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PRIMARY RESEARCH PAPER

Composition of native and introduced mtDNA lineages in *Coregonus* sp. in two Austrian lakes: evidence for spatio-temporal segregation of larvae?

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Abstract We hypothesized that there is spatio-temporal genetic (mtDNA) structure of native and introduced larval whitefish (*Coregonus* sp.) in two Austrian lakes (Traunsee and Hallstättersee). Larval whitefish were sampled from 12 sites in each lake and screened for variation in the mtDNA NADH-1 gene. Based on the sequencing of adult fish together with existing GenBank sequences, an RFLP protocol was developed to assign haplotypes from larval samples into one of two divergent lineages. All but one site (pelagic) in Traunsee contained both haplotypes, thus there was no support for spatial segregation of mtDNA groups in that lake. However, weekly sampling from December to May in Traunsee revealed a temporal pattern, with the native haplotypes dominating in December and January before the appearance of the introduced Baltic clade. In Hallstättersee, only three of the 12 sites sampled revealed haplotypes from the introduced clade and thus spatial segregation seems operative on that lake. Our results imply that differences in the spawning ecology of the two groups maintain sufficient reproductive isolation to be reflected in distinct larval occurrence in space and time highly consistent with genetic differences on the mtDNA level. If the two lineages were highly introgressed, we would expect to find little or no correspondence between spatio-temporal patterns in larval distribution and the differentiated mtDNA lineages.

Keywords Coregonids · *Coregonus renke* · *Coregonus maraena* · RFLP · NADH1-gene · NADH3-gene

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Introduction

Most commercially exploited European whitefish (*Coregonus sp.*) populations are supported by stocking programmes in continental Europe (e.g. Luczynski & Ritterbusch-Nauwerck, 1995; Turkowski, 2002; Douglas & Brunner, 2002). In Austria, *Coregonus* is native to lakes situated at the northern slopes of the Alps (Gassner et al., 2003; Kottelat & Freyhof, 2007) presumably colonizing the region after the Last Glacial Maximium (LGM). In the thirteenth or fourteenth century, lakes south of the Alps were stocked with fish from lakes on the northern slopes of the Austrian Alps (Diem, 1964). Non- native *Coregonus maraena* Bloch (1779), vernacular name 'Maraene', were introduced to Austrian pond aquaculture at the beginning of the 1950s and since then are stocked annually into many

Austrian lakes. These fish originate from Polish Lake Miedwie (Madü-See in German) and were established as a hatchery strain of high commercial value in Czech pond aquaculture at the end of the nineteenth century (Šusta, 1898; IUCN, 1997). Contemporary fishery managers in Austria still draw distinction between introduced Maraene and native Reinanke in terms of the source of stocking material and in nature based on a suite of phenotypic traits (Ritterbusch-Nauwerck & Lahnsteiner, 2005).

It has been hypothesized that hybridization and introgression in combination with the favoured use of the introduced Baltic lineage by fishery managers might have led to a collapse of native lineages (Luczynski & Ritterbusch-Nauwerck, 1995). However, spatial or temporal differences in spawning among native and introduced lineages of *Coregonus* might provide the basis for reproductive isolation. Spawning runs of *Coregonus* into lake tributaries are very common in Scandinavia (Næsje et al., 1986; Salojärvi & Huusko, 1990; Salojärvi & Mutenia, 1992; Heikinheimo et al., 2000) and have also been reported in a major tributary of Lake Constance, the Alpenrhein (Rhulé & Kindle, 1992). In Austria, spawning migrations of native whitefish occur in the rivers Traun (tributary to Traunsee) and the Koppentraun (tributary to Hallstättersee) (Haempel, 1916; Neresheimer & Ruttner, 1928; Haempel, 1930; Wanzenböck et al., 2002a, b). Hallstättersee has been stocked with the introduced Baltic lineage since the middle of the last century but no stocking has been carried out since 1980 (Jagsch, 1992). Traunsee has been stocked with Baltic Maraene, with fish from Lake Constance and recruits from a breeding programme carried out with native fish. Traunsee is further interesting in that both normal and dwarf growth forms of the native lineage are found (Benda, 1949).

