

Evolutionary Ecology Research, 2012, **14**: 487–502

Does water depth or diet divergence predict progress towards ecological speciation in whitefish radiations?

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ABSTRACT

Question: Is the extent of genetic divergence between sympatric whitefish ecotypes – a proxy for progress towards speciation – related to the extent of ecological divergence in spawning depth or diet?

Study system: Whitefish (*Coregonus* spp.) that have diversified into two or more sympatric ecotypes in subalpine Swiss lakes. Sympatric ecotypes vary in the extent of reproductive isolation.

Analytical methods: We measured the degree of spawning depth differentiation based on the depth-at-capture of different ecotypes. We estimated diet differentiation between ecotypes as Mahalanobis distances from stable isotopes. We compared each of these to genetic differentiation measured from AFLP data, using modified correlation tests and phylogenetically independent contrasts to account for non-independence of comparisons in lakes with more than two ecotypes.

Results: We found that the magnitude of divergence in spawning depth was generally – albeit only marginally significantly – associated with the extent of genetic divergence between sympatric ecotypes. This effect was clearly stronger than the effect of diet divergence, which was not associated with genetic differentiation. Furthermore, there was no evidence for an interactive effect of depth and diet divergence on progress towards speciation.

Keywords: AFLP, *Coregonus* spp., parapatric speciation, stable isotope analysis, sympatric speciation.

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INTRODUCTION

It is becoming increasingly apparent that ecological speciation and adaptive radiation are important processes contributing to species diversity. Ecological speciation occurs when reproductive barriers emerge either as a by-product or as a direct consequence of divergent selection between populations adapting to different environments (Schluter, 2001). While many cases of ecological speciation have been documented (e.g. McKinnon and Rundle, 2002; Nosil *et al.*, 2002; Bernatchez *et al.*, 2010), we have a limited understanding of why it occurs in some systems but not others. In particular, variation in progress towards speciation is largely unexplained (Hendry, 2009; Nosil *et al.*, 2009). The question of what factors promote or constrain the establishment and maintenance of reproductive barriers is therefore of fundamental importance for understanding speciation.

During ecological speciation, natural selection typically results in divergence in populations' ecological niches – the ways in which they obtain resources and interact with their environments. Niche divergence is likely to be especially important for sympatric or parapatric speciation without geographic isolation. This is because both divergent selection and an association between mate choice and traits under selection are typically required to overcome the homogenizing effects of gene flow. Different components of the niche may be more or less likely to undergo divergence during the process of ecological speciation. One useful contrast is divergence between habitats versus divergence in dietary resources that may be partitioned by sympatric species within these habitats. A distinction is often made in the ecological literature between the α - and β -niches, analogous to α -diversity (local diversity) and β -diversity (turnover between habitats). The α -niche captures niche differences among locally coexisting taxa such as partitioning of diet items or microhabitats, while the β -niche describes species' positions along 'macrohabitat' gradients such as climate or altitude (Ackerly *et al.*, 2006; Silvertown *et al.*, 2006). Either α - or β -niche divergence may be a primary factor in speciation, with divergence in local resources and habitats roughly corresponding to models of sympatric and parapatric speciation, respectively (Dieckmann and Doebeli, 1999; Doebeli and Dieckmann, 2003).

A number of case studies indicate that divergence along environmental gradients can play a key role in speciation (e.g. Richman and Price, 1992; Schneider *et al.*, 1998). In most aquatic environments, water depth is an especially important gradient. Depth habitat has a spatial component and is thus more strongly associated with the β -niche, although speciation by depth divergence can occur over a relatively small spatial extent. Speciation involving divergence in depth has been described in Lake Victoria cichlids (Seehausen *et al.*, 2008; Seehausen and Magalhaes, 2010) and coregonid fishes in Europe and North America (Turgeon *et al.*, 1999; Helland *et al.*, 2008; Vonlanthen *et al.*, 2009). In intraspecific studies in which fish are sampled at multiple sites, the depth difference between sites is sometimes a good predictor of the genetic differentiation between samples (Corrigan *et al.*, 2011). While most studies have taken place in lakes, recent evidence suggests that depth-based divergence plays a role in speciation in marine environments as well (Crow *et al.*, 2010; Ingram, 2011).

Water depth is simultaneously correlated with a number of potentially important environmental features, including light intensity and quality, temperature, pH, and oxygen availability. Because of the spatial component and strong ecological gradients associated with depth, any divergence in depth habitat between populations may reduce gene flow as a by-product of local adaptation to one or more environmental gradients. Where it results in spatial separation of spawning locations, the depth gradient may promote the occurrence of

‘magic traits’: phenotypes that mediate both divergent natural selection and assortative mating (Servedio *et al.*, 2011). In some cases, adaptation to different depth environments may directly promote divergence in mating preference through sensory drive (Seehausen *et al.*, 2008). For example, in Lake Victoria cichlids, the strength of the correlation between depth habitat and colour phenotype is related to the extent of genetic differentiation between populations (Seehausen and Magalhaes, 2010). Despite growing evidence that water depth can play a role in speciation, its importance has only rarely been tested (Seehausen *et al.*, 2008; Vonlanthen *et al.*, 2009; Ingram, 2011). In particular, it is largely unknown whether the magnitude of depth segregation is predictive of the extent of progress towards speciation.

