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REPORT

Genetic assessment of spatiotemporal evolutionary relationships and stocking effects in grayling (*Thymallus thymallus*, Salmonidae)

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Abstract

Domestically reared introduced (or escaped) individuals can have detrimental genetic effects on the indigenous populations into which they are released. Consequently, numerous studies have attempted to estimate whether non-native specimens have contributed to the gene pool of wild populations. So far, the key limiting factor of such studies has been their lack of appropriate baseline genetic material. Here, microsatellite DNA analyses of historical scale samples and contemporary wild and introduced populations were used to assess spatiotemporal population structure and stocking effects among endangered Lake Saimaa (eastern Finland) grayling (*Thymallus thymallus*, Salmonidae). Significant decreases in genetic differentiation were detected between wild and introduced populations since the commencement of stocking in 1986. Accordingly, up to 15% of the contemporary wild grayling were confidently identified to be of pure hatchery origin, and recent hybridization between the hatchery and indigenous individuals appeared likely. Despite these clear genetic imprints of stocking, the contemporary populations exhibited evolutionary relationships congruent with the sampling locations, and up to 73% of contemporary individuals were identified to be of pure indigenous origin. The use of historical baseline material should prove efficient for monitoring gene flow between domesticated and wild populations in other species also, e.g. salmonids, game animals and plants.

Keywords

Admixture, conservation, historical scale samples, individual assignment tests, introductions, introgressive hybridization, microsatellites.

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INTRODUCTION

Gene flow from domesticated populations into the wild, whether accidental (escapees from domestically reared populations) or deliberate (introductions), may pose serious threats to natural species via decreasing the overall inherited diversity, reduction of fitness due to outbreeding depression and loss of local adaptations (Avise & Hamrick 1996). In light of these facts, numerous studies have utilized DNA polymorphisms in an attempt to estimate the effect that non-native individuals have had on the genetic composition of wild indigenous populations. Such results can be applied, e.g. for prioritizing the conservation of populations that have retained most of their indigenous genetic structure (e.g. Hindar *et al.* 1991; Largiadèr & Scholl 1996; Wayne 1996; Beaumont *et al.* 2001).

A common approach to achieve this goal has been to assess genetic differences between contemporary wild and introduced populations and to infer their levels of admixture based on "diagnostic" marker alleles (e.g. Gottelli *et al.* 1994). An alternative strategy has been to utilize individual multilocus genotype-based statistical methods to identify specimens of indigenous and introduced origin and, subsequently, estimate their proportions in the wild (reviewed in Hansen *et al.* 2001a). However, the key limiting factor in most such studies so far has been that they lacked samples from wild populations in their natural state, i.e. prior to being potentially affected by introductions. An optimal way to overcome this severe limitation would be to utilize archival genetic material collected prior to introductions as baseline data (Nielsen *et al.* 1997; reviewed in Hansen *et al.* 2001a).

Because of their complex life history characteristics, the detrimental effects of introductions utilizing genetically non-native individuals may be especially commonplace in salmonid fish populations (reviewed in Allendorf & Waples 1996). This is exemplified in European grayling (*Thymallus thymallus*, Salmonidae), which are being domestically cultured in vast numbers and increasingly released into the wild (Makkonen *et al.* 2000). Grayling may display substantial genetic differentiation across short hydrological distances (Koskinen *et al.* 2001), and are prone to local adaptations (Haugen & Vøllestad 2000).

Mainly because of destruction of suitable spawning habitat, pollution and overfishing, population sizes of *T. thymallus* inhabiting the 4380 km^2 Lake Saimaa water system (eastern Finland) have been declining in recent decades (Makkonen *et al.* 2000). Consequently, the Finnish fisheries authorities and local water owners have supplemented several parts of Lake Saimaa with hatchery-reared grayling. Extensive numbers of individuals have been introduced from broodstocks created using founders caught from a single spawning site since 1986 (Fig. 1). Particularly noteworthy is the fact that geographically (and potentially genetically) distinct populations have been mixed in a largely controlled manner, and that these populations can be assumed to have been unaffected by stocking prior to the recorded introductions (Makkonen *et al.* 2000); such an assumption can not be made in many other managed species, which may have been stocked for hundreds of years (e.g. Nielsen *et al.* 1997). Hence, Lake Saimaa grayling may provide an exceptionally good model species for studying the genetic effects of introductions in a natural setting.

Here, we report a study of the spatial and temporal evolutionary relationships of Lake Saimaa grayling, based on analyses of 10 microsatellite DNA loci. Our specific objectives were to use historical (collected prior to stocking) and contemporary samples to quantify temporal changes in *T. thymallus* evolutionary relationships caused by stocking. We further applied the genetic data to identify the origin (indigenous or hatchery) of wild-caught individuals, and to assess whether hybridization has potentially occurred between specimens from the natural and hatchery populations.

MATERIALS AND METHODS

Sampled populations

A total of 795 specimens, collected from nine different locations within Lake Saimaa (536 individuals) and seven hatchery broodstocks (259 individuals), were included in the study. An additional 37 wild-caught *T. thymallus*, originating from the Eger River (Germany), were included as an outgroup sample for phylogenetic analyses (Table 1; Fig. 1). All sampling of wild individuals was carried out by fly fishing, gill nets or seines between 1986 and 2001 in May to September (Table 1). The Lieksanjoki and Vuoksi populations represent river spawning grayling forms, whereas all other populations spawn in lakes (Makkonen *et al.* 2000).

Extensive stocking has been carried out in Lake Saimaa since 1986, initially using hatchery broodstocks founded with spawning individuals originating from Puruvesi, and more recently Lieksanjoki, Etelä-Saimaa and Vuoksi (Fig. 1). The Lieksanjoki, Etelä-Saimaa and Vuoksi broodstocks have been used exclusively for local supplementation. Conversely, the Puruvesi hatchery populations have been used for introducing a total of 108 181 grayling into Pielinen (between 1989 and 1998), 51 832 individuals into Etelä-Saimaa (1986 to 1999) and 14 110 grayling into Höytiäinen (1989 to 1998) (Fig. 1; Sundell 2000; Sundell *et al.* 2001). Three year-classes of the Puruvesi broodstocks, founded in 1986, 1990 and 1995, were included in this study (PurHat-86, PurHat-90 and PurHat-95; Table 1; Fig. 1). However, introductions into the above regions have utilized additional year classes also, founded with individuals caught from the same Puruvesi location (Fig. 1), from which samples were not available.

