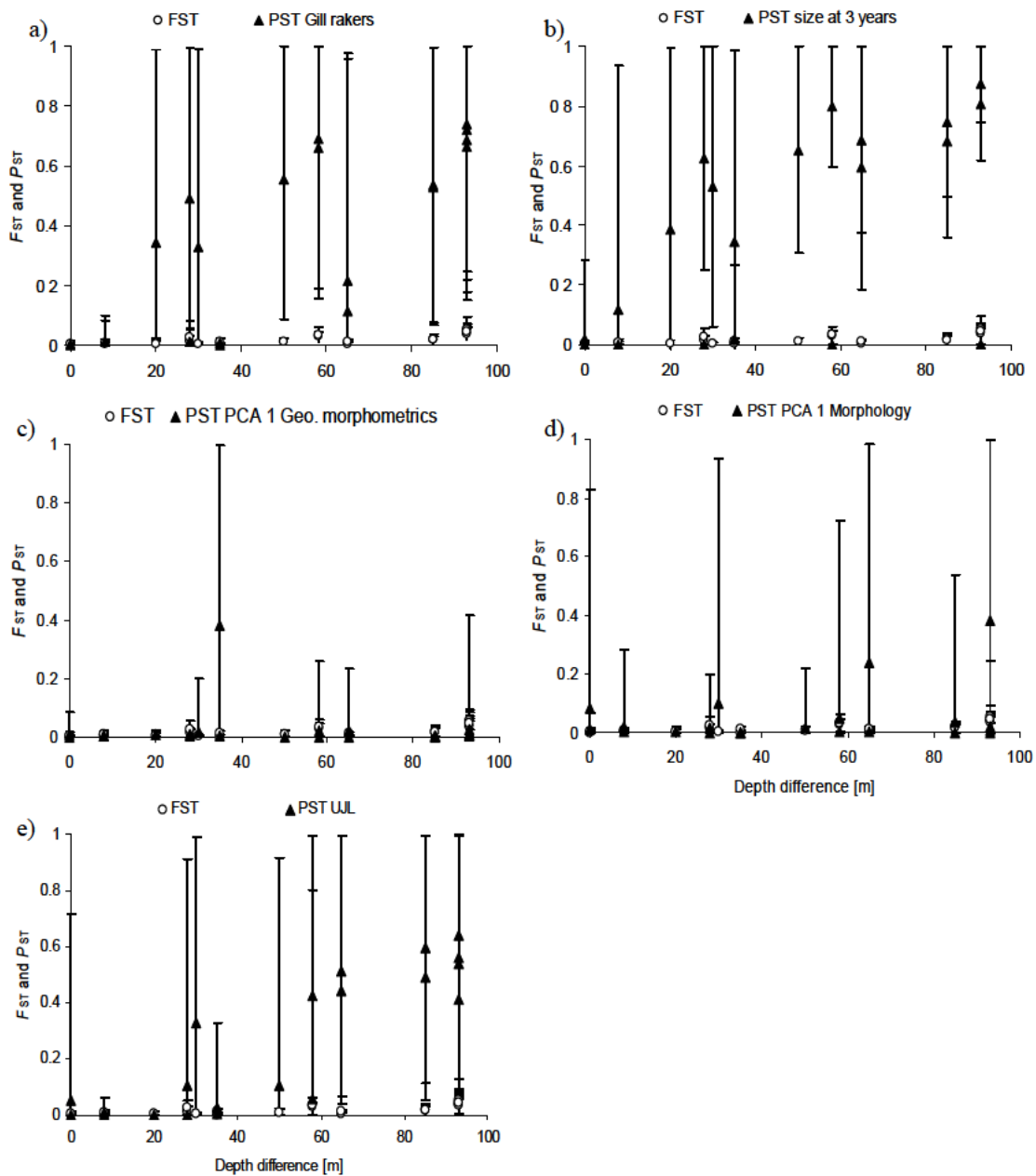
Figure 4. P_{ST} - F_{ST} comparisons for various population pairs: Black triangles represent P_{ST} distances for gill raker counts (a), estimated body size at age of three years (b), first principal component scores of shape analysis (c) and linear morphometrics (d), upper jaw length ratio (e), all with corresponding 95% confidence intervals. Open circles represent F_{ST} values. All distances are plotted against the depth differential between spawning sites. The following populations were included in this analysis: 2m, 10m, 30m, 60m, 95m, Deep 1, and Shallow 1.
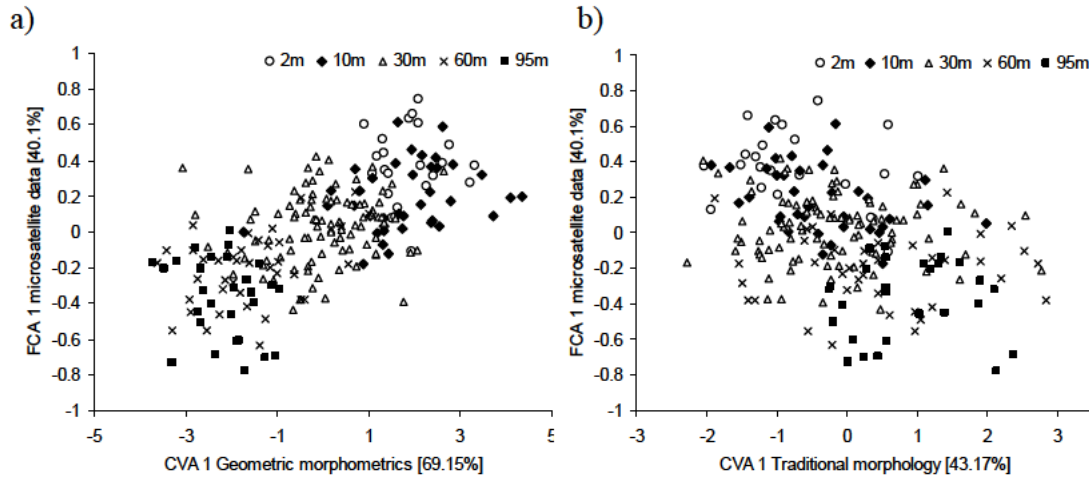
Figure 5. The scores on the first axis of a factorial correspondence analysis using 14 microsatellite loci plotted against the scores on the first canonical variate of geometric morphometrics (a) and linear morphometric distances (b). One outlier, included in the correlation analyses, is out of range of the graph in panel a (-3.70/1.31).

DISCUSSION

We analyzed the effects of differences in water depth, spawning time and geographical distance between spawning sites on the distribution of variation in neutral genes and ecologically relevant traits in whitefish of Lake Neuchâtel, Switzerland. The whitefish of this lake are part of a larger postglacial adaptive radiation involving some 30 different species (Steinmann, 1950). We found strong parallel clines in multiple neutral loci, and several phenotypic traits along gradients of spawning site depth. Both genetic and phenotypic diversity were structured exclusively by water depth at spawning site, but neither by spawning time nor by geographical distance, or any two-way interactions between variables. Within a given spawning depth, populations are fully genetically contiguous across the length of the lake with no indication of isolation-by-distance. Samples taken from five different spawning depth zones, however, were significantly differentiated both phenotypically and at five out of 14 microsatellite loci. These results suggest a dominant role for isolation-by-adaptation along the water depth gradient in Coregonid adaptive radiation. In agreement with theory suggesting that populations can differentiate along ecological gradients in parapatry despite gene flow (Endler, 1973), adaptive differentiation along the water depth gradient is likely maintained by divergent selection, as indicated by very large P_{ST} values obtained for gill raker counts and other morphometric variables related to feeding ecology. That the phenotypic cline has its parallel in a genotypic cline, suggests that gene flow restriction in Lake Neuchâtel whitefish is a by-product of divergent selection among different spawning depths. At present we do not know how suites of phenotypic traits, associated with specific feeding ecologies, translate into adaptation or preference for a particular spawning depth. Finally, it remains unclear, whether what we see is a stable balance between selection and gene flow, an incipient forward speciation process, or speciation reversal due to recent anthropogenic habitat alteration causing previously distinct species to collapse into one single gene pool. Below we discuss these alternatives.

Scenarios for origin of the cline

Several scenarios can potentially explain the origin of the parallel clines of allele frequencies at neutral loci and in phenotypes. Lake Neuchâtel underwent environmental changes in the past decades (see below), which could have led to an erosion of previously stronger genetic differentiation of species. Such speciation reversal has been reported in several other fish and is typically associated with anthropogenic habitat changes, such as eutrophication (Seehausen, 2006a). A recent event of increased gene flow is expected to generate an increased incidence of linkage disequilibrium between neutral loci, detectable for several generations even when mating within the population is random (Weir, 1979). We did indeed observe a slight but significantly larger frequency of linkage disequilibrium in populations spawning at 30 and 60m depth (Table 1), whereas we did not observe any difference between depth ranges in deviations from HWE or the magnitude of F_{IS} (Table 1).

The power of microsatellite markers to detect these signs when hybridizing populations are only weakly genetically differentiated and have high numbers of alleles at each locus, can however be constraining. Indeed, our analyses of simulated demographically admixed populations based on the two populations from the extremes of the depth range did not recover any signs of increased admixture in measures of HWE deviations, F_{IS} or linkage disequilibrium (Table 1). Hence, even though slightly increased linkage disequilibrium was observed in the populations caught at 30 and 60m that may be considered indicative of recent admixture, the analyses using simulated admixed populations suggests that these differences might as well be due to a type I error. Variances in gill raker counts were somewhat elevated at intermediate depths, which could also be an indication for recent hybridization (Fig. 3b), but elevated adaptive variance in the middle of a cline is also predicted by divergent selection alone, independent of the historical origins of the cline (Gavrilets, 2004). Perhaps the strongest indication of relatively larger admixture at intermediate depth is the significant association between microsatellite allele frequencies and adaptive traits at intermediate but not at extreme depths. Nevertheless, given these data, we cannot make any robust conclusions regarding alternative hypotheses for the origin of the cline.

If introgressive hybridization was occurring without divergent selection, we would expect the phenotypic and genetic differentiation along the spawning depth continuum to break down very quickly, and if divergent selection was not quite strong, differentiation at unlinked neutral loci could not possibly be maintained (Barton & Hewitt, 1985). We found no evidence of physical linkage between any of the phenotype traits and any of the microsatellite loci, including the five that showed significant depth clines (Table 6). Hence, it is unlikely that hitchhiking of neutral loci linked to loci under selection can explain the significant population differentiation observed at microsatellite loci. Instead, these data may suggest sufficiently reduced gene flow along the depth gradient to permit differentiation at unlinked loci by drift, consistent with the porous genome hypothesis of selection-driven speciation (Wu, 2001).

Specifically, the parallel clines in microsatellite loci and adaptive phenotypic traits along the spawning depth gradient is consistent with the isolation-by-adaptation hypothesis, where a reduction of gene flow due to divergent selection between environments can facilitate genetic differentiation at other loci by drift (Nosil *et al.*, 2008). Incomplete sympatric or parapatric speciation, namely a quasi-equilibrium between divergent selection and homogenising gene flow between two or more incipient species therefore seems a plausible explanation for our data (Rueffler *et al.*, 2006). The observation of highest migration rate estimates from the population at 30m

towards all other populations may lend some support to the incipient speciation hypothesis, as the opposite pattern might be expected in a scenario of recent admixture and hybridization happening at intermediate spawning depths. Experimental work (Endler, 1973), mathematical modelling (Endler, 1973; May *et al.*, 1975; Gavrilets, 2004) and simulations (Endler, 1973; Doebeli & Dieckmann, 2003) suggest that adaptive divergence between populations and speciation can occur despite gene flow, particularly when many demes form a cline along a smooth environmental gradient. Some theoretical (Slatkin, 1985) and empirical work in wild populations (Hendry *et al.*, 2002; Moore & Hendry, 2005), however, show that divergence and speciation in parapatry can be severely constrained by even moderate amounts of gene flow. The number of demes involved, the smoothness of the environmental gradient and the strengths of divergent selection all greatly influence the effects of gene flow on population differentiation, and thus the rate of population divergence along environmental gradients (Endler, 1973).Many clinal speciation models and experiments used explicitly linear arrangements of demes which generate synergistic effects of both divergent selection and IBD. Even weak divergent selection can then generate phenotypic and genotypic clines if a sufficiently large number of demes are involved because many demes in close proximity cause a self-cancelling of gene flow along a gradient (Endler, 1973).

Evidence for divergent natural selection

Either of the two scenarios to explain the Lake Neuchatel whitefish cline, forward speciation and maintenance of partial differentiation after recently increased gene flow require the occurrence of divergent selection. The P_{ST} - F_{ST} comparisons show that between-population divergence in body size, gill raker numbers, and upper jaw length is significantly larger than expected under neutrality. Both growth and gill raker numbers are known to be highly heritable in Salmonid fishes in general and in whitefish in particular (Nilsson, 1992; Gjedrem, 2000; Bernatchez, 2004). That the P_{ST} - F_{ST} difference increases with increasing spawning depth differential therefore suggests increasing differentials of divergent selection with increasing water depth difference. Linearity of this trend, however, is only given in the shallow part of the spawning depth range (2-60m). In areas deeper than 60m, the relationship flattens and morphology does not diverge further. That all three divergent phenotypic traits, body size, gill raker counts, and upper jaw length are related to feeding ecology implicates divergent selection between divergent feeding regimes as a key driver of population differentiation. Gill raker numbers are often strongly divergent between sympatric whitefish species (Bernatchez, 2004; Ostbye *et al.*, 2005) and as mentioned above, densely rakered fish are thought to feed more efficiently on zooplankton while sparsely rakered ones are thought to be more efficient on benthic foods (O'Brien, 1987; MacNeill & Brandt, 1990; Link & Hoff, 1998). Experimental data supporting this hypothesis, however, are remarkably absent. Divergent selection between feeding regimes is nevertheless consistent with commonly proposed mechanisms of ecological speciation in whitefish (Schluter, 1996; Bernatchez, 2004; Kahilainen *et al.*, 2004; Kahilainen & Ostbye, 2006; Landry *et al.*, 2007) and other fish in postglacial lakes (Schluter, 1993; Skulason *et al.*, 1993; Schluter, 1995; Taylor, 1999). However, the divergence that we demonstrated here is between fish from spawning grounds at different water depth. There is no evidence that whitefish specialize for feeding at the same water depth that they spawn at. Neither is there any evidence that the morphological traits with clinal variation are related to spawning ecology. It may be plausible that body size somehow influences spawning depth, but this remains to be investigated in more detail. Therefore, no obvious mechanism can be invoked that

translates a spawning habitat depth gradient into one related to feeding ecology. Thus, and despite the fact that divergent selection on feeding ecology is likely involved, other unknown mechanisms related to spawning ecology or the interaction between spawning and feeding ecology might be implicated in the adaptive radiation of whitefish in central European lakes.

Implications for conservation of species and adaptive diversity in whitefish
Previous studies reported two (Wagler, 1937; Steinmann, 1950; Douglas *et al.*, 1999) or three (Dottrens & Quartier, 1949; Bargetzi, 1960) phenotypically, ecologically and genetically distinct whitefish species in Lake Neuchâtel. All previous studies, however, obtained fish from commercial fishermen fishing during the spawn fishery at the extreme ends of the depth and temporal distribution: early and shallow for *Coregonus palea,* and deep and late for *Coregonus candidus* (Wagler, 1937; Steinmann, 1950; Douglas *et al.*, 1999). In our study, we find individual fish that match the species described by these authors when we only compare fish sampled at the extremes of the spawning depth and time distributions. However, we sampled the spawning distributions more continuously and demonstrated additional phenotypic and genotypic variation tightly associated with spawning depth in a continuous fashion. Because we could not detect any strong genetic or phenotypic discontinuities between spawning depths and/or times, it is difficult to determine the actual number of different demes or incipient species residing in Lake Neuchâtel. Our data should not be mistaken as evidence for a single admixed whitefish population. On the contrary, we show that every water depth is inhabited by a different set of phenotypes and genotypes with restricted gene flow between them. Independent of the exact species status, these populations should therefore be considered distinct genetically differentiated ecotypes.

The apparent lack of strong isolation barriers between these ecotypes makes the diversity of whitefish in this lake prone to respond very quickly to changes in their environment. Organic pollution (eutrophication) of this lake (and many other Swiss lakes) during the 1950s to the early 1980s may have indeed had such an effect. The resulting oxygen depletion in deep water areas (Stadelmann *et al.*, 1997), likely rendered deep waters unsuitable for whitefish (Müller & Stadelmann, 2004). This would have multiple effects on the coexistence of depth-partitioned whitefish species: it reduced available niche diversity, and area size in the deep part of the habitat continuum, it shortened the selection gradient and simultaneously forced genotypes adapted to deep-spawning to spawn shallower than they would otherwise. All these effects potentially reduced relative population sizes and increased gene flow between ecotypes. The large-scale loss of whitefish diversity in other Swiss lakes during increased eutrophication supports this hypothesis (Vonlanthen *et al.* unpubl.).

A final consideration that needs to be taken into account is that for economic reasons, professional whitefish fisheries tends to target large and small whitefish, while intermediate fish sizes are fished much less heavily. The fishing mortality is therefore not evenly distributed among fish of different body size. It is conceivable that this management scheme enhances the fitness of genotypes of intermediate morphology and therefore influences the fitness landscape that would otherwise prevail in the lake.

At this stage we cannot infer whether whitefish diversity in Lake Neuchâtel is in the process of collapsing or expanding, but it is clear that the ecotypic diversity in this lake has a genetic basis, and that the persistence of the ecotype complex in this lake is highly sensitive to changes in the lake and its fisheries management. Future analyses of historical collections of whitefish scale samples from this lake will

hopefully shed more light onto the temporal trends in genetic structure and ecological diversity.

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Chapter 4

Demographic admixture, isolation by adaptation and speciation along an environmental gradient in Alpine whitefish

Manuscript

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ABSTRACT

The investigation of patterns and mechanisms of ecological speciation in emerging model systems is needed to better understand the process of ecological speciation. One such emerging model system is coregonine fish of the northern hemisphere where multiple adaptive radiations have occurred in many lakes after the last glacial retreat. Here we present an in-depth investigation of whitefish diversity in a Swiss lake. Our results show that during spawning, whitefish are phenotypically and genetically structured in a cline-like pattern along the water depth gradient. This distribution is however not continuous, but consists of three genetically and phenotypically distinct species that overlap in their depth distribution with one species being genetically and phenotypically intermediate. The genetic variation among all individuals is strongly correlated with body size, suggesting isolation by adaptation and that mate choice based on size, or any other trait correlated with size, might be implicated in the process of speciation. Both clinal- and hybrid speciation scenarios are plausible explanations for the observed pattern. The historical occurrence of intermediate whitefish and the fact that the intermediate species has private alleles suggest that its origin is not due to recent changes in the environment or stocking, but they could have however played a role in the recent spread of this species.

INTRODUCTION

Ecological speciation was suggested very early in the history of evolutionary biology (Darwin, 1859, Fisher, 1930), but received much less attention during the modern synthesis (Morell, 1999, Coyne & Orr, 2004). This has, however, changed in recent years and ecological speciation is now recognized as an important speciation mechanism (Schluter, 2000, Rundle & Nosil, 2005, Funk *et al*., 2006, Schluter, 2009). Speciation is ecological when reproductive isolation directly evolves as a consequence of divergent selection on traits between environments (Schluter, 2001). It does not only occur in allopatry, but can also arise in sympatry or narrow parapatry (Dieckmann & Doebeli, 1999, Doebeli & Dieckmann, 2000, Ito & Dieckmann, 2007). Theoretical evidence suggests that speciation despite gene flow is especially likely to occur when divergent selection directly interacts with a reduction in gene flow in heterogeneous environments when a high number of demes form a cline along ecological gradients (Endler, 1973, May *et al*., 1975, Doebeli & Dieckmann, 2003, Gavrilets, 2004). Simultaneously with ecological divergence, reproductive isolation can be achieved by extrinsic post-zygotic or pre-zygotic mechanisms (Rundle & Nosil, 2005). Both may evolve well before intrinsic hybrid dysfunction evolves (Seehausen *et al*., 1997, Seehausen *et al*., 2008). Therefore, ecological speciation is reversible as long as intrinsic genetic incompatibilities have not evolved (Seehausen *et al*., 1997, Seehausen *et al*., 2008). As a consequence, changes in the environment can have severe effects on species diversity that has arisen from ecological speciation. In particular, a loss of habitat heterogeneity can flatten the adaptive landscape of a species flock (Gavrilets, 2004) leading to an increase in gene flow.

Increased gene flow between species can have different outcomes. (i) It can lead to a complete fusion of species, also known as speciation reversal (Seehausen *et al*., 1997, Taylor *et al*., 2006, Seehausen, 2006, Grant & Grant, 2008, Seehausen *et al*., 2008). This has been shown for several taxa (Todd & Stedman, 1989, Seehausen *et al*., 1997, Taylor *et al*., 2006, Seehausen *et al*., 2008), but one of the most striking examples can be found within the Lake Victoria cichlid radiation, where a combination

of the introduction of a top predator and increased eutrophication coinciding with a reduction in visibility led to a complete collapse of several cichlid species complexes (Seehausen *et al.*, 1997). (ii) It can lead to an increase in adaptive genetic variance, which could be the basis for the formation of new species facilitated by transgressive segregation (Rieseberg *et al.*, 1999). (iii) It could generate a new phenotypically intermediate species (Grant & Grant, 1992, Arnold, 2006, Mallet, 2007) without the loss of parental species (Demarais *et al.*, 1992, Rieseberg, 1997, Giessler *et al.*, 1999, Buerkle *et al.*, 2003). (iv) Finally, a stable cline between selection and geneflow could emerge (Endler, 1977, Moore, 1977, Doebeli & Dieckmann, 2003, Gavrilets, 2004).

North temperate lakes are young ecosystems that emerged after the last glacial maxima about 15'000 years BP (Hantke, 1991). After glacial retreat, several fish species colonized the newly available habitats. Some taxa, especially those belonging to the family Salmonidae, have subsequently radiated into many new species within a large number of lakes (Schluter, 1996, Taylor, 1999, Hudson *et al.*, 2007). Whitefish (*Coregonus spp.*) underwent multiple adaptive radiations in parallel and are the most diverse species complex within the Salmonidae (Bernatchez, 2004; Kottelat & Freyhof, 2007). Numerous lakes in central and northern Europe harbour multiple sympatric species (Hudson *et al.*, 2007), which are likely adapted to divergent feeding and spawning habitats (Ostbye *et al.*, 2005, Vonlanthen *et al.*, 2009). Most species are divergent in various phenotypic traits with growth rate and gill raker number being most divergent (Steinmann, 1950, Bernatchez *et al.*, 1999, Ostbye *et al.*, 2006). Feeding adaptations range from benthic specialists with few and short gill rakers to zooplanktivorous pelagic specialists with numerous and long gill rakers (O'Brien, 1987, McNeely *et al.*, 1990, Link & Hoff, 1998). In the large pre-alpine lakes of Switzerland, the different species spawn in a range of depths, from shallow to over 200m, and their spawning period can range from August to April. During lake stratification, all spawning is confined to the cold water below the thermocline. It has been suggested that spawning depth of whitefish species is tightly associated with their phenotypes: small whitefish tend to spawn in deep water, while large whitefish spawn in shallower water (Steinmann, 1950, Kottelat & Freyhof, 2007, Vonlanthen *et al.*, 2009).