In other cases, divergence in the dietary (α) niche may be a more important component of speciation than habitat divergence. Dietary divergence appears to be a key element of speciation in some lacustrine fish populations, including threespine stickleback (Schluter, 1993), crater lake cichlids (McKaye *et al.*, 2002; Barluenga *et al.*, 2006), arctic char (Gislason *et al.*, 1999), and Sulawesi silversides (Roy *et al.*, 2007). The use of benthic prey items such as insect larvae and pelagic prey items such as zooplankton favour different foraging behaviours and morphology. Disruptive selection resulting from intraspecific competition or a bimodal resource distribution may favour ecological divergence and potentially reproductive isolation. The dietary niche may also be involved in the occurrence of magic traits, if dietary divergence results in assortative mating. This can occur because mating is based on visible phenotypes such as body size that are associated with diet differences (Nagel and Schluter, 1998), or because mating is directly associated with separation in foraging microhabitat or behaviour (Snowberg and Bolnick, 2008).

While either depth habitat or diet may be the primary axis of divergence during non-allopatric speciation in fish, these axes may also interact. For example, simultaneous divergence on both axes will increase the dimensionality of speciation. If populations are divergent on multiple niche axes, it is thought that speciation is more likely to go to completion (Nosil *et al.*, 2009). Even in systems such as crater lake cichlids where sympatric divergence in diet is thought to be the primary factor, divergence in breeding depth appears to play some role in reproductive isolation (McKaye *et al.*, 2002). Similarly, many cases of divergence along depth gradients are thought to involve at least some divergence in diet, even if only because the composition of potential prey items changes with depth (Vonlanthen *et al.*, 2009; Seehausen and Magalhaes, 2010). In other cases, such as *Coregonus albula* and *C. fontanae* in Lake Stechlin, divergence in depth seems to be accompanied by little if any dietary differentiation (Helland *et al.*, 2008). Given a system with sufficient variation in both ecological and genetic divergence among populations, we should be able to test whether divergence in depth, in diet, or their interaction is the better predictor of progress towards speciation.

Whitefish radiations in Swiss lakes

Whitefish (genus *Coregonus*: *C. lavaretus* and *C. clupeiformis* species complexes) have diversified into between two and at least six sympatric ecotypes in many large and deep lakes in the northern hemisphere (Lu and Bernatchez, 1999; Hudson *et al.*, 2007, 2011; Bernatchez *et al.*, 2010; Siwertsson *et al.*, 2010). We use the term ‘ecotype’ to refer to any population with characteristic morphology associated with its ecology (diet and/or habitat). This definition spans a continuum from weakly differentiated morphs with low genetic distinctiveness to reproductively isolated species. This variation makes whitefish a useful system in which to test for ecological correlates of progress towards speciation.

One centre of whitefish diversity is in deep lakes on the northern slopes of the European Alps. This region houses a monophyletic radiation consisting of about 40 distinct ecotypes in the *Coregonus lavaretus* species complex, many of which have been recognized as distinct species (Kottelat and Freyhof, 2007). The species complex derives from a hybridogenic founding population, and includes many ‘sub-radiations’ that are endemic to individual lakes or groups of historically connected lakes (Hudson *et al.*, 2011). These sub-radiations are generally monophyletic, implying that most speciation has occurred within lakes. As in other European lakes (Sivertsson *et al.*, 2010), lake depth is a good predictor of historic whitefish ecotype diversity. However, lakes with extensive anthropogenic eutrophication have subsequently seen ecotype extinctions associated with reduced oxygenated depth (Vonlanthen *et al.*, 2012). The genetic, phenotypic, and ecological population structure has been the subject of detailed investigation in some of these lakes. These studies indicate that genetic divergence between ecotypes is typically associated with some degree of divergence in spawning depth and trophic morphology (Vonlanthen *et al.*, 2009; B. Lundsgaard-Hansen *et al.*, submitted). Here we extend these studies across multiple lakes by testing whether the magnitude of divergence in depth or diet better predicts genetic differentiation between sympatric ecotypes.

METHODS

Whitefish were sampled from each of six lakes located throughout Switzerland using gill nets placed at multiple depths. Lakes included in this sample were: Constance ($n = 5$ ecotypes), Lucerne ($n = 3$), Neuchâtel ($n = 2$), Thun ($n = 5$), Walen ($n = 2$), and Zurich ($n = 2$) (detailed information about lakes and sample sizes are given in Table 1). For this study, we used a total of 834 individuals collected between 2004 and 2010 in conjunction with other sampling. In two lakes (Lucerne and Neuchâtel), fish were sampled systematically along water depth gradients during the spawning period. In the remaining lakes, sampling was targeted to the known spawning grounds of the different ecotypes based on previous sampling or the knowledge of local fishermen. In Lakes Lucerne, Neuchâtel, and Thun, fish were collected at multiple times from multiple sites to distinguish within-ecotype genetic structure from genetic differences between ecotypes. No substantial geographical or temporal genetic structure within ecotypes has been observed (Vonlanthen *et al.*, 2009; Lundsgaard-Hansen *et al.*, submitted; D. Bittner *et al.*, unpublished data). The spawning location and morphology of collected fish were used to assign them to the ecotypes established by earlier taxonomic work (Kottelat and Freyhof, 2007; see also Vonlanthen *et al.*, 2012). For each fish collected, we recorded the length, weight, sex, and depth of capture. The first gill arch on the left side was removed for later gill raker counts, and a sample of muscle tissue was preserved in 100% ethanol for DNA analysis.

We quantified progress towards speciation based on the extent of genetic divergence between sympatric populations. Hudson *et al.* (2011) sampled 561 polymorphic AFLP loci from a total of 48 species and ecotypes of *Coregonus*. AFLP data have both advantages and disadvantages compared with other genetic markers. Many are likely to contain regions under selection, and thus to lack the (presumed) neutrality of markers such as microsatellites. However, they can be taken as a representative sample of the whole genome, and thus may be able to pick up on heterogeneous genomic divergence that would be detectable only under more restricted conditions with strictly neutral loci (Thibert-Plante and Hendry, 2010).