Scale samples from Pielinen (Pie-89-91; collected between 1989 and 1991; Table 1; Fig. 1) consisted of individuals aged 4+ to 8+, and scale samples from Etelä-Saimaa (Esa-86; collected in 1986; Table 1; Fig. 1) consisted of individuals aged 3+ to 6+. Because stocking of Pielinen and Etelä-Saimaa commenced in 1989 and 1986, respectively (Fig. 1), the scale samples represented populations unaffected by any stocking activities (stocking was performed using 1+ individuals).

DNA extraction and microsatellite analyses

DNA from tissue samples was extracted using a salt extraction protocol (Aljanabi & Martinez 1997). The following 10 microsatellites were utilized: *BFRO004*, *BFRO005*, *BFRO007*, *BFRO011*, *BFRO013*, *BFRO018*, *Cocl23*, *Ogo2*, *Str73INRA*, and *Str85INRA*. Further information of the microsatellites, their original references and detailed description of their multiplex-PCR and electrophoresis protocols is available in Koskinen & Primmer (2001). DNA from scale samples (Table 1) was extracted from three to eight scales per individual using a commercial tissue extraction kit (QIAGEN) following the manufacturer's instructions, except that the final elution step was performed with only 100 μL of distilled water (in order to increase the DNA concentration). To avoid PCR problems that may be associated with historical material, potentially leading to allelic "drop-out" events (Nielsen *et al.* 1999), each microsatellite was amplified separately for the scale samples, using primer amounts and PCR profiles reported in

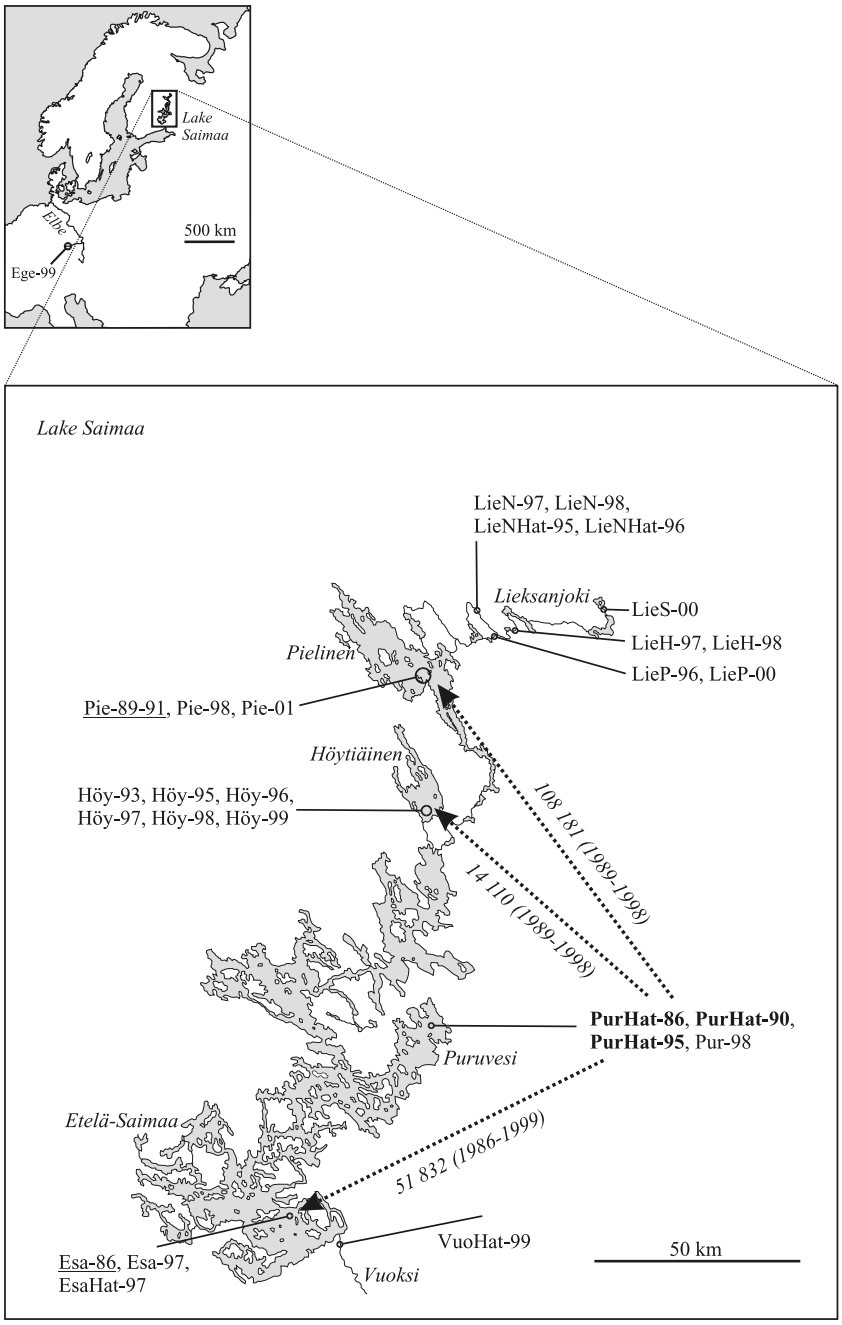

Figure 1 Map presenting the origins of the Lake Saimaa and Eger River European grayling (*Thymallus thymallus*, Salmonidae) populations utilized in the study. The Puruvesi hatchery broodstocks (marked in bold) have been used for introducing grayling into the Pielinen, Höytiäinen and Etelä-Saimaa regions. The numbers of stocked specimens and the years when the introductions took place are indicated. The historical Pielinen and Etelä-Saimaa populations (underlined) were sampled prior to the commencement of the stocking activities.

Koskinen & Primmer (2001). The criterion for including an individual for subsequent analyses was that seven out of 10 of an individual's microsatellites could be amplified successfully.