Many central European lakes underwent strong environmental changes during the last few decades (Lang & Reymond, 1996). The most severe change was organic pollution, which led to oxygen depletion in deep areas during lake stratification (Müller & Stadelmann, 2004). Whitefish diversity has been reduced by about 55% by speciation reversal that occurred due to the loss of reproduction habitats (see Chapter 5). Whitefish radiations inhabiting these ecosystems thus offer excellent opportunities to investigate the mechanisms underlying ecological speciation and the effects of environmental changes on biodiversity dynamics. In Lake Lucerne, Switzerland (Fig. 1), originally four ecologically differentiated native whitefish species were recognized based on divergent phenotypic characters: the fast-growing and sparsely-rakered Balchen (*C. sp. "Bodenbalchen"*), the slow-growing and densely-rakered Albeli (*C. zugensis*), the phenotypically intermediate Edelfisch (*C. nobilis*), and one species (*C. sp. "Alpnacherfelchen"*) seemingly endemic to adjacent Lake Alpnach, which is connected to Lake Lucerne by a 300m long, 100m wide and relatively shallow channel (Steinmann, 1950, Svarvar & Müller, 1982). Compared to other lakes, Lake Lucerne has only been moderately impacted by organic pollution during the last century (Liechti, 1994). However, one species, *C. nobilis*, became rare during this eutrophic phase and was thought to be extinct for several years (Muller, 2007). In addition, a new winter-spawning whitefish, seemingly phenotypically intermediate between *C. zugensis* and *C. sp. "Bodenbalchen",* was recently observed (Michel, 1996).

Here we quantitatively assess the distribution of winter spawning whitefish in Lake Lucerne along a depth gradient. Previous samplings often targeted known species, likely omitting unknown species. This could have lead to a biased assessment and to an underestimation of the number of species present, but also to an overestimation of the overall divergence among species (Vonlanthen *et al.*, 2009). We investigate how whitefish diversity is distributed along this depth gradient using both molecular and morphological techniques. We test whether the phenotypically intermediate whitefish represents a new species or whether diversity is continuously distributed along the depth gradient. We finally test which alternative evolutionary mechanisms might be implicated in the emergence of a new whitefish species and whether recent environmental changes might have played a role.

MATERIAL & METHODS

Sampling

Lake Lucerne is a pre-alpine lake in central Switzerland (Fig. 1). It has a volume of 11.8 km^3 with a mean depth of 114 m, a maximum depth of 214 m and a shore length of 114 km. It is an oligotrophic lake where whitefish (*Coregonus sp.*), perch (*Perca fluviatilis*), pike (*Esox lucius*) and roach (*Rutilus rutilus*) are the most abundant fish species (Stadelmann, 2007). Whitefish were sampled from several spawning locations in multiple years (Table 1). Quantitative sampling was performed on multiple dates covering the spawning time of the two winter spawning species (sampling dates: 19.11.07; 26.11.07; 5.12.07; 11.12.07; 18.12.07). Benthic gill nets with mesh sizes of 25 mm, 35 mm, and 45 mm, each with a surface of 250 m^2, were set in five different depths (2, 10, 20, 30, and 40 m) covering the common spawning depths of the two known species, as well as intermediate depths (Fig. 1b). One additional sampling using 45 mm mesh size was performed in 2 m depth at the same location as the quantitative sampling on the 31.12.2005. To test for geographical consistency in the distribution of Lake Lucerne whitefish species, several other spawning sites were sampled within a narrow depth range using mesh sizes of either 26 mm or 45 mm (Fig. 1). All nets were set for about 20 hours overnight. All fish were weighed, total length and body length were measured, and a piece of muscle tissue was preserved in absolute ethanol for DNA analysis. Scales were collected from all fish. Aging was performed using a scale reading lens and was based on annual growth rings that can be identified on scales. The first gill arch was preserved in 70% ethanol and the number of gill rakers from the left gill arch was counted using a magnifying lens or a microscope. A sampling summary is given in Table 1.

DNA extraction and microsatellite amplification

DNA was extracted using a Qiagen® Bio Sprint 96 extraction robot according to manufacturer's standard extraction protocol. All fish were genotyped at ten microsatellite loci: Cocl-Lav49, Cocl-Lav61, Cocl-Lav6, Cocl-Lav68, Cocl-Lav10, Cisco-157, Cocl-Lav4, Cocl-Lav45, Cocl-Lav18, BWF2 (Patton *et al.*, 1997, Turgeon *et al.*, 1999, Rogers *et al.*, 2004). Forward primers were labelled with three differently coloured fluorescent dyes for multiplexing. Because of overlapping allele ranges, two pre-PCR multiplex primer sets were used. The first loci set included Cocl-Lav49, Cocl-Lav61, Cocl-Lav6, Cocl-Lav68, Cocl-Lav10 and Cisco-157. The second set included Cocl-Lav4, BWF2, Cocl-Lav45 and Cocl-Lav18 (Bittner, 2009). The PCR amplification was performed using the Qiagen® Multiplex PCR Kit in a 10.2 µl

reaction volume of 5 μl Multiplex Master Mix, 3 μl H_2O, 1 μl Primer Mix and 1.2 μl DNA extraction product. PCR was run on a Techne TC-412 thermocycler applying the following profile: Initial denaturation for 15 min at 95°C followed by 30 cycles with 30 sec at 94°C, 90 sec at 57°C, 90 sec at 72°C, and a final extension of 10 min at 72°C. All PCR products were resolved on a Beckman coulter®, CEG™ 8000 following manufacturer's protocol. Scoring was done by eye using the CEQ™ 8000, GENETIC ANALYSIS SYSTEM SOFTWARE v. 9.0.
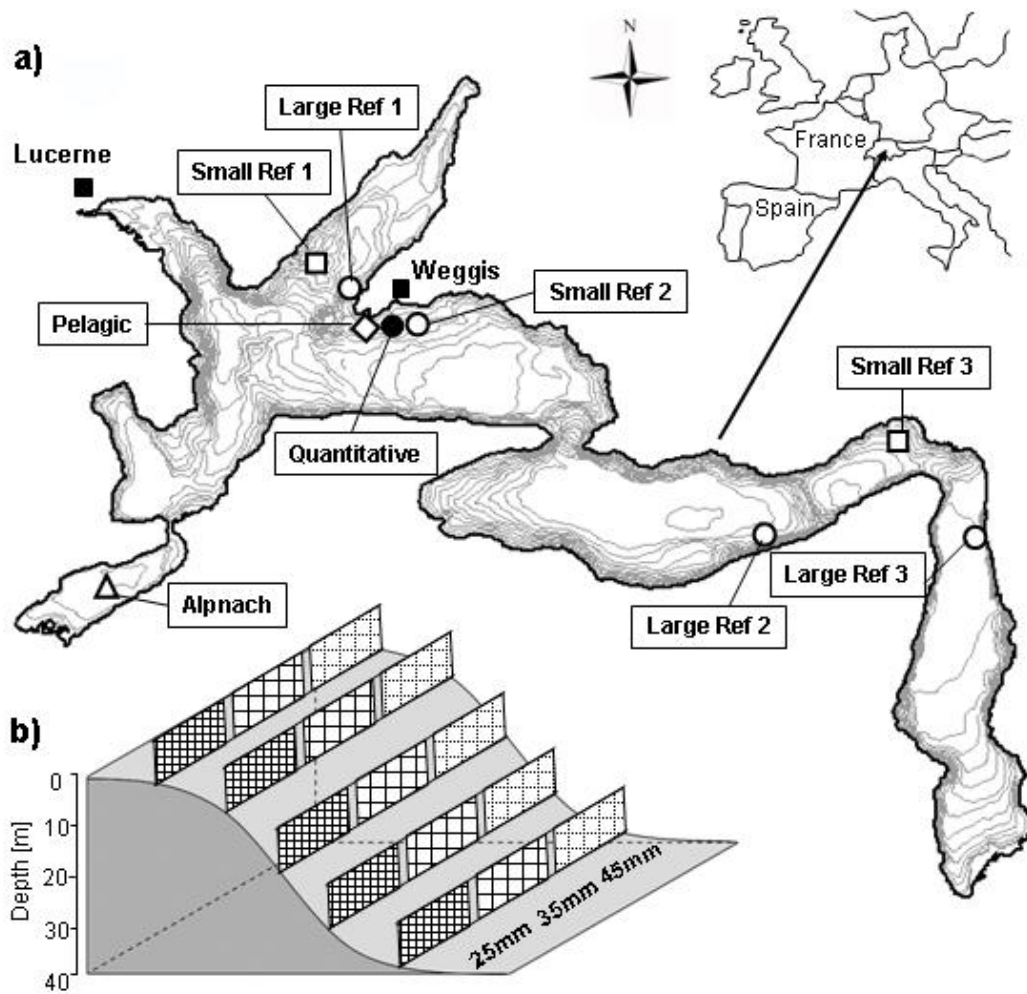
Figure 1 Whitefish sampling strategy in Lake Lucerne. (a) The open circles correspond to reference sites of the large size class, including a few fish from the intermediate size class (see Table 1). Open squares correspond to reference sampling locations of the small size class. The open triangle corresponds to whitefish sampled in Lake Alpnach,. The open diamond corresponds to whitefish caught in the open water. A detailed summary of sample sizes and GPS locations is given in Table 1. (b) Sampling design for the quantitative sampling (bold circle). Three mesh sizes (25, 35, 45 mm) were set in five different depths (2, 10, 20, 30, and 40 m) on five different dates covering the spawning time range of all winter spawning whitefish species.

Table 1. Sampling summary for all whitefish caught. Given are the name for each population (Name) and the GPS location (GPS), the sampling dates (Sampling date), the total number of whitefish caught on each sampling location (Sample size), the number (N) of three-year-old whitefish (3 year old), which were included in the population genetic analyses (Genetics) along with all the Alpnach samples, and the samples sizes corresponding to the size classes defined in the results (Small, Int., and Large).

Name	GPS		Sampling date	Sample size	N 3 year old	N Small	N Int.	N Large	N Genetics
	North	East							
Quantitative	47°01'36.37"	8°25'31.56"	19.11-18.12.2007	268	149	107	34	14	149
Supp. Large	47°01'36.37"	8°25'31.56"	31.12.2007	8	6		14	5	6
Pelagic	47°01'34.00"	8°24'47.00"	19.11-18.12.2007	66	19				19
Large Ref 1	47°01'44.71"	8°23'42.23"	13.12.2005	41	16	-	-	16	16
Large Ref 2	46°58'31.81"	8°33'05.74"	22.12.2005	30	6	-	-	6	6
Large Ref 3	46°58'24.01"	8°36'29.52"	14+22.12.2005	63	16	-	6	10	16
Small Ref 1	47°02'37.04"	8°23'11.80"	19.11+29.12.2005	30	9	9	-	-	9
Small Ref 2	47°01'40.95"	8°25'42.53"	21.11.2005	30	9	9	-	-	9
Small Ref 3	46°59'53.11"	8°35'05.27"	21.12.2005	63	12	12	-	-	11
Alpnach	46°57'52.11"	8°19'10.49"	02.12.2004	20	-	-	-	-	20
Total				619	242	137	54	51	241

Data Analysis

Sampling

We first used bubble plots to graphically investigate the data obtained by quantitative sampling along the time and depth gradients using the three different gill net mesh sizes. The deviations from normality in body size and gill rakers of all ripe fish obtained in each mesh size were tested using a Shapiro-Wilk test (Shapiro & Wilk, 1965). If deviations from normality were observed, the fit of a mixture of two or three normal distributions was compared based on Akaike's Information Criterion corrected for sample size, AIC_c, using the software DISCMIXTUREPROGS v 0.4 for analysis of mixture models using discretisation (Brewer, 2003, Burnham & Anderson, 2004). To test whether the size gaps observed in fish catches could be due to size selectivity of gill netting, skewed normal selectivity curves were estimated for each mesh size using the Dsn function provided in the sn package for R (http://azzalini.stat.unipd.it/SN) and summed to estimate total selectivity across all mesh sizes (Azzalini & Capitanio, 1999, Fujimori & Tokai, 2001). To test whether deviations from normality in body size were the results of admixture of differently aged fish or of admixture of different species, the age of fish from three size classes defined from selectivity curves obtained above were compared using t-tests. The size frequencies of fish aged two to six years are shown in histograms.

Sources of population genetic structure

We used four potential sources of population structure (sampling date, sampling depth, gill net mesh size and body length) of three-year-old fish in a hierarchical analysis of molecular variance (AMOVA: Excoffier *et al*., 1992) to test which sources of structure accounted for the largest fraction of genetic variance across samples. Additionally, we used the Bayesian clustering algorithm implemented in STRUCTURE v. 2.0 to estimate the number of genetic groups, K, and posterior probabilities of each individual to belong to a certain group (Pritchard *et al*., 2000). Ten runs of each K from 1 to 6 were performed with an initial burn in period of 100'000 steps and a MCMC with

1'000'000 using default run parameters. All further analyses were based on the best structuring found in these analyses.

Genetic structure within populations

Observed and expected heterozygosities were calculated for each locus within each population using ARLEQUIN v. 3.11 (Excoffier *et al.*, 2005). Deviations from Hardy-Weinberg equilibrium (HWE) for each population across all loci were tested with a Fishers Exact Test using GENEPOP v. 4.0 (Raymond & Rousset, 1995) with 1'000'000 steps in the Markov chain and 5'000 dememorization steps. F_{IS} values (Weir & Cockerham, 1984) for each locus in each population and for each population across all loci, the number of alleles (A_N) and allelic richness (A_R) for each population were calculated in FSTAT v. 2.9.3 (Goudet, 2001). Deviations from linkage equilibrium between all pairs of loci for each population were tested using ARLEQUIN. Significance levels of F_{IS} and deviations from HWE were corrected for multiple testing using a sequential Bonferroni correction (Rice, 1989).

Genetic structure among populations

To estimate the genetic differentiation among populations, multi-locus pairwise F_{ST}-values (Weir & Cockerham, 1984) were calculated in ARLEQUIN. A principal component analyses (PCA) based on individual allele frequencies of an equal number of samples for each population was performed to visualize population genetic differentiation between individuals. Equal sample sizes for each population were required because sample sizes were unequal, which would likely influence the outcome of a PCA. The sub-sampling of individuals was performed using the re-sampling procedure as implemented in POPTOOLS for MICROSOFT EXCEL 2003. Additionally, to maximise allelic variation among populations, a factorial correspondence analysis using all sampled individuals using the grouping defined above was performed in GENETIX v. 4.04 (Belkhir *et al.*, 1996-2002). The relationship between genetic variation and body length was assed by a regression of the PCA scores from the first axis against body length among all individuals and within each population. In order to estimate whether sampling depth, body length or a combination of both best explained the genetic variation amongst individuals, a multiple regression was performed using the PCA scores from the first axis or variation as dependent variable *versus* body length and depth as covariates. To test whether relationships between genetic variation and body length can be explained by hitchhiking of microsatellite loci on genomic regions under selection instead of isolation by adaptation, we performed a locus-by-locus inspection of global F_{ST} values and regressed the first PCA axis of genetic variation on body length. Finally, to compare the intermediate population to potential hybrids resulting from a cross between the parental species, an F1 hybrid population was simulated for the genetics data using the resample procedure implemented in Poptools for Microsoft Excel 2003.

RESULTS

Sampling results

In total, 268 whitefish were caught in the quantitative sampling design. The body sizes ranged from 160 mm to 420 mm (Fig. 2). Whitefish were caught on all sampling dates with a peak observed on December 11th (Fig. 2a+b). They were distributed among all

Table 2. Summary of tests for normal distribution and mixture models of two or three normal distributions for two morphological traits, size and gill rakers. The data was analysed using the three potential groupings of the quantitative sampling (Grouping variable), and divided into each possible class (Grouping classes). Shown for each grouping are: the summary statistics for the Shapiro-Wilk's test for normality (W), the degrees of freedom (d f.) and the corresponding p-value. If the distribution did significantly diverge from a normal distribution, the fit based on Akaike's Information criterion of a single (AICc1), a mixture of two (AICc2), or three (AICc3) normal distributions is given. If ΔAIC_{23} (AICc2 - AICc3) is greater than 4, then a mixed model of three normal distributions is more likely. If it is smaller than -4 and if ΔAIC_{12} (AICc1- AICc2) is larger than 4, then a mixture of two normal distributions explain the data best. If ΔAIC_{23} (AICc2- AICc3) is between 4 and -4, a mixture of two or three normal distributions are equally likely.

Trait	Grouping variable	Grouping classes	W	d.f.	p-value	AICc1	AICc2	AICc3	ΔAIC_{12}	ΔAIC_{23}
Body size	Mesh size	25mm	0.783	168	**<0.001**	3197	773	771	2424	2
		35mm	0.927	91	**<0.001**	3247	820	807	2427	13
		45mm	0.928	9	0.466	-	-	-	-	-
	Date	19.11.2007	0.914	27	**<0.05**	1387	267	267	1121	-1
		26.11.2007	0.955	27	0.289	-	-	-	-	-
		05.12.2007	0.858	51	**<0.001**	2769	489	472	2279	18
		11.12.2007	0.786	89	**<0.001**	5210	879	840	4331	39
		18.12.2007	0.909	74	**<0.001**	4615	760	726	3855	34
	Depth	2m	0.953	10	0.700	-	-	-	-	-
		10m	0.958	20	0.505	-	-	-	-	-
		20m	0.908	56	**<0.001**	2799	528	531	2271	-4
		30 m	0.884	117	**<0.001**	5340	1070	1051	4270	19
		40m	0.663	65	**<0.001**	2479	549	535	1931	14
Gill rakers	Mesh size	25mm	0.896	157	**<0.001**	851	670	677	181	-8
		35mm	0.975	89	0.086	-	-	-	-	-
		45mm	0.913	9	0.335	-	-	-	-	-
	Date	19.11.2007	0.908	27	**<0.05**	209	143	152	66	-9
		26.11.2007	0.921	27	**<0.05**	223	159	155	64	4
		05.12.2007	0.927	49	**<0.01**	366	261	251	106	10
		11.12.2007	0.922	81	**<0.001**	561	396	402	165	-6
		18.12.2007	0.938	71	**<0.01**	515	366	367	149	-1
	Depth	2m	0.929	10	0.441	-	-	-	-	-
		10m	0.933	20	0.179	-	-	-	-	-
		20m	0.970	49	0.240	-	-	-	-	-
		30n	0.899	114	**<0.001**	770	547	550	223	-3
		40m	0.851	62	**<0.001**	368	274	280	94	-6

sampled depths (2-40 m) with body size decreasing and gill raker number increasing with depth (polynomial regression: N=268, df=267, R^2=0.47, $p<0.001$; linear regression: N=255, df=254, R^2=0.33, $p<0.001$; Fig. 2c+d). Mesh sizes where highly selective for body size (Fig. 2e) but the sizes of fish caught in different mesh sizes did overlap. Both body size distribution and gill raker counts did significantly differ from normality in several mesh sizes, at several dates and in several depths (Table 2). A mixture model of two or three overlapping normal distributions best explained the observed pattern when deviating from normality (Table 2). The estimated selectivity curves overlapped between mesh sizes and the calculated selectivity across all mesh sizes does not show any distinct gap that could otherwise explain the multimodality observed across all mesh sizes (Fig. 3a). The deviation from normality observed in several mesh sizes suggest either admixture of different age classes or species within mesh sizes and across all samples. The comparison of ages of all whitefish from all mesh sizes indeed shows that the age differs between the three size classes (C_1:150-

245 mm, C_2: 246-315 mm and C_3: 316-430 mm) defined using the selectivity curves of the different mesh sizes (Fig. 3b). Fish from the second group, however, are younger than those from the first and the third group (t-tests: C_1 with C_2: t=4.643, d.f.=244, $p<0.001$; C_1 with C_3: t=-4.018, d.f.=173, $p<0.001$; C_2 with C_3: t=-4.539, d.f.=111, $p<0.001$), supporting the occurrence of multiple species. The size distribution of 3-year-old fish shows a tri-modal distribution, suggesting 3 distinct groups (Fig. 3c, Fig. S1). The boundaries between the distinct peaks were set at a size of 235 mm and 320 mm. Gill rakers did not reveal a tri-modal pattern but fish of intermediate size were also intermediate in gill raker counts (Fig. 3d).
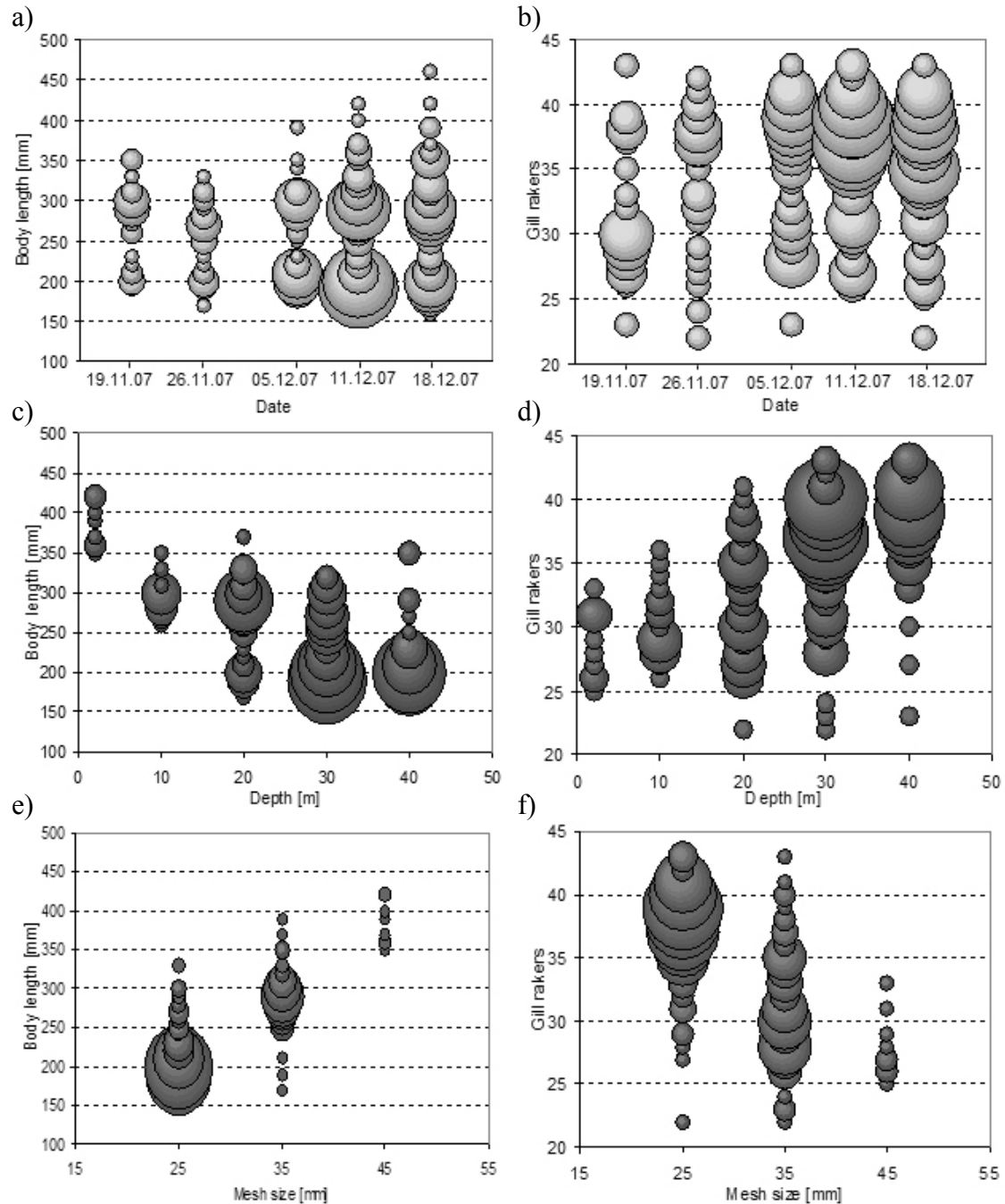
Figure 2. Results of a quantitative sampling of 268 whitefish along a depth and time gradient using three monofilament gill net mesh sizes. Body size (a) and gill raker counts (b) of whitefish caught at five different sampling dates. Body size (c) and gill raker counts (d) of whitefish caught at different depths. Body size (e) and gill raker counts (f) of whitefish caught in different mesh sizes.
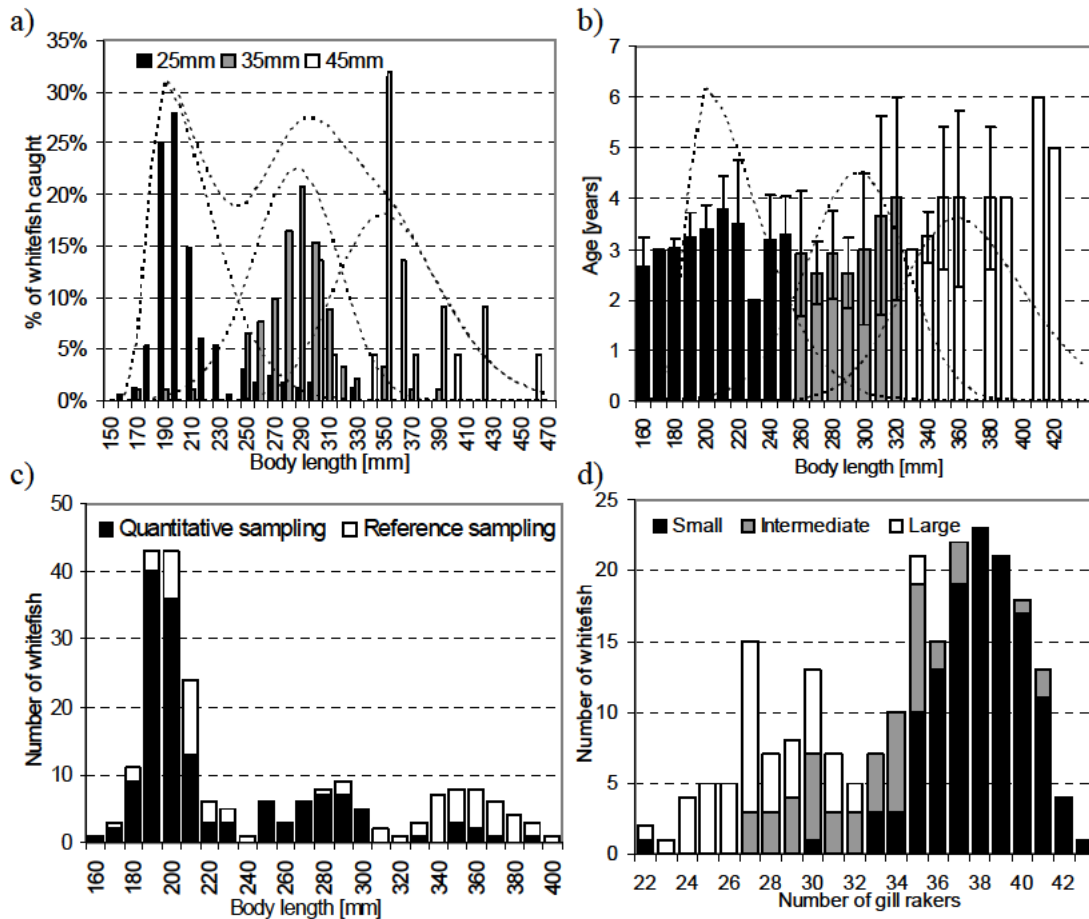
Figure 3. (a) Histogram showing the % number of whitefish caught for each gill net mesh size with whitefish structured by size classes. Dashed lines represent the selectivity curves for each gill net mesh size and across all mesh sizes assuming a right skewed normal distribution. (b) Histogram showing the average age of whitefish for a given size class with the corresponding standard deviations. Dashed lines represent the selectivity curves for each mesh size used. Black bars stand for fish with the highest likelihood to be caught with a 25 mm mesh size, grey bars for fish to be caught with 35 mm, and white bars for fish to be caught with 45 mm. (c) Body length histogram of 242 three-year-old whitefish from Lake Lucerne. Size histograms for other age classes are given in Fig. S1. (d) Histogram for gill raker counts of the three size classes (Small: 160-235 mm; Intermediate: 236-320 mm; Large: 321-392 mm).