We used the subset of Hudson and colleagues’ (2011) AFLP dataset corresponding to the 19 ecotypes used in the present study. This reduced dataset contained 139 individuals

Table 1. Description of lakes and whitefish ecotypes sampled for this study

#	Lake	Maximum depth (m)	Species/ecotype	N_{AFLP}	Mean depth \pm s.d. (m)	Mean $\delta^{15}\text{N} \pm$ s.d. (‰)	Mean $\delta^{13}\text{C} \pm$ s.d. (‰)	Mean GRN \pm s.d. (‰)
1	Constance	254	<i>C. sp.</i> 'Alpenrhein'	5	1.0 \pm 0.0 (5)	12.48 \pm 0.22 (5)	-28.24 \pm 0.13 (5)	32.4 \pm 2.1 (5)
2			<i>C. arenicolus</i>	6	8.5 \pm 0.0 (6)	13.95 \pm 0.20 (5)	-28.49 \pm 0.32 (5)	27.0 \pm 4.6 (3)
3			<i>C. macrophthalmus</i>	6	15.0 \pm 0.0 (6)	13.07 \pm 0.49 (7)	-27.80 \pm 0.25 (7)	37.7 \pm 2.8 (7)
4			<i>C. wartmanni</i>	6	1.5 \pm 0.0 (6)	12.93 \pm 0.61 (8)	-27.91 \pm 0.27 (8)	36.6 \pm 2.0 (8)
5			<i>C. sp.</i> 'Weissfelchen'	7	5.0 \pm 0.0 (7)	13.47 \pm 0.78 (7)	-27.27 \pm 0.58 (7)	30.1 \pm 2.7 (7)
6	Lucerne	214	<i>C. sp.</i> 'Bodenbalchen'	10	6.5 \pm 5.8 (51)	8.48 \pm 0.69 (13)	-28.01 \pm 0.29 (11)	27.7 \pm 2.8 (62)
7			<i>C. nobilis</i>	7	164.6 \pm 9.8 (41)	8.12 \pm 0.36 (7)	-28.50 \pm 0.16 (7)	37.3 \pm 1.7 (41)
8			<i>C. zugensis</i>	9	35.6 \pm 9.5 (132)	8.24 \pm 0.76 (13)	-27.94 \pm 0.20 (13)	37.9 \pm 2.6 (135)
9	Neuchatel	152	<i>C. candidus</i>	7	95.0 \pm 0.0 (57)	13.39 \pm 0.58 (11)	-27.87 \pm 0.19 (11)	32.2 \pm 2.0 (60)
10			<i>C. palea</i>	7	34.4 \pm 29.1 (68)	13.28 \pm 0.51 (11)	-28.12 \pm 0.31 (10)	29.9 \pm 3.1 (68)
11	Thun	217	<i>C. albellus</i>	6	108.0 \pm 21.7 (44)	7.34 \pm 0.73 (8)	-28.10 \pm 0.21 (8)	38.4 \pm 3.6 (44)
12			<i>C. alpinus</i>	8	82.1 \pm 33.5 (63)	7.80 \pm 0.66 (9)	-27.93 \pm 0.24 (9)	21.3 \pm 3.9 (64)
13			<i>C. sp.</i> 'Balchen'	5	41.6 \pm 44.7 (5)	7.01 \pm 0.49 (4)	-27.51 \pm 0.16 (4)	27.6 \pm 3.6 (5)
14			<i>C. fattioi</i>	11	23.2 \pm 19.4 (54)	6.97 \pm 0.59 (14)	-27.69 \pm 0.24 (13)	33.2 \pm 3.5 (57)
15			<i>C. sp.</i> 'Felchen'	4	58.0 \pm 47.0 (3)	7.12 \pm 0.38 (4)	-27.89 \pm 0.43 (4)	38.5 \pm 4.2 (4)
16	Walen	145	<i>C. duplex</i>	9	4.6 \pm 3.3 (58)	6.82 \pm 0.55 (7)	-28.52 \pm 0.29 (8)	26.2 \pm 2.2 (59)
17			<i>C. heglingsus</i>	8	43.4 \pm 5.7 (127)	6.29 \pm 0.40 (10)	-28.14 \pm 0.24 (10)	35.4 \pm 1.9 (133)
18	Zurich	136	<i>C. duplex</i>	11	3.9 \pm 1.7 (27)	14.31 \pm 0.66 (9)	-28.34 \pm 0.66 (9)	28.8 \pm 2.1 (11)
19			<i>C. heglingsus</i>	7	43.8 \pm 5.1 (42)	14.52 \pm 0.53 (6)	-27.68 \pm 0.29 (6)	37.0 \pm 2.0 (7)

Note: N_{AFLP} gives the sample size for genetic analysis. Ecotype means, standard deviations, and sample sizes (in parentheses) are given for depth of capture (untransformed), $\delta^{15}\text{N}$, $\delta^{13}\text{C}$, and gill raker number (GRN).

(4–11 per ecotype; Table 1) and 449 polymorphic loci. We used the program AFLP-SURV (Vekemans, 2002) to estimate ecotype AFLP allele frequencies using the square-root method. In the absence of information on levels of inbreeding, this method assumes Hardy-Weinberg equilibrium, but is relatively robust to deviations from this assumption (Bonin *et al.*, 2007). We calculated pairwise Nei's genetic distance between all sympatric ecotypes in our dataset, and explored using F_{ST} as an alternative measure of genetic divergence. As lakes involved in this study appear to have been colonized fairly rapidly following the last glacial retreat (Hudson *et al.*, 2011), we expect variation in genetic distance among ecotypes within a lake to represent variation in progress towards speciation (i.e. absence of interbreeding) rather than the amount of time available for divergence.