In Traunsee, it is presumed that only the native normal growth form ascends tributaries to spawn (Lahnsteiner & Wanzenböck, 2004), whereas local fisherman believe that both native and introduced lineages spawn in near shore littoral areas. Temporal segregation of dwarf and normal growth forms of whitefish is reported in Swiss lakes (Kirchhofer & Lindt-Kirchhofer, 1998; Douglas & Brunner, 2002) and in Traunsee (Hassan, 2000). In Traunsee, the primary spawning period of the dwarf form is in early autumn (mainly October), and the appearance of larvae is expected in December. The normal growth form is reported to have a very limited spawning period lasting from mid-November until mid-December (Neresheimer & Ruttner, 1928; Hassan, 2000, Wanzenböck et al., 2002a, b). The introduced Baltic lineage spawns from the end of November until the end of December, resulting in an overlap of the spawning time with the normal native growth form.

A standard approach to infer spawning habitat use and timing is to catch adult, running ripe (ready-to-spawn) fish in different habitats and at different times. However, this indirect method can be challenged as ripe fish may quickly swim to other places to spawn or delay the spawning act. Newly hatched larvae sampled in different habitats of a lake during different times can also be evaluated genetically to determine if they do indeed belong to reproductively isolated units or correspond to similar genetic substructure seen with adult fish. Thus, spawning habitat and timing may be delineated more precisely.

In the present study we have combined mtDNA sequence analysis of the NADH-1 gene with a diagnostic RFLP (restriction fragment length polymorphism) protocol to study the composition of mtDNA lineages in larval whitefish. Furthermore, we evaluate the potential of genetic structure, using mtDNA, related to the temporal (Traunsee) and spatial (Traunsee & Hallstättersee) occurrence of larval whitefish.

Materials and methods

Study area

The studied lakes, Traunsee and Hallstättersee, are situated in the River Traun drainage basin in the lake district Salzkammergut east of Salzburg, Austria (Fig. 1). Both are typical glacially formed, oligotrophic, sub-alpine lakes with rather low retention times due to the relatively large flow of the River Traun (Traunsee 69.4 $m^3 s^{-1}$, Hallstättersee 53.4 $m^3 s^{-1}$). Both lakes exhibit steeply sloped shorelines but otherwise differ considerably in terms of physical characteristics (Table 1). Whitefish, which serve as the main commercial target species, is the dominant fish species in both lakes. In addition to whitefish, the fish communities comprise mainly Arctic charr *Salvelinus alpinus* L., European perch *Perca fluviatilis* L., northern pike *Esox lucius* L. and several cyprinid species.