Sample structuring

Of the four structuring variables, date, depth, mesh size and size class of three-year-old fish, size class explained most genetic variation with a global F_{ST} of 0.051 (Table 3). Date did not explain any genetic variance. Depth explained a significant amount of genetic variation, suggesting some genetic structuring by depth; the global F_{ST} was, however, one magnitude smaller than that between size classes (Global F_{ST}=0.025). Mesh size, with a global F_{ST} of 0.046, explained a relatively large portion of variation.

This was not unexpected as size selectivity of gill nets generates three size classes that roughly coincide with the size classes that explained most genetic variation. However, the global F_{ST} was smaller than the one observed between size classes, probably due to some admixture of different sizes within a given mesh size (see Fig. 3a). STRUCTURE analyses were not effective in discriminating between the

Table 3 AMOVA results for the alternative structuring of whitefish sampled in a quantitative sampling. Alternative grouping of samples, sample sizes (N), degrees of freedom (d.f.), global F_{ST} and p-value are given.

Grouping	N	d.f.	Global F_{ST}	p-value
Date	268	4	0.003	0.887
Depth	268	4	0.025	<0.001
Mesh size	268	2	0.046	<0.001
Size classes	174	2	0.051	<0.001

three groups, but the distribution of assignment likelihoods seems to support the occurrence of three genetically distinct groups defined by body size (Fig. S2). Subsequent analyses were therefore performed with populations structured by size classes.

Table 4. For each population we report the grouping name and their size range, the sample size (N), observed (H_O) and expected heterozygosity (H_E), significance level of deviation from HWE for each population across all loci (P-HWE), Allelic richness (A_R), inbreeding coefficient (F_{IS}) and its significance level (p-F_{IS}), and number of deviations from linkage equilibrium (N_{LD}) at a significance level of p=0.05. The simulated population was not included in the Total/overall calculations.

Grouping	Size range at age 3 years	N	H_O	H_E	P-HWE.	A_R	F_{IS}	P-F_{IS}	N_{LD} $p<0.05$
Small	160-235mm	107	0.48	0.50	n.s.	4.58	0.045	n.s.	2
Intermediate	236-320mm	48	0.54	0.58	<0.001	4.81	0.08	<0.05	5
Large	321-381mm	19	0.52	0.56	n.s.	4.30	0.076	n.s.	4
Ref_Small	160-235mm	29	0.49	0.50	n.s.	4.56	0.029	n.s.	3
Ref_Large	321-381mm	32	0.60	0.60	n.s.	4.71	0.008	n.s.	1
Alpnach	-	20	0.54	0.57	n.s.	4.70	0.032	n.s.	1
SimF1	-	30	0.61	0.57	n.s.	4.80	-0.067	n.s.	3
Total/overall		255	0.53	0.55		4.61	0.045		16 (5.92%)

Genetic diversity within populations

No deviations from HWE and no significant F_{IS} values were observed in any population, except for the intermediate size class where both deviations from HWE and F_{IS} were significant (Table 4). Over all populations, 16 deviations from linkage equilibrium were observed, which corresponds to 5.92% of all pairwise comparisons and therefore is slightly higher than expected by chance. These deviations are highest in the intermediate size class, which could be explained by demographic admixture or gene flow. In the remaining populations, type 1 errors instead of physical linkage of microsatellite loci seem more likely (Ohtha, 1982). This is consistent with a genetic linkage map for North American whitefish (*C. clupeaformis*), which included six of the ten loci used in this study (Rogers *et al*., 2007). Allelic richness ranged from 4.30 to 4.81 and was highest in the medium size class; however, these differences were not significant. Over all loci, 70 alleles were observed: 47 were shared among all populations; four private alleles were found in the large and intermediate size class; eight in the small size class (Fig. 4d).

Table 5 Pairwise F_{ST} values between Lake Lucerne whitefish species based on 10 microsatellite loci (below diagonal) and the corresponding p-value (above the diagonal). Sample sizes are given in Table 2.

	Small	Medium	Large	Ref_Small	Ref_Large	Alp	SimF1
Small	-	***	***	n.s.	***	***	***
Medium	0.035	-	*	***	***	**	n.s.
Large	0.109	0.015	-	***	n.s.	***	*
Ref_Small	-0.002	0.025	0.096	-	***	***	***
Ref_Large	0.136	0.034	-0.005	0.118	-	***	***
Alp	0.071	0.022	0.041	0.054	0.054	-	***
SimF1	0.027	-0.004	0.018	0.022	0.035	0.037	-

*** $p<0.001$; ** $p<0.01$; * $p<0.05$; n.s. non significant
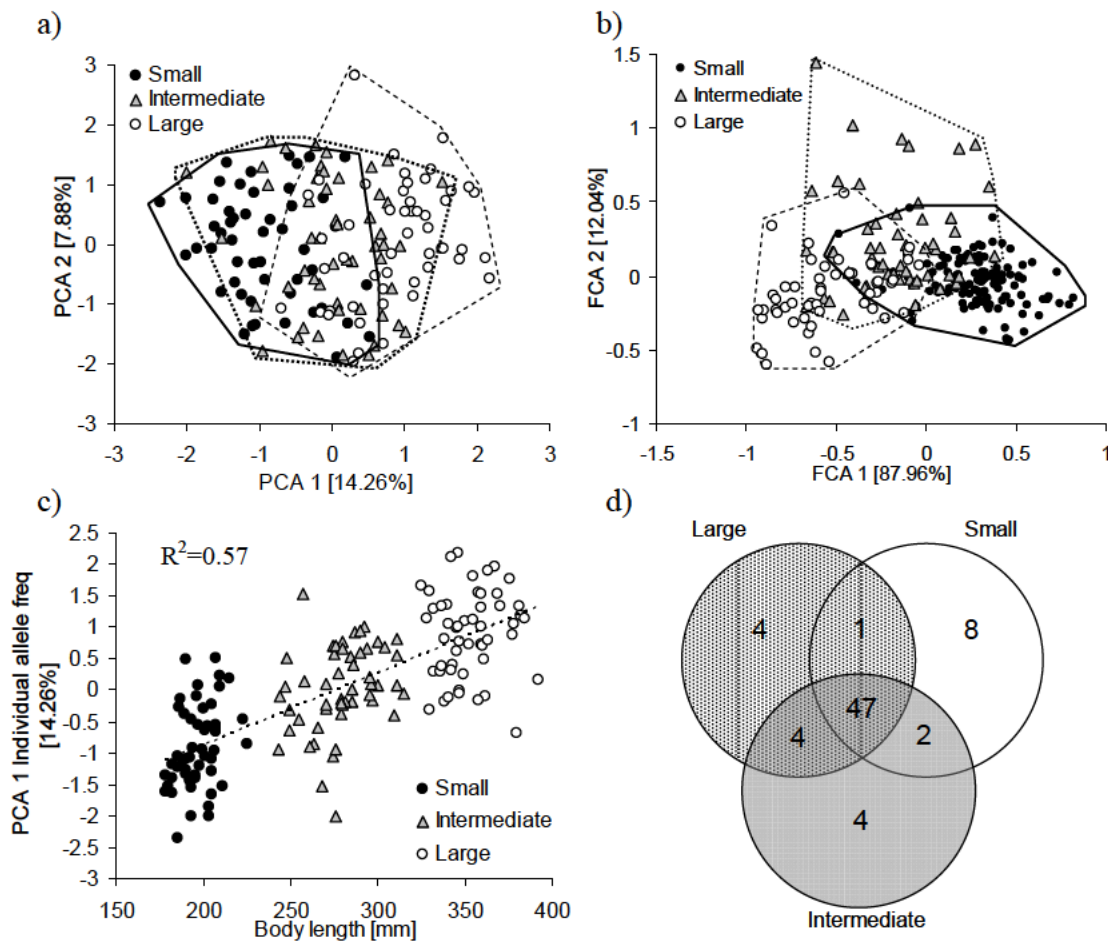
Figure 4. (a) Scatter plot showing the spread of individuals of the three size classes (N=150) along the first two principal component axes of multilocus allele frequencies, (b) and along the first two factorial correspondence axes where individuals were grouped into the three size classes in order to maximize the variation among groups (N=241). (c) The scores of the first axis from a principal component analysis based on individual allele frequencies plotted against body length of three-year-old whitefish. The regression results are given in Table 6. (d) Analysis of shared and private alleles for each population. Each circle represents the number of microsatellite alleles found in 50 randomly sampled individuals for each species. The overlapping regions correspond to alleles that are shared among the species.

Genetic diversity among populations

Multilocus pairwise F_{ST} values among populations ranged from 0 to 0.136 and were highest between the small and large size classes (between 0.096 and 0.136) and non-significant when compared within the same size class from different locations. F_{ST} values were also non-significant between simulated F1 hybrids of small and large whitefish and intermediates (Table 5). F_{ST} between the intermediate size class and the small and large size classes varied between 0.015 and 0.035, suggesting that they are genetically intermediate to the small and large ones. This is confirmed in the principal component and factorial correspondence analyses (Fig. 4a+b). There is a strong linear relationship between size and PC1 of individual allele frequencies across size classes (Fig. 4c, Table 6). This is also significant within the small and intermediate size classes (Table 6), suggesting either physical linkage between putatively neutral markers and genes coding for size or a strong signal of isolation by adaptation along an adaptive continuum (Nosil *et al.*, 2008). Because nine out of ten microsatellite loci display this pattern (See Table S1), belonging to at least six different linkage groups (Rogers *et al.*, 2007), it seems rather unlikely that this pattern is the result of physical linkage between microsatellite loci and some genes under selection. Besides size,

genetic PC1 was also significantly related to water depth (N=154, R^2=0.276, $p<0.001$), but water depth did not explain any remaining variance in a multiple regression including body size as a variable.

Table 6. Linear regression analyses between the first principal component scores from individual allele frequencies and body length of three-year-old fish. Given are the size classes analysed (Size class), the correlation coefficient (R^2) with corresponding p-value, and the slope (slope) with corresponding p-value.

Size class	R^2	d f.	p-value	slope	p-value
Small	0.12	50	<0.05	0.021	<0.05
Intermediate	0.10	50	<0.05	0.012	<0.05
Large	0.00	50	n.s.	0.003	0.627
Overall	0.57	152	<0.001	0.011	<0.001

DISCUSSION

Quantitative sampling revealed that the winter spawning whitefish of Lake Lucerne are phenotypically and genetically structured in a cline-like pattern along the water depth gradient on their spawning grounds. However, body length is not normally distributed at each depth, suggesting an admixture of species of different size along the depth gradient. Over all samples, a clear tri-modal distribution of body length of three-year-old fish was observed, which cannot be explained by gill net selectivity. The hierarchical AMOVA analysis based on microsatellite genotypes confirms the three size clusters and the individual principal component analysis showed that the intermediate size class is composed of genetically intermediate fish (Fig. 4c). Furthermore, genetic variation among all individuals and within two of the three size classes is strongly correlated with body size, suggesting isolation by adaptation based on size (Nosil *et al*., 2008). The occurrence of a strong relationship between genetics and size together with admixture of different size classes within an age class on the spawning site further suggests that mate choice based on size, or any other trait correlated with size, might be implicated in the process of speciation in whitefish. At least two different speciation scenarios may predict such a pattern - clinal speciation along an environmental gradient (Endler, 1973, Doebeli & Dieckmann, 2003, Gavrilets, 2004) or hybrid speciation (Arnold, 2006, Mallet, 2007). The historical occurrence of intermediate whitefish (Steinmann, 1950) and the fact that the intermediate species has private alleles suggest that its origin is not due to recent changes in the environment or stocking.

Presence of three species structured along an environmental gradient
The observation that whitefish were morphologically and genetically structured in a cline-like fashion along the sampled depth gradient is consistent with other data that show that sympatric whitefish species often spawn at different water depths with the larger species typically spawning in shallower sites (Steinmann, 1950, Smith & Todd, 1984, Vonlanthen *et al*., 2009). Generally, the small species (*C. zugensis*, Fig. 5) is highly abundant and predominates at depths of 40 m (Fig. 2c). The fast growing species (provisionally called *C. sp. "Bodenbalchen,"* Fig. 5) is encountered in far smaller numbers and almost exclusively in very shallow depths of less than 10 m (Fig 2c). These two previously known species can be clearly separated by morphology, genetics and spawning habitat.

Importantly, the observed cline is not a result of demographic admixture of these two isolated species, but instead is due to the presence of a third species; one that is morphologically and genetically distinct, but intermediate in body size and allele frequencies, which is dominant at intermediate depths of 10 to 30 m where it overlaps to some extent with *C. zugensis* (Fig 2c). This intermediate species, which we provisionally refer to as *C. sp. "Schwebbalchen"* (Fig. 5) is distinct in body length, gill raker numbers, and microsatellite allele frequencies (Fig. 4a, b, c), and may be distinct in other morphological traits, including head shape and fin coloration that were not analysed in detail (Fig. 5).Because three monofilament gill net mesh sizes have been used for sampling, a tri-modal distribution in body length could be the result of size-selective fishing. The sum of the skewed normal selectivity curves estimated for each mesh size however shows that no large gap exists in size selectivity across the three mesh sizes combined. Furthermore, deviations from normality and the better fit of mixture models of two or three normal distributions for the size of whitefish caught within a single mesh size (Table 2) support the occurrence of several phenotypic groups, even within individual mesh sizes. Had we sampled from a continuous underlying distribution of body sizes, uni-modal distributions of size within each mesh size would have been expected.
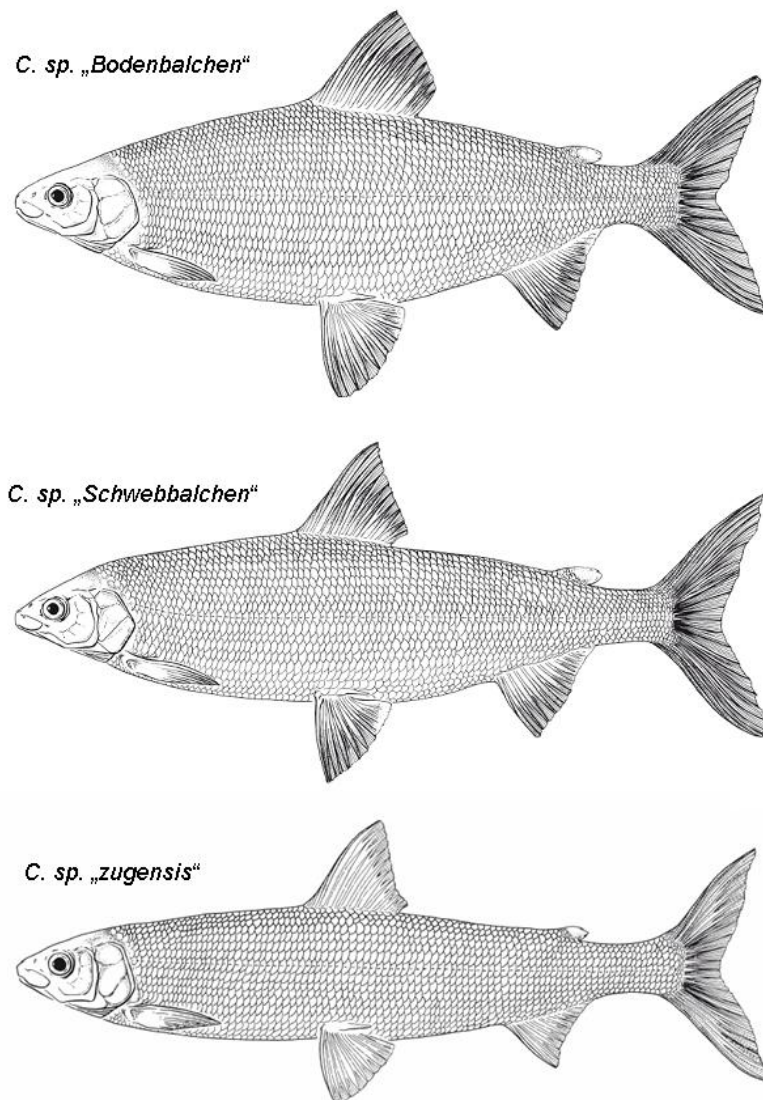
Figure 5. Drawings of the three winter spawning species from Lake Lucerne © Verena Kälin.

A role of behavioural mate choice?

We find fish of different size classes admixed on the spawning ground at several depths, especially *C. sp. "Schwebbalchen"* and *C. zugensis*. Furthermore, we observe a strong pattern of isolation by adaptation for size, or some other trait correlated with size, both across all individuals and within species. Taken together, and assuming that whitefish were spawning where we caught them (we analysed only ripe or almost ripe fish; over 90% had a maturation degree of 5 or 6 according to Smolina, 1920), these observations suggest that mate choice based on body length, or some other phenotypic or behavioural trait related to size, maintains the genetic differentiation observed between species. The significant regressions within the small and intermediate sized whitefish suggest that size selective mating even occurs within species with selection strength decreasing with increasing size (Table 6). A strong tendency to mate assortatively based on size might explain why whitefish frequently speciate into small and large species in the absence of geographical isolation. The evolution of assortative mating can be dramatically facilitated when natural selection acts on a trait that has the potential to directly act as mate choice signal, as that resolves the antagonism between recombination and selection ("magic-trait model of speciation": Gavrilets, 2004). Growth rate in whitefish has been proposed to be under divergent natural selection (Ostbye *et al.*, 2006, Rogers *et al.*, 2007) and there is repeated empirical evidence for size-dependent assortative mating in salmonids and many other fish (Foote & Larkin, 1988, Sigurjonsdottir & Gunnarsson, 1989, McKinnon *et al.*, 2004). Experiments to test the hypothesis of size-assortative mating between the whitefish species of Lake Lucerne are sorely needed.

Recent or ancient hybrid or clinal speciation?

C sp. "Schwebbalchen" is intermediate not only in terms of gill raker counts and body size (Fig. 3c+d), but also in terms of microsatellite allele frequencies (Fig. 4a+b), and is not genetically different from a simulated F1 hybrid. Such a pattern could be the result of at least two different mechanisms - clinal speciation along an environmental gradient (Endler, 1973, Doebeli & Dieckmann, 2003, Gavrilets, 2004) or hybridisation following secondary contact (Schwarz *et al.*, 2005, Gompert *et al.*, 2006, Arnold, 2006, Mallet, 2007, Rieseberg & Willis, 2007). Based on our results we cannot differentiate between the two as both mechanisms implicate gene flow from both extreme species into the intermediate species, and therefore predict intermediate allele frequencies at neutral loci and perhaps intermediate genetically-based phenotypic traits. We found a heterozygote deficiency (Tab 4), which instead suggests admixture of different species. However, this seems rather unlikely as all fish analysed were three years old and a bimodal distribution in body size would be expected if the intermediate population consisted of an admixture of the two extreme species. To differentiate between a hybrid origin and simultaneous ecological speciation along an environmental gradient, more detailed population genetic end environmental data, especially about ecological niche use of the three species, is necessary. Independent from the underlying mechanisms, the strong relationship between genetic variation and body size, both among species and also within species, suggests that a strong pattern of reproductive isolation by size is present, consistent with the isolation by adaptation hypothesis (Nosil *et al.*, 2008). Here adaptation is related to size, which likely represents adaptation to different feeding niches, but also to spawning depth (Kottelat & Freyhof, 2007, Rogers & Bernatchez, 2007, Vonlanthen *et al.*, 2009). Additionally, the large genetic differentiation observed between the two extreme species further suggests that the selective pressures maintaining these differences must be strong, as a

break down of the divergent species would otherwise be expected (Seehausen *et al.*, 2008).