We calculated the mean square-root transformed depth of capture (hereafter 'depth^{1/2}') for each ecotype. The transformation has two purposes. First, it removes a positive relationship between the mean and variance of ecotypes' depth distributions. Second, it helps to linearize relationships between depth and environmental gradients such as temperature and light intensity. A given increment of absolute depth usually results in much greater environmental turnover in shallower than deeper water, and the transformation helps to reduce this effect. We estimated the degree of depth differentiation between pairs of sympatric ecotypes as the absolute difference between their mean depth^{1/2}, divided by a measure of variance in depth^{1/2}. As some ecotypes had zero variance in depth of capture, we calculated the latter value as the mean within-ecotype variance in depth^{1/2}, weighted by sample size. This variance (1.84) may be biased downward by the targeting of sampling to the centre of most ecotypes' depth distributions. However, as all comparisons were standardized to the same variance, any such bias does not qualitatively affect our results.

We used stable isotope analysis to quantify the extent of diet divergence among sympatric ecotypes. Stable isotopes of carbon and nitrogen are increasingly used as measures of niches in ecological studies (Fry, 2006). The ratio of heavy to light stable isotopes in a consumer is reflective of its diet, and isotopes have the advantage of measuring diet over longer time periods (days to years, depending on the tissue) than short-term stomach content data. As long as distinct resources vary in their isotope values [as is generally the case in lake food webs (Post, 2002)], isotopic differences between sympatric populations imply consistent differences in their diets. Isotope data have successfully been used to demonstrate temporally consistent trophic niche differences among coregonine fish species (Schmidt *et al.*, 2011).

Stable isotope analysis was carried out on scales collected from 4 to 14 individuals per ecotype ($N_{total} = 159$; Table 1). Scales were used because they integrate over a much longer time period than tissues such as muscle that have higher turnover rates. Scale isotope values therefore capture feeding differences over the lifetime of the fish rather than tracking seasonal fluctuations in baseline isotope values within lakes (Perga and Gerdeaux, 2003, 2005). The use of scale tissue also avoids potential biases in $\delta^{13}C$ values that could result from differences in lipid content among ecotypes (Post *et al.*, 2007). Non-replacement scales were taken from below the lateral line, washed, and acidified (1.0 M HCl for 2 min). Samples were dried (80°C for 24 h) and homogenized, and weighed (0.4 mg) into a tin cup. Stable isotope ratios were determined using a NC2500 elemental analyser coupled to an Isoprime isotope ratio mass spectrometer, and converted to the conventional 'delta' notation ($\delta^{13}C$ and $\delta^{15}N$). We calculated the mean, variance, and covariance of $\delta^{15}N$ and $\delta^{13}C$ values for each ecotype.

We did not have baseline data (isotope values of primary consumers) necessary to convert isotope data to standardized ecological variables such as trophic position and percentage of benthic carbon (Post, 2002). Instead, we measured isotope distances between sympatric

ecotypes relative to variation and covariation within ecotypes [for an alternative approach to standardizing isotopic divergence, see Kaeuffer *et al.* (2012)]. Specifically, we measured diet differentiation as the square-root of the Mahalanobis distance (Mahalanobis, 1936):

$$D = [(\mu_1 - \mu_2)' \Sigma_{1,2} (\mu_1 - \mu_2)]^{1/2},$$

where μ_1 and μ_2 are vectors of ecotype means for each dimension (in this case, $\delta^{15}\text{N}$ and $\delta^{13}\text{C}$), and $\Sigma_{1,2}$ is the average of the covariance matrices of the two ecotypes. Mahalanobis distance is a convenient measure of divergence in sets of characters that differ in units and dimensionality, as it puts all sets of traits on a comparable scale (Arnegard *et al.*, 2010). The standardization provides a meaningful estimate of isotopic niche divergence in the absence of baseline data, although the potential to detect isotopic differentiation still relies on there being sufficient variation in the isotopic values of potential prey items. Such variation can be assumed in large and deep lakes (Perga and Gerdeaux, 2005). Our measure of depth divergence can also be interpreted as Mahalanobis distances, though this distinction is unnecessary for a one-dimensional niche measure with intra-ecotype variances assumed to be constant. These and all further calculations and statistical analyses were done in the R environment (R Development Core Team, 2011).

In addition to the genetic, depth, and isotopic distances between ecotypes, we also examined a measure of phenotypic divergence. We calculated the mean and variance of gill raker number for each ecotype, and calculated Mahalanobis distances between each sympatric ecotype pair. Gill raker number is an important, typically heritable ecological trait in *Coregonus* and other fishes, with high numbers of gill rakers generally associated with consumption of smaller (i.e. zooplanktonic) prey (Kahilainen *et al.*, 2011). This trait provides a convenient measure of ecologically relevant morphological divergence, as variability in gill raker number among ecotypes is correlated with overall variability in body form (Vonlanthen *et al.*, 2012). Therefore, we use gill raker number distance in place of genetic distance to measure the relationship between adaptive morphological divergence and divergence in depth or dietary niche.

We tested for relationships between depth and/or isotopic divergence and genetic differentiation using two complementary approaches. Analysis of our dataset is complicated by the fact that some lakes contain three or more ecotypes while others contain a single pair. As we are not interested in comparisons between ecotypes in different lakes, the matrices of distances between ecotypes are incomplete. It is inappropriate to simply analyse distances as data points, as multiple distances from the same lake are clearly non-independent. However, the incompleteness of the matrices precludes analysis using standard Mantel permutation tests of correlations between distance measures (Mantel, 1967). On the other hand, treating lakes as replicates and averaging all depth, isotope, and genetic distances among sympatric ecotypes would discard a considerable amount of information about variation among ecotypes within lakes.