Genetic diversity and Hardy–Weinberg and linkage equilibrium tests

The POP100GENE computer program (available at: http://www.ensam.inra.fr/URLB/pop100gene/pop100gene.html) was employed to calculate allele numbers (A), observed heterozygosity (H_O) and expected gene diversity (H_E) estimates. GENEPOP version 3.2a (Raymond & Rousset 1995) was used to conduct exact probability tests for deviations from Hardy–Weinberg equilibrium (H–W-E) and exact tests for deviations from genotypic linkage equilibrium (L-E). Corrections for multiple significance tests were performed by applying Fisher's method and a sequential Bonferroni type correction (Rice 1989). H–W-E and L-E tests across populations (within loci) were carried out using

Table 1 Details and diversity indices of European grayling (*Thymallus thymallus*, Salmonidae) populations included in the study

Population details						Diversity indices[1]		
River/lake	Location	Code	Sampling/founding time	Description[2]	Sample size	A	H_O	H_E
Lieksanjoki	Naarakoski[3]	LieN-97–98	May 1997–98	Wild (t)	16	2.6 (0.8)	0.40 (0.23)	0.40 (0.22)
Lieksanjoki	Naarakoski	LieNHat-95	May – June 1995	Hatchery (t)	40	3.0 (0.8)	0.44 (0.22)	0.41 (0.20)
Lieksanjoki	Naarakoski	LieNHat-96	May – June 1996	Hatchery (t)	35	2.9 (1.3)	0.40 (0.20)	0.39 (0.21)
Lieksanjoki	Haapavitja	LieH-97	May 1997	Wild (t)	25	2.8 (1.1)	0.35 (0.21)	0.36 (0.21)
Lieksanjoki	Haapavitja	LieH-98	May 1998	Wild (t)	40	3.0 (1.3)	0.38 (0.21)	0.38 (0.19)
Lieksanjoki	Pudaskoski	LieP-96	September 1996	Wild (s)	31	2.5 (1.1)	0.43 (0.20)	0.44 (0.20)
Lieksanjoki	Pudaskoski	LieP-00	September 2000	Wild (t)	41	3.0 (0.9)	0.46 (0.20)	0.47 (0.21)
Lieksanjoki	Saarikoski	LieS-00	August 2000	Wild (t)	24	3.1 (0.9)	0.50 (0.23)	0.49 (0.21)
Pielinen	Koliranta[3]	Pie-89–91	May 1989–91	Wild (s)	30	3.0 (1.4)	0.41 (0.28)	0.42 (0.28)
Pielinen	Koliranta	Pie-98	May – June 1998	Wild (t)	77	4.5 (1.5)	0.45 (0.26)	0.50 (0.23)
Pielinen	Koliranta	Pie-01	May 2001	Wild (t)	79	4.3 (1.3)	0.45 (0.26)	0.47 (0.24)
Höytiäinen	Höytiäinen[3]	Höy-93–99	May – October 1993–99	Wild (s)	60	3.9 (1.2)	0.57 (0.21)	0.55 (0.18)
Puruvesi	Puruvesi	Pur-98	May 1998	Wild (t)	31	3.7 (1.6)	0.54 (0.19)	0.54 (0.18)
Puruvesi	Puruvesi	PurHat-86	May – June 1986	Hatchery (t)	19	3.5 (1.0)	0.53 (0.14)	0.55 (0.12)
Puruvesi	Puruvesi	PurHat-90	May – June 1990	Hatchery (t)	35	3.9 (1.5)	0.47 (0.17)	0.50 (0.16)
Puruvesi	Puruvesi	PurHat-95	May – June 1995	Hatchery (t)	39	3.7 (1.6)	0.54 (0.22)	0.52 (0.21)
Etelä-Saimaa	Etelä-Saimaa	Esa-86	May 1986	Wild (s)	37	3.3 (1.4)	0.45 (0.24)	0.48 (0.22)
Etelä-Saimaa	Etelä-Saimaa	Esa-97	May 1997	Wild (t)	27	3.7 (1.3)	0.50 (0.22)	0.53 (0.21)
Etelä-Saimaa	Etelä-Saimaa	EsaHat-97	May – June 1997	Hatchery (t)	64	3.3 (1.4)	0.54 (0.22)	0.50 (0.21)
Vuoksi	Vuoksi	VuoHat-99	May – June 1999	Hatchery (t)	27	2.8 (0.9)	0.36 (0.23)	0.35 (0.23)
Eger (Germany)	Eger River	Ege-99	Unknown	Wild (t)	37	3.5 (2.2)	0.37 (0.34)	0.37 (0.31)

[1]Allele number (A), observed heterozygosity (H_O) and expected gene diversity (H_E) estimates are averages across the 10 microsatellite loci. The corresponding standard deviations are indicated in parentheses.

[2]Samples consisted of tissue (t) or dried scales (s).

[3]Populations were formed by pooling samples collected from the same locations in different years.

samples which fulfilled the following criteria: they (1) included at least 25 mature individuals; (2) were collected from spawning grounds during spawning time in a single year (May to June within Lake Saimaa; Seppovaara 1982); and (3) were collected from regions known to be unaffected by stocking of grayling from other locations, or regions unaffected by stocking at the time when the samples were collected. Populations LieH-97, LieH-98, Pur-98 and Esa-86 fulfilled all of the above criteria. H–W-E-tests across loci (within populations) were carried out for all populations (Table 1).

Genetic differentiation and relationships between populations

The program GENEPOP was used to test for genic differentiation between populations. Corrections for multiple significance tests were performed as indicated above. GENEPOP was also employed to calculate pairwise and global (across populations) F_{ST} estimates. Potential differences in the pairwise F_{ST} estimates of the contemporary natural populations vs. populations used for the introductions, and samples collected prior to commencement of stocking vs. populations used for the introductions, were tested using Wilcoxon's signed rank test. Genetic differentiation between populations was further quantified with the D_A genetic distance of Nei *et al.* (1983). The D_A distances were used to construct a Neighbour-Joining (N-J) phylogram (Saitou & Nei 1987), which was artificially rooted with the German Eger River population. Statistical support estimates for the nodes of the N-J tree were obtained by resampling the microsatellites across 2000 bootstrap replicates. The D_A estimation, phylogram construction and bootstrapping procedures were conducted using a computer program kindly provided by Jean-Marie Cornuet and Sylvain Piry (Laboratoire de Modélization et Biologie Evolutive, INRA, Montpellier, France).

Individual assignment tests

Individual assignment tests were applied with an aim to identify pure representatives of indigenous and hatchery populations within the supplemented regions of Lake Saimaa where: (1) introductions had been carried out from locations geographically distinct from the areas where the grayling were stocked (and were thereby expected to potentially allow for detection of indigenous or hatchery individuals); and (2) historical samples collected prior to stocking were available. The individual assignment tests were conducted using the program GENECLASS version 1.0.02 (Cornuet *et al.* 1999). We employed the Bayesian statistical approach (Rannala & Mountain 1997), because earlier analyses revealed that the different assignment procedures available in GENECLASS delivered very similar results in Lake Saimaa *T. thymallus* (Koskinen *et al.* 2001). Although the analyses of GENECLASS include the possibility of "direct assignment" of individuals, statistically most rigorous conclusions can be obtained by the "exclusion method" (Cornuet *et al.* 1999), which was therefore the only method used here. The exclusion method was applied to identify wild indigenous and Puruvesi hatchery specimens within the Pielinen and Etelä-Saimaa regions (Fig. 1), using the locally caught historical and contemporary samples and all Puruvesi broodstocks as reference populations and utilizing "self classification" of individuals as indicated in Table 2.