A recent emergence could have been induced by an increase in gene flow caused by organic pollution leading to introgressive hybridisation between the two previously isolated species (Hubbs, 1955, Seehausen *et al.*, 1997, Seehausen, 2006, Taylor *et al.*, 2006, Arnold, 2006). During eutrophication, the deep waters in polluted lakes experienced oxygen and light depletion, reducing reproductive success of deep spawning species that may have lead to a shift of parts of the deep water spawning populations into shallower waters (Chapter 5). This reduced the spatial isolation of spawning habitats and thus increased gene flow. On the other hand, whitefish are artificially bred for stocking for management purposes where the different species are unintentionally hybridized. The observation of a slightly enhanced number of shared alleles between the intermediate *C. sp. "Schwebbalchen"* and large *C. sp. "Bodenbalchen"* tend to support some effect of stocking on gene flow between these two species (Fig. 4d). The size classes of such potential hybrids are however remarkably absent from catches, as shown by the distinct gap observed between the two size classes (Fig. 3c). This suggests strongly reduced fitness of those hybrids or, maybe more generally, of all artificially bred fish. The observation of private alleles in the intermediate *C. sp. "Schwebbalchen"*, the fact that winter spawning whitefish have been observed prior to the pollution and heavy stocking phase (Steinmann, 1950), and the fact that Lake Lucerne has only moderately been impacted by organic pollution, rather suggest that the intermediate species emerged prior to stocking and eutrophication. It could however be that stocking and/or eutrophication fostered the spread of this species. They are today indeed very abundant, even more abundant than the large *C sp. "Bodenbalchen"*, while they were apparently rare in the past (Steinmann, 1950).

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SUPPLEMENTARY MATERIAL

Table S1. Locus by Locus inspection of the pattern of genetic differentiation. Shown are the Global F_{ST} with corresponding p-value, the linkage group this locus corresponds to(Rogers *et al.*, 2007), and the regression results between PC 1 of individual allele frequencies for each locus against body size.

Locus	Global F_{ST}	p-value	Linkage group	R^2	p-value
Cocl 49	0.0609	<0.001	-	0.08	*p<0.001*
Cocl 61	0.27624	<0.001	-	0.31	*p<0.001*
Cocl 6	0.11546	<0.001	18+26	0.21	*p<0.001*
Cocl 68	0.11448	<0.001	19	0.21	*p<0.001*
Cocl 10	0.0508	<0.001	23	0.14	*p<0.001*
C2-157	0.00854	n.s.	10	0.03	*p<0.05*
Cocl 4	0.00489	n.s.	12	0.00	n.s.
BWF 2	0.04116	<0.001	-	0.06	*p<0.01*
Cocl 45	0.03238	<0.001	-	0.06	*p<0.01*
Cocl 18	0.00218	n.s.	14	0.03	*p<0.05*
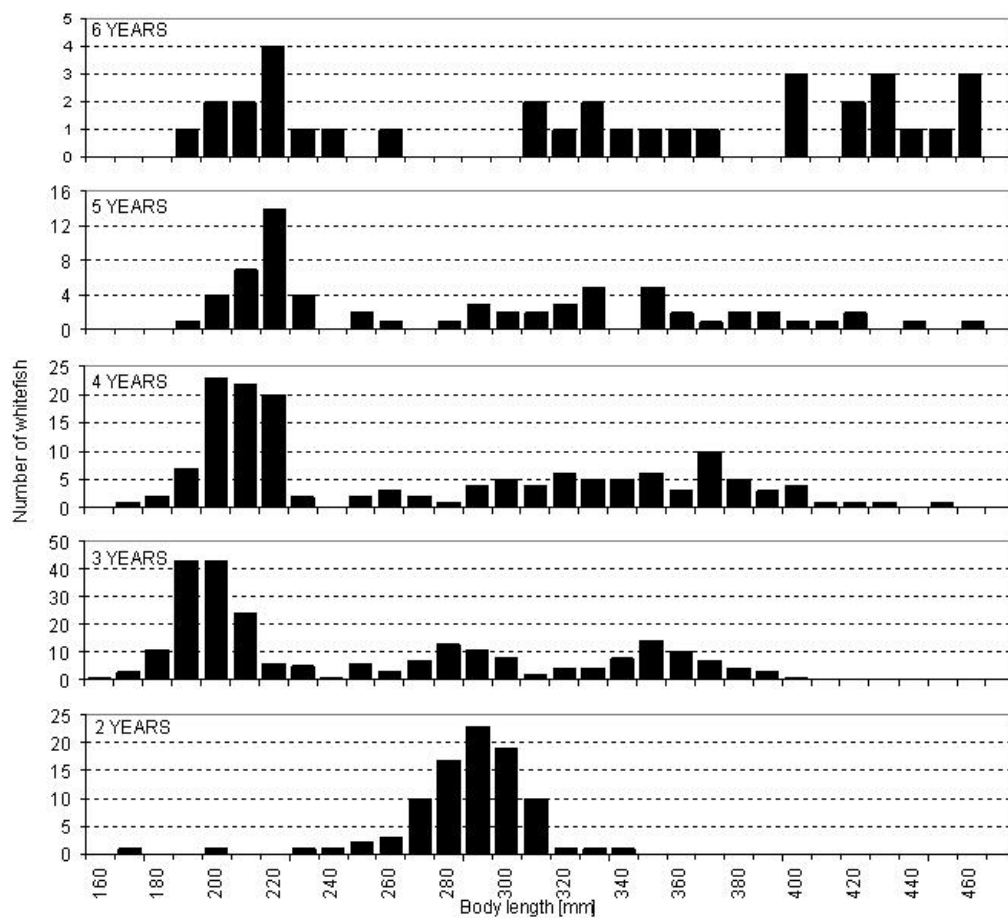
Figure S1. Body size histograms of 583 winter spawning whitefish from Lake Lucerne for the different age classes.
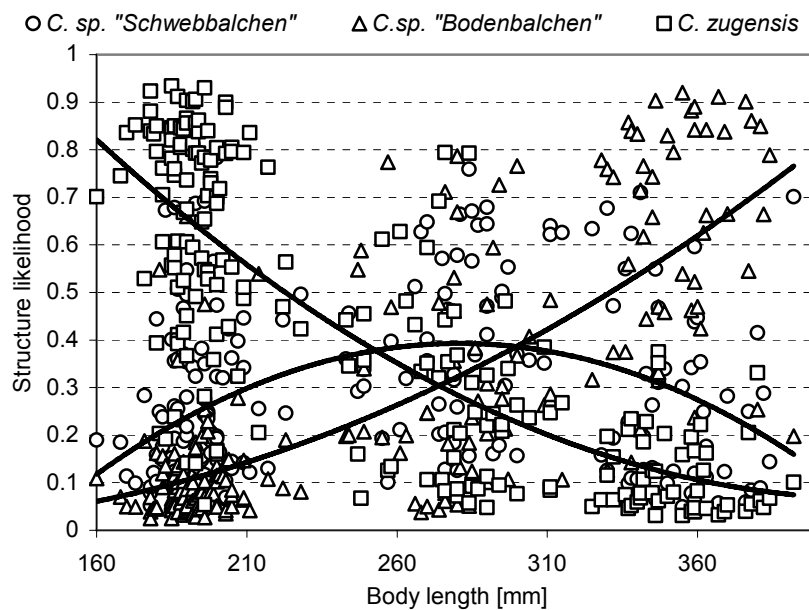
Figure S2. Structure likelihoods for K=3 populations computed over all whitefish samples (N=466) but shown for three-year-old fish only (N=212) against body length [mm]. Bold lines correspond to second degree polynomial fits for each group.

Chapter 5

Multidimensional ecological divergence and different stages of speciation in Alpine whitefish

Manuscript

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ABSTRACT

It is common to observe cases in nature where divergent selection generates only incomplete reproductive isolation and thus does not lead to speciation. This may be due to the nature of selection, namely due to variation in the strength of divergent selection or in the number of traits subjected to divergent selection. However, evidence for this remains scarce. Here we demonstrate the occurrence of different stages in speciation in the adaptive radiation of Alpine whitefish. Using partial Mantel correlations between genetic and ecological distances among individuals based on four ecological traits, we show that up to three different traits were significantly correlated to genetic differentiation. While the same traits are important in the majority of the cases, the relative importance of the different traits varies among lakes. We find that divergent selection during adaptive radiation likely acts on several ecological traits but we cannot distinguish between the multifarious and stronger selection hypotheses. As a consequence, any change in the environment that relaxes divergent selection on any one of the important ecological traits will weaken reproductive isolation among species.

INTRODUCTION

Investigating mechanisms of speciation is a central issue in evolutionary biology. In recent years, significant advances in understanding the processes leading to the origin of new species have been made (Coyne & Orr, 2004, Gavrilets, 2004, Butlin et al., 2009). Amongst other mechanisms, ecological speciation has received much attention (Schluter, 2000, Dieckmann et al., 2004, Gavrilets, 2004, Schluter, 2009). It posits that divergent selection between ecological niches drives the evolution of reproductive isolation, and predicts that ecologically divergent pairs of populations will exhibit greater levels of reproductive isolation than ecologically more similar populations (Schluter, 2000, Rundle & Nosil, 2005). It is however common to observe cases in nature where divergent selection generates only incomplete reproductive isolation, resulting in weak or no differentiation at neutral genetic loci (Rueffler et al., 2006, Nosil, 2007, Seehausen et al., 2008b). It generally remains unclear what factors explain this variation in the stages of ecological speciation (Nosil et al., 2009). Several non-ecological factors have been suggested, including the genetic architecture of traits under selection, mate choice traits, time since divergence started and the extend of gene flow (Kirkpatrick & Ravigne, 2002, Coyne & Orr, 2004, Gavrilets, 2004, Forister et al., 2007, Rasanen & Hendry, 2008). Alternatively, two ecological explanations for variation in the completeness of ecological speciation have been proposed (Nosil et al., 2009): (1) variation in the strength of divergent selection acting on a given trait ("stronger selection" hypothesis), i.e. that speciation is more likely to go to completion if selection is stronger on an individual trait; (2) variation in the number of traits that selection acts on ("multifarious selection" hypothesis), i.e. that speciation is more likely to go to completion if selection acts on a larger number of traits associated with multiple divergent niche dimensions. The first hypothesis is based on the expectation that stronger selection is more effective at generating reproductive isolation. The latter is based on the principle that different dimensions of divergent adaptation have additive effects on reproductive isolation (Nosil et al., 2009). In the adaptive landscape metaphor, the completion of speciation would in scenario (1) be the results of a deeper fitness valley between two adaptive peaks in a single dimension or, in scenario (2) the result of the generation of peaks that are separated in multiple fitness dimensions

(Nosil & Harmon, 2009). It is important to note that these two hypotheses do not mutually exclude each other, and it is even likely that both mechanisms operate simultaneously.

Several different stages of speciation are sometimes observed in the same species complex (Hendry *et al.*, 2002, Seehausen *et al.*, 2008b, Nosil & Sandoval, 2008, Nosil *et al.*, 2009, Berner *et al.*, 2009), making these examples powerful model systems for exploring the causes of variation in the progress towards speciation (Seehausen, 2009). A previously understudied system in this regard is the radiation of whitefish in central Europe. Whitefish radiations (*Coregonus clupeaformis* and *C. lavaretus* complexes) in the northern hemisphere are an emerging model system in ecological speciation and adaptive radiation research (Bernatchez, 2004, Hudson *et al.*, 2007). Whitefish have colonized freshwater areas of formerly glaciated regions that became ice free only 15'000-20'000 years ago (Steinmann, 1950, Taylor, 1999). Multiple species coexisting in several lakes across the northern hemisphere (Steinmann, 1950, Behnke, 1972, Reshetnikov, 1988, Pigeon *et al.*, 1997, Svärdson, 1998, Hudson *et al.*, 2007) and parallel evolution (Landry *et al.*, 2007, Ostbye *et al.*, 2006, Pigeon *et al.*, 1997) suggest that multiple independent adaptive radiations have occurred. Particularly dense geographical clustering of many independent lake radiations is found along the northern edge of the Alps in central Europe. Strong cytonuclear phylogenetic discordance observed all over the Alpine range suggests that two distant glacial refugial lineages hybridized in secondary contact prior to, or during the re-colonisation of the Alpine range (Hudson *et al.*, submitted). This admixed ancestral stock radiated independently in each of the larger lakes in the region, forming between one and five endemic species in each of them that are generally genomically monophyletic (Hudson *et al.*, submitted). The parallel evolution in sympatry of several phenotypically and genetically distinct whitefish species despite opportunity for gene flow suggests that divergent selection maintains the distinctiveness of species.

Adaptation to divergent food resources, *e.g.* benthic *versus* pelagic food items, has been suggested as the major driving force behind ecological speciation of fish in lakes (Schluter, 1996, Taylor, 1999), including whitefish (Bernatchez, 2004, Kahilainen & Ostbye, 2006). However, sympatric whitefish species are also divergent in several other ecological, behavioural and morphological aspects. Different sympatric species commonly spawn at different depths and at different times (Steinmann, 1950, Vonlanthen *et al.*, 2009), feed at different depths within the benthic (Rogers *et al.*, 2002, Helland *et al.*, 2007) or pelagic zones *(*Helland *et al.*, 2007) and show physiological adaptations to their foraging habitats (Rogers *et al.*, 2002, Ohlberger *et al.*, 2008,). Finally, there is evidence that different whitefish species may be affected differently by parasites (Valtonen *et al.*, 1988). Hence, it is likely that ecological speciation in whitefish is driven by multiple selection gradients, making this a suitable system to test whether variation in the completeness of speciation is associated with variation in the multifariousness of selection or in its overall strength. Estimates of selection are difficult to obtain and no data exist for whitefish. However, differences in phenotypic divergence may be taken as a surrogate for variation in selection. The sympatric whitefish species in central European lakes vary in growth rate, spawning time, spawning habitat, colour, morphology and gill raker number and density (Wagler, 1937, Rufli, 1978, Steinmann, 1950, Kottelat & Freyhof, 2007). Recent population genetic studies generally agreed with former taxonomic classifications, confirming that sympatric ecotypes are typically genetically differentiated species (Douglas *et al.*, 1999, Douglas & Brunner, 2002, Bittner, 2009, Hudson *et al.*, submitted). However, these studies have also shown that the degree of

divergence at neutral genetic loci between species within lakes or between lakes can vary by several orders of magnitude (Vonlanthen *et al.*, 2009, Bittner, 2009).

Here we use the central European whitefish system to test whether variation in the number of divergent ecological traits contributes to explain variation in the stages of speciation. We analysed population genetic structure and divergence in four ecological traits in fish from four large pre-alpine lakes. (1) *Spawning time*: it influences the time of fry emergence and might also influence survival through different mechanisms like food availability, predation or over wintering mortality (Cushing, 1990, Sogard, 1997, Svensson & Sinervo, 2000, Woods *et al.*, 2009). (2) *Spawning depth*: it requires physiological or behavioural adaptations to live at different water depths, either in adults (Weaver *et al.*, 1996, Rogers *et al.*, 2002), or in eggs (Woods *et al.*, 2009). (3) *Gill raker number*: it is linked to feeding ecology (Svärdson, 1979, Bernatchez, 2004, Kahilainen & Ostbye, 2006). (4) *Growth rate*: it reflects physiological adaptations to different feeding and predation regimes (Rogers *et al.*, 2002, Helland *et al.*, 2007, Ohlberger *et al.*, 2008). We first analyzed the population genetic structure of all species within the four studied lakes using ten neutral microsatellite loci. We then examined whether divergence in multiple ecological traits is implicated in shaping population genetic structure within lakes by applying an individual based partial Mantel correlation. We finally tested whether the multifarious selection hypothesis explains the different stages in speciation observed.

MATERIALS AND METHODS

Study system

Lake Neuchâtel
In Lake Neuchâtel whitefish genetic and morphological diversity is organised along a spawning depth gradient ranging from 2m depth to more than 90m depths (see Vonlanthen *et al.* 2009). Spawning takes place between the beginning of December and mid February. Originally, two species were described (*C. palea, C. candidus*). Phenotypes corresponding to these can today be found at the opposite extremes of the gradient.

Lake Lucerne
Lake Lucerne is believed to harbour in its main basin four endemic species, a winter littoral spawning large species (*C. sp "Bodenbalchen"*), a winter deep spawning small species (*C. zugensis*), a winter spawning species that spawns at intermediate depths (*C. sp. "Schwebbalchen"*) and a summer deep spawning species of intermediate size (*C. nobilis*) (Steinmann, 1950) (see Chapter 4).

Lake Walen
Lake Walen was proposed to harbour up to four whitefish species (Steinmann, 1950) but only two can still be distinguished today. The first is a small and deep spawning species with an extended spawning period ranging from August to December (*C. heglingus*). Steinmann (1950) distinguished two different species within this group, a summer and a winter spawning species. The second is a large species that spawns in winter in the littoral zone (*C. duplex*). Finally, a large species was thought to spawn at greater depths or even in pelagic areas of the lake (*C. zuerichensis*), but is no longer found today.

Lake Thun

Lake Thun is one of the most species rich lakes known in central Europe. Five distinct whitefish species have been observed (Bittner, 2009): a large winter littoral spawning whitefish (*C. sp "Balchen"*), an average sized winter spawning species that spawns in intermediate depths (*C. fatioi*), an average to small size winter deep spawning species (*C. sp. "Tiefenalbock"*), and two summer and deep spawning, small species (*C. albellus* and *C. alpinus*) where one feeds pelagically and the other benthically. In this study we did not include *C. sp. "Tiefenalbock"* because we could not obtain samples representative.

Sampling strategy

Fish were caught in their spawning season using benthic gill nets of different mesh sizes on spawning sites known to the commercial fishermen. There was one exception; the littoral sample (Sample 36, Table S1) from Lake Walen was caught from the lake shore at night using spot lights and seines. This is an old and traditional method to catch the littoral spawning species that spawn in less than 0.5m depth where gill netting is less efficient. To be able to test for a contribution of isolation by distance, we sampled each species from several spawning sites at distant geographical locations within the same lake. Based on reports of fishermen, an extended spawning period was expected for *C. heglingus* in Lake Walen. Sampling of the offshore area was thus repeated five times monthly between August and December. Additionally, a depth gradient was sampled at several times through one spawning season in Lake Lucerne (see Chapter 3.). We included only fish that were mature or very close to maturation (maturation degree 5 and 6 according to Smolina, 1920). Each fish was measured, weighed, a piece of muscle tissue was preserved in absolute ethanol for DNA analysis and scales were collected for determination of age and growth rate. A summary of the sampling design is given in Table S1 and locations are shown in Fig. 1.
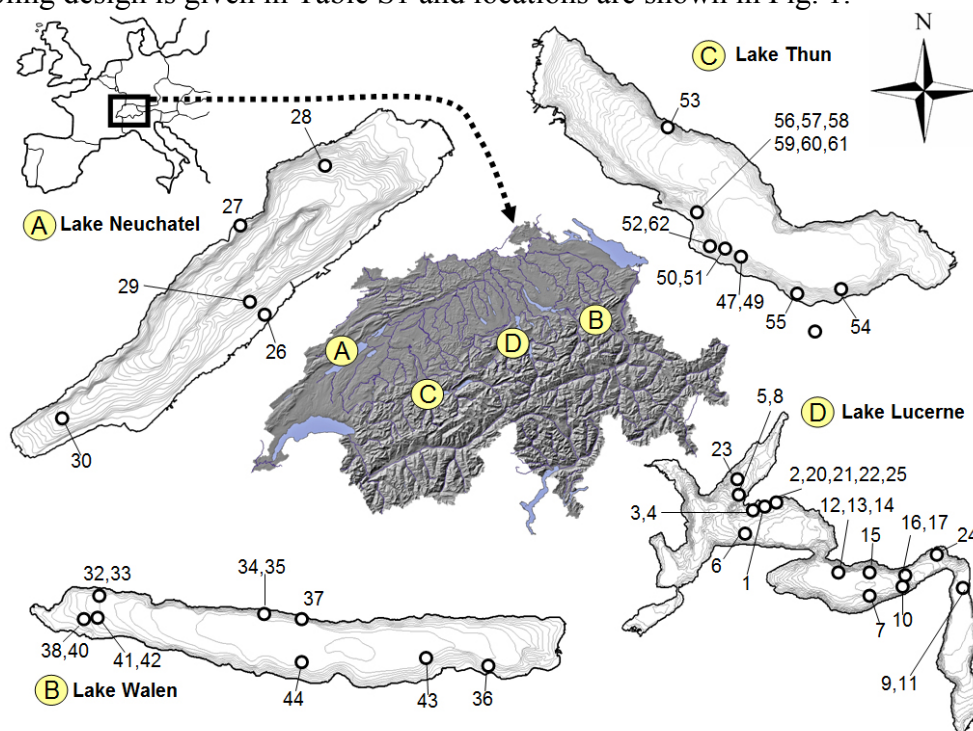

Figure 1. Map of Switzerland showing the sampling locations in the four studied lakes. Circles represent sampling sites and the associated numbers correspond to sample ID given in Table S1. Details about GPS locations of samples, depth of samples and sampling dates are shown in Table S1.

DNA extraction and microsatellite amplification

Total DNA was extracted using a QIAGEN BioSprint 96 extraction robot with corresponding standard extraction method. All specimens were genotyped at ten microsatellite markers (Patton *et al.*, 1997; Turgeon *et al.*, 1999; Rogers *et al.*, 2004). Forward primers of the following loci were labelled with fluorescent dyes: Cocl-Lav4, Cocl-Lav-49 and Cocl-Lav61 with Cy5; Cocl-Lav6, Cocl-Lav10, Cocl-Lav45, Cocl-Lav68 and BWF2 with Cy5.5; Cocl-Lav18 and Cisco-157 with Cy7. Because of overlapping allele size ranges of many markers, two different loci sets were generated for pre-PCR multiplexing. The first set included Cocl-Lav-49, Cocl-Lav61, Cocl-Lav6, Cocl-Lav68, Cocl-Lav10, and Cisco-157. The second set included Cocl-Lav4, Cocl-Lav224, BWF2, Cocl-Lav45 and Cocl-Lav18 (Bittner, 2009). Multiplex PCR amplification was performed using QIAGEN® (Basel, Switzerland) Multiplex PCR kit according to the manufacturer's protocols. PCR were carried out in 10μl reactions volumes containing 5μl QIAGEN Multiplex PCR Master mix, 3μl ddH$_2$O, 1μl DNA (20ng/μl) and 1μl primer mix (2pmol/μl each primer). The thermocycler profile began with an initial denaturation step at 95°C for 15min, followed by 30 cycles of 30 sec at 94°C, 90sec at 57°C, 90 sec at 72°C and ended with a final extension of 10 min at 72°C. Fragments from each PCR product was resolved on an automated capillary loading DNA sequencer (CEQ 8000, Beckmann and Coulter, Fullerton, CA, USA) following manufacturer protocols and guidelines and scored using the provided fragment analysis software (Beckman Coulter, Fullerton, CA, USA). All allele designations were verified by eye.

Fish morphology

Gill raker numbers were counted on the first gill arch from the left side of the head of each fish. The age and the growth rate of each fish were estimated scalimetrically, whereby fish size for each year was back-calculated using the distance between the scale centre and each annular ring. The relationship between length at age (L_i) and scale size was estimated by a linear regression using the following equation (Caranhac, 1999):

$$L_i = -\frac{a}{b} + \left(L_c + \frac{a}{b}\right) * \frac{R_i}{R_c}$$

where *a* and *b* are the intercept and the slope of the regression, R_c is the scale radius and R_i the distance from the scale centre to the year ring, L_c is the body length (Smale & Taylor, 1987, Caranhac, 1999).

Data analysis

Population genetic analyses

For population genetic analyses, the fish were clustered into putative species using morphology (mainly gill raker counts and back-calculated size at the age of three years), spawning location and spawning time. Expected (H_E) and observed (H_O) heterozygosities were calculated for every species using ARLEQUIN version 3.1 (Excoffier *et al.*, 2005). Deviations from Hardy-Weinberg equilibrium (HWE) were tested with exact tests (Guo & Thompson, 1992) for each locus and population using GENEPOP version 3.4 (Raymond & Rousset, 1995) with 1'000'000 steps in the Markov chain and 5'000 dememorization steps. Inbreeding coefficients (F_{IS}; Weir &

Cockerham, 1984) were calculated at each locus within each species and over all loci for all species and tested for significant deviation from zero using FSTAT version 2.9.3 (Goudet, 2001). Significance levels of both, HWE and F_{IS} were corrected for multiple comparisons using sequential Bonferroni corrections (Rice, 1989). FSTAT was also used to calculate allelic Richness (A_R), and the number of alleles (A_N). Deviations from linkage equilibrium between all pairs of loci for each population were estimated using ARLEQUIN.