We developed two distinct solutions to this problem. The first approach directly analyses the distances between populations. This is done using a standard Pearson's correlation test after first modifying the degrees of freedom (Haag *et al.*, 2005). The number of rows in the matrix (ecotypes) is treated as the effective sample size, so correlation tests use degrees of freedom (d.f.) = 17 (19 - 2). Simulations confirm that this modification does not bias estimates of the correlation or increase Type I error. The second approach uses a modified version of the method of phylogenetically independent contrasts [PICs (Felsenstein, 1985)]. We used the genetic distance matrices to construct neighbour-joining phylogenetic trees for the

populations within each lake, treating each alternative rooting of the tree as equally likely to allow model-averaging (Burnham and Anderson, 2004) to account for phylogenetic uncertainty. We calculated sets of unstandardized PICs (modified to allow the use of Mahalanobis distances) for each ecological axis, and compared these contrasts to branch lengths in the tree as a measure of genetic distance. Unlike for standard PIC analyses where branch lengths represent time or neutral genetic divergence, in this case we expect them to reflect the extent of interbreeding between sympatric ecotypes. Both methods are described in detail in the Appendix (see evolutionary-ecology.com/data/2725appendix.pdf).

RESULTS

Estimated Nei's genetic distance between pairs of sympatric ecotypes varied from 0 to 0.043, while F_{ST} varied from 0 to 0.193. Stable isotope biplots (Fig. 1) show variable degrees of isotopic niche overlap among ecotypes both among and within lakes. Ecotype means and standard deviations for depth, $\delta^{15}\text{N}$, $\delta^{13}\text{C}$, and gill raker number are presented in Table 1. When ecological data were converted to a comparable scale using Mahalanobis distance, the distribution of depth distances between sympatric ecotypes (mean = 2.28, range 0.17–7.71) was similar to the distribution of isotopic distances (mean = 2.02, range 0.38–7.29).

There was a positive correlation between spawning depth distance and genetic distance ($r=0.45$; Fig. 2A). After modifying the degrees of freedom to account for the non-independence of distance measures, this correlation approached statistical significance

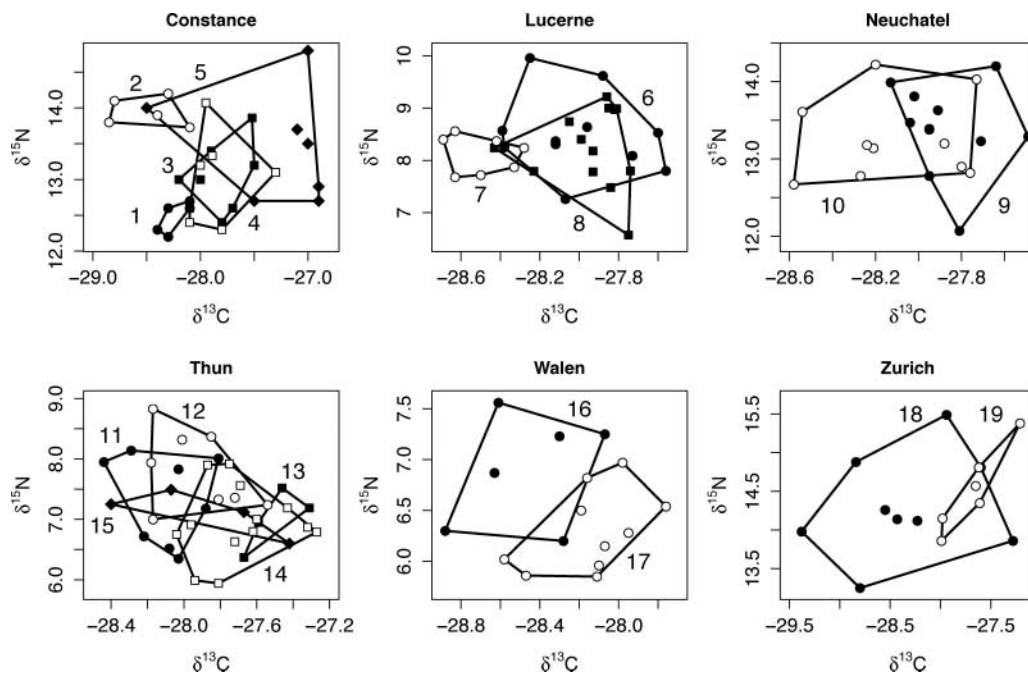


Fig. 1. Stable isotope data ($\delta^{15}\text{N}$ and $\delta^{13}\text{C}$) for the 19 ecotypes from the six lakes used in this study. Each ecotype within a lake is represented by a distinct combination of symbol and shading, and enclosed by a convex hull. Numbers beside convex hulls correspond to the ordering of ecotypes in Table 1.