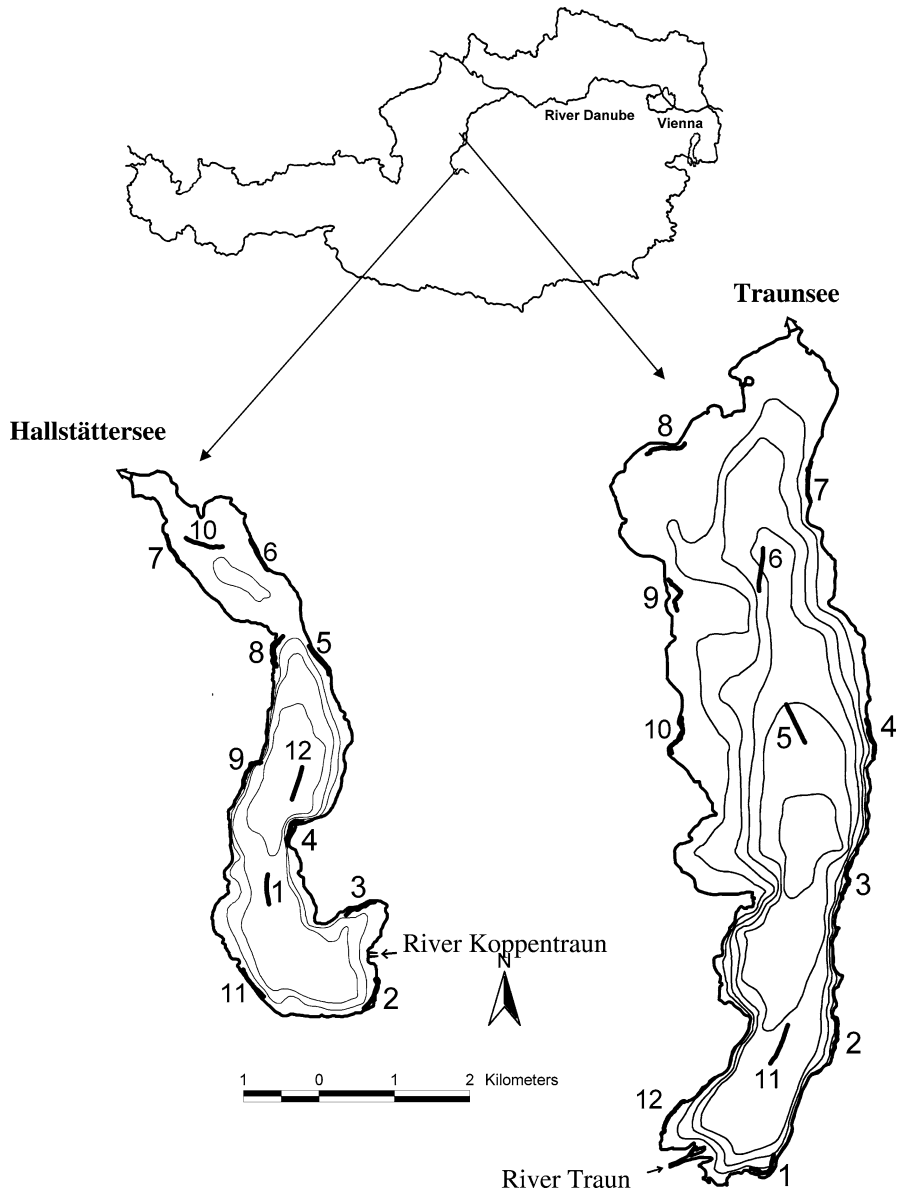

Fig. 1 Location of the studied lakes Traunsee and Hallstättersee, Austria. The sampling sites are depicted by numbered thick lines. Depth contours are given in 40-m increments

Altogether 17 fish species are present in Traunsee and 13 species in Hallstättersee (Gassner et al., 2003).

Sampling strategies

Larval and juvenile whitefish were sampled with a boat-propelled push net (Lahnsteiner & Wanzenböck, 2004). Twelve sampling sites were selected to cover the diversity of habitats and presumed spawning areas in each lake (Lahnsteiner & Wanzenböck, 2004) (Fig. 1). Nine sites were situated near shore, but in both lakes two of these stations had a pelagic rather than littoral character due to steeply sloping banks (Traunsee: stations two and three, Hallstättersee: stations four and nine). At both lakes, three stations were situated offshore and could be characterized as pelagic. At Traunsee weekly samples were taken, whereas Hallstättersee was sampled on 16 March 2004 (Table 2), as only one temporal peak of larval emergence is known from this lake (Lahnsteiner & Wanzenböck, 2004). In addition to lake sampling, drifting larvae from the River Traun inflow into both lakes (named the Koppentraun in Hallstättersee) were collected on four occasions (Table 2). The drift net

Table 1 Limnological characteristics of Traunsee and Hallstättersee (Gassner et al., 2006)

Characteristic	Traunsee	Hallstättersee
Height above sea level (m)	423	508
Drainage area (km^2)	1422	646
Lake area (km^2)	24.4	8.6
Maximum depth (m)	191	125.2
Mean depth (m)	89.7	64.9
Volume (10^6 m^3)	2188.7	588.1
Outflow (m^3 s^{-1})	69.4	35.4
Theoretical retention time (years)	1	0.5
Mean total phosphorus content (mg m^{-3})	3.4	8.7
Oxygen level in the hypolimnia (mg l^{-1})	10	8.5
Average Secchi depth (m)	5.4	7.9
Trophic classification	Oligotrophic	Oligotrophic

was built of a rectangular metal frame (51 cm × 44 cm) and a conical ichthyoplankton net (mesh size—750 µm at the front, 650 µm at the rear). A collecting bucket with a removable bottom was fixed at the rear end of the net. The construction was exposed for fifteen minutes, emptied and pushed into the river again. Upon capture the larvae were separated from plankton and other organic matter, counted and preserved in 96% ethanol. The catch per unit of effort (CPUE) for push net fishing was standardized to the number of larvae 100 m^{-3} of the water filtered.