Genetic mixture analysis

A recently developed Bayesian Markov Chain Monte Carlo (MCMC) based method, implemented in the program STRUCTURE (Pritchard *et al.* 2000), allows for probabilistic estimation of the proportion of an individual's genome originating from given reference populations (Pritchard *et al.* 2000). The method attempts to cluster individuals into K panmictic groups (where K may be unknown *a priori*) by

Table 2 Percentage of individuals assigned to, and excluded from ($P \leq 0.05$), Lake Saimaa European grayling (*Thymallus thymallus*, Salmonidae) reference populations. (a) Pielinen (Pie) and Puruvesi (Pur) individuals assigned into at least one of the Pie populations while being excluded from all of the Pur populations, and assigned into at least one of the Pur populations while being excluded from all of the Pie populations; (b) Etelä-Saimaa (Esa) and Puruvesi individuals assigned into at least one of the Esa populations while being excluded from all of the Pur populations, and assigned into at least one of the Pur populations while being excluded from all of the Esa populations

(a)

Population	*n*	Assigned to Pie and excluded from Pur	Assigned to Pur and excluded from Pie
Pie-89–91	30	83	0
Pie-01	79	73	4
PurHat-86	19	0	84
PurHat-90	35	0	80
PurHat-95	39	0	82

(b)

Population	*n*	Assigned to Esa and excluded from Pur	Assigned to Pur and excluded from Esa
Esa-86	37	41	0
Esa-97	27	48	15
PurHat-86	19	0	32
PurHat-90	35	0	54
PurHat-95	39	0	62

minimizing H–W and gametic phase disequilibrium within groups. Arguably, it may be possible not only to detect pure representatives of the formed clusters, but also potential hybrids between the K differentiated groups (Pritchard *et al.* 2000).

We applied STRUCTURE to assess the proportion of indigenous wild and Puruvesi hatchery genomes within the historical and contemporary Pielinen and Etelä-Saimaa populations. Specifically, posterior distributions of individuals' admixture coefficients (q) and their 90% probability limits were estimated for reference data sets consisting of either historical or contemporary Pielinen or Etelä-Saimaa populations and one of the Puruvesi broodstocks (the analyses were made using each one of the Puruvesi broodstocks as a reference population). This approach allowed us to assess whether the posterior distribution of q in the Pielinen and Etelä-Saimaa locations had changed since the commencement of stocking. The data sets were modelled assuming that they included 1, 2, . . . , or 5 populations. The posterior probability estimates for K with the different *a priori* assumptions on K were then calculated for each run. Following the recommendations of Pritchard *et al.* (2000), we used a burn-in period of 50 000 steps followed by 100 000 MCMC replicates.

RESULTS

Microsatellite analysis, intrapopulation diversity and Hardy–Weinberg and linkage equilibrium

The microsatellite results obtained from analyses of the tissue as well as the scale samples were unambiguously scorable and reproducible. Therefore, allelic drop-out events were not likely. The mean allele numbers across the microsatellites ranged from 2.5 to 4.5 in the Lake Saimaa populations (Table 1). The intrapopulation observed and expected heterozygosity varied from 0.35 to 0.57 and 0.35 to 0.55, respectively (Table 1).

Exact probability tests across populations did not reveal any significant ($P \leq 0.05$) deviations from Hardy–Weinberg equilibrium at the 10 microsatellite loci. Exact test for genotypic linkage equilibrium for each locus pair across populations (Fisher's method) indicated five significant ($P \leq 0.05$) deviations from L-E out of 45 possible tests. However, none of these remained significant after applying the Bonferroni-type correction for multiple tests. Exact probability tests across loci demonstrated that one of the wild-caught contemporary Pielinen populations (Pie-98) and the Etelä-Saimaa hatchery population (EsaHat-97) exhibited significant ($P \leq 0.05$) deviations from the H–W-E after correcting for multiple tests. These populations were excluded from the subsequent analyses that assume H–W-E.

Genetic interpopulation relationships of *T. thymallus* within Lake Saimaa

Despite the fact that the Pielinen and Etelä-Saimaa regions have been extensively supplemented using the Puruvesi hatchery broodstocks (Fig. 1), genic differentiation between the historical and contemporary Pielinen populations was not statistically significant. Similarly, the historical and contemporary Etelä-Saimaa populations were not statistically significantly differentiated (Appendix). In contrast, highly significant ($P \leq 0.01$) genic differentiation was observed for all between-region pairwise comparisons of *T. thymallus* samples collected from the Lieksanjoki, Pielinen, Puruvesi, Etelä-Saimaa and Vuoksi regions after correcting for multiple tests (Appendix). Interestingly, differentiation was often significant ($P \leq 0.05$) even between populations within the above regions across very short hydrological distances, for instance between the Lieksanjoki Pudaskoski (LieP-96 and LieP-00) and Haapavitja (LieH-97 and LieH-98) populations separated by < 10 km (Appendix; Fig. 1).

In line with the above results, the F_{ST} and D_A estimates between the different Lake Saimaa regions were substantial, i.e. as high as 0.47 (mean = 0.24; Appendix) and 0.51 (mean = 0.24; results not shown), respectively. Marked differentiation was observed between the Pielinen and Etelä-Saimaa populations and the populations utilized in their supplementation from Puruvesi (Appendix). For instance, the contemporary Pielinen population (Pie-01) and the hatchery populations used for stocking Pielinen (PurHat-86, PurHat-90 and PurHat-95) had pairwise F_{ST} estimates ranging from 0.17 to 0.20 (Appendix).

The level of genetic differentiation between the wild populations and populations used for their supplementation has decreased over time (Fig. 2). The temporal decrease in F_{ST} averaged 0.06–0.03 across all 10 loci, corresponding to 15.0% to 18.2% (mean = 16.6%) in the Pielinen vs. Puruvesi comparisons and 38.5% to 44.4% (mean = 41.9%) in the Etelä-Saimaa vs. Puruvesi comparisons (Fig. 2). Wilcoxon's signed rank test across loci indicated the changes to be statistically significant ($P \leq 0.05$) for all of the comparisons (Fig. 2).