The genetic differentiation among species was investigated by estimating multilocus F_{ST} values with 20'000 bootstrap replicates (Weir & Cockerham, 1984) using ARLEQUIN. To test the hypothesis that species of different ecotype within lakes are genetically closer than species of the same ecotype from different lakes, hierarchical analyses of molecular variance (Excoffier et al., 1992) were performed using ARLEQUIN. Two different models were tested: First, species were grouped by lake of origin; second, species were grouped by ecotype i.e in the following categories: small body size and deep spawning with numerous gill rakers; large body size and shallow spawning with few gill rakers; intermediate in body size, gill rakers and spawning depth; small body size, deep spawning and few gill rakers; and finally, intermediate body size, numerous gill rakers, summer and deep spawning. The exact groupings are given in the legend to Table 3. To visualize the phylogenetic relationships among species, a neighbour-joining (NJ) tree (Saitou & Nei, 1987) was generated based on Cavalli Sforza & Edwards chord distances (D_C; Cavalli-Sforza & Edwards, 1967). D_c was calculated in GENEDIST, the 1'000 Boostrap pseudo-replicates were performed across loci using SEQBOOT and the final tree was calculated using CONSENSE. All phylogenetic analyses were performed in PHYLIP version 3.65 (Beerli & Felsenstein, 2001).

Testing for divergence in multiple ecological traits
To test how many ecological traits explained a significant component of the genetic differentiation within the different lakes, we calculated partial Mantel correlations (Mantel, 1967, Smouse et al., 1986) between individual genetic distances and the individual Euclidean distances in each ecological trait. Individual genetic distances were used because the identification of populations to species is based on the same traits. Genetic distances among all individuals within each lake were calculated from microsatellite allele frequencies using Rousset's individual distance (Rousset, 2000) in SPAGEDHI version 1.2 (Hardy & Vekemans, 2002). The Mantel correlations were calculated using the software ZT as implemented in MANTEL TESTER (Bonnet & Van der Peer, 2002). Four ecological traits were chosen that were known to vary among species. The first two, spawning depth and spawning time are related primarily to reproductive ecology and phenology (Steinmann, 1950, Vonlanthen et al., 2009), while the other two, number of gill rakers and body size at the age of three years, are directly related to trophic ecology (Steinmann, 1950, Rogers et al., 2002, Bernatchez, 2004, Kahilainen & Ostbye, 2006, Ohlberger et al., 2008). Additionally, to test for the null hypothesis of isolation by distance, geographic distance among individuals was included in the Mantel correlations. Finally, Pearson correlations among the different trait were calculated to test how and which direction these are related.

Testing for "multifarious" versus "stronger" selection
Nosil and colleagues (2009) recently suggested a method to test the alternative, but not mutually exclusive, hypotheses of "more multifarious" *versus* "stronger" selection

explaining the progress towards speciation. Ideally, the test uses multiple species pairs for which experimental data exists that measures selection on multiple ecological traits (Nosil *et al.*, 2009). A principal component approach relating genetic differentiation to data from reciprocal transplant experiments was proposed to calculate the Levene's index as a measure of multfariousness of selection. Such experimental data does however not exist in most cases. Nosil *et al.* (2009) therefore suggested, as an alternative, to use trait divergence as a surrogate for divergent selection.

Here, we first tested whether the divergence in multiple ecological traits or rather variation therein could explain the different stages of speciation in whitefish. For this purpose, we used a multiple regression between F_{ST} and the divergence in the four ecological traits for a total of 14 sympatric (same lake) species pairs. Trait divergence for each species pair was calculated as the average difference in each of the traits (spawning time, spawning depth, gill rakers, and body size at the age of three years; Table 6). This average difference for each trait was then scaled to a value between zero and one, where one represents the maximal divergence observed among all pairwise comparisons.

To test for multifariousness of selection, we grouped the species pairs first into two (seven pairwise comparisons in each group) and then into three groups (four pairwise comparisons in each group, two were excluded to keep sample sizes constant) based on the degree of reproductive isolation observed (pairwise F_{ST} values). A principal component analysis was then performed on the variation in the four ecological traits for each group. The Levene's index was subsequently calculated from the variation explained by the different PC axes.

RESULTS

Population genetic analyses
No significant deviations from HWE and no significant F_{IS}-values were observed at any of the microsatellite loci in any of the species after sequential Bonferroni correction (Table 1). Forty-four (8.1%) tests for deviations from linkage equilibrium

Table 1. Population genetic summary statistics for 12 whitefish species from four lakes: Given are the sample sizes (N), observed (H_O) and expected heterozygosities (H_E) with corresponding P-value for deviations from HWE, allelic richness (A_R), the inbreeding coefficient (F_{IS}) with corresponding P-value level, and the number of deviations from linkage equilibrium out of a total of 45 pairs of loci observed at a significance level of $p<0.05$. After Bonferroni correction, none of the F_{IS} values and deviation from HWE remained significant.

Lake	Species	N	H_O	H_E	P-value	A_R	F_{IS}	P-value	N_{LD} $p<0.05$
Neuchâtel	*C. palea*	45	0.53	0.54	0.93	4.72	0.012	0.37	3
	C. candidus	111	0.56	0.55	0.72	4.86	-0.024	0.13	3
Walen	*C. duplex*	118	0.45	0.46	0.47	3.73	0.027	0.13	0
	C. heglingus	442	0.47	0.48	0.95	3.84	0.03	0.01	2
Lucerne	*C. sp. "Bodenbalchen"*	131	0.58	0.58	0.77	4.84	0.009	0.30	2
	C. sp. "Schwebbalchen"	118	0.57	0.59	0.02	5.43	0.027	0.08	8
	C. zugensis	270	0.48	0.49	0.19	5.02	0.027	0.03	3
	C. nobilis	83	0.48	0.48	0.79	4.05	-0.006	0.43	3
Thun	*C. sp. "Balchen"*	30	0.52	0.52	0.52	4.26	0.003	0.45	2
	C. fatioi	85	0.53	0.54	0.35	4.27	0.019	0.23	6
	C. albellus	60	0.46	0.48	0.38	4.15	0.037	0.13	5
	C. alpinus	77	0.49	0.47	0.37	3.37	-0.034	0.14	7
Total/		1570	0.51	0.52		5.28			44 (8.1%)

Table 2. Pairwise F_{ST} values observed among all whitefish species based on ten microsatellite loci are shown below diagonal and the corresponding significance levels above the diagonal.

Lake	Nr	Species	A	B	C	D	E	F	G	H	I	J	K	L
Neuchâtel	A	C. palea	-	***	***	***	***	***	***	***	***	***	***	***
	B	C. candidus	0.05	-	***	***	***	***	***	***	***	***	***	***
Walen	C	C. duplex	0.13	0.05	-	***	***	***	***	***	***	***	***	***
	D	C. heglingus	0.22	0.12	0.13	-	***	***	***	***	***	***	***	***
Luceme	E	C. sp "Bodenbalchen"	0.07	0.06	0.11	0.18	-	***	***	***	***	***	***	***
	F	C. sp. "Schwebbalchen"	0.10	0.04	0.08	0.10	0.04	-	***	***	***	***	***	***
	G	C. zugensis	0.19	0.09	0.14	0.10	0.13	0.04	-	***	***	***	***	***
	H	C. nobilis	0.16	0.08	0.14	0.10	0.10	0.04	0.03	-	***	***	***	***
Thun	I	C. sp. "Balchen"	0.08	0.09	0.16	0.22	0.09	0.10	0.20	0.19	-	*	***	***
	J	C. fatioi	0.05	0.07	0.12	0.19	0.07	0.07	0.17	0.15	0.01	-	***	***
	K	C. albellus	0.13	0.12	0.17	0.17	0.13	0.11	0.18	0.16	0.16	0.12	-	***
	L	C. alpinus	0.08	0.05	0.09	0.21	0.07	0.08	0.16	0.15	0.11	0.08	0.15	-

*** $p<0.001$; ** $p<0.01$; * $p<0.05$; n.s.=not significant

Table 3. Summary of the AMOVA results. Species were either not grouped or grouped by their lake of origin or by ecotype (Grouping). Shown are the F_{CT} (the variance explained among groups), the F_{SC} (the variance explained among populations within groups), the F_{ST} (global F_{ST} among all populations with no grouping), and all corresponding P-values. Grouping by lakes lead to the following five groups: Group 1: A, B; Group 2: C, D; Group 3: E, F, G, H; Group 4: I, J, K, L. Grouping by ecotype lead to the following groups: Group 1:A, C, E, I; Group 2: B, D, G, K; Group 3: F, J; Group 4: H; Group 5:L. A label to species designation is given in Table 2.

Grouping	F_{CT}	df	P-value	F_{SC}	df	P-value	F_{ST}	d.f.	p-value
No grouping	-	-	-	-	-	-	0.121	11	<0.001
By lake	0.042	3	<0.05	0.089	8	p<0.001	-	-	-
By ecotype	0.019	4	n.s.	0.108	7	p<0.001	-	-	-

Table 4. Results of the Mantel correlations calculated among whitefish individual pairwise trait divergence and Pearson correlations among individuals traits for Lake Thun, Lake Neuchâtel, Lake Lucerne and Lake Walen (below the diagonal) with corresponding significance levels (above the diagonal: *** = $p<0.001$; ** = $p<0.01$; * = $p<0.05$; n.s.=non significant).

		Mantel correlations				Pearson correlations			
		Gill rakers	Spawning time	Spawning depth	Body size	Gill rakers	Spawning time	Spawning depth	Body size
Lake Thun	Gill rakers	-	***	***	n.s.	-	***	n.s.	*
	Spawning time	0.155	-	***	***	0.334	-	***	***
	Spawning depth	0.089	0.617	-	***	-0.025	-0.771	-	***
	Body size	0.015	0.579	0.367	-	0.161	0.787	-0.666	-
Lake Neuchatel	Gill rakers	-	***	***	***	-	***	***	***
	Spawning time	0.341	-	***	***	0.567	-	***	***
	Spawning depth	0.450	0.783	-	***	0.640	0.899	-	***
	Body size	0.016	0.492	0.668	-	-0.520	-0.699	-0.795	-
Lake Lucerne	Gill rakers	-	***	***	***	-	***	***	***
	Spawning time	-0.075	-	***	***	-0.231	-	***	**
	Spawning depth	0.085	0.928	-	*	0.455	-0.912	-	***
	Body size	0.604	-0.093	0.055	-	-0.795	0.141	-0.376	-
Lake Walen	Gill rakers	-	***	***	***	-	***	***	***
	Spawning time	0.254	-	***	***	-0.600	-	***	***
	Spawning depth	0.778	0.390	-	***	0.872	-0.621	-	***
	Body size	0.691	0.207	0.777	-	-0.811	0.564	-0.876	-

were significant at $p<0.05$. Linkage disequilibrium was especially high in Lake Thun species (20 deviations observed instead of nine that would be expected by chance) and in *C. sp. "Schwebbalchen"* of Lake Lucerne. Occasional interspecific gene flow is a likely explanation for the deviations. Gene flow between *C. alpinus* and *C. albellus* in Lake Thun has indeed been demonstrated (Bittner, 2009), and there is likely also gene flow between *C. sp. "Schwebbalchen" and C. sp. "Bodenbalchen"* in Lake Lucerne (see Chapter 3). The ten loci showed a wide range of variability, with five to 20 alleles observed within species and 126 alleles over all loci and all species. Within species, the mean number of alleles per locus ranged from 3.9 to 8.1, H_E ranged from 0.46 to 0.59, and A_R ranged from 3.37 to 5.43 (Table 1). We found low to high levels of genetic differentiation between species with pairwise F_{ST} values ranging between 0.01 and 0.22 (Table 2). The AMOVA results show that structuring samples by lake explained more genetic variation than structuring by ecotype (Table 3), consistent with the hypothesis that every lake contains a replicate adaptive radiation, sometimes referred to as the "species flock" scenario (Douglas *et al.*, 1999). The neighbour-joining tree confirms this pattern where each lake is monophyletic (Fig. 2). The statistical support for the lake-monophyly is however not very strong.

Figure 2. Neighbour-joing tree for twelve whitefish species collected in five different pre-alpine lakes based on Cavalli-Sforza *D*c distances (Cavalli-Sforza & Edwards, 1967) derived from ten microsatellite loci. Bootstrap values based on 1000 replicates are given for each branch.

Testing for divergence in multiple ecological traits
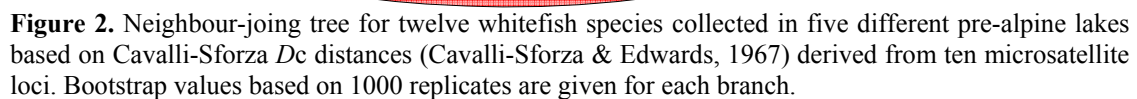
Pairwise Mantel correlations among the four different niche-related traits reveal significant correlations in every lake (Table 4), even though strength of correlations varies among lakes. Using partial Mantel correlations, significant correlations between ecological and genetic distances were observed in all four lakes whereas isolation by distance was not observed in any of the lakes (Table 5). Three traits were significant in Lakes Walen and Thun, two traits in Lake Lucerne and one trait in Lake Neuchâtel. Among all traits, spawning depth and gill rakers affected genetic distance in three out of four lakes, size in two lakes, and spawning time only in Lake Thun.

Table 5. Mantel and partial Mantel correlations of Rousset's individual genetic distance (Rousset, 2000) and individual Euclidean distances of gill raker counts, body length at the age of three years, spawning depth and spawning time. Shown are the lakes analysed (Lake), the sample size (N), the first significant axis (Factor 1) with corresponding correlation coefficient (r) and significance level (P-value). The same results are given for each round of partial mantel correlations and summed for the total r estimation (Sum r).

Lake	N	Factor 1	r	P-value	Factor 2 and controlling for Factor 1	r	P-value	Factor 3 and controlling for Factors 1+2	r	P-value	Sum r
Thun	179	Spawning time	0.204	<0.001	Gill rakers	0.103	<0.001	Depth	0.085	<0.001	0.392
Neuchâtel	118	Depth	0.140	<0.01	-	-	-	-	-	-	0.140
Walen	183	Depth	0.319	<0.001	Size	0.108	<0.01	Gill rakers	0.071	<0.05	0.498
VWS	426	Size	0.363	<0.001	Gill rakers	0.107	<0.001	-	-	-	0.470

Testing for "multifarious" versus "stronger" selection
The multiple regression calculated between pairwise genetic distance and the divergence in the four ecological traits (Table S2) was significant for gill rakers (N=14; $R2=0.49$, $p<0.01$; Fig. 3a). None of the three remaining traits (pawning depth, spawning time, and body size) explained any significant residual variance. The principal component analyses performed on differences in the four traits shows that multiple PC axes explain a significant proportion of variance in niche divergence between species (Fig. 4a,b). The Levene's indexes calculated from these results were higher when genetic differentiation was higher (Fig 4c,f), consistent with the hypothesis of multifarious niche divergence.
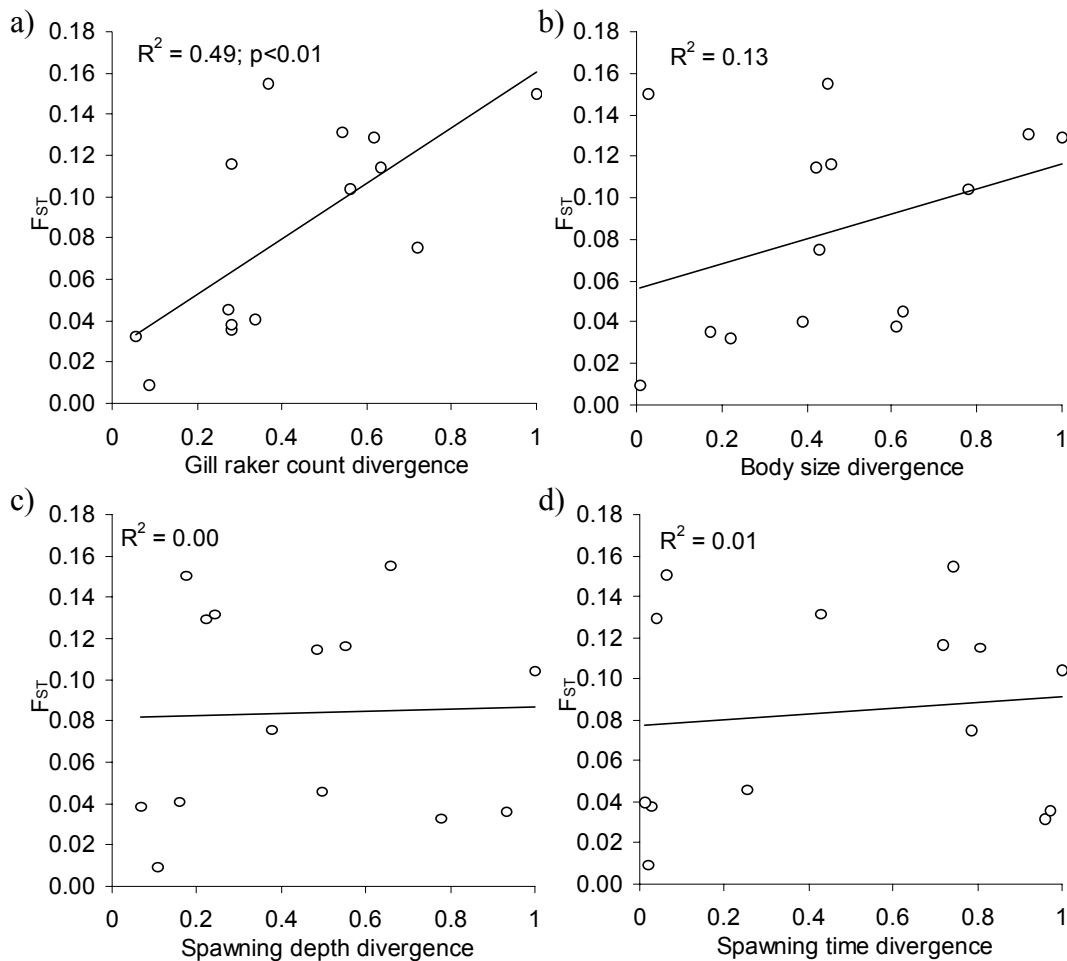
Figure 3. Linear regession between gill raker divergence (a), body size divergence (b), spawning depth divergence (c), and spawning time divergence of 14 sympatric species pairs; and pairwise genetic differentiation (F_{ST}).

DISCUSSION

We analysed population genetic, meristic and ecological data from 1570 whitefish sampled from four different lakes in central Europe. The analysis of molecular variance and the phylogenetic tree (based on Cavalli-Sforza chord distances) shows that divergent ecotypes from the same lake are generally more closely related than similar ecotypes from different lakes, suggesting, congruent with other studies (Douglas *et al.*, 1999, Hudson *et al.*, submitted),that species diversity has evolved
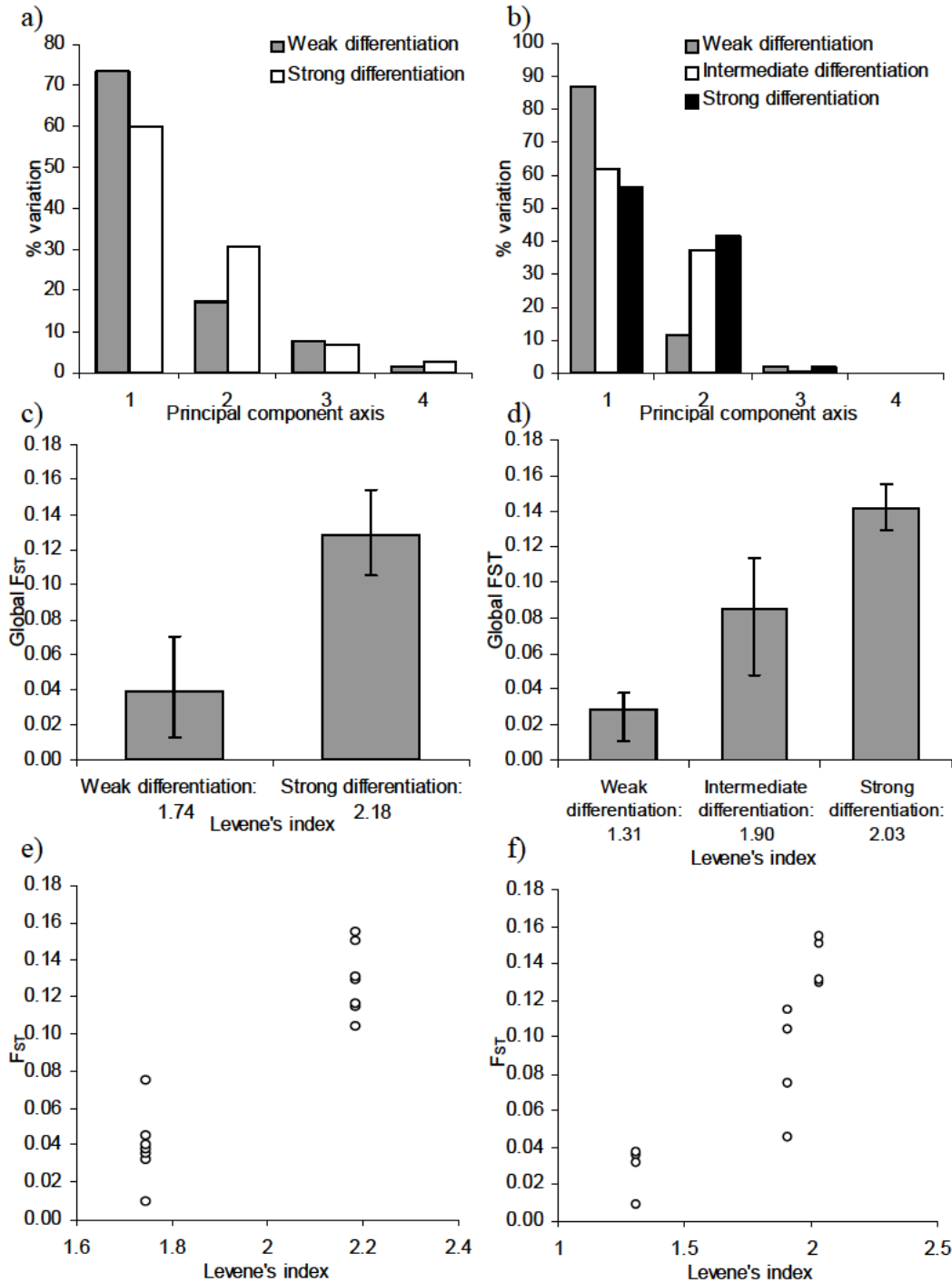
Figure 4. Tests for multifarious selection. (a,b) Percent variation between species differences explained by the different principal component axes calculated from scaled euclidean distances from four traits (Spawning day, Spawning depth, Gill rakers, and Body size) for weakly, intermediately and strongly differentiated species pairs. (c,d) Average pairwise F_{ST} observed with corresponding Levene's Index as measure for multifariousness of niche divergence. (e,f) Linear regression calculated between pairwise F_{ST} values and Levene's Index. A positive relationship indicates that divergence between species is indicative for multifarious niche divergence.

through replicate adaptive radiations. The population genetic analyses further revealed highly variable degrees of neutral genetic differentiation between species both within and among lakes, confirming the existence of different stages in speciation of different whitefish species and incipient species pairs. Using partial Mantel correlations between genetic and ecological distances among individuals, and thus removing confounding effects of non-independence of the different traits, we show that up to three different ecological traits significantly explain genetic differentiation of sympatric species within lakes. We find that while some traits are important in most lakes, the relative importance of the different traits differs among lakes. Furthermore, we find a positive relationship between the Levene's Index of dimensionality of phenotypic divergence and the stages towards speciation. This suggests that divergent selection acts on multiple ecological traits, but the analysis neither allows distinguishing between multifarious and stronger selection in individual speciation events, nor between these two possibilities and selection in different directions in different speciation events. Altogether, these results suggest that any change in the environment that relaxes divergent selection along either one of the important ecological dimensions will weaken reproductive isolation and genetic differentiation among species

Ecological dimensions of divergence
We found strong correlations among the four ecological variables showing that the four niche axes may not be independent from each other (Table 4). For example, spawning time and spawning depth seem always correlated, and sometimes very tightly. This is partly because summer spawning whitefish tend to spawn deeper than winter spawning whitefish, and this results in a negative correlation in all lakes that have summer spawning whitefish (lakes Lucerne, Thun, and Walen). In Lake Neuchâtel where no summer spawning occurs, this correlation is positive, suggesting that spawning first occurs shallow and becomes deeper as time advances. On the other hand, spawning depth is negatively correlated with body size in all lakes, suggesting that deep spawning whitefish tend to have smaller body sizes than shallow spawning whitefish. Additionally, in some lakes (lakes Lucerne, Walen, Neuchâtel) gill raker numbers are in some cases (in lakes Lucerne, Walen, Neuchâtel) strongly negatively correlated with body size, suggesting that adaptation to different feeding niches involves divergent selection both on gill raker morphology and size.