Genetic analysis

A diagnostic RFLP protocol was designed based on a sample of 29 adult fish from various lake locations including the two inflow areas. Whole genomic DNA was extracted from ethanol-preserved fin clips with a standard high salt protocol (Sambrook et al., 1989). The whole NADH-dehydrogenase-1 mtDNA gene (ND1) was PCR amplified using self-designed primers Tt-ND1-F1: 5′-GTA ATT GCG AAA GGC CTA AG-3′ and Tt-ND1-R1: 5′-CCC CTA TTA GCC ACG CTA TC-3′. In order to place our results in the framework of a large-scale phylogeographic study on *Coregonus,* we additionally sequenced a selected number of individuals for partial segments of both the NADH-3 and cytochrome b genes using the primers and conditions reported in Østbye et al. (2005). Reactions were performed in 25 µl volumes in a Gene Amp PCR System 9700. Initial denaturation was 94°C for 3 min. PCR was carried out in 34 cycles of denaturation at 94°C for 30 s, annealing at 54°C for 40 s and extension at 72°C for 60 s. Amplified DNA templates of 29 individuals (Traunsee—14, Hallstättersee—9, tributary—6) were purified using ExoSap-it and sequenced in both directions with the PCR primers using Applied Biosystems Big Dye Terminator kit following the manufacturer's protocols. Sequence products were cleaned using Sephadex G50 and visualized on an ABI-3100 (Applied Biosystems) genotyping apparatus. New mtDNA sequences generated in this study have been deposited in GenBank, Accession nos.: FM211035 to FM211042.

Sequences were entered into MEGA (Kumar et al., 2001) for phylogenetic analysis and a bifurcating tree was constructed based on both parsimony and distance (Kimura two-parameter model) approaches. The distance-based tree was constructed with a Neighbour-Joining (NJ) algorithm and both NJ and parsimony trees were supported with 1000 bootstrap replicates. Both trees were rooted with sequences available from *C. peled* and *C. albula*. (GenBank nos.: DQ399871 and DQ399870). The genetic relation of closely related haplotypes produced within this work was also viewed with a 95% parsimony

Table 2 Sampling design, larval catches and number of genetically assessed larvae

Location	Method	Date	No. of hauls	No. of larvae caught	mtDNA screening		
					Total analysed	Clade A	Clade B
Traunsee	Push net	18 Dec. 2003–24 May 04	288	4849 (2.78/100 m^{-3})	313 (6.5%)	228 (73%)	85 (27%)
Hallstättersee	Push net	16 March 2004	12	622 (8.05/100 m^{-3})	106 (17%)	98 (93%)	8 (7.5%)
River Traun	Drift net	8 March–10 March 2004	45	81	23 (28%)	20 (87%)	3 (13%)
R. Koppentraun	Drift net	16 March 2004	2	161	17 (10.5%)	16 (94%)	1 (6%)
Total			347	5713	459 (8%)	362 (79%)	97 (21%)

network made with the program TCS ver. 1.13 (Clement et al., 2000).

Results

Sampling

A total of 5713 larval *Coregonus* were captured from the two lakes including the inflow locations (Table 2). As Traunsee was sampled across 24 weekly intervals, a large number of samples were taken from this lake. With the use of a random number generator as well as additional consideration given to sites or dates where few fish were caught, approximately 500 larvae were chosen for screening with an RFLP protocol.

Sequencing

The entire ND1 gene, comprising 975 bp, was read in 29 individuals. A total of nine GenBank sequences (DQ399863 to DQ399869, DQ399871, AB034824; Kohlmann et al., 2007) was added to this dataset and aligned using MultAlin ver 5.4.1 (Corpet, 1988). From this combined alignment, there were a total of 54 variable sites of which 39 were parsimony informative defining 15 haplotypes. Excluding the out-group, there were 13 haplotypes, defined by 25 variable sites of which 14 were parsimony informative. There were 22 synonymous and 3 non-synonymous substitutions. The NJ and parsimony trees showed very similar results so only the NJ tree is shown, in which only unique haplotypes are depicted (Fig. 2). There was a 3.2% net difference between the out-group taxa, *C. peled* and *C. albula* and the in-group sequences. Within the in-group, two clades (A & B) are relatively well supported and represent mtDNA lineages with a net difference of 0.6%, based on seven fixed positions between the two clades (Fig. 2). Twelve of the 14 sequences from Traunsee, 8 of the 9 sequences from Hallstättersee, and all 6 larvae sequenced in the river Koppentraun could be assigned to Clade A. Clade B included a sequence corresponding to the most frequent Baltic drainage haplotype (H2) reported in Kohlmann et al. (2007), in which their work sequenced a longer PCR product.

