Expressed Sequence Tag-Linked Microsatellites as a Source of Gene-Associated Polymorphisms for Detecting Signatures of Divergent Selection in Atlantic Salmon (*Salmo salar* L.)

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The prediction that selection affects the genome in a locus-specific way also affecting flanking neutral variation, known as genetic hitchhiking, enables the use of polymorphic markers in noncoding regions to detect the footprints of selection. However, as the strength of the selective footprint on a locus depends on the distance from the selected site and will decay with time due to recombination, the utilization of polymorphic markers closely linked to coding regions of the genome should increase the probability of detecting the footprints of selection as more gene-containing regions are covered. The occurrence of highly polymorphic microsatellites in the untranslated regions of expressed sequence tags (ESTs) is a potentially useful source of gene-associated polymorphisms which has thus far not been utilized for genome screens in natural populations. In this study, we searched for the genetic signatures of divergent selection by screening 95 genomic and EST-derived mini- and microsatellites in eight natural Atlantic salmon, *Salmo salar* L., populations from different spatial scales inhabiting contrasting natural environments (salt-, brackish, and freshwater habitat). Altogether, we identified nine EST-associated microsatellites, which exhibited highly significant deviations from the neutral expectations using different statistical methods at various spatial scales and showed similar trends in separate population samples from different environments (salt-, brackish, and freshwater habitats) and sea areas (Barents vs. White Sea). We consider these ESTs as the best candidate loci affected by divergent selection, and hence, they serve as promising genes associated with adaptive divergence in Atlantic salmon. Our results demonstrate that EST-linked microsatellite genome scans provide an efficient strategy for discovering functional polymorphisms, especially in nonmodel organisms.

Introduction

The prediction that selection affects the genome in a locus-specific way (Cavalli-Sforza 1966) affecting also the flanking neutral variation, known as genetic hitchhiking (Maynard Smith and Haigh 1974), enables the use of polymorphic markers in noncoding regions to detect the footprints of selection (Lewontin and Krakauer 1973). Thus, identification of loci that differ substantially in diversity (Schlötterer 2002a; Kauer, Dieringer, and Schlötterer 2003) and/or in population divergence (Beaumont and Nichols 1996; Vitalis, Dawson, and Boursot 2001; Beaumont and Balding 2004) from the rest of the genome can be flagged as ''outlier'' loci which are potentially affected by selection (reviewed by Schlötterer et al. 2002b; Luikart et al. 2003; Storz (in press)).

Recently, multi-locus screens based on genomic microsatellites have been applied in humans (Kayser, Brauer, and Stoneking 2003; Storz, Payseur, and Nachman 2004) and traditional model organisms (Schlötterer 2002a; Kauer, Dieringer, and Schlötterer 2003). However, the strength of the selective footprint on microsatellite locus depends on the distance from the selected site and will decay with time due to recombination (Wiehe 1998). Therefore, the utilization of polymorphic markers closely linked to coding regions of the genome would have a higher probability of detecting the footprints of selection and be more cost effective, as more gene-containing regions are covered (Vigouroux et al. 2002) compared to more conventional approaches using random selection of polymorphic markers. In addition, close linkage between a polymorphic marker and a transcribed gene further simplifies subsequent sequence analysis of the closest candidate gene, especially when the full genome sequence and/or a high-density linkage map of the study species is not available, as is the case for most nonmodel organisms. Therefore, the occurrence of highly polymorphic microsatellites in the untranslated regions of expressed sequence tags (ESTs) (Li et al. 2004) is a potentially useful source of gene-associated polymorphisms. Thus far however, the use of such gene-associated markers has been limited to linkage mapping studies (e.g., Ruyter-Spira et al. 1996) and an evaluation of their use for potentially identifying genes involved in local adaptation in natural populations is lacking. Given that the number of ESTs publicly available in species other than traditional model organisms is increasing rapidly (e.g., Rise et al. 2004), these loci have the potential to serve as a rich source for gene-associated polymorphisms and present a promising alternative to methods that utilize anonymous markers such as amplified fragment length polymorphisms (AFLP) (e.g., Wilding, Butlin, and Grahame 2001; Campbell and Bernatchez 2004), especially in species with relatively low gene density and high recombination rates.