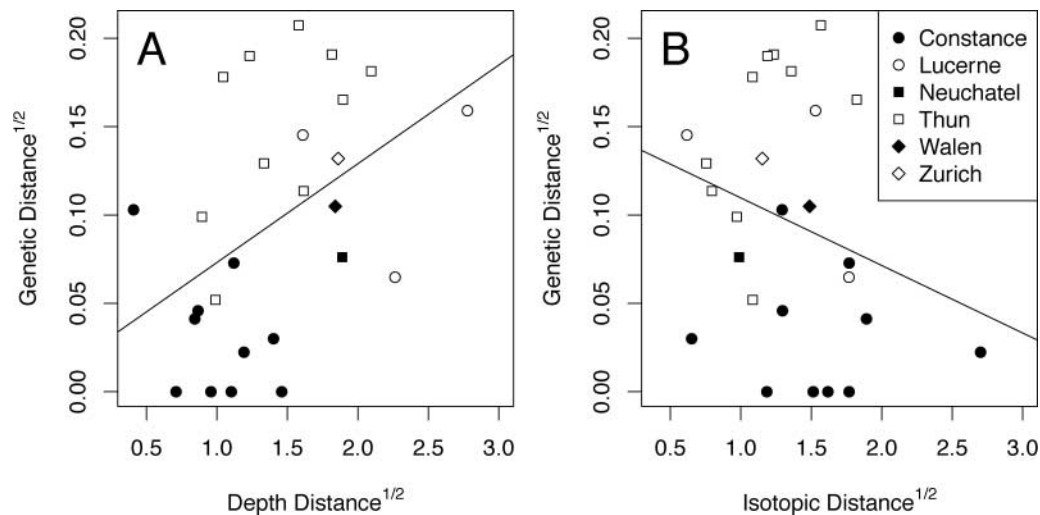


Fig. 2. All pairwise genetic distances between sympatric ecotypes versus all pairwise differences in (A) spawning depth and (B) diet as measured by stable isotopes. Distances between ecotypes are square-root transformed, and different lakes are labelled by shading and symbol as indicated in the legend. Least-squares regression lines are shown to illustrate the general relationships.

($t_{17} = 2.07$, $P = 0.054$). Isotope distance, on the other hand, showed a non-significant negative correlation with genetic distance ($r = -0.26$, $t_{17} = -1.11$, $P = 0.28$; Fig. 2B). The results were effectively identical when genetic distance was calculated using F_{ST} in place of Nei's genetic distance (depth: $r = 0.45$, $t_{17} = 2.09$, $P = 0.052$; isotope: $r = -0.27$, $t_{17} = -1.14$, $P = 0.27$). Depth distance and isotope distance were not themselves correlated ($r = -0.02$, $t_{17} = -0.07$, $P = 0.94$).

Trophic morphological distance (gill raker distance) was not correlated with either depth distance ($r = 0.03$, $t_{17} = 0.11$, $P = 0.92$) or isotope distance ($r = 0.06$, $t_{17} = 0.23$, $P = 0.82$). Gill raker distance and genetic distance showed a trend towards a positive correlation ($r = 0.37$, $t_{17} = 1.62$, $P = 0.12$).

The contrasts-based analysis was generally consistent with the direct analysis of the distance matrices. In the linear model with depth contrasts as the sole predictor of genetic distance, depth had a positive effect on genetic distance ($\beta = 0.038$; Fig. 3A), and the slope was positive in all 147 alternative tree configurations. However, between uncertainty in parameter estimation and in tree rooting, this parameter estimate was associated with a relatively high standard error (s.e. = 0.027), so the t -test revealed only a weakly positive trend ($t_{11} = 1.39$, $P = 0.15$). The univariate model with depth as a predictor had the lowest mean ΔAIC_c at 0.93, but was only incrementally better supported than the null model with no predictors (mean $\Delta AIC_c = 1.21$). Depth effects were not altered substantially based on the inclusion of isotope contrasts or the interaction term in the model (Table 2).

Isotope contrasts had a negative but non-significant relationship with genetic distance ($\beta = -0.040$, s.e. = 0.049, $t_{11} = -0.80$, $P = 0.28$; Fig. 3B), also consistent with the direct analysis of distances. As was the case for depth contrasts, the estimate and significance of isotope contrasts were largely unaffected by the inclusion of other terms in the model. The univariate model with isotope contrasts as a predictor was less well supported (mean

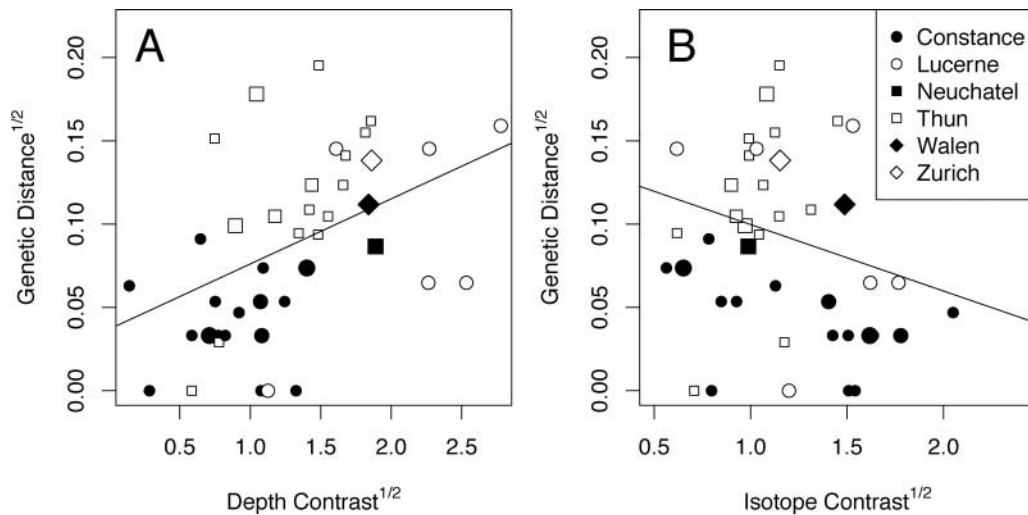


Fig. 3. All unique independent contrasts for genetic divergence versus (A) depth contrasts and (B) isotope contrasts, across all alternate rootings of the within-lake neighbour-joining trees. Symbol size is proportional to the natural logarithm of the number of times each unique contrast occurs in the set of 147 tree configurations (e.g. contrasts from the three lakes with only two ecotypes appear in all analyses). Distances between ecotypes are square-root transformed, and different lakes are labelled by shading and symbol as indicated in the legend. Lines correspond to the average slope and intercept for the univariate analysis of genetic divergence versus depth or isotope contrasts across all configurations.