A total of 246 bp for the ND3 and 282 bp for the cytochrome b gene in 10 individuals from each lake (5 from Clade A and 5 from Clade B) were aligned,

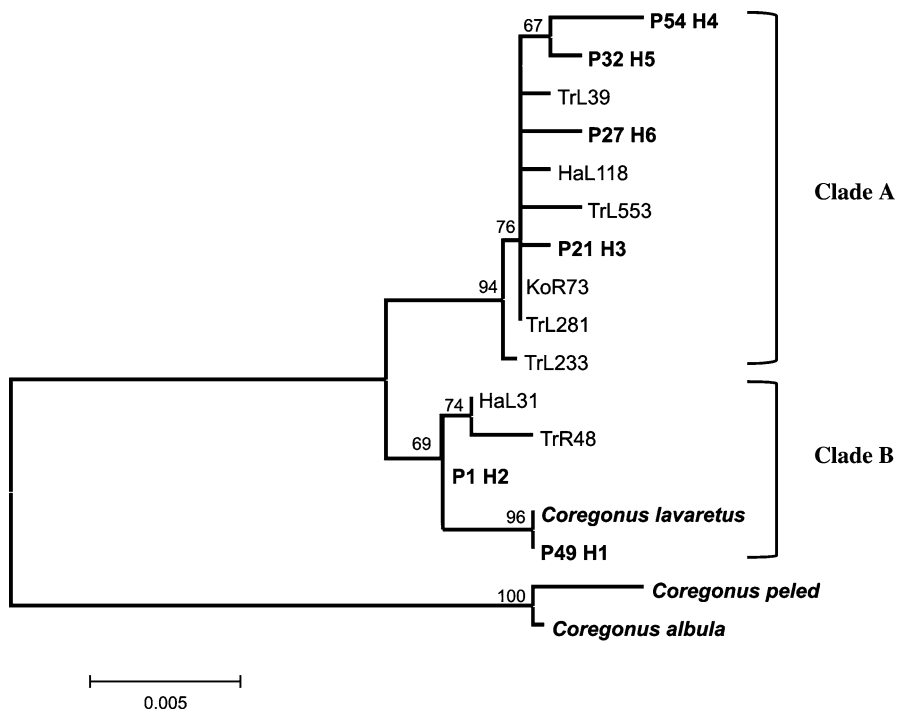

Fig. 2 Neighbour-Joining dendrogram of the complete mtDNA ND1 haplotypes. Depicted are the unique haplotypes found among the 29 individuals sequenced. The sample designations are TrL, Traunsee; HaL, Hallstättersee; KoR, River Koppentraun; TrR, River Traun; P, Polish lakes. Node support is shown as the per cent of 1000 boostrap replicates

corresponding to the haplotype data in Østbye et al. (2005). One parsimony network was produced with this data and could be directly compared to the network shown in Fig. 2 of Østbye et al. (2005). Two clusters of haplotypes can be seen, separated by eight mutational steps, and corresponding to the Clades 3-1 (North European clade) and 3-3 (South European clade) reported in Østbye et al. (2005). These clades clearly correspond to our Clade A (3-1) and Clade B (3-3). All five individuals from Clade A sampled from Hallstättersee as well as four individuals from Traunsee corresponded to the most frequent and central haplotype in Clade 3-1 (A1) shown in Østbye et al. (2005). One additional haplotype, not previously reported, was found in Traunsee. Four individuals from Clade B sampled from Hallstättersee and three from Traunsee corresponded to the central haplotype of Clade 3-3 (R1) in Østbye et al. (2005), which was the dominant clade found in Central Europe (termed Southern Europe) in that work. The three remaining individuals each carried a haplotype one mutational step divergent from this R1 haplotype.

Restriction fragment length polymorphism (RFLP)

An RFLP protocol was developed to assign individuals to one of the two mtDNA clades identified from the sequence analysis. The enzymes AciI and HgaI (New England BioLabs) each cut the ND1 gene at one of the seven diagnostic positions defining the two clades and thus were chosen for the protocol. The whole ND1 gene was amplified as described above and used as a template in the digest. Restriction digestions were carried out at an incubation time of 3 h at 37°C in 20 µl volumes using 10 µl of the PCR amplified DNA, 0.25 µl of the enzyme and 2 µl buffer. The digests were visualized on 2% agarose gel stained with ethidium bromide at 80 V for 3 h. For Clade A the following fragment patterns have been obtained: AciI—four cuts, thus creating five fragments of the template (697, 122, 68, 46, 42 bp) and HgaI did not cut the sequence. The template of Clade B has been cut by both enzymes: AciI—five cuts, creating six fragments (494, 203, 122, 68, 46, 42 bp) and HgaI—one cut resulting in two fragments (507, 468 bp). However fragments smaller than 100 bp were not distinguished on the gel.