Salmonid fishes are good candidates for assessing the efficiency of using EST-linked microsatellites for genome screens as (1) the large diversity in behavior, immunology, life-history patterns, and other traits among local salmonid populations at various geographical scales has been widely recognized as evidence of adaptation to the local environment (Taylor 1991; Adkison 1995) and (2) a large number of EST sequences are publicly available (Rise et al. 2004).

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Key words: Adaptation, nonneutral evolution, divergent selection, microsatellite DNA, genetic hitchhiking, outlier loci, EST, Atlantic salmon.

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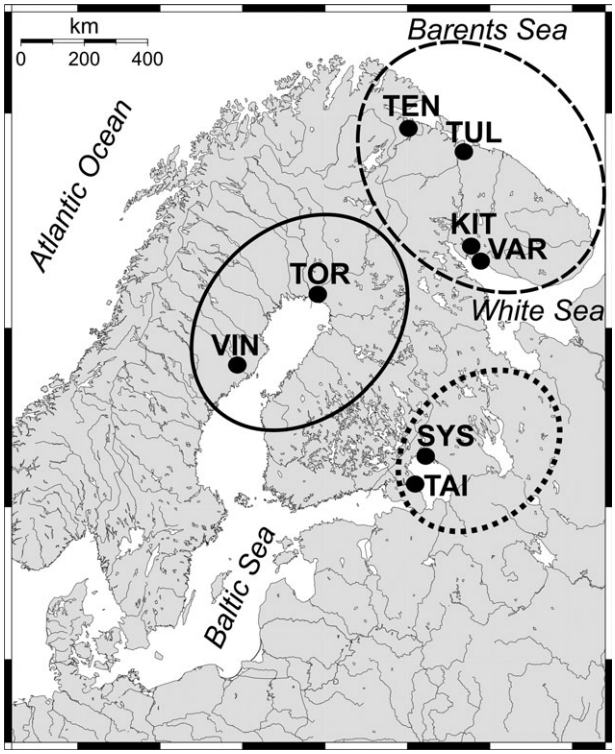

FIG. 1.—Map of Northern Europe showing locations of the studied Atlantic salmon populations. Populations inhabiting salt- (TEN, R. Teno/Tana; TUL, R. Tuloma; VAR, R. Varzuga; KIT, R. Kitsa), brackish (VIN, R. Vindelälven; TOR, R. Torne/Tornionjoki), and freshwater (TAI, R. Taipale; SYS, R. Syskynjoki) habitats during the adult feeding phase are surrounded by dashed, solid, and dotted circles, respectively.

In addition, despite their tendency to evolve local adaptations (Taylor 1991), the number of genes and genomic regions that have been found to associate with adaptive or fitness-related traits in salmonids is limited (e.g., Danzmann, Jackson, and Ferguson 1999; Sakamoto et al. 1999; Langefors et al. 2001; Tao and Boulding 2003).

In this study, we aimed to detect genetic signatures of selection in free-living populations of Atlantic salmon (*Salmo salar* L.) by screening 95 tandem repeat markers to identify genes and genomic regions potentially important for local adaptation. More specifically, we used genomic and EST-associated mini- and microsatellites to scan eight wild salmon populations sampled from different spatial scales inhabiting similar and contrasting natural environments (salt-, brackish, and freshwater habitat) in order to detect molecular signatures of divergent selection. We compared the consistency of the results obtained using four different neutrality tests and evaluated the robustness of the results across a large spatial scale by assessing whether the outlier loci possessed similar trends in different population pairs.