$\Delta AIC_c = 2.30$) than the model with depth, as was the additive depth + isotope model (mean $\Delta AIC_c = 2.16$).

The interactive effect of depth contrasts and isotope contrasts was weak ($\beta = 0.019$, S.E. = 0.099) and non-significant ($t_9 = 0.19$, $P = 0.38$). The interactive depth \times isotopes model was the only one that could be effectively excluded from the set of credible models on the basis of AIC_c (mean $\Delta AIC_c = 6.01$).

As measured by the sum of the mean Akaike weights of models including the term, no term in the analysis had high statistical importance (i.e. approaching a value of 1). Depth contrasts had an importance value of 0.528, compared with 0.387 for isotope contrasts and 0.024 for the interaction term.

Also consistent with the analysis of distance matrices, neither depth contrasts nor isotopic contrasts were predictive of gill raker number contrasts. The null model with no predictor variables provided a better fit (mean $\Delta AIC_c = 0.136$) than any model including depth or isotopic contrasts (all mean $\Delta AIC_c > 2$; see Table 3 for full results). The statistical importance inferred from summed mean Akaike weights was low for depth contrasts (0.298), isotope contrasts (0.264), and their interaction (0.034).

DISCUSSION

Our study of genetic differentiation among sympatric whitefish ecotypes suggests that ecological axes differ in their importance for speciation. Depth divergence showed a marginally significant positive association with genetic divergence, while there was no

Table 2. Summary of regression models of genetic divergence among sympatric whitefish ecotypes against contrasts in depth and stable isotopes

Model	ΔAIC_c	w_A	Depth			Isotope			Depth \times Isotope		
			Estimate	s.e.	P-value	Estimate	s.e.	P-value	Estimate	s.e.	P-value
~ (null)	1.21	0.270	—	—	—	—	—	—	—	—	—
~ Depth	0.93	0.343	0.038	0.027	0.15	—	—	—	—	—	—
~ Isotope	2.30	0.202	—	—	—	-0.040	0.049	0.28	—	—	—
~ Depth + Isotope	2.16	0.161	0.040	0.027	0.13	-0.046	0.043	0.21	—	—	—
~ Depth \times Isotope	6.01	0.024	0.036	0.032	0.20	-0.045	0.046	0.23	0.019	0.099	0.38

Notes: All models included an intercept term, which is not presented or interpreted. Small sample-corrected Akaike Information Criterion (relative to the lowest scoring model; ΔAIC_c), Akaike Weights (w_A), and parameter estimates are averaged across all 147 tree configurations. Standard errors are estimated by combining standard errors of parameter estimates and the variance in parameter estimates across tree configurations. P-values are from *t*-tests with d.f. = 13 minus the number of terms in the model.

Table 3. Summary of regression models of contrasts in gill raker number among sympatric whitefish ecotypes against contrasts in depth and stable isotopes

Model	ΔAIC_c	w_A	Depth			Isotope			Depth \times Isotope		
			Estimate	s.e.	P-value	Estimate	s.e.	P-value	Estimate	s.e.	P-value
~ (null)	0.14	0.530	—	—	—	—	—	—	—	—	—
~ Depth	2.06	0.206	0.173	0.300	0.33	—	—	—	—	—	—
~ Isotope	2.38	0.172	—	—	—	0.044	0.555	0.39	—	—	—
~ Depth + Isotope	4.86	0.058	0.182	0.305	0.32	0.022	0.567	0.39	—	—	—
~ Depth \times Isotope	7.51	0.034	0.078	0.370	0.38	-0.045	0.046	0.23	0.492	1.365	0.36

Notes: Column headings and interpretation of results are as in Table 2.

tendency for genetic distance to increase with dietary divergence. These results were generally consistent between the direct analysis of distance data and the modified independent contrasts-based analysis.

As we had predicted, depth distance was positively related to the extent of genetic divergence between whitefish ecotypes ($r = 0.45$; Figs. 2A, 3A). However, certain caveats must be kept in mind during interpretation of the results. First, the results were at best marginally statistically significant. It may be that the effect is real and that the low statistical support reflects simply low power. Our sample sizes are relatively small at all scales, as the dataset includes six lakes containing at most five sympatric ecotypes, with only a few individuals of each ecotype measured for most traits. This will limit the precision of our estimates of ecotype means and, especially, variances, while also reducing our ability to detect any true association between measures of divergence. Furthermore, our analyses account for the non-independence of population comparisons in multi-ecotype lakes, but do not allow us to thoroughly measure the contribution of differences between lakes and variation in divergence within lakes. The positive relationship between depth and genetic divergence seems to result largely from differences between lakes (e.g. ecotypes in Lake Thun are generally more divergent in both depth habitat and genotype than ecotypes in Lake Constance), but there was some indication that variation within lakes contributed (Fig. 3A). In light of these considerations, we tentatively interpret the results as supporting a greater role for divergence in depth than diet in whitefish genetic differentiation, but caution that increased sampling of lakes and of individuals within lakes will be required before the question can be settled with confidence.

Assuming for the moment that the suggestive relationship between depth divergence and genetic distance is real, how should we interpret this pattern? As this is a strictly correlative study, we cannot be sure that greater spatial or environmental separation results in reduced gene flow, rather than reduced gene flow allowing depth differentiation (Räsänen and Hendry, 2008). Perhaps more likely, these processes may occur simultaneously. Intra-population variation in depth habitat may allow some initial genetic differentiation, which may then facilitate further depth separation, and so on. While we acknowledge that we cannot assign a causal relationship between ecological divergence and gene flow, our correlative approach remains valid as a means of comparing the importance of multiple ecological axes for divergence between ecotypes.