A total of 459 larvae (8% of the sample) were screened with the RFLP protocol (Table 2). In both lakes, Clade A haplotypes were dominant with 93% ($N = 98$) occurring in Hallstättersee and 73% ($N = 228$) in Traunsee (Table 2). The temporal trend in Traunsee was analysed by grouping samples into two-week intervals. The temporal distribution of the two clades over these intervals differed significantly (K–S test, $P = 0.002$; Fig. 3) with Clade A occurring much more frequently during the first winter months (December and January). An initial total catch peak occurred in February when substantial numbers of Clade B individuals first appeared; a second much larger catch peak occurred in April, whereby both Clades were found in all samples.

In Traunsee, there was an additional temporal pattern to be seen between offshore and near shore sites (Fig. 3). Along the nine near shore sampling sites, the percentage of Clade B haplotypes varied little while they were infrequent to non-existent at the three offshore sites.

In Hallstättersee, Clade B haplotypes were only found at three of the eastern shoreline sample sites (Fig. 4) and were at their highest frequencies at stations five (36%) and six (18%).

At both river inflows most of the observed larvae could be assigned to Clade A. In the River Traun 20 of the 23 (87%) and in the River Koppentraun 16 of

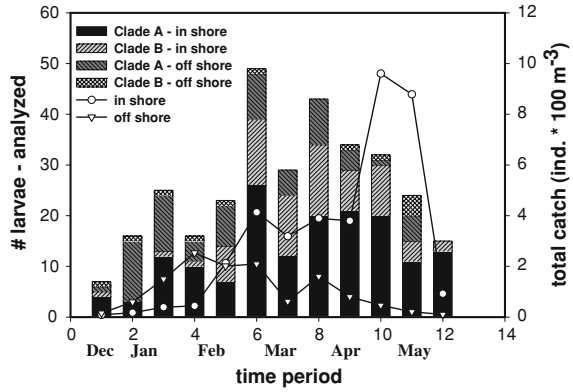

Fig. 3 Proportion of Clade A and Clade B mtDNA haplotypes found in larval whitefish during the course of the catching period and across 12 sampling sites in Traunsee. The left *y*-axis shows the number of analysed larvae indicated by bars and the right *y*-axis shows the catch per unit of effort (CPUE) standardized to the number of larvae 100 m^{-3} separated into near shore and offshore sampling sites

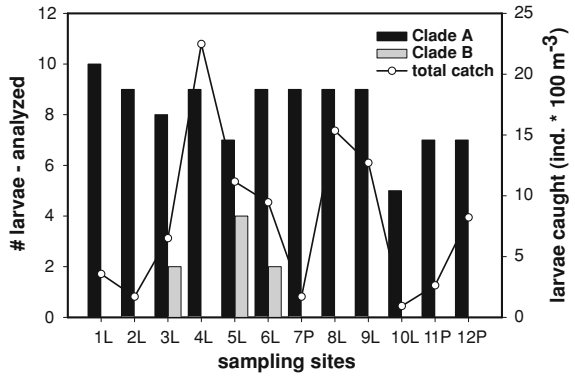

Fig. 4 Proportion of Clade A (*black bars*) and Clade B (*grey bars*) haplotypes at the 12 sampling sites of Hallstättersee. The L stands for near shore sites, the P for offshore. The left *y*-axis shows the number of analysed larvae indicated as bars and the right *y*-axis shows the catch per unit of effort (CPUE) standardized to the number of larvae 100 m^{-3}

the 17 (94%) screened larvae could be assigned to Clade A.