Material and Methods

Study Populations

Because the spatial scale of selection is expected to vary among different loci, we sampled four closely related wild population pairs (Barents Sea: R. Teno/Tana and R. Tuloma; White Sea: R. Varzuga and R. Kitsa; Baltic Sea: R. Vindelälven and R. Torne/Tornionjoki; landlocked: R. Taipale and R. Syskynjoki) inhabiting distinct natural environments (salt-, brackish, and freshwater habitat) to be able to detect divergent selection at relatively similar and contrasting environments and both small and large spatial scales (average distance between populations 171 and 671 km, respectively) (fig. 1). In total, 200 individuals were analyzed (24–28 specimens per population). Total DNA was extracted from ethanol-preserved fin clips using salt extraction protocol as outlined in Aljanabi and Martinez (1997).

EST Database Mining and Micro- and Minisatellite Genotyping

In total, 58,146 Atlantic salmon EST sequences present in the GenBank database were scanned for di-, tri- and tetranucleotide microsatellite repeats using TANDEM REPEATS FINDER v.3.01 (Benson 1999) with the following parameters: match 2; mismatch 7; indel 7; and minimum alignment score 50. Because EST databases are redundant (i.e., contain many overlapping sequences from the same gene), identified microsatellite-containing ESTs were clustered using CAP3 program with a 40-base pair overlap and 95% identity criterion in order to identify homologous loci (Huang and Madan 1999). Primers flanking 8 tetra- and 126 dinucleotide repeat sequences were designed using PRIMER3 software (Rozen and Skaletsky 2000). Similarity search of microsatellite-containing EST sequences was conducted using BlastN and BlastX with the default parameters as described in Altschul et al. (1990). Detailed amplification procedures and primer sequences are described in Vasemägi , Nilsson, and Primmer (in press). Altogether, 75 EST-associated microsatellites that gave high-quality amplification products were selected for further population-wide genotyping using a MegaBACE™ 1000 capillary sequencer (Amersham Biosciences, Buckinghamshire, UK). We also included three histocompatibility complex–linked mini- (*MHCIIα*; Stet et al. 2002) and microsatellites (*MHCI*, *TAP2B*; Grimholt et al. 2002) in the screening panel as they have been shown to associate with pathogen resistance and mate choice in Atlantic salmon (Landry et al. 2001; Langefors et al. 2001; Miller et al. 2004) and are hence good a priori candidates as loci potentially under selection. In addition, the same individuals were analyzed with 17 genomic microsatellite loci (Tonteri et al. 2005; A. Tonteri and C. Primmer, unpublished data).

Genetic Diversity and Differentiation Measures

Conformance to Hardy-Weinberg (H-W) equilibrium expectations was tested using exact tests (Guo and Thompson 1992) as implemented in GENEPOP 3.1b (Raymond and Rousset 1995). Gene diversity (Nei 1978) and pairwise F_{ST} estimates according to Weir and Cockerham (1984) were calculated with the software Microsatellite-Analyser (Dieringer and Schlötterer 2003). The significance of F_{ST} estimates among populations was tested by permuting individuals between samples. Ninety-five percent confidence intervals (CI) of the mean F_{ST} estimates were obtained by bootstrapping (1,000 replicates) over loci. Heterogeneity

in F_{ST} estimates among loci was quantified by calculating 2.5th, 25th (Q_1), 75th (Q_3), and 97.5th percentiles from the observed F_{ST} values. Because one of the neutrality tests applied (see below) assumes that no mutations have occurred after the divergence of two populations from the common ancestor population (Vitalis, Dawson, and Boursot 2001), we determined the spatial scale where stepwise-like mutations, in addition to genetic drift, have contributed to genetic differentiation among studied populations by testing whether $R_{ST} = F_{ST}$ using allele size randomization procedure (10,000 permutations) as implemented in SPAGeDi 1.1 (Hardy and Vekemans 2002). If the observed R_{ST} is significantly larger than the randomized R_{ST}, the stepwise-like mutations have contributed to the observed differentiation pattern (Hardy et al. 2003).