The D_A genetic distance based N-J phylogram revealed relatively well supported phylogenetic population groups, that coincided with the geographical origins of the samples: the Pielinen, Etelä-Saimaa and Lieksanjoki populations each formed monophyletic clusters with bootstrap estimates of 100%, 96% and 67%, respectively (Fig. 3). In addition, all of the Puruvesi populations grouped together, with bootstrap support of 64%; however, the Höytiäinen *T. thymallus* were also placed within the Puruvesi assemblage (Fig. 3). Some internal substructures were also revealed. For instance, within the Lieksanjoki region, the Pudasjoki tributary samples (LieP-96 and LieP-00) were distinct from all of

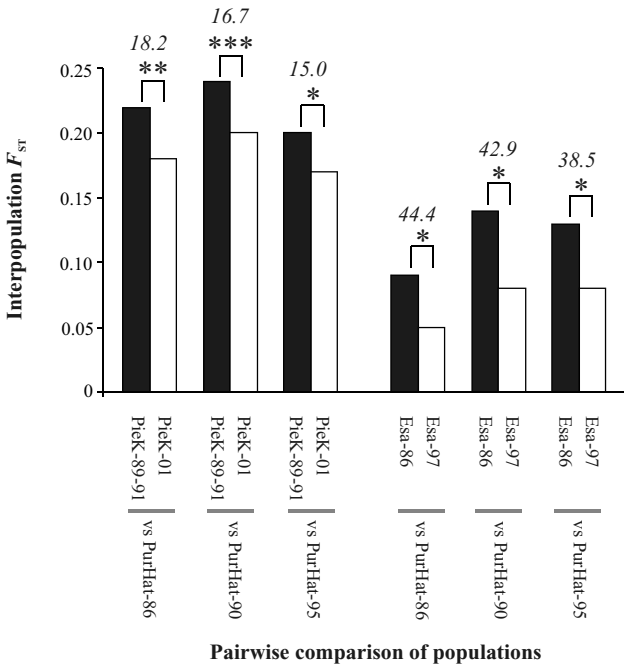

Figure 2 Temporal changes in F_{ST} estimates between Lake Saimaa Pielinen and Etelä-Saimaa European grayling (*Thymallus thymallus*, Salmonidae) populations versus the Puruvesi hatchery broodstocks used for stocking grayling into Pielinen and Etelä-Saimaa. Pie-89-91 and Esa-86 represent historical baseline samples collected prior to the commencement of stocking activities. Pairwise F_{ST} estimates for each historical (black bars) and contemporary (white bars) population versus each of the three Puruvesi broodstocks are indicated. The numbers in italics list how much the pairwise wild-hatchery F_{ST} estimates had decreased over time. The probability values for statistical significance of change in F_{ST} were obtained using Wilcoxon's signed rank test across loci. *, $0.01 < P \leq 0.05$; **, $0.005 < P \leq 0.01$; ***, $P \leq 0.005$.

the remaining Lieksanjoki grayling with a bootstrap estimate of 98% (Fig. 3). An important result of the N-J phylogram was that the contemporary and historical populations within Pielinen (Pie-89–91 and Pie-01) and Etelä-Saimaa (Esa-86 and Esa-97) formed highly supported groups (bootstrap support of 100% and 96%, respectively), rather than mixing among the Puruvesi broodstocks which have been used for supplementing these regions (Fig. 3).

Origins and the level of hatchery-indigenous admixture of historical individuals in supplemented regions of Lake Saimaa

The exclusion method, implemented in GENECLASS, indicated that 100% and 92% of the historical Pielinen and Etelä-Saimaa individuals, respectively, had the highest likelihood of originating from the local reference populations (results not shown). In addition, 83% and 41% of the historical Pielinen and Etelä-Saimaa individuals, respectively, were not only assigned to at least one of the local reference populations, but also confidently excluded ($P \leq 0.05$) from all of the Puruvesi broodstocks (Table 2). None of the historical specimens were assigned to any of the hatchery Puruvesi broodstocks while being excluded from the local wild populations (Table 2), suggesting that none of the historical Pielinen and Etelä-Saimaa grayling were of pure hatchery origin, a sensible conclusion given that the samples were collected prior to the possibility of human-mediated gene flow.

The genetic mixture analyses, implemented in STRUCTURE, for the data sets consisting of one of the wild populations and one of the Puruvesi broodstocks indicated that the posterior probability estimates for K were always the highest with an *a priori* assumption of $K = 2$. All of the reported values (Fig. 4) are thereby based on the assumption of a two-population model. In the case of the historical Pielinen samples, the genetic mixture analysis strongly supported the individual assignment test results by revealing admixture coefficients (q) ~ 1 with narrow probability limits for 29/30 (97%) of the individuals (Fig. 4). This was the case regardless of which Puruvesi broodstock was included in the reference data set. Within Etelä-Saimaa, a proportion of the historical specimens exhibited estimates of q near or below 0.5; however, these estimates were uninformative in that they always had probability limits spanning most, or all, of the 0–1 range (results not shown).

Origins and the level of hatchery-indigenous admixture of contemporary individuals in supplemented regions of Lake Saimaa

Seventy-three percent and 48% of the contemporary Pielinen and Etelä-Saimaa specimens, respectively, were assigned to at least one of the local populations and confidently excluded ($P \leq 0.05$) from all of the Puruvesi broodstocks (Table 2). Hence, the proportions of the contemporary specimens confidently identified to be of indigenous origin were similar to the historical samples. In contrast, however, 4% of the contemporary Pielinen and 15% of the contemporary Etelä-Saimaa individuals were assigned to at least one of the Puruvesi broodstocks and excluded from all local reference populations (Table 2), a result strongly suggesting that these fish were of pure hatchery origin.