Discussion

This work represents the first attempt to evaluate the mtDNA genetic structure of larval *Coregonus* in Austria, and relates this structure to spatio-temporal occurrence. Two distinct mtDNA lineages (Clade A & Clade B) were revealed for the assessed lakes. We presume that the dominant Clade A represents the native (Alpine) lineage in these lakes, although Clade B is the dominant clade in Central Europe as depicted in Østbye et al. (2005). The sample sites representing this clade in Østbye et al. (2005) were all located in Switzerland, in the Rhine drainage, whereas our sample sites are in the Danubian drainage. The architecture of mtDNA lineages for freshwater fishes in the Rhine and Danube differs, as well as inferences concerning post-glacial colonization (Nesbø et al., 1999; Weiss et al., 2002). While the post-glacial colonization of Central Europe by whitefish is not well explained, it may have easily involved multiple routes or waves of expansion that differ between the Rhine and Danube drainages. We presume that the higher percentage of Clade B haplotypes in Traunsee compared to Hallstättersee reflects the more intensive use of Maraene stocks in the management of that lake. However, the predominate Clade in both lakes was Clade A, corresponding to the most frequent haplotype A1 in Østbye et al. (2005). This haplotype is predominantly found in north Fennoscandia and to a lower extent in Alpine lakes of Switzerland. We associate Clade B with Baltic 'Maraene' as it corresponds to the most common haplotype H2 found in Polish lakes (Kohlmann et al., 2007).

More importantly, there appears to be some spatio-temporal mechanisms operating in Traunsee that prevent the complete mixture of these lineages. The prevalence of Clade A haplotypes in the two inflow samples supports the notion that the tributary spawning areas are predominately or exclusively used by native whitefish stocks. Both Haempel (1916) and Neresheimer & Ruttner (1928) reported ripe individuals ascending the inflowing rivers of both lakes, decades before the lakes were stocked with Baltic, Lake Miedwie, individuals.

Spawning sites located in shallow nearshore areas are typical for many European lakes. Inshore larval catches corroborate their proximity to the spawning grounds (Karjalainen et al., 2002; Lahnsteiner & Wanzenböck, 2004). The apparent spatial pattern in Hallstättersee with larvae of Clade B solely caught at three eastern littoral sampling sites corresponds with the spawning sites of Maraene as reported by local fishermen. The spatial pattern may be additionally affected by the apparent temporal shift (with Clade A appearing first) in Traunsee. As mentioned above, the sympatric existence of native normal and dwarf populations is reported (Neresheimer & Ruttner, 1928) differing in spawning times: predominantly in October for the dwarf form and for the native normal growth form from mid-November until mid-December. The spawning time of the introduced Maraene is similar to the native normal growth form. In their extensive study on the spatio-temporal distribution of larval whitefish, Lahnsteiner & Wanzenböck (2004) described distinct patterns of larval temporal distribution in Traunsee and Hallstättersee. In Traunsee, the first larvae were caught in December in accordance with the spawning time of the dwarf form. As expected, early larvae could be assigned to Clade A because of the earlier spawning time of the native dwarf form. In accordance with the later spawning time of the native normal growth form and of Maraene, an initial catch peak was found in February, when the first Clade B individuals appeared, and peak catches occurred in April with both clades in all samples.

The results validate the presence of two different mtDNA lineages (Clade A and Clade B), with a spatio-temporal pattern, indicating that the introduced lineage did not cause a collapse of the native lineage. The findings lend support to the hypothesis that different spawning times or localities may be a mechanism preventing or limiting hybridization of native and introduced lineages. The presence of Clade B haplotypes in Central Europe need not solely stem from anthropogenic transport and stocking as the lineage is broadly distributed and may have entered Central Europe concomitantly with Clade A during one or more ice age-related expansion events. However, our spatio-temporal patterns overall, as well as the dominance of Clade A in Hallstättersee, are more easily explained if it is assumed that Clade B entered these lakes through anthropogenic activities. If the two mtDNA clades were shared in this region ancestrally, or if high levels of introgression between the introduced and the native lineages occurred, one would not expect to find any spatio-temporal patterns in their occurrence.

Furthermore, our spatio-temporal results complement information on ecological niche segregation with regard to spawning activity of sympatric whitefish lineages (Lu & Bernatchez 1999; Lahnsteiner & Wanzenböck 2004). Such data are important when studying niche partitioning of genetically distinct whitefish populations since the larval and early juvenile phase is considered a decisive period in a fish's life history (Chambers & Trippel, 1997). Mortality rates during the early life history phase are generally high, facilitating enhanced selection pressures, which might potentially be linked with local adaptation along ecological gradients and ultimately to speciation processes.

Presently, a more detailed multi-locus screening of *Coregonus* throughout Austria is underway and should be able to more definitively describe the level of population structure in several lakes where both native and introduced lineages are presumed to occur. These studies aim to promote the conservation and sustained management of native lineages.

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