The Mantel regression analyses relating genetic differentiation among individuals and differentiation in the four ecological traits that all have significant heritable components (Rogers *et al.*, 2002, Bernatchez, 2004, Rogers *et al.*, 2007), show that multiple phenotypic and ecological dimensions of divergence are implicated in whitefish speciation. While spawning depth explained most of the variation in the genetic differentiation of whitefish in lakes Walen and Neuchâtel, spawning time explained more in Lake Thun, and body size in Lake Lucerne whitefish. Among the four niche dimensions, the water depth of the spawning site explained genetic differentiation in three of the four studied lakes, and was the dominant dimension in two of the lakes. Gill rakers explained genetic differentiation in three of four lakes, but it was never the dominant trait. Body size explained genetic differentiation in two lakes, and was the dominant trait in one, and spawning time explained genetic differentiation in one lake where it was also the dominant trait. All traits therefore contributed to genetic differentiation among individuals at least in some lakes. The observation that the traits explaining most variation in individual genetic differentiation and that correlations among these traits differ markedly among lakes could have two alternative but non-exclusive explanations. First, whitefish populations

in different lakes could experience different adaptive landscapes. Alternatively, whitefish populations could evolve differently in each lake in response to similar adaptive landscapes. The former case would imply different ecological conditions, while the latter would imply different genetic constraints. Both mechanisms are likely to be important in shaping adaptive radiations, but all radiations included in this study share either a common ancestral population, or closely related ancestral populations of similar genetic composition (Hudson *et al.*, submitted). Different adaptive landscapes seem therefore to be a more parsimonious explanation for the differences observed.

Evidence for multifarious selection in whitefish speciation?

The multiple regression analysis between genetic differentiation measured in several sympatric species pairs and their divergence in four ecological traits revealed significant correlations only with gill rakers (Fig. 3). This may suggest that stronger divergence in the single ecological dimension of gill raker counts best explains the observed variation in the stages of speciation. This result is consistent with the suggestion that divergence in gill raker counts might be associated with adaptation to divergent feeding resources (Bernatchez, 2004, Kahilainen & Ostbye, 2006), which is purported to be the main axis of ecological divergence in whitefish (Svärdson, 1979). An alternative possibility could be that other axes of ecological divergence vary more strongly between lakes lakes, and that this analysis which is based on sympatric species pairs and tests for relationships across lakes, can therefore not detect other axes of ecological trait divergence. The partial mantel correlation results obtained on individuals, showing that individual genetic divergence within lakes is indeed explained by multiple ecologically divergent traits, tends to supports this view.

Independently of whether niche divergence is multidimensional or not, divergent selection explaining variation in stages of speciation can either be variable in its "multifariousness" or in its "strength" (Nosil *et al.*, 2009). Our results show a positive relationship between the Levene's Index as estimation of the multifariousness of selection and genetic differentiation between whitefish species (Fig. 4). This may be consistent with the hypothesis that the stage of speciation is predicted by multifarious selection (Nosil *et al.*, 2009). However, considering how divergence in different traits influences a principal component analysis of multiple species pairs, a higher Levene's Index does not necessarily represent evidence for multifarious selection on any given speciation event. Indeed the Levene's Index is expected to be high when pairwise differences are highest in different traits in different species pairs, which would be consistent with stronger selection on a single trait in each given speciation event, but on different traits in different speciation events. The higher Levene's Index observed when genetic differentiation is also high might thus either indicate stronger divergent selection on different traits, or more multifarious selection. Either way, they analysis suggests that multiple axes of divergent selection are implicated in whitefish adaptive radiation.

The inference of selection from phenotypic and environmental data however suffers from additional limitations. Similar to the Q_{ST}-F_{ST} comparisons for inferring selection (Whitlock, 2008), it potentially confounds effects of additive genetic divergence with environmental effects. Furthermore, it assumes that the taxon pairs do not differ strongly in the genetic variance-covariance (G) matrix because trait divergence is a function of both selection and the G matrix (Lande, 1979, Schluter, 2000, Nosil & Harmon, 2009). It however seems rather unlikely that these limitations constrain the interpretation for whitefish radiations. First, all traits included in this study have been demonstrated to have a strong heritable component in North American

lake whitefish (Rogers *et al.*, 2002, Bernatchez, 2004, Rogers *et al.*, 2007). Second similar demographic history for all central European radiations can be expected which makes it rather unlikely that G matrices differ markedly among radiations and species pairs (Douglas *et al.*, 1999, Hudson *et al.*, submitted; and results shown above).

Diversity loss due to break down of niche dimensions?

Our results have important implications for conservation biology and fisheries management. The data suggest that any change in the environment that relaxes divergent selection on either one of the important traits will weaken reproductive isolation and genetic differentiation among species (Seehausen *et al.*, 2008a, Schluter, 2009,). This has indeed occurred in many central European lakes, where eutrophication has led to a partial or complete collapse of at least one of the major dimensions of species divergence, namely spawning depth, and this has caused a sudden erosion of species distinctiveness and associated functional phenotypic diversity by increasing gene flow between species (see Chapter 6). Organic pollution lead to an increase in bioavailable phosphorous, which in turn lead to increased productivity and associated increase in oxygen demand at the sediment surface due to decomposition processes. The resulting oxygen depletion, which is particularly strong at greater depths, shortened the spawning depth gradients and reduced temporal and spatial pre-mating isolation (see Chapter 6). Furthermore, the increased productivity lead to faster growth in some small whitefish species (Kirchhofer, 1995, Muller & Bia, 1998) thus reducing the size differences among species. Organic pollution has caused partial collapse of three of the four niche dimensions that we identified as important in speciation, likely explaining loss of species diversity through speciation reversal in all polluted lakes.

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SUPPLEMENTARY MATERIAL

Table S1. Summary of the samples included in this study. The sample number corresponds to the numbers in Fig. 1. The letters correspond to the following species: Lake Neuchâtel: A) *C.palea*; B) *C. candidus*; Lake Walen: C) *C. duplex*; D) *C. heglingus*; Lake Lucerne: E) *C. sp "Bodenbalchen"*; F) *C. sp. "Schwebbalchen"*; G) *C. zugensis*; H) *C. nobilis*; Lake Thun: I) *C. sp. "Balchen"*; J) *C. fatioi*; K) *C. albellus*; L) *C. alpinus*. Shown are also the GPS positions of each sampling site, the sampling dates and depths. Finally, the number of samples included in population genetic analyses (N Genetics) and for the individual Mantel correlations (N Mantel) are given. The number of individuals included in Mantel correlations was lower than that for the species analyses because fish that were not caught on their spawning grounds, and fish with missing data were excluded.

Lake	Sample Number	Species	GPS North	GPS East	Sampling Date	Sampling Depth	N Genetics	N Mantel
Lucerne	1	E, F, G	47°01'36.37"	8°25'31.56"	19.11-18.12.2007	2, 10, 20, 30, 40	176	164
	2	E	47°01'36.37"	8°25'31.56"	31.12.2007	2	8	8
	3	E,F	47°01'34.00"	8°24'47.00"	19.11-18.12.2007	10	20	-
	4	G	47°01'32.54"	8°24'39.45"	19.11.2004	40	22	-
	5	E	47°01'44.71"	8°23'42.23"	10.12.2004	8	11	-
	6	E,F	47°00'20.92"	8°23'38.15"	06.07.2005	10	7	-
	7	E,F	46°58'06.61"	8°30'50.44"	31.08.2005	10	7	-
	8	E,F	47°01'44.71"	8°23'42.23"	13.12.2005	5	41	41
	9	E,F	46°58'24.01"	8°36'29.52"	14.12.2005	3	30	28
	10	E,F	46°58'31.81"	8°33'05.74"	22.12.2005	3.5	30	29
	11	E,F	46°58'24.01"	8°36'29.52"	22.12.2005	3.5	31	30
	12	H	46°59'16.99"	8°28'55.53"	29.07.2004		22	-
	13	H	46°59'16.99"	8°28'55.53"	21.07.2005	165	9	5
	14	H	46°59'16.99"	8°28'55.53"	29.07.2005	180	8	8
	15	H	46°59'13.75"	8°31'00.88"	02.08.2005	165	21	19
	16	H	46°59'09.78"	8°33'24.80"	10.08.2005	150	10	9
	17	H	46°59'09.78"	8°33'24.80"	07.08.2006	150	13	-
	18	G	NA	NA	Aug. 2005	NA	4	-
	19	G	NA	NA	06.07.2005	NA	9	-
	20	G	47°01'40.95"	8°25'42.53"	13.07.2005	NA	16	-
	21	G	47°01'40.95"	8°25'42.53"	07.09.2005	NA	11	-
	22	G	47°01'40.95"	8°25'42.53"	21.11.2005	40	25	22
	23	G	47°02'37.04"	8°23'11.80"	14.12.2005	50	31	30
	24	G	46°59'53.11"	8°35'05.27"	21.12.2005	50	9	7
	25	G	47°01'37.77"	8°25'58.94"	29.12.2005	60	31	26
							602	**426**
Neuchâtel	26	A	46°52'26.05"	6°52'08.06"	7.12 2005 - 12.01.2006	2	24	23
	27	A	46°56'06.84"	6°50'35.87"	09.12.2004	2	21	-
	28	B	46°58'57.06"	6°57'12.99"	19.01.2006	95	20	28
	29	B	46°53'04.88"	6°51'19.15"	7.12 2005 - 12.01.2006	60	52	38
	30	B	46°48'52.78"	6°42'08.90"	19.01.2006	95	30	29
							147	**118**

Lake	Sample Number	Species	GPS		Sampling date	Sampling depth	N Genetics	N Mantel
			North	East				
Walen	31	C	NA	NA	09.08.2005	NA		-
	32	C	47° 8'19.66"	9°07'19.45"	07.12.2005	10	19	9
	33	C	47° 8'19.66"	9°07'19.45"	01.12.2006	10	10	1
	34	C	47° 7'51.11"	9°11'53.83"	Dec.2006	2	21	20
	35	C	47° 7'51.11"	9°11'53.83"	Dec.2006	2	14	14
	36	C	47° 6'56.05"	9°16'07.39"	Dec.2006	NA	24	-
	37	C	47° 7'44.20"	9°12'28.82"	14.12.2004	7	20	14
	38	D	47° 7'47.60"	9°07'08.16"	09.08.2005	45	15	14
	39	D	NA	NA	Aug 1999/2000	50	24	-
	40	D	47° 7'47.60"	9°07'08.16"	09.08.2005	45	15	14
	41	D	47° 7'51.71"	9°07'26.92"	15.09.2006	45	142	22
	42	D	47° 7'51.71"	9°07'26.92"	18.10.2006	50	95	8
	43	D	47° 7'05.24"	9°14'58.07"	01.11.2006	38	135	55
	44	D	47° 7'01.82"	9°12'21.73"	14.12.2004	55	16	12
							560	**183**
Thun	45	J	NA	NA	04.08.2005	NA	25	-
	46	J	NA	NA	04.08.2005	NA	9	-
	47	L	46°40'11.82"	7°43'37.84"	06.09.2005	115	36	29
	48	J	NA	NA	06.09.2005	NA	1	-
	49	K	46°40'11.82"	7°43'37.84"	06.09.2005	115	31	27
	50	L	46°40'21.42"	7°43'16.03"	08.09.2005	65	7	6
	51	K	46°40'21.42"	7°43'16.03"	08.09.2005	65	8	5
	52	J	46°40'31.03"	7°42'34.04"	16.12.2005	30	33	30
	53	J	46°42'53.82"	7°41'33.39"	16.12.2005	4	17	16
	54	I	46°39'40.13"	7°47'39.22"	19.12.2005	4	10	10
	55	I	46°39'25.96"	7°45'08.50"	19.12.2005	4	20	18
	56	L	46°41'03.92"	7°42'20.26"	21.12.2005	65	3	-
	57	K	46°41'03.92"	7°42'20.26"	21.12.2005	65	11	1
	58	K,L	46°41'03.92"	7°42'20.26"	11.08.2006	42.5		13
	59	L	46°41'03.92"	7°42'20.26"	23.08.2006	42.5	23	9
	60	L	46°41'03.92"	7°42'20.26"	01.09.2006	65	8	5
	61	K	46°41'03.92"	7°42'20.26"	04.09.2006	125	10	10
	62	J	46°40'31.03"	7°42'34.04"	16.12.2004	NA		-
Total							**252**	**179**

Table S2. Summary of pariwise population genetic distances and scaled average trait divergence between species pairs.

Species 1	Species 2	Pairwise F_{ST}	Day	Depth	Gill raker	Body size
C. sp. "Balchen"	*C. fatioi*	0.01	0.022	0.106	0.086	0.007
C. zugensis	*C. nobilis*	0.03	0.961	0.776	0.054	0.219
C. sp. "Schwebbalchen"	*C. nobilis*	0.04	0.973	0.934	0.281	0.172
C. sp "Bodenbalchen"	*C. sp. "Schwebbalchen"*	0.04	0.027	0.066	0.280	0.609
C. sp. "Schwebbalchen"	*C. zugensis*	0.04	0.012	0.158	0.335	0.391
C. palea	*C. candidus*	0.05	0.252	0.496	0.271	0.625
C. fatioi	*C. alpinus*	0.08	0.785	0.377	0.720	0.429
C. sp "Bodenbalchen"	*C. nobilis*	0.10	1.000	1.000	0.561	0.781
C. sp. "Balchen"	*C. alpinus*	0.11	0.806	0.483	0.634	0.423
C. fatioi	*C. albellus*	0.12	0.720	0.551	0.280	0.456
C. sp "Bodenbalchen"	*C. zugensis*	0.13	0.039	0.224	0.616	1.000
C. duplex	*C. heglingus*	0.13	0.430	0.242	0.542	0.920
C. albellus	*C. alpinus*	0.15	0.065	0.174	1.000	0.026
C. sp. "Balchen"	*C. albellus*	0.16	0.741	0.658	0.366	0.449

Chapter 6

Anthropogenic eutrophication drives mass extinction by speciation reversal in adaptive radiations

Submitted

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ABSTRACT

Biodiversity provides innumerable cultural and economic benefits to humans (Singh, 2002, Worm *et al*., 2006) but human activity is increasing the rate at which species go extinct (Sala *et al*., 2000, Thomas *et al*., 2004). Meeting the critical challenge of reducing extinction rates requires identifying and protecting the ecological conditions and evolutionary processes which produce and maintain biological diversity (Chapin *et al*., 2000, Rosenzweig, 2001). Species can go extinct through two fundamentally different processes, by the sequential extirpation of populations due to habitat loss or overexploitation, or by loss of genetic distinctiveness through introgressive hybridization (Rhymer & Simberloff, 1996). A special case of the latter is speciation reversal (Seehausen, 2006), whereby changes in the form or strength of natural selection increase introgressive hybridization among historically sympatric species, thus eroding the ecological and genetic differences that distinguish closely related but ecologically divergent species (Seehausen *et al*., 1997, Taylor *et al*., 2006). We analysed historical and modern data on species richness in replicated adaptive radiations of salmonid fish (whitefish; *Coregonus* spp.) in central European lakes and provide evidence that anthropogenic eutrophication drove a mass extinction through speciation reversal by reducing the ecological niche space that generated these species.

INTRODUCTION AND METHODS

Adaptive radiation involves the evolution of ecological diversity within rapidly multiplying lineages (Schluter, 2000). The resulting diversity is often the outcome of ecological speciation, whereby traits under divergent natural selection, or genetically correlated traits, contribute to reproductive isolation (Schluter, 2000, Coyne & Orr, 2004, Rundle & Nosil, 2005, Schluter, 2009, Gavrilets & Losos, 2009). When reproductive isolation between ecologically differentiated populations is maintained by the temporal or spatial clustering of breeding aggregations, adaptive radiation occurs through the correlated partitioning of ecological and reproductive niche space. Because speciation occurs rapidly during adaptive radiation, gene flow between species is not typically inhibited by intrinsic post-zygotic isolation but rather by divergent natural and sexual selection (Schluter, 2009). Environmental changes that reduce the diversity of ecological or reproductive niches will thus relax the selective forces that drive speciation, causing extinction by speciation reversal (Seehausen, 2006, Hendry *et al*., 2006, Taylor *et al*., 2006).

Fishes of recently de-glaciated lakes of the northern hemisphere have become model systems to study adaptive radiation because several evolutionary lineages are undergoing adaptive radiation in multiple lakes (Schluter, 1996, Rundle *et al*., 2000, Ostbye *et al*., 2006). These radiations are characterized by the correlated partitioning of ecological (food and foraging habitat) and reproductive (time and location) niche space (McPhail, 1993, Skulason & Smith, 1995, Schluter, 1996, Bernatchez, 2004). Though it is clear that organic pollution alters freshwater ecosystems and fish communities world-wide (Carpenter *et al*., 1998, Verschuren *et al*., 2002), it is unclear and underappreciated how it affects adaptive radiations of lacustrine fishes (Seehausen *et al*., 1997).
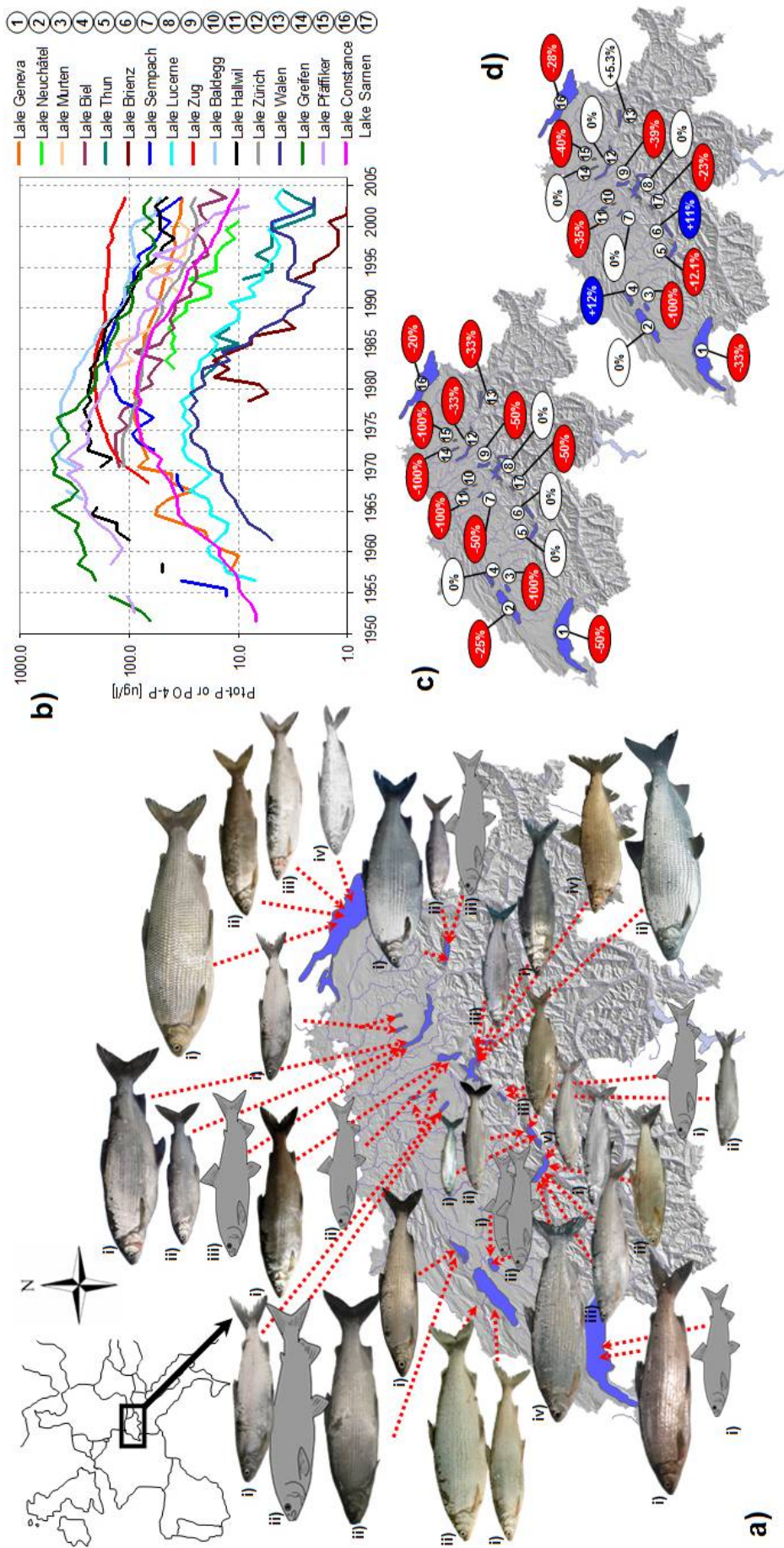
Fig 1.

Figure 1. (a) Whitefish species diversity in Swiss lakes. We use the Taxonomy of (Kottelat & Freyhof, 2007) and use local common names for so far undescribed, or taxonomically questionable species (lakes are identified in brackets). (1) Lake Geneva: (i) *C. fera* (ii) *C. hiemalis* †; (2) Lake Neuchâtel: (i) *C. candidus* (ii) *C. palea*; (3) Lake Murten: (i) *C. cf. palea* † (ii) *C. cf. confusus* †; (4) Lake Biel: (i) *C. confusus* (ii) *C. palea*; (5) Lake Thun: (i) *C. albellus* (ii) *C. alpinus* (iii) *C. fatioi* (iv) *C. sp. "Balchen"* (v) *C. sp. "Felchen"* ; (6) Lake Brienz: (i) *C. albellus* (ii) *C. sp. "Felchen"* (iii) *C. sp. "Balchen"*; (7) Lake Sempach (i) *C. suidteri* (ii) *C. cf. "Bodenbalchen"*; (8) Lake Lucerne: (i) *C. nobilis* (ii) *C. sp. "Bodenbalchen"* (iii) *C. zugensis* (iv) *C. sp. "Schwebbalchen"*; (9) Lake Zug: (i) *C. sp "Zugerbalchen"* (ii) *C. cf. zugensis* †; (10) Lake Baldegg (i) *C. cf. suidteri*; (11) Lake Hallwil (i) *C. cf. suidteri*; (12) Lake Zürich: (i) *C. duplex* (ii) *C. heglingus* (iii) *C. zuerichensis*; (13) Lake Walen: (i) *C. duplex* (ii) *C. heglingus* (iii) *C. zuerichensis*; (14) Lake Greifen: (i) *C. cf. duplex*; (15) Lake Pfäffiker: (i) *C. cf. zuerichensis*; (16) Lake Constance: (i) *C. arenicolus* (ii) *C. macrophthalmus* (iii) *C. wartmanni* (iv) *C. gutturosus* † (v) *C. sp. "Weissfelchen"*; (17) Lake Sarnen: (i) *C. cf. suidteri* (ii) *C. cf. zugensis* †. (b) 50 year trends in Phosphorus concentration [µg/l] during lake overturning from 16 of the 17 lakes included in this study. (c) Species richness changes in 17 lakes. (d) Functional diversity (gill raker counts) change in 16 lakes. Red ellipses represent lakes with more than 10% diversity loss; blank ellipses represent little or no changes, while blue ellipses indicate lakes with an increase in diversity of more than 10%. The observed functional diversity increase in Lake Biel can be attributed to recent colonization by a species from Lake Thun (*C. albellus*) (Bittner, 2009) and the increase in Lake Brienz to the fact that Steinmann (25) failed to identify one species (*C. sp. "Balchen"*).