Within Pielinen, the genetic mixture analysis supported the assignment test results by revealing that a majority of the contemporary Pie-98 and Pie-01 specimens were of indigenous origin ($q \sim 1$, with narrow probability limits), and a proportion of hatchery origin ($q \sim 0$, with narrow probability limits) (Fig. 4). Interestingly, the posterior distribution of q of the contemporary Pie-01 individuals was also different from that observed with the historical samples (and the Pie-98 samples) in that numerous Pie-01 grayling

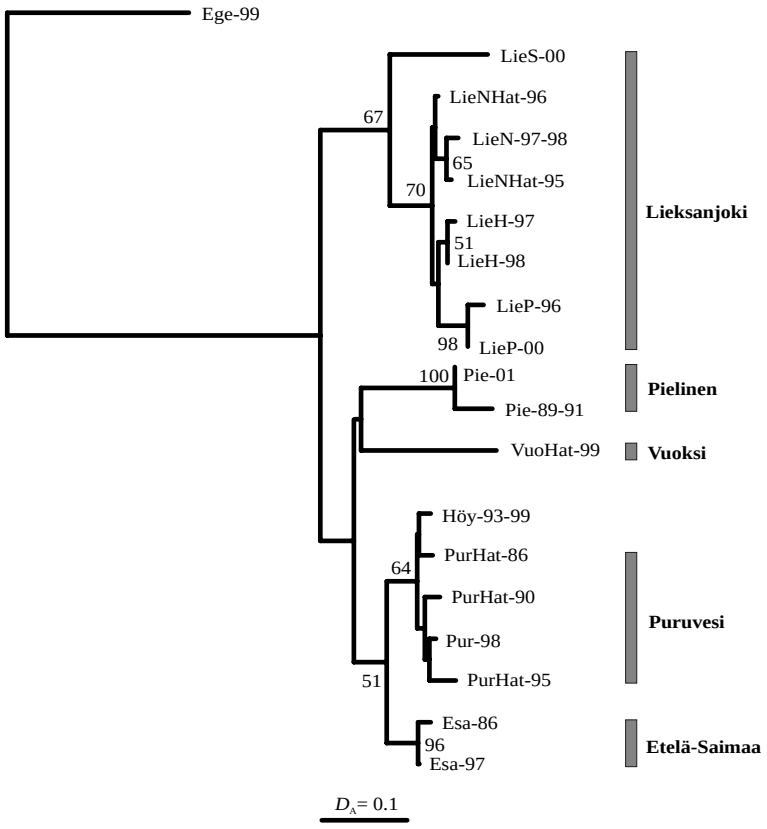

Figure 3 A Neighbour-Joining phylogram of Lake Saimaa European grayling (*Thymallus thymallus*, Salmonidae) populations based on the D_A genetic distances. Numbers beside the branches represent bootstrap support obtained by resampling the microsatellites across 2000 replicates (only estimates above 50% are indicated). Grey vertical bars indicate geographical origins of the populations (see Fig. 1).

exhibited an estimate of q near 0.5, implying hybridization between the indigenous wild and hatchery populations (Fig. 4). Although only results from the data sets including the PurHat-86 broodstock as a reference are presented (Fig. 4), the results were the same regardless of which Puruvesi hatchery population was included in the analysis. Within Etelä-Saimaa, similar posterior distributions for q were observed for the contemporary grayling as for the historical samples, i.e. all of the estimates of q were uninformative in that they had probability limits spanning most, or all, of the 0–1 range (results not shown).

The mean estimate of q across individuals within a given population can be used as an estimate of the total admixture proportion of the population (Pritchard *et al.* 2000). The mean q in the historical and contemporary Pielinen populations ranged from 0.97 to 0.98 and 0.87 to 0.90, respectively, depending on which Puruvesi broodstock was included in the reference data set. In Etelä-Saimaa, the mean q in the historical population varied from 0.81 to 0.93, whereas with the contemporary population the estimates ranged from 0.68 to 0.85. These summary statistics corresponded to a 7–11% increase in the mean level of hatchery-indigenous admixture within Pielinen, and an 8–16% increase in the mean level of hatchery-indigenous admixture within Etelä-Saimaa (results not shown).

DISCUSSION

Domestically reared individuals have been introduced into the wild in numerous culturally and economically important species. Intentional introductions of non-native specimens have probably been most extensive in salmonids (Ryman *et al.* 1995), including species such as Atlantic salmon (*Salmo salar*), cutthroat trout (*Oncorhynchus clarki*) and rainbow trout (*O. mykiss*) (reviewed in Hindar *et al.* 1991). In light of the emerging awareness that human-mediated mixing of genetically distinct populations may pose severe conservation problems (Hindar *et al.* 1991; Waples 1991; Ryman *et al.* 1995; Avise & Hamrick 1996), it has become important to monitor accurately whether the populations used for the introductions and the local indigenous populations were initially genetically differentiated, and whether changes in the genetic composition of the indigenous populations have occurred. Such results can be applied, e.g. for prioritizing the conservation of populations that have retained most of their natural genetic structure. In this study, we utilized an approach that enabled exceptionally rigorous quantification of stocking effects, through analysis of historical baseline samples in addition to contemporary genetic material.

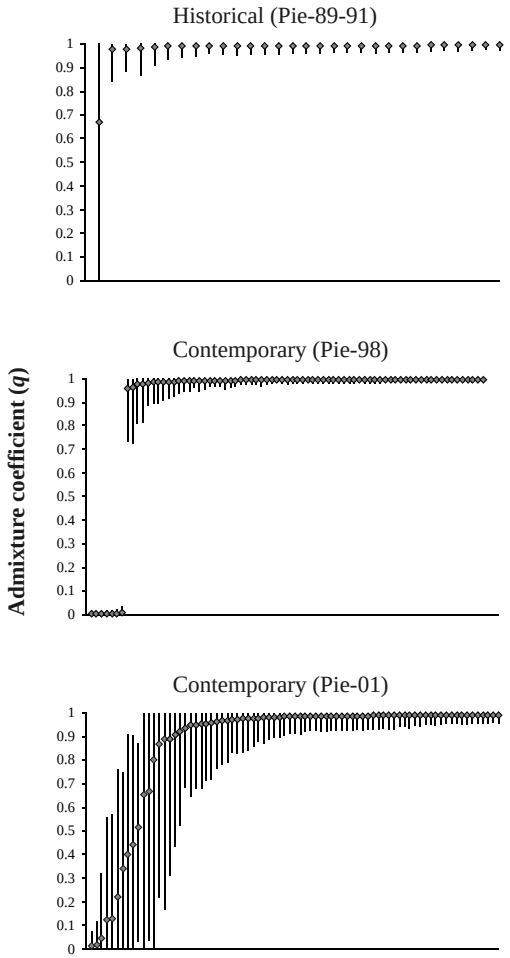

Figure 4 Posterior distribution of individual admixture coefficients (q) and their 90% probability limits in the historical Pielinen (Pie-89-91) and the contemporary Pielinen (Pie-98 and Pie-01) populations. The PurHat-86 broodstock was included in the historical and contemporary data sets, however, the results were the same regardless of which Puruvesi hatchery stock was used as a reference population. An estimate of $q = 1$ denotes pure indigenous genotype, $q = 0$ pure hatchery genotype and $q = 0.5$ may indicate first generation hybrid between the indigenous and hatchery populations. The individuals have been ordered from left to right with increasing estimates of q.