Methods for Detection of Divergent Selection

Spatially varying divergent selection is expected to increase genetic differentiation between populations and reduce variability at linked loci. To search for the signatures of divergent selection we applied three methods, which identify outlier loci based on various estimators of population divergence (Beaumont and Nichols 1996; Vitalis, Dawson, and Boursot 2001; Beaumont and Balding 2004) and an empirical approach based on reduction in genetic diversity (Schlötterer 2002a; Kauer, Dieringer, and Schlötterer 2003). Because of the explorative nature of multi-locus screens, we did not apply the extremely conservative Bonferroni correction for the obtained significance values, but instead, we initially report all loci that fall outside 99% from the neutral expectations. Additionally, we evaluated the status of identified candidate loci by assessing whether the putative outliers possess similar trends in separate (albeit not statistically independent) population samples from different environments (salt-, brackish, and freshwater habitats) and sea areas (Barents vs. White Sea). As all applied neutrality tests are based on different assumptions and parameters, the detection of outlier loci simultaneously with more than one statistical approach will strengthen the candidate status of particular locus.

The first method (hereafter referred to as the ''F_{ST}-test'') developed by Beaumont and Nichols (1996) calculates Cockerham and Weir's (1993) estimator of F_{ST} for each locus in the sample, and coalescent simulations based on a symmetrical island model of population structure are used to generate data sets with the mean F_{ST} similar to the empirical distribution. To calculate approximate P values for each locus, 100,000 independent loci were generated and simulated distribution of F_{ST} was then compared to the observed F_{ST} values conditional on heterozygosity to identify potential outliers as implemented in the software FDIST 2 (http://www.rubic.reading.ac.uk/~mab/software/fdist2.zip). Sample sizes were set to 24 individuals per population in all simulations. Because our pairwise sampling strategy at the large geographical scale (salt-, brackish, and freshwater comparisons; Barents vs. White Sea) likely violates the assumption of equal migration rate, individual populations within each category were pooled together (i.e., R. Vindelälven and Torne/Tornionjoki samples were pooled to construct a brackish water data set) and two subpopulations were simulated assuming stepwise mutation model. Loci with unusually high F_{ST} values conditional on heterozygosity were regarded as potentially under divergent selection.

The second likelihood-based method that uses hierarchical-Bayesian model (hereafter the Bayes test), developed by Beaumont and Balding (2004), has similar characteristics compared to the F_{ST}-test of Beaumont and Nichols (1996) but uses more information from the raw data and does not assume the same value of F_{ST} for each subpopulation. Therefore, this method should be more suitable when some populations exhibit lower variability or reduced immigration than others, which is likely the case in our data set at a large spatial scale. We applied the Bayes test to identify potential outliers from neutrality associated with different environments (salt-, brackish, and freshwater habitat comparison) and sea areas (Barents vs. White Sea). It should be noted that the Bayes test is not a pairwise test because all populations in a particular analysis are treated separately. We did not apply the Bayes test to closely related population pairs at the local scale as simulations by Beaumont and Balding (2004) showed that there was no advantage to combine F_{ST}- and Bayes tests (both based on F_{ST} estimation) when the same number of subpopulations were used (i.e., there was considerable growth of false positives compared to very few additional ''truly'' selected loci). We identified outlier loci potentially subject to divergent selection and their corresponding posterior ''P values'' from the proportion of positive locus-effect parameters α_i among 2,000 Markov chain Monte Carlo outputs as outlined by Beaumont and Balding (2004).

The third coalescence-based simulation approach (subsequently referred as the F-test), developed by Vitalis, Dawson, and Boursot (2001), relies upon a population-split model from the common ancestor population and uses the population-specific parameters of population divergence, F (conditional on the number of alleles), to identify putative outlier loci affected by selection. The expected joint distributions of F_{pop1} and F_{pop2} were generated by performing 100,000–500,000 coalescent simulations for each pairwise comparison using the software DETSEL v.1.0 (Vitalis et al. 2003). The following nuisance parameters were used in different combinations to generate null distributions with similar number of allelic states as in the observed data set: mutation rate (infinite allele model [IAM]) 0.005, 0.001, and 0.0001; ancestral population size 500, 1,000, and 10,000; population size before the split 50 and 500; time since an assumed bottleneck event 50, 100, and 200 generations; time since the population split 50 and 100 generations. The loci with six or more alleles were grouped together as the joint distribution of F_{pop1} and F_{pop2} becomes tighter when the number of alleles increases (Vitalis, Dawson, and Boursot 2001). Loci that fall outside the specified ''probability region'' compared to the simulated data points are reported as potentially being affected by selection.

The fourth empirical approach (hereafter referred to as the *lnRH* test) identifies loci that differ in variability from the reminder of the genome by calculating the ratio of gene diversity in two populations (Kauer, Dieringer, and Schlötterer 2003). It has been demonstrated that *lnRH* is approximately normally distributed under neutrality (Kauer,

Table 1
Genetic Diversity (A_M, mean number of alleles; H, gene diversity) and Divergence Estimates (R_{ST}, genetic differentiation based on allele size; F_{ST}, genetic differentiation based on allele identity; F, population-specific divergence) of the Studied Loci in Atlantic Salmon Populations from Different Spatial Scales and Environmental Conditions (salt, brackish, and freshwater habitats)

	A_M		H		R_{ST}		F_{ST}				F	
Comparison	Pop1	Pop2	Pop1	Pop2	Obs.	Perm.	Mean	95% CI	Q_1 & Q_3	2.5th, 97.5th Percentile	Pop1	Pop2
Small scale												
White Sea: KIT (pop1) versus VAR (pop2)	6.7	6.6	0.62	0.61	0.03 NS	0.02	0.02	0.01–0.02	0–0.03	0–0.09	0.01	0.02
Barents Sea:TEN (pop1) versus TUL (pop2)	6.7	6.0	0.61	0.61	0.04 NS	0.03	0.03	0.02–0.04	0–0.05	0–0.16	0.01	0.04
Baltic Sea: VIN (pop1) versus TOR (pop2)	5.0	5.9	0.52	0.54	0.04 NS	0.04	0.05	0.04–0.07	0.01–0.07	0–0.22	0.08	0.03
Landlocked:TAI (pop1) versus SYS (pop2)	4.7	3. 5	0.49	0.45	0.14 NS	0.13	0.14	0.10–0.14	0.04–0.19	0–0.46	0.11	0.17
Large scale												
Barents Sea (pop1) versus White Sea (pop2)	6.3	6. 7	0.61	0.61	0.06**	0.03	0.04	0.03–0.05	0.01–0.05	0–0.13	0.03	0.05
Brackish (pop1) versus saltwater (pop2)	5.4	6.5	0.53	0.61	0.16***	0.08	0.09	0.08–0.11	0.03–0.13	0–0.40	0.17	0.04
Brackish (pop1) versus freshwater (pop2)	5.4	4.1	0.53	0.47	0.22***	0.12	0.13	0.10–0.15	0.04–0.20	0–0.46	0.12	0.18
Saltwater (pop1) versus freshwater (pop2)	6.5	4.1	0.61	0.47	0.23***	0.12	0.14	0.11–0.16	0.06–0.21	0–0.45	0.06	0.24

NOTE.—Observed R_{ST} > permuted R_{ST}, one-sided test, NS, nonsignificant $P > 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$; 95% CI mean; Q_1 & Q_3, first and third quartile.

Dieringer, and Schlötterer 2003). Therefore, after standardization (mean = 0; SD = 1) 95% of neutral loci are expected to have values between −1.96 and 1.96 (99% CI between −2.58 and 2.58; 99.9% CI between −3.29 and 3.29). In the cases when a locus was monomorphic in one population, we added a single different allele to the sample in order to avoid the heterozygosity value being zero.

Results

Genetic Diversity and Population Differentiation

Both EST-associated and genomic microsatellites showed relatively similar levels of genetic variation (median gene diversity across populations 0.57 and 0.70, respectively; median number of alleles across populations 4.8 and 5.9, respectively; Mann-Whitney U test, $P > 0.05$) and differentiation among populations (global F_{ST} 0.11 and 0.12, respectively; Mann-Whitney U test $P > 0.05$), indicating that both types of markers were generally affected by the same kind of evolutionary forces across the genome. Gene diversity and number of alleles differed significantly (Wilcoxon's signed rank test: gene diversity $P < 0.05$; number of alleles, $P < 0.001$) among populations from salt-, brackish, and freshwater habitats (table 1). The results of genetic diversity estimates and H-W testing for each locus and population are available in Appendix 1 (Supplementary Material online). Genetic differentiation measured across loci was highly significant (F_{ST}, $P < 0.001$) between all studied populations, and the level of divergence varied considerably between the geographically proximate population pairs ranging from 0.02 (White Sea: R. Kitsa vs. R. Varzuga) to 0.14 (landlocked: R. Taipale vs. R. Syskynjoki). Pairwise F_{ST} values between more distantly related pairs were, on average, larger (table 1). Observed multi-locus R_{ST} values were significantly higher than permuted R_{ST} estimates across a large spatial scale (between salt-, brackish, and freshwater habitat comparisons; Barents vs. White Sea), suggesting that stepwise-like mutations have contributed to the micro- and minisatellite divergence at this scale (table 1).

Tests for Selection at a Local Geographical Scale

In total, 18 EST-linked and 4 genomic microsatellites were identified as outliers in four geographically proximate population pair comparisons at the 99% P level using one or more neutrality tests (table 2 and fig. 2). Five EST-associated microsatellite loci (*CA047944*, *CA062621*, *CA054978*, *CA054565*, *CA061621*) exhibited significant deviations from the neutral expectations with all three statistical approaches (table 2). The EST locus similar to glycogen debranching enzyme (*CA058586*) was an outlier in two separate population pairs (table 2). Hence, we consider these six loci as the most promising candidates affected by divergent selection at a small geographical scale. Two *MHC*-linked markers were also identified as putative outliers in two pairwise population comparisons (table 2).

Tests for Selection Across a Broad Geographical Scale

In total, 21 EST-linked and 4 genomic microsatellites were identified as outliers ($P < 0.01$) with one or more statistical approach in the large-scale comparisons (table 3 and fig. 3). Fourteen loci deviated from the neutral expectations in more than one habitat/sea area comparison. Five EST-associated microsatellite loci (*CA058586*, *CA048136*, *CA060208*, *CA062621*, *CA039588*) exhibited significant departures from the neutral expectations in at least three out of four outlier tests within a single comparison (table 3). EST locus similar to glycogen debranching enzyme

Table 2
Candidate Loci for Adaptive Genetic Divergence Between Geographically Proximate (small spatial scale) Atlantic Salmon Populations

Acc. no/ Locus Name	KIT Versus VAR			TEN Versus TUL			VIN Versus TOR			TAI Versus SYS			Homology/Gene
	F_{ST}	*F*	*lnRH*	F_{ST}	*F*	*lnRH*	F_{ST}	*F*	*lnRH*	F_{ST}	*F*	*lnRH*	
CA054978	0.15**	***	2.59**										—
CA058586	0.07	***	3.38***							0.20	**	−2.23*	*Oryctolagus cuniculus* glycogen debranching enzyme
CA769358	0.04	***	2.97**										*Oncorhynchus mykiss* viral haemorrhagic septicaemia virus-induced protein-5
CB514369	0.07	**	−2.15*										—
CA062621				0.25**	***	−2.08*							—
CB514761				0.16*	***								*Mus musculus* Ariadne-1 protein homolog (ARI-1)
CA042465				0.10	**	−2.98**							—
CA047944							0.22*	***	3.11**				—
CA054565							0.21*	**	2.55*				—
CA061261							0.12*	**	2.37*				—
Ssa14							0.41**	***					Anonymous microsatellite
Ssa171							0.14	***	−2.1*				Anonymous microsatellite
CA046540							0.06	**	−2.08*				*Oncorhynchus mykiss* vitellogenin receptor (vtg receptor gene)
MHCII							0.15*	**					Major histocompatibility complex class II alpha chain
CA047146							0.05	***					—
CA058902										0.46*	***		—
CA047146										0.39*	***		—
SSOSL438										0.39	**	2.98**	Anonymous