The European Alps harbour one of the highest density of replicate radiations of whitefish, with between one and five endemic species (Kottelat & Freyhof, 2007, Hudson *et al*., submitted) in over a dozen lakes (Fig. 1a). Across all lakes, the ecological diversity of at least 41 species was assessed and described in considerable detail in a monograph published nearly 60 years ago (Steinmann, 1950). However, at the time of their description, eight species were already believed extinct (Steinmann, 1950). Many of the lakes lie in densely populated regions, and nutrient pollution of lakes was suggested as the main culprit (Steinmann, 1950). Lake pollution increased dramatically in the decades that followed, but its effect on whitefish diversity remained untested. Each of the whitefish radiations is characterized by ecologically differentiated species that grow to different sizes and spawn at different depths and times (Steinmann, 1950). Winter spawning occurs at a range of different water depths (from 1m to over 200m), whereas spawning during summer stratification is restricted to cold, well-oxygenated water below the thermocline (typically below ~20m). Fertilized eggs sink to the lake floor where they settle in the layer of organic sediment, and can only develop and hatch if the water-sediment interface is well-oxygenated (Müller & Stadelmann, 2004). By the mid 20th century organic pollution had increased primary production in all of the study lakes (Fig. 1b). The associated increase in microbial decomposition rates resulted in oxygen depletion in the water-sediment interface and a reduction or complete failure in whitefish recruitment (Müller & Stadelmann, 2004). With the implementation of stricter environmental regulations and the reduction of phosphorous loads by sewage treatment in the 1980s, the lakes are now returning to their natural trophic state (Fig. 1b).

We predicted that lower oxygen levels at the water–sediments interface decreased the availability of deep water spawning habitat, which reduced the strength of reproductive isolation between deep and shallower spawning sympatric species, and drove extinction through speciation reversal. To test our hypothesis we collected data on historical and present species richness in every whitefish lake of Switzerland. We use two different measures of historical diversity, and three of present diversity: (1) Species richness (see Supplementary Material); (2) Total range of gill raker numbers of the first gill arch historically reported (Steinmann, 1950), and measured between 2005 and 2008. Gill raker range provides a comparable genetically based measure of ecological functional diversity because it is related to trophic ecology (Link & Hoff,

1998) and has a high heritability (h^2) of 0.79 (Bernatchez, 2004). To allow comparisons between historical and contemporary data and to avoid bias in sample size, we estimated gill raker range by sampling normal distributions generated from measured gill raker counts when sample sizes were unequal (see Supplementary Material); (3) For contemporary data, we used molecular population genetic data based on 10 microsatellite loci to estimate number of species and extent of genetic differentiation in seven lakes.

RESULTS AND DISCUSSION

Historical gill raker range is highly correlated with pre-eutrophication estimates of species richness among lakes (N=17 lakes, r=0.88, $p<0.001$; supplementary Fig. 2). Present gill raker range is similarly correlated with observed numbers of species present today (N=12 lakes, r=0.95, $p<0.001$; supplementary Fig. 2), and also with population genetics-based estimates of current species numbers (N=7 lakes, r=0.97, $p<0.001$; supplementary Fig. 2). Hence gill raker range provides a good surrogate for species richness that is independent of taxonomic revisions.

A linear regression reveals that historical diversity was positively related to maximum lake depth (N=17 lakes, species richness: $R^2=0.51$, $p<0.01$; functional diversity: $R^2=0.32$, $p<0.05$, Fig. 2a) as predicted by the reproductive niche space hypothesis. Lakes historically containing summer and deep-spawning species are significantly deeper than lakes that did not ($N_{Summer}=8$, $N_{NoSummer}=9$: t=3.15, df=15, $p<0.01$; $N_{Deep}=11$, $N_{NoDeep}=6$: t=4.44, df=15, $p<0.001$). Together these results suggest that prior to eutrophication, lake depth affected reproductive niche availability and the degree of reproductive isolation among ecologically differentiated species. Stepwise multiple regression with maximum lake depth, minimum oxygen concentration observed between 1994 and 2006 at lake bottom during stratification (in mg/L), and maximum phosphorus concentrations observed between 1951 and 2004 during lake turnover (P_{max}, in μg/L), reveals that the present number of genetically distinct species also depends on lake depth (N=7 lakes, $R^2=0.64$, $p<0.05$, Fig. 2d) (see Supplementary Material). However, the degree of genetic differentiation between the species in a lake, which varies widely, is unrelated to depth, but is instead well predicted by the severity of nutrient pollution (multiple regression: N=6 lakes, $R^2=0.80$, $p<0.05$, Fig. 2c). One lake for which genetic data was available has been conservatively excluded from this analysis because it does not hold multiple species. Furthermore, current species and functional diversity, in contrast to historical diversity and the present number of genetically differentiated species, is unrelated to depth, but instead is predicted by the O_2min concentration (multiple regression: species richness: N=17 lakes, $R^2=0.62$, $p<0.001$; functional diversity: N=16 lakes, $R^2=0.57$, $p<0.001$, Fig. 2b). The discrepancy in depth explaining the present number of genetically differentiated species and the functional diversity of the number of species defined using morphological traits is likely due to the fact that the genetic results are based on lakes that have on average been less affected by eutrophication than lakes not included in genetic analyses.

Together, these results suggest that the temporal and spatial range of available spawning habitat controls functional diversity and genetic differentiation among species, and that anthropogenic eutrophication has weakened differentiation and reduced diversity. Indeed, lakes that still hold summer spawning species have significantly higher O_2min at the bottom than those without ($N_{Summer}=5$, $N_{NoSummer}=12$; t=2.18, df=15, $p<0.05$).
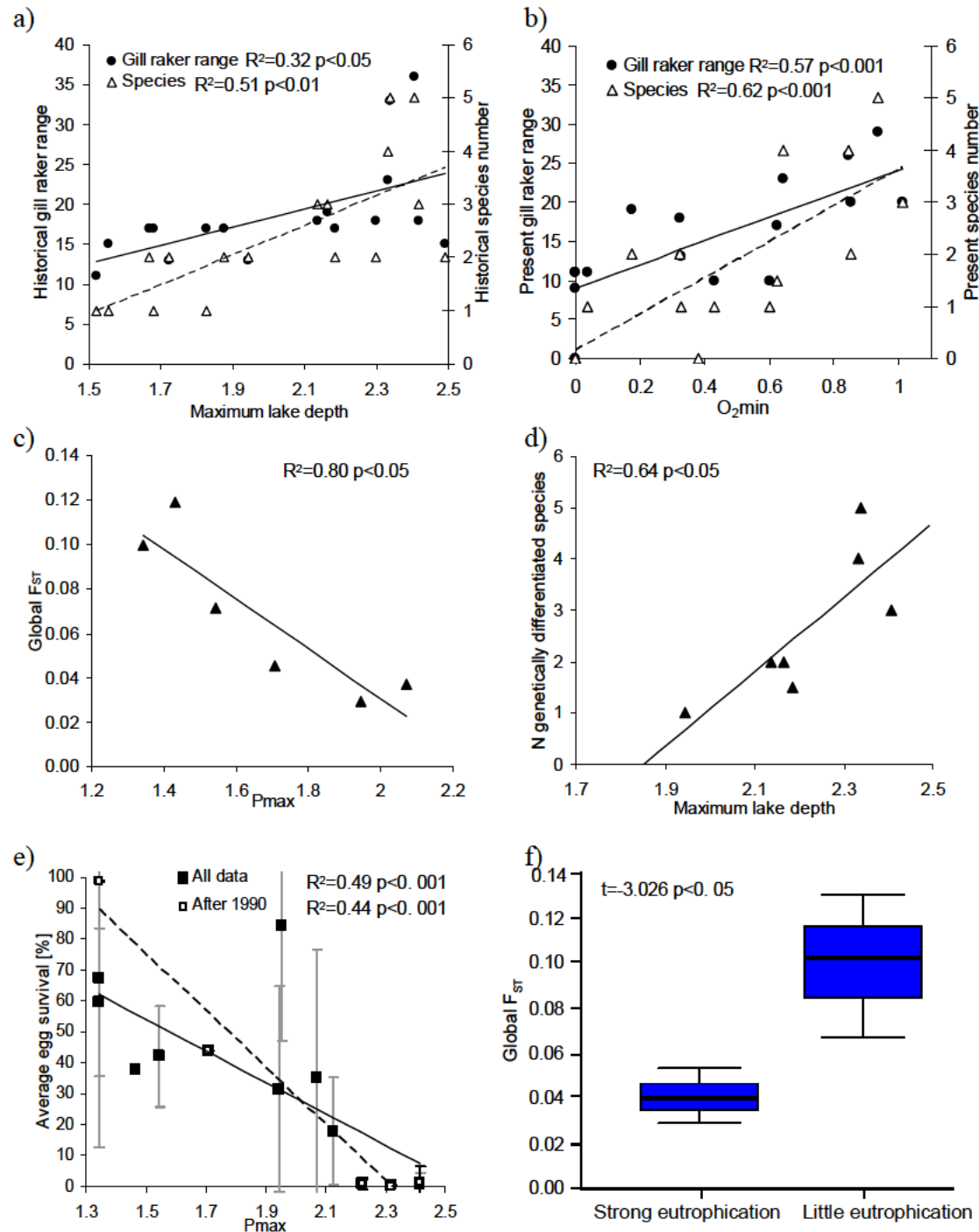
Figure 2. (a) Historical whitefish diversity in central European lakes measured as species numbers and gill raker range against the maximum depth of lakes. (b) Present diversity measured as species numbers and gill raker range against annual minimum oxygen concentration measured at the lake bottom during stratification (O_2min). (c) Global F_{ST} values of neutral genetic differentiation among species within lakes against the maximum phosphorous concentration (P_{max}). (d) Present numbers of genetically differentiated species against maximum depth of lakes. (e) The relationship between P_{max} and the proportion of viable whitefish eggs among all eggs collected in dredge samples from 12 different lakes. The error bars represent the standard deviations of multiple measures taken over several years. The sample sizes (Number of eggs analysed/ Number of samples taken in different years) are as follows (From left to right and top to bottom): Lake Thun (2223/3), Lake Sarnen (675/6), Lake Walen (90/1), Lake Lucerne (399/3), Lake Neuchâtel (32/1), Lake Constance (7169/15), Lake Geneva (1400/2), Lake Zürich (649/2), Lake Biel (1404/3), Lake Sempach (5106/16), Lake Zug (1852/6), Lake Hallwil (4513/13). (f) Comparison of global F_{ST} values of overall differentiation among species within lakes that have been strongly and more subtly influenced by eutrophication.

Similarly, lakes that still hold deep spawning species have higher O_2min levels and lower phosphorus concentrations than those without (N_{Deep}=8, N_{NoDeep}=9: O_{2min}: t=2.22, df=15, p<0.05; P_{max}: t=-2.43, df=15, p<0.05). To test the hypothesis that eutrophication affects recruitment success and reproductive niche availability, we collected eggs from the sediments of whitefish spawning grounds in 12 lakes between 1968 and 2008. The proportion of live eggs in our samples decreased dramatically with the phosphorous concentration (Fig. 2e). Survival was close to zero when P_{max} exceeded 150µg/L. Interestingly, egg survival rate was better predicted by the maximum phosphorous concentration (P_{max}) measured during the peak of the eutrophic phase, than by phosphorous levels measured in the year the eggs were sampled (P_s) (N=74 samples; P_{max}: R^2=0.49, p<0.001; P_s: R^2=0.21, p<0.001). When we restrict this analysis only to samples taken well after the eutrophication peak (1990 to 2008), the pattern remains the same (N=29 samples; P_{max}: R^2=0.44, p<0.001; P_s: R^2=0.09, p=0.109). This suggests organic waste from historic pollution continues to negatively affect recruitment and reproductive niche availability many years after water quality began to improve.

Today, the majority of the lacustrine whitefish radiations in Switzerland display much lower species and functional diversity than 100 years ago (Fig. 1c+d). We observed an average species loss of 33% and a functional diversity loss of 17.6% during the past century (species richness: t=-6.154; df=16, p<0.001; functional diversity: t=-2.587; df=15, p<0.05; Fig. 1c+d). The degree of loss in species richness is strongly related to P_{max} (multiple regression: N=17, R^2=0.616, p<0.001). In several small and highly eutrophic lakes, natural reproduction ceased completely and indigenous whitefish species went extinct (Steinmann, 1950). They have been replaced by mixed captive breeding stocks which explains why functional variation is still present in some of them (Müller & Stadelmann, 2004). The surviving species in three formerly eutrophic lakes show an average global genetic differentiation that is 59% lower relative to that observed among species in three lakes that had never been eutrophic. Including in the calculation of loss of species richness (See Supplementary Material) the average reduction of genetic differentiation (for the 6 lakes with multiple species for which we have genetic data), we find that only 45% of the original species richness survived a century of lake pollution. This is a conservative estimate of actual loss because we assume that there was no loss of genetic differentiation in the three least eutrophic lakes, and we assume no loss of genetic differentiation amongst surviving species in all those lakes for which we have no population genetic data.

These results suggest that species richness has been reduced by extinction through speciation reversal, with total extirpation of whitefish in the most polluted lakes. The speciation reversal scenario is supported by the following patterns in population genetic data: (1) There is a strong negative correlation between global genetic differentiation of species within lakes and the extent of nutrient pollution in recent lake history (Fig. 2c); (2) Among six lakes with similar maximum depths, global genetic differentiation among species is significantly higher in lakes that were less affected by nutrient pollution (Fig. 2f; t=-3.026, df=4, p<0.05). Importantly, maximum lake depth and historical whitefish diversity did not differ between polluted and unpolluted groups of lakes (Maximum depth: t=0.35, df=4, p=0.74; Species richness: t=0.71, df=4, p=0.55; Historical gill raker range: t=0.05, df=4, p=0.97); (3) As an illustrative example, the closely situated and connected Lakes Zürich and Walen share the same whitefish species pairs, a small deep-spawning (*C. heglingus*) and a large shallow-spawning species (*C. duplex;* Fig. 1). Genetic differentiation between species clearly exceeds that within species between lakes, suggesting a common origin for each species (Fig. S3). However, global multilocus F_{ST} between sympatric species

is substantially larger in oligotrophic Lake Walen (0.131) than in more eutrophic Lake Zürich (0.041).

In addition to the molecular genetic evidence, the pattern of loss in gill raker diversity is consistent with extinction through speciation reversal. In all radiations, large species with few gill rakers spawn in the shallows while small species with high gill raker counts spawn deep (Steinmann, 1950, Vonlanthen *et al*., 2009). If eutrophication were driving extinction through extirpation of deep spawning populations, diversity loss should occur predominantly on the high end of the gill raker distribution. If, on the other hand, extinction were due to speciation reversal by hybridization, diversity should be lost from both ends of the gill raker distribution. To test these distinguishing predictions we compared the difference between historical and recent data from seven lakes that lost diversity. In support of the speciation reversal scenario, diversity is being likely lost from both the lower (mean gill raker loss= -3.86; t=-4.23; df=6; $p<0.01$) and upper (mean gill raker loss=-2.86; t=-3.33; df=6; $p<0.05$) ends of the gill raker distributions. In addition to the loss of suitable breeding habitat, this functional diversity loss might also have been accelerated by stocking practices which may artificially increase gene flow among species.

All this strongly suggests that eutrophication drives the erosion of species distinctiveness and associated functional phenotypic diversity by increasing gene flow among species that previously partitioned a wider range of reproductive niches. Divergent selection can maintain some ecological differentiation among populations distributed along a spawning niche gradient when the gradient is sufficiently long and deeper sediments are well oxygenated down to significant lake depths (Vonlanthen *et al*., 2009). Shortening the gradient by increasing pollution, however reduces temporal and spatial pre-mating isolation to the extent that selection can no longer counteract the effects of increasing gene flow between ecologically differentiated species (Seehausen *et al*., 2008). Finally, if even shallow water sediments become anoxic, whitefish are completely extirpated from lakes.

In summary, our data suggest that anthropogenic eutrophication has caused a mass extinction of fish species by significantly altering the conditions which drive and maintain species richness in fish adaptive radiations. Differences in the detail of the mechanisms not withstanding, we note a striking parallel to the diversity loss in cichlid fish (Seehausen *et al*., 1997), Great Lake ciscos (Seehausen, 2006), and sticklebacks (Taylor *et al*., 2006). We estimated that 55% of the whitefish diversity and 17.6% of the functional genetically based morphological diversity in central Europe's large lakes has been lost in less than a century. This occurred largely unnoticed despite the fact that these fish are ecologically dominant and constitute a commercially important fishery in central European lakes. Though human activity may drive extinction through extirpation or increasing hybridization, the importance of extinction through speciation reversal may be underestimated for two reasons. First, the process involves subtle changes in the distributions of phenotypic and genetic variation without obvious changes in geographical distributions. Speciation reversal will therefore typically be more difficult to detect than either sequential population extirpation or hybridization following translocation (Rhymer & Simberloff, 1996, Seehausen *et al*., 2008). Second, speciation reversal is a potentially rapid process; collecting the relevant data will often require luck or keen foresight (Taylor *et al*., 2006). Thus a significant proportion of regional biota could collapse rapidly (Seehausen *et al*., 1997, Seehausen *et al*., 2008) where species rich biota have arisen in geologically recent times through adaptive radiation. Together with evidence from similar study systems, the case of central European whitefish suggests that species richness of lake fish radiations might be lost globally due to anthropogenic activities that disrupt the evolutionary process

responsible for driving adaptive radiation. It remains to be confirmed if similar mechanisms may apply to other aquatic organisms (Brede *et al*., 2009) and the potentially complex effects of simultaneous rapid diversity loss at several interacting trophic levels in aquatic food webs requires investigation. It is likely that implosive loss of diversity of ecologically dominant species such as large planktivorous fish has tractable effects on other components of aquatic ecosystems (Goldschmidt *et al*., 1993, Harmon *et al*., 2009). In the face of population growth and climate change, recognizing the evolutionary impacts of anthropogenic environmental change presents a critical challenge. Ecosystem services depend on ecosystem function, and the latter can only be understood if we understand the evolutionary mechanisms generating ecological diversity (Seehausen, 2009, Harmon *et al*., 2009).

SUPPLEMENTARY MATERIAL

Adaptive radiation and ecological speciation in whitefish

Whitefish (*Coregonus* spp.) are undergoing adaptive radiations through ecological speciation in many postglacial lakes of the northern hemisphere (Bernatchez, 2004, Hudson *et al*., 2007). Located at the southern edge of their global distribution range, the large lakes of the European Alps contain an unparalleled concentration of whitefish radiations, with between one and five endemic species in over a dozen lakes (Fig. 1a; Hudson *et al*., submitted). Within lake morphological differences among species are associated with ecological divergence along two principal axes: consumable resources (Rufli, 1978, Kahilainen & Ostbye, 2006) and foraging habitat (Helland *et al*., 2007, Ohlberger *et al*., 2008). Gill raker number is a highly heritable meristic trait (h^2=0.79) that differs consistently between species foraging predominantly on pelagic zooplankton (high count) and those feeding on benthic macroinvertebrates (low count) (Schluter, 1996, Bernatchez, 2004, Ostbye *et al*., 2005). Reproductive isolation among whitefish species is maintained by divergence in spawning site depth, time and possibly mate choice (Chapters 2, 3, 4). In species-rich radiations, spawning behaviour involves a wide range of seasons and water depths (Fig. S1). Winter spawning occurs at all depth ranges during lake mixing when water temperature varies little with depth. Summer spawning occurs during lake stratification and is confined to the deep, cold and well-oxygenated water below the thermocline (Fig. S1). Generally large whitefish with low gill raker numbers spawn in the shallow littoral regions while small species have high densities and spawn in deep areas. Two exceptions to this rule are two low gill raker species in Lakes Thun and Constance that spawn deep (Steinmann, 1950).

Sampling

Between 2005 and 2008 we collected 2161 whitefish specimens from 15 lakes using monofilament gill nets (mesh sizes 14-60mm). We collected a minimum of 20 individuals of each known species from every lake except for *C. heglingus* in Lake Zürich of which only 17 were caught, and *C. sp. "Felchen"* in Lake Thun which could not be obtained. In most lakes, we collected fish from known spawning grounds. In five lakes (Lakes Sempach, Walen, Lucerne, Thun and Neuchâtel) we collected fish at multiple times from multiple locations. Finally, we sampled systematically along the entire spawning depth gradient (Vonlanthen *et al*., 2009) in two lakes, Neuchâtel and Lucerne. All fish were mature or nearly mature [maturation degree 5 and 6 (Smolina, 1920)]. We recorded the length, weight and sex of every fish. A piece of muscle tissue was removed and preserved in 100% ethanol for DNA analysis. The first gill raker arch was removed from every individual for gill raker count analysis (Table S1).