Spatiotemporal evolutionary relationships between *T. thymallus* populations within Lake Saimaa

The results of this study revealed that the Puruvesi grayling stocked into the wild had contributed to the genetic structure of the Pielinen and Etelä-Saimaa populations. Namely, consistent and significant reductions in genetic differentiation (F_{ST}) of up to 44% were evident between the wild Pielinen vs. hatchery Puruvesi populations and wild Etelä-Saimaa vs. hatchery Puruvesi populations since the commencement of stocking in 1986 (Fig. 2). Whether reproductive admixture between the indigenous and hatchery populations has in fact taken place, or whether Lake Saimaa is simply inhabited by some hatchery fish that fail to reproduce in the wild, will be discussed later.

Despite the substantial temporal genetic changes, the grayling populations had, nevertheless, retained clear imprints of their indigenous genetic population structure. For instance, the contemporary and historical Pielinen populations were not statistically significantly differentiated (Appendix) and formed highly supported monophyletic clusters in the N-J phylogram (Fig. 3). This was the case also with the contemporary and historical Etelä-Saimaa populations. Although requiring further study, it is therefore possible that many of the hatchery individuals stocked into the wild survived poorly. These suggestions parallel results in other salmonids implying natural selection acting against introduced fishes (e.g. Skaala *et al.* 1996; Poteaux *et al.* 1998; Hansen *et al.* 2000). The potential reasons for such fitness differences remain largely unknown, however, inability of the introduced individuals to effectively avoid predators has been suggested (Einum & Fleming 1997).

Lake Saimaa is currently inhabited by a number of genetically substantially differentiated populations that form five main groups coinciding relatively well with the geographic origins of the samples (Fig. 3). This finding reinforces the results from an earlier study (Koskinen *et al.* 2001) and provides additional information towards understanding the population structure of grayling across the entire Lake Saimaa water system. Such knowledge assists in the development of appropriate conservation guidelines for *T. thymallus*, and suggests that the locations corresponding to the five main population groups (Fig. 3) should be exclusively managed using hatchery populations created from locally caught founders. Moreover, some consideration of population structuring within each region is warranted. Although more detailed assessment of fine-scale differences requires further study, such substructures were exemplified by the substantially genetically differentiated Lieksanjoki populations (Fig. 3). Although genetic data have been applied for making management recommendations in numerous species (Avise & Hamrick 1996), the case of Lake Saimaa grayling is exceptional in that the distinct main groups are hydrologically very close to each other. For instance, Lieksanjoki is located 35 km from the Pielinen spawning site and Vuoksi is only 20 km from the Etelä-Saimaa population (Fig. 1).

It now appears that the historical management strategies of introducing individuals from the Puruvesi broodstocks into Pielinen and Etelä-Saimaa have resulted in the mixing of not only geographically, but also genetically, distinct populations. Such practices may eventually decrease the overall genetic diversity and compromise local adaptations of the populations involved (reviewed in Allendorf &

Waples 1996). Although we are currently investigating whether adaptive differences exist between the Lake Saimaa grayling populations, it should be noted that such differences are certainly possible, given that correlation between neutral molecular and quantitative genetic variation is commonplace (reviewed in Merilä & Crnokrak 2001), and that adaptive differentiation has also been observed between other grayling populations across short waterway distances (Haugen & Vøllestad 2000). Hence, it is positive to note that, based primarily on the results presented here, the Finnish fisheries authorities and water owners have recently ceased utilizing the Puruvesi broodstocks for supplementing the Pielinen and Etelä-Saimaa regions.

Genetic detection of the origins of individuals in stocked regions of Lake Saimaa

Approaches based on individual multilocus genotypes provided further resolution of stocking effects by enabling confident assessment of the origins of grayling within the Pielinen and Etelä-Saimaa regions. We were able to show that 4% and 15% of the contemporary Pielinen and Etelä-Saimaa individuals, respectively, were excluded from all of the local populations but not from the Puruvesi broodstocks, indicating that these individuals were most likely of hatchery origin, or pure descendants of hatchery fish. In contrast, none of the historical Pielinen and Etelä-Saimaa individuals were excluded from the local wild populations while being assigned to the hatchery populations, an observation very well in line with the idea that Puruvesi hatchery individuals now inhabit parts of Lake Saimaa because of stocking.

However, substantial proportions of the contemporary Pielinen and Etelä-Saimaa grayling most likely represented the indigenous populations unaffected by the introductions: 73% of the contemporary Pielinen individuals could be assigned to the local reference populations and excluded from all of the Puruvesi hatchery broodstocks. Furthermore, 48% of *T. thymallus* presently inhabiting Etelä-Saimaa could be excluded from all of the Puruvesi hatchery populations and assigned to Etelä-Saimaa (Table 2). These findings reinforce the population-level inferences that Lake Saimaa grayling have retained much of their indigenous genetic structure.

Although the population-level analyses and individual assignment tests indicated that stocking had resulted in accumulation of hatchery genomes into the indigenous populations, they nevertheless did not given an indication whether the introductions had resulted in *inherited* population structure changes. Can introgressive hybridization between the hatchery and indigenous grayling be revealed in the wild? The Bayesian clustering method, introduced by Pritchard *et al.* (2000), was specifically applied to address this question. An interesting result was that many of the Pielinen specimens, captured in 2001, exhibited an intermediate estimate of q, but this was not the case with the historical *T. thymallus*, all but one of which had $q \sim 1$, with narrow probability limits (Fig. 4). Such a pattern is exactly what one would expect if hybridization had occurred following the introductions (Pritchard *et al.* 2000). It could be argued that the probability limits of q were so wide for many of the contemporary Pielinen grayling that they may as well be of pure indigenous or hatchery origin, but exhibit uninformative genotypes, rather than being hybrids. This problem was already pointed out by Pritchard *et al.* 2000), and also observed with other empirical studies using the method (Beaumont *et al.* 2001; Hansen *et al.* 2001b). Although we can not exclude this possibility, results from the historical samples indicated that pure indigenous fishes should primarily exhibit $q \sim 1$, with narrow probability limits (Fig. 4), suggesting that the contemporary individuals with intermediate estimates of q are quite possibly hatchery × indigenous hybrids. It was also interesting that, although a proportion of the Pielinen individuals caught in 1998 were clearly of hatchery origin ($q \sim 0$), none of the specimens in this population had intermediate estimates of q (Fig. 4). This observation suggests that hybridization between the Puruvesi hatchery and Pielinen indigenous grayling has occurred only after 1998. Averaging the estimates of q suggested that the level of hatchery × indigenous admixture, at the population level of resolution, had increased 7–11% within Pielinen and 8–16% within Etelä-Saimaa, which is well in line with the observed decrease in genetic differentiation between populations over time (see above).