Evidence is accumulating that water depth gradients frequently play a role in the adaptive diversification of both freshwater and marine fish. There are a number of reasons why water depth divergence may be a particularly important predictor of progress towards speciation. Many environmental gradients covary with water depth, and the multidimensional nature of the depth gradient has the potential to cause strong divergent selection acting on populations at different depths. The multiple environmental axes might either increase the total strength of divergent selection on one aspect of the phenotype, or may lead to 'multifarious' selection acting on multiple genetically independent dimensions of the phenotype (Nosil *et al.*, 2009). Both spatial isolation and sensory adaptation involving depth may result directly in reduced gene flow and the evolution of reproductive isolation between populations. While our data do not allow us to identify which features of the depth gradient promote genetic divergence, they further point to whitefish as a promising system in which to pursue this question.

Our stable isotope measures of diet did not indicate any tendency for the magnitude of dietary divergence between ecotypes to be correlated with progress towards speciation. If

anything there appears to be a weak negative relationship between isotope distance and genetic distance ($r = -0.26$; Figs. 2B, 3B), although we cannot see an obvious interpretation for such an effect. The correlation is mainly driven by a single pair of ecotypes in Lake Constance (*C. arenicolus* and *C. sp.* 'Alpenrhein') that are genetically similar but have very different isotope values (Figs. 1, 2B). When this distance is removed from the analysis, the relationship is further weakened ($r = -0.16$, $t_{17} = -0.67$, $P = 0.51$). Thus, there appears to be no robust relationship between the magnitude of isotopic divergence between ecotypes and the extent of their genetic differentiation.

Our isotope results should be interpreted with caution, as small sample sizes limit the precision of estimates of ecotype means and covariances, and as the absence of baseline data makes the meaning of differences between ecotypes ambiguous (Fry, 2006). While there was some overlap in $\delta^{15}\text{N}$ and $\delta^{13}\text{C}$ values of sympatric ecotypes, on average the extent of divergence in isotopes was comparable to divergence in depth, when standardized by intra-ecotype (co)variances. This suggests that isotopically distinct food sources are present in the lakes, and that these are partitioned to some extent between whitefish ecotypes. Lakes in Switzerland, as elsewhere, show the expected $\delta^{13}\text{C}$ difference between benthic and pelagic prey, but also show extensive temporal variation in isotope values (Perga and Gerdeaux, 2005). As we did not detect the expected correlation between gill raker divergence and isotopic distance, isotopic differences among ecotypes may reflect temporal and/or spatial variation in prey isotope values in addition to benthic versus pelagic feeding.

If we assume that the isotope variation is indicative of dietary variation, there are a number of reasons why diet divergence may be unrelated to the extent of progress towards speciation. First, disruptive selection on diet may be weak or absent, so there may simply be no pressure to diverge in this dimension. However, the fact that there is considerable dietary diversity among ecotypes suggests that divergence in diet is not constrained by stabilizing selection or a lack of genetic variation. Alternatively, ecological conditions may favour divergence, but there may be no genetic mechanism for mate choice to become linked to diet. Body size differs between some ecotypes and may plausibly be involved in both foraging adaptation and assortative mating (Nagel and Schluter, 1998), but internal traits such as gill rakers that are more closely linked to foraging are unlikely to be used in mate choice.

In addition to the lack of any positive effect of isotopic divergence on genetic divergence, there was no evidence for a depth \times isotope interaction. This is somewhat surprising, as divergence occurring on more ecological dimensions should be a good predictor of progress towards speciation (Nosil *et al.*, 2009). However, as discussed above, divergence in depth habitat may itself be highly multivariate in nature, and divergence in depth may be sufficient to allow genetic differences to build up whether or not it is accompanied by substantial dietary divergence. The measure of depth habitat used in this study is the depth at which spawning occurs; this is similar to foraging depth for some ecotypes, but others may forage in a greater, or simply different, range of depths. Detailed sampling outside the spawning season should help to clarify the relationship between foraging depth, spawning depth, and diet.

Our analysis suggests that water depth rather than diet is the primary ecological axis associated with genetic divergence between whitefish ecotypes, but it does not tell us what factors determine whether – and to what extent – speciation will occur. For example, variation in ecotype number in Scandinavian whitefish is related to both environmental factors such as lake depth and productivity, and to time since colonization (Siwertsson *et al.*, 2010). In the case of Swiss whitefish, lake depth is also associated with ecotype diversity (Vonlanthen *et al.*, 2012), but some variation in the extent of ecotype divergence may result

from recent habitat modification by humans. For example, the low differentiation among ecotypes in Lake Constance appears to result in part from an increase in gene flow due to anthropogenic pollution over the past century (Vonlanthen *et al.*, 2012). Much as the availability of diverse depth habitats can promote speciation, the compromising of the depth gradient may be associated with failure to speciate or even speciation reversal. Ongoing study of adaptive radiation in whitefish will further reveal the contribution of different features of the depth gradient to the speciation process.

ACKNOWLEDGEMENTS

We thank D. Roy for help with sample collections and processing, and B. Matthews, L. Revell, and K. Wagner for discussions about the data and analyses. T.I. was supported by a travel grant from the European Science Foundation program 'Frontiers of Speciation Research' ('Frospects') and by the National Science and Engineering Research Council of Canada (NSERC). Data collection was supported by Eawag Action field grant AquaDiverse to O.S.

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