Quantifying relative diversity of whitefish in each lake

Whitefish diversity in the northern Alps is characterized by parallel ecological speciation occurring in multiple lakes (Douglas *et al*., 1999, Douglas *et al*., 2005, Vonlanthen *et al*., 2009, Hudson *et al*., submitted). In 1950, Steinmann published a detailed and data rich monograph on taxonomy, ecology and evolution of whitefish in Swiss lakes (Steinmann, 1950).
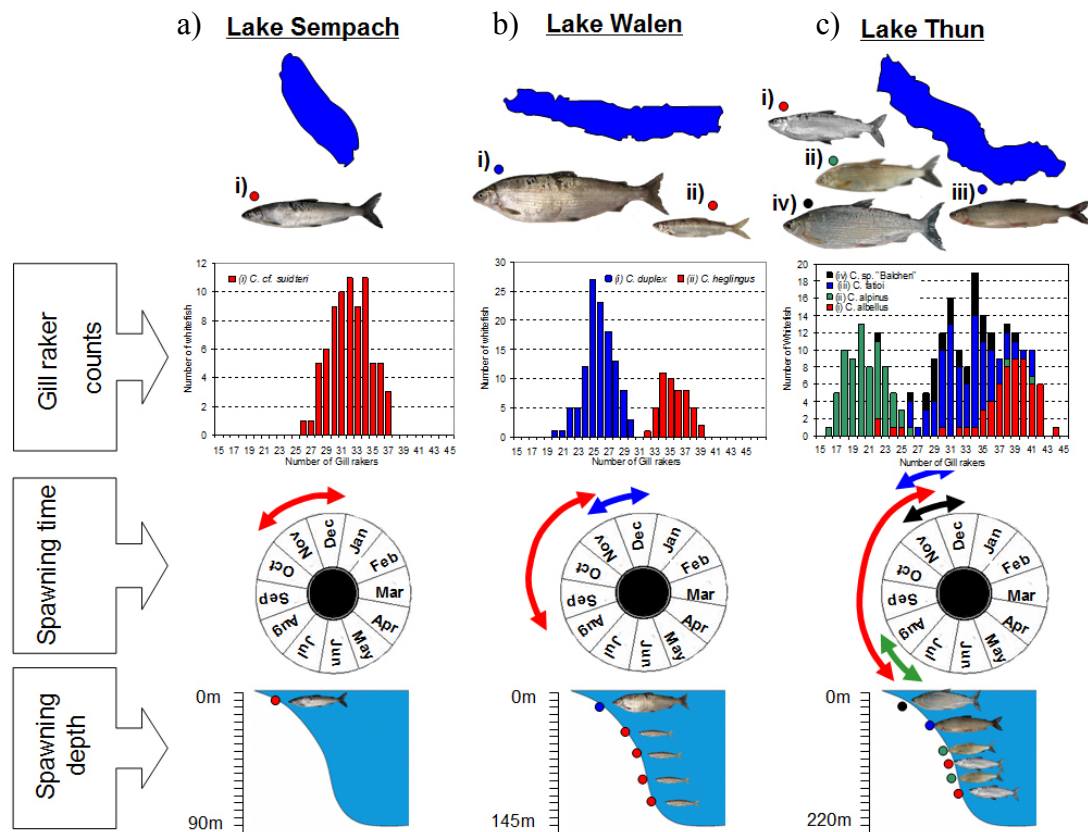
Figure S1. Shown are representative examples of ecological and reproductive niche divergence between whitefish species from three lakes. Arrow colours of the spawning times correspond to the colours and species in the distribution of gill raker count plots. (a) Lake Sempach harbours only one undifferentiated population (*C. cf. suidteri*). The population has an intermediate and unimodal range of gill raker counts, spawns in winter and only in shallow water. This pattern is widespread among lakes harbouring only one species. (b) Lake Walen has two distinct species (i) *C. duplex* and (ii) *C .heglingus*, with a bimodal distribution of gill raker counts. *C. heglingus* has an extended spawning period and spawns in deep waters below ~25m of depth while *C. duplex* spawns in winter and exclusively shallow, i.e. in depths less than ~10m. (c) The Lake Thun radiation contains five different species of which the following four where included in this study (i) *C. albellus*, (ii) *C. alpinus*, (iii) *C. fatioi* and (iv) *C. sp.* "*Balchen*". Together, they cover a very large range in gill raker numbers and occupy a wide range of spawning times and depths.

A more recent taxonomical treatment (Kottelat & Freyhof, 2007), used Steinmann's monograph as a benchmark. Steinmann defined species principally by morphological and meristic traits, and by differences in spawning ecology (Steinmann, 1950). Recent genetic and morphological studies often confirmed and sometimes refined these classifications (Douglas *et al*., 1999, Vonlanthen *et al*., 2009, Hudson *et al*., submitted). Whitefish alpha-taxonomy has however been unstable through time and authors, like in other recent species radiations (Fatio, 1890, Wagler, 1937, Steinmann, 1950, Kottelat, 1997, Kottelat & Freyhof, 2007). Fortunately, Steinmann provided high quality data on the gill raker distributions for most large lakes of Switzerland (Steinmann, 1950). The number of gill rakers on the first gill arch varies among species and is highly heritable (Bernatchez, 2004). We find that the historically observed range of gill raker counts from a lake is tightly correlated with the number of historically recognized species ($R^2 = 0.78$; Fig. S2; Steinmann, 1950). The gill raker range measured from our modern collections is similarly tightly correlated to the present number of species in a lake and also to the number of genetically differentiated species in a lake (Fig. S2). Unequal sample sizes can however greatly influence the

range in gill raker count observed. Sample sizes for lakes Geneva, Sempach, Zug, Hallwil, Greifen, Pfäffiker, Constance and Sarnen were expected to be lower than historical sample sizes (Table S1). We therefore estimated gill raker normal distributions and subsequently sampled 100 random individuals from these distributions for each species in each of these eight lakes. We then use the per-lake range in gill raker numbers and the per-lake number of historically and presently described species to compare historical pre-eutrophication with modern post-eutrophication diversity in these whitefish radiations.
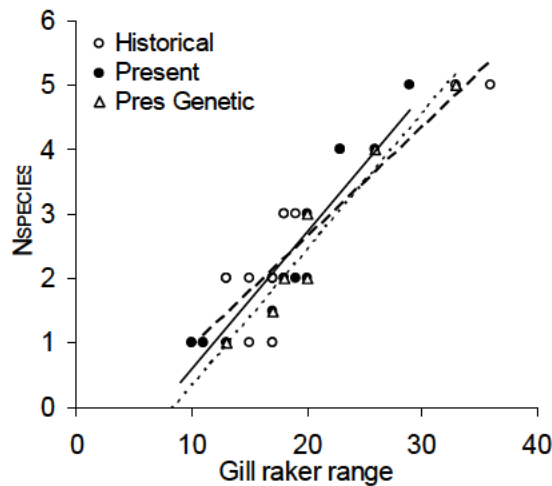
Figure S2. The correlation between the historically recognized number of whitefish species (Steinmann, 1950) and historic gill raker range for 17 Swiss lakes and the correlation between presently observed genetically and phenotypically different-tiated whitefish species and the gill raker range observed in seven and 16 lakes. The range in gill raker count is highly correlated with species numbers: Historical species: N=17 lakes, r= 0.88, $p<0.001$; Present species: N=12 lakes, r=0.95, $p<0.001$; Present genetic species N=7 lakes, r=0.97, $p<0.01$.

Environmental variables

There is rich data on many biotic and abiotic variables available for all the large lakes of central Europe. Because they can affect the availability of spawning and feeding habitat (Landry *et al.*, 2007, Vonlanthen *et al.*, 2009), we predicted that lake depth, minimum oxygen concentration at the lake bottom close to the sediment water interface, and nutrient load would influence whitefish species richness (see main text and Fig. S1). Lake depths and maximum phosphorus content (Ptot-P [mg/m^3]) data were obtained from (Liechti, 1994). O$_2$ depth profiles [mg/l] were obtained from the following federal, cantonal and private offices: EAWAG for Lakes 7, 8, 10, 11, and 17; Internationale Gewässerschutzkomission für den Bodensee (IGKB) for lake 16; Base de données INRA de Thonon-les-Bains and CIPEL-INRA for lake 1; AWEL, Amt für Abfall, Wasser, Energie und Luft – Baudirektion Kanton Zürich for lakes 12, 13, 14, and 15; Gewässer- und Bodenschutzlabor des Kantons Bern for lakes 5 and 6; Les 3 lacs (Cantons de Fribourg, Neuchâtel et Berne) for lakes 2, 3 and 4; Amt für Umweltschutz des Kantons Zug for lake 9. As maximum phosphorus concentration, we took the highest value observed in time series covering the period 1951 to 2004, including the onset and peak of the eutrophic phase and the re-oligotrophication that began in the 1980s. As minimum oxygen concentrations we took the lowest values observed at the lake bottom at the deepest point of each lake between 1994 and 2006, after the onset of re-oligotrophication. Oxygen data from the peak eutrophication period were not available. However, because of the latency in the recovery of lake sediments from organic pollution, our data carry a strong and distinct signature of differential pollution.

Table S1. Species and functional diversity from 17 large central European lakes. The number of historically recognized whitefish species [N Historical species;(Steinmann, 1950)]; the number of presently observed phenotypically distinct species (N Present species); the number of genetically distinct species observed at present (N Genetic species), where 1.5 represents a species cline observed in Lake Neuchâtel (Vonlanthen et al., 2009); the percent loss in the number of Species (% Species loss); the percent reduction in global genetic differentiation (% Loss of genetic differentiation) calculated as 1- (Present Global F_{ST}/ Historical Global F_{ST}) where historical F_{ST} is assumed to be equal to the average of the Global F_{ST} observed in the three least impacted lakes Walen, Lucerne and Thun (Mean Global F_{ST}=0.101); the percent diversity loss (% Diversity loss) calculated as (100- % Species Loss)*(100-% Reduction Global F_{ST}); the historically observed range in the gill raker count (Historical Gill raker range), the range in gill raker counts observed today (Modern gill raker range), the loss in functional diversity (% Functional loss); the number of individuals used for present day gill raker analysis (N Modern gill raker range) and the historically used sample sizes (N Historical gill raker range); the minimum oxygen concentration measured at the deepest point of each lake (Min O_2); the maximum phosphor concentration observed during the eutrophic period (P_{tot}-P); and the maximum lake depth (Maximum lake depth). Gill raker ranges and sample sizes that were adjusted are labelled with a *.

Lake	Species diversity and genetic differentiation						Functional ecological diversity					Min O_2 [mg/l]	P_{tot}-P [µg/l]	Maximum lake depth [m]
	N Historical species	N Present species	% Species loss	N Genetic Species	% Loss of genetic differentiation	% Diversity loss	Historical gill raker range	Modern gill raker range	% Functional loss	N Historical gill raker range	N Modern Gill Raker Range			
Lake Geneva	2	1	-50%			-50%	15	10*	-33.3%	61	24*	3	90	309
Lake Neuchatel	2	1.5	-25%	1.5	-47%	-60%	17	17	0.0%	?	341	3.2	50	152
Lake Biel	2	2	0%			0%	17	19	11.8%	?	49	0.5	132	74
Lake Murten	2	0	100%			-100%	17	0	-100.0%	?		0	150	45.5
Lake Thun	5	5	0%	5	0%	0%	33	29	-12.1%	471	331	7.6	21	217
Lake Brienz	3	3	0%			0%	18	20	11.1%	>123	100	9.3	17	261
Lake Sempach	2	1	-50%	1		-50%	13	13*	0.0%	>12	76*	1.12	165	87
Lake Lucerne	4	4	0%	4	0%	0%	23	23	0.0%	180	730	3.4	34	214
Lake Zug	2	1	-50%			-50%	18	11*	-38.9%	?	20*	0.09	208	198
Lake Baldegg	1	0	-100%			-100%	17			?		1.4	517	66
Lake Hallwil	1	0	-100%			-100%	17	11*	-35.3%	?	20*	0	260	47
Lake Zürich	3	2	-33%	2	-59%	-73%	18	18	0.0%	76	66	1.1	119	136
Lake Walen	3	2	-33%	2	0%	-33%	19	20	5.3%	?	236	6.1	26	145
Lake Greifen	1	0	-100%			-100%	11	11*	0.0%	?	50*	0	507	32.3
Lake Pfäffiker	1	0	-100%			-100%	15	9*	-40.0%	?	19*	0	367	35
Lake Constance	5	4	-20%	3	-70%	-76%	36	26*	-27.8%	694	79*	6	87	254
Lake Sarnen	2	1	-50%			-50%	13	10*	-23.1%	?	20*	1.7	21	52
Total/average	41	28	-33%		-29%	-55%			-17.6%		2161			

Population genetic analysis of species differentiation

Detailed information about laboratory methods, including DNA extractions, microsatellite amplification and genotyping are given in (Vonlanthen *et al.*, 2009) with the difference that only ten loci were not used here. Within each lake, we identified species using morphological traits (mainly gill raker counts and back-calculated size at the age of three years), spawning location and spawning time. Between 17 and 426 individuals of each species (a total of 1679 individuals) were genotyped at ten microsatellite loci. For each species, expected (H_E) and observed (H_O) heterozygositiy were calculated using Arlequin 3.1 (Excoffier *et al.*, 2005). Deviations from Hardy-Weinberg equilibrium (HWE) were tested with exact tests (Guo & Thompson, 1992) for each locus and species using GENEPOP 3.4 (Raymond & Rousset, 1995) with 1,000,000 steps in the Markov chain and 5,000 dememorization steps. Inbreeding coefficients (F_{IS}: Weir & Cockerham, 1984) were calculated across all loci for all species and tested for significant deviations from zero using FSTAT version 2.9.3 (Goudet, 2001). Significance levels for deviation from HWE and for F_{IS} were corrected for multiple comparisons using the sequential Bonferroni method (Rice, 1989). Deviations from linkage equilibrium between all pairs of loci for each species were tested using ARLEQUIN 3.1 (Excoffier *et al.*, 2005) with a significance level of $p < 0.01$. The global genetic differentiation of species within each lake was calculated using a hierarchical analysis of molecular variance (Excoffier *et al.*, 1992) using ARLEQUIN 3.1 (Excoffier *et al.*, 2005). Finally, allelic richness (A_R) was calculated in FSTAT version 2.9.3 (Goudet, 2001).

No significant deviations from HWE and no significant F_{IS} were observed in any of the 18 species. 22 out of 810 pairwise linkage tests were significant at a significance level of 0.01. Because they consisted of different pairs of loci in different populations, we conclude that they were the result of a type one error or due to random genetic drift in finite populations instead of physical linkage between loci (Ohtha, 1982). The global genetic differentiation within lakes ranged from 0.030 (Lake Constance) to 0.131 (Lake Walen). Allelic richness within species ranged from 3.1 (*C. alpinus*, Lake Thun) to 4.78 (*C. Sp. "Schwebbalchen"* Lake Lucerne). Population genetic summary statistics are provided in Table S2.

Egg survival data

Whitefish spawn either directly over the lake bottom or in the water column, depending on the species. The eggs of all species settle to the sediment surface, where they develop until hatching, requiring a well oxygenated sediment surface (Steinmann, 1950). Whitefish eggs were collected from the lake bottom in 12 lakes at multiple times between 1968 and 2003. Eggs were collected using a sled dredge (Elster, 1933), a diver-operated suction apparatus in deeper water and a kick net for shallow areas. Details of the sampling methods can be found in (Müller, 1992). Sampling was done between early January and early March, just before the anticipated beginning of mass hatching of the corresponding whitefish species in each lake. All eggs were taken to the laboratory, sorted and classified into six classes: (1) Developing normally [D_N]; (2) developing but embryo deformed [D_D]; (3) unfertilized [U]; (4) dead/undeterminable [T]; (5) empty with a small hole [E] (a sign of invertebrate predation); (6) empty/split open (hatched) [H]. To assess reproductive success we calculated the percentage of eggs that developed normally [containing almost hatched embryos of developmental

stage 12-13 (Luczynski & Quoss, 1984)] plus hatched egg shells among all possibly fertilized eggs (Sum of D_N, D_D, T and H) with the following equation:

$$\frac{D_N + H}{D_N + D_D + T + H} \times 100$$

Since not all dead eggs might have been fertilized, the true percentage of viable eggs may be somewhat higher than our estimates.

Table S2. Population genetic summary statistics for 18 species from seven lakes: sample sizes (N), the global F_{ST} value calculated over all species within each lake (Global F_{ST}), observed (H_O) and expected heterozygosity (H_E), significance of deviation from HWE, the inbreeding coefficient (F_{IS}) with corresponding significance level and the number of deviations from linkage equilibrium out of a total of 55 pairs of loci observed at a significance level of $p<0.01$.

Lake	Species	N	Global F_{ST}	H_O	H_E	Sig.	A_R	F_{IS}	Sig.	N_{LD} $p<0.01$
Sempach	*C. suidteri*	60	-	0.50	0.53	n.s.	4.31	0.043	n.s.	0
Neuchâtel	*C. palea*	45	0.054	0.53	0.54	n.s.	4.18	0.012	n.s.	2
	C. candidus	59		0.56	0.56	n.s.	4.33	-0.012	n.s.	0
Walen	*C. duplex*	98	0.131	0.44	0.46	n.s.	3.30	0.039	n.s.	0
	C. heglingus	426		0.47	0.48	n.s.	3.49	0.031	n.s.	1
	C. sp. "Bodenbalchen"	137		0.57	0.59	n.s.	4.37	0.02	n.s.	0
Lucerne	*C. sp. "Schwebbalchen"*	118	0.068	0.57	0.59	n.s.	4.78	0.027	n.s.	4
	C. zugensis	276		0.48	0.49	n.s.	4.36	0.024	n.s.	0
	C. nobilis	83		0.48	0.48	n.s.	3.72	-0.006	n.s.	2
	C. sp. "Balchen"	30		0.52	0.52	n.s.	3.86	0.015	n.s.	0
Thun	*C. fatioi*	85	0.103	0.53	0.54	n.s.	4.05	0.028	n.s.	3
	C. albellus	60		0.46	0.48	n.s.	3.74	0.037	n.s.	3
	C. alpinus	77		0.49	0.47	n.s.	3.10	-0.034	n.s.	3
Lake Zürich	*C. duplex*	45	0.041	0.51	0.54	n.s.	3.99	-0.006	n.s.	1
	C. heglingus	17		0.54	0.54	n.s.	3.96	0.04	n.s.	0
Lake Constance	*C. arenicolus*	23		0.51	0.53	n.s.	4.18	0.037	n.s.	1
	C. macrophthalmus	20	0.030	0.56	0.56	n.s.	4.40	0.015	n.s.	2
	C. wartmanni	20		0.51	0.53	n.s.	3.71	0.041	n.s.	0
Total/overall		1679		0.51	0.52		3.99			22 (2.72%)
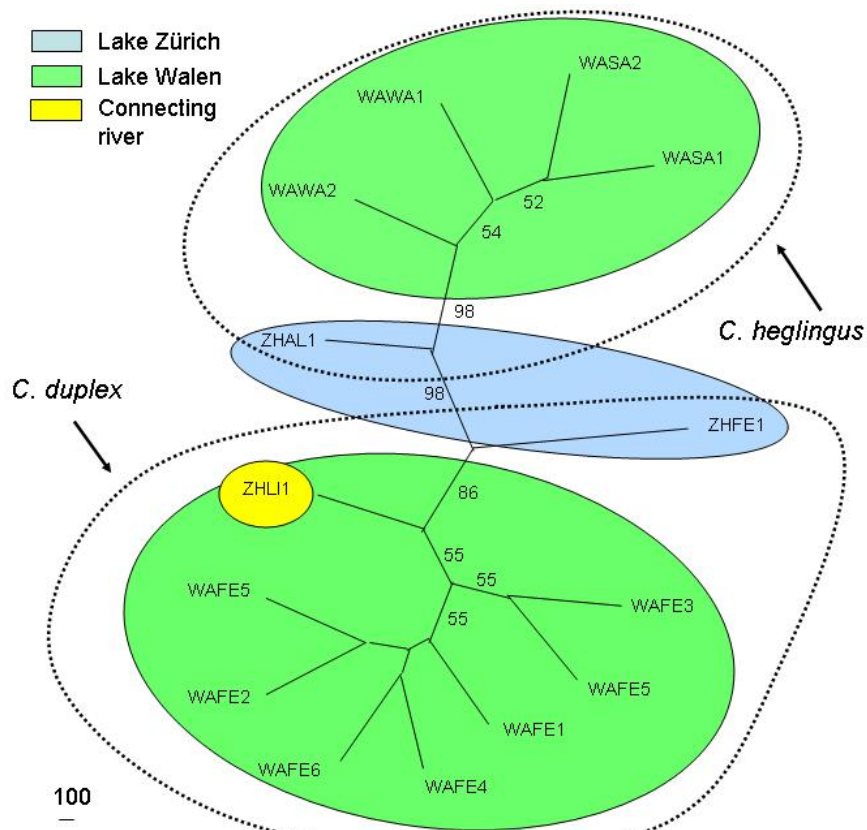
Figure S3. Phylogenetic signature of introgression in eutrophic Lake Zurich. Lake Walen and Lake Zürich are connected sister lakes containing the same pair of whitefish species endemic to this system. *C. duplex* is a large and shallow spawning, *C. heglingus* a small and deep spawning species. A neighbour joining consensus tree based on Cavallis-Sforza chord distance (D_C) with 1000 bootstrap replicates reveals that while the two species are still reciprocally monophyletic, the sympatric populations from recently eutrophic Lake Zürich (blue background) are genetically much closer to each other than are the sympatric populations from never eutrophied Lake Walen (green background). Also included are whitefish spawning in the river "Linthkanal" that connects the two lakes (yellow background).

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EDUCATION

-2004-   University of Bern, Zoological Institute, Bern, Switzerland, **PhD** in Aquatic Ecology and Evolutionary Biology Title: On speciation and its reversal in adaptive radiations - The central European whitefish system Supervision: Prof. Dr. Ole Seehausen

- 2003-04   University of Bern, Zoological Institute, Bern, Switzerland, **Master of Science** in Biology with Major in Zoology, November 2004. Title: Genetic analysis of potential postglacial watershed crossings by the bullhead (Cottus gobio L.) Supervision: PD Dr. Carlo Largiadèr

PROFESSIONAL EXPERIENCE

-2008-   Eawag: Research assistant

-2004-05   Aqua-Sana: Private consulting office in aquatic ecology as collaborator

-2003-04   University of Bern: Centre for Fish and Wildlife Health FIWI. Research assistant

SELECTED ACTIVITIES

2009   Oral presentation at the EAWAG seminar series. Title: *Speciation and extinction in central European whitefish: 15'000 years of evolution vs. 100 years of pollution*

2009   Oral presentation at the Institute for Limnology in Mondsee, Austria. Title: *Adaptive radiation and diversity loss in central European whitefish*

2009   Oral presentation at the Fischereiverwaltertagung, Switzerland. Title: *Populationsgenetische und morphologische Untersuchung der Nasen (Chondrostoma nasus)*

2008   Oral presentation at the University of Besançon, France. Title: *Etude génétique des populations de poissons*

2008   Oral presentation at the International coregonid symposium in Winnipeg, Canada. Title: *Divergence along a steep ecological gradient in whitefish (Coregonus lavaretus spp.)*

2007   Oral presentation at the International symposium of the European ichtyological society in Duborvnik, Croatia. Title: *Evidence for a species continuum along an ecological gradient with hybridization in whitefish (Coregonus lavaretus spp.)*

2007   Oral presentation at the CMPG Seminar series, University of Bern, Switzerland: Title: *Evidence for a species continuum along an ecological gradient with hybridization in whitefish (Coregonus lavaretus spp.)*

2007   Oral presentation in the evolutionary Biology seminar series at the University of Lausanne,Switzerland. Title: *Evidence for a species continuum along an ecological gradient with hybridization in whitefish (Coregonus lavaretus spp.)*

2006.   Oral presentation at the Institut für Seenforschung in Langenargen, Germany. Title: *Sympatrische Artentstehung bei Felchen (Coregonus lavaretus) in mitteleuropäischen Seen*

2005   Oral presentation at the International coregonid symposium in Olztyn, Poland. Title: *Ecological Factors and their Influences on Speciation in Whitefish (Coregonus sp.)*

SERVICE AS REVIEWER

Molecular Ecology, Journal of Fish Biology, Journal of Zoological Systematics and Evolutionary Research, Journal of Knowledge and Management of Aquatic Ecosystems, Biological Journal of the Linnean Society.

PUBLICATIONS

PEER REVIEWED JOURNALS

Vonlanthen, P., Excoffier, L., Bittner, D., Persat, H., Neuenschwander, S. & Largiadèr, C.R. 2007. Genetic analysis of potential postglacial watershed crossings in central Europe by the bullhead (Cottus gobio L.). Molecular Ecology 16: 4572-4584

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Zimmerli, S., Bernet, D., Burkhardt-Holm, P., Schmidt-Posthaus, H., Vonlanthen, P., Wahli, T. & Segner, H. 2007. Assessment of fish health status in four Swiss rivers showing a decline of brown trout catches. Aquatic Sciences 69: 11-25.

NON-PEER REViEWED JOURNALS AND REPORTS FOR GOVERNMENTAL AGENCIES

Hudson A., Vonlanthen P., Lundsgaard-Hansen B., Denis R., Seehausen O. (2008) Untersuchungen zur Verwandtschaft der Felchen aus dem Lago di Como, di Lugano und Maggiore

Vonlanthen P., Lundsgaard-Hansen B., Hudson A., Seehausen O. (2008) Untersuchungen zur Verwandtschaft der Felchen aus dem Zürichsee, dem Walensee und dem Linthkanal

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