The possibility of rigorously identifying the origins of individual grayling offers fisheries managers a tool to separate representatives of the indigenous populations and Puruvesi hatchery stocks among wild individuals captured, prior to founding a hatchery broodstock. By enabling exclusion of non-native genomes from the created broodstocks, such a possibility may prove useful for conservation of the natural genetic integrity of, e.g. the Pielinen population, where a proportion of the contemporary grayling are clearly of Puruvesi hatchery origin (see above). Genetic detection of the origins of salmonids and consequent selection of individuals utilized for founding hatchery broodstocks has also been applied in the supplementation programmes of the winter run chinook salmon (*O. tshawytscha*) spawning in the rivers of California's Central Valley (Banks *et al.* 2000).

The importance of historical baseline genetic material in monitoring gene flow between domesticated and wild populations

Several points in this study exemplified the benefits of utilizing historical baseline samples to accurately measure

the genetic effects of introductions. Without samples collected prior to introductions, it naturally becomes impossible to estimate changes in genetic differentiation over time (Fig. 2). Sometimes contemporary genetic relationships of introduced and wild populations may shed light to this issue (Brunner *et al.* 1998); however, as exemplified in Fig. 3 and the Appendix, the introduced and wild populations may appear very distinct, although substantial stocking effects have, in fact, taken place (Table 2; Figs 2 and 4). Some studies have been able to estimate "potential maximum introgression rates" based on diagnostic marker alleles (e.g. Poteaux *et al.* 1998). This approach also would benefit from baseline samples – otherwise, by definition, only the potential maximum rates of introgression can be measured, not the actual effect of the introductions. Various forms of individual assignment tests have recently been described and such tests may prove very useful for stocking-effect assessment (reviewed in Hansen *et al.* 2001a), especially with access to historical samples (Nielsen *et al.* 1997). In this study, the use of historical baseline populations as reference data sets avoided the common problem of having to use solely the contemporary samples as reference populations; in such situations, the contribution of the introduced specimens may severely alter the allele frequencies of the wild reference populations, leading to erroneous assignment results (reviewed in Hansen *et al.* 2001a).

Conclusions

This study demonstrates that analyses of historical baseline material enables rigorous quantification of stocking effects. Although the utilized analyses enabled clear detection of stocking-related genetic changes, the wild populations had retained much of their indigenous genetic composition. These data provide evidence that natural populations can maintain a substantial proportion of their indigenous genetic structure under intensive introductions, and aid in the development of management strategies for Lake Saimaa grayling. Finnish fisheries managers now have a tool to identify the origins of specimens in the wild, enabling the selection of pure indigenous fish to create hatchery broodstocks.

Microsatellite DNA loci and the use of historical baseline material should also prove efficient for monitoring gene flow between domesticated and wild populations in other species. Such studies may be most relevant in the field of fisheries (Ryman *et al.* 1995); however, similar applications may prove important, e.g. for studies of introgression between domesticated and wild Canidae (reviewed in Wayne 1996), Felidae (Beaumont *et al.* 2001), game animals (Thulin *et al.* 1997) and plants (Ellstrand 1992).

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APPENDIX

Pairwise F_{ST} estimates and test results for genic differentiation (*$P \leq 0.05$) between populations of European grayling (*Thymallus thymallus*, Salmonidae). The population abbreviations are given in column 1, together with a population number. On space grounds, only the population number is given in the table head

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19
1 LieN-97–98																			
2 LieNHat-95	0.00																		
3 LieNHat-96	0.02	0.03*																	
4 LieH-97	0.03	0.06*	0.09*																
5 LieH-98	0.03	0.05*	0.08*	0.01															
6 LieP-96	0.11*	0.10*	0.13*	0.11*	0.11*														
7 LieP-00	0.10*	0.11*	0.12*	0.10*	0.09*	0.01													
8 LieS-00	0.20*	0.22*	0.21*	0.20*	0.22*	0.26*	0.21*												
9 Pie-89–91	0.38*	0.37*	0.38*	0.41*	0.39*	0.41*	0.36*	0.35*											
10 Pie-01	0.33*	0.33*	0.34*	0.36*	0.36*	0.36*	0.32*	0.32*	0.00										
11 Höy-93–99	0.23*	0.24*	0.22*	0.26*	0.26*	0.28*	0.23*	0.23*	0.22*	0.19*									
12 Pur-98	0.24*	0.26*	0.24*	0.29*	0.29*	0.30*	0.25*	0.24*	0.25*	0.21*	0.01*								
13 PurHat-86	0.25*	0.27*	0.25*	0.30*	0.29*	0.30*	0.24*	0.24*	0.22*	0.18*	0.00	0.02							
14 PurHat-90	0.28*	0.30*	0.28*	0.32*	0.31*	0.32*	0.26*	0.25*	0.24*	0.20*	0.03*	0.02	0.02*						
15 PurHat-95	0.29*	0.30*	0.29*	0.32*	0.31*	0.34*	0.28*	0.25*	0.20*	0.17*	0.06*	0.05*	0.05*	0.04*					
16 Esa-86	0.27*	0.27*	0.25*	0.32*	0.31*	0.33*	0.29*	0.31*	0.19*	0.16*	0.09*	0.10*	0.09*	0.14*	0.13*				
17 Esa-97	0.27*	0.28*	0.26*	0.32*	0.31*	0.32*	0.26*	0.27*	0.19*	0.16*	0.05*	0.06*	0.05*	0.08*	0.08*	0.01			
18 VuoHat-99	0.41*	0.38*	0.39*	0.45*	0.43*	0.42*	0.40*	0.47*	0.33*	0.28*	0.27*	0.31*	0.31*	0.31*	0.31*	0.24*	0.26*		
19 Ege-99	0.54*	0.53*	0.55*	0.55*	0.54*	0.51*	0.47*	0.53*	0.54*	0.50*	0.45*	0.49*	0.48*	0.49*	0.50*	0.50*	0.49*	0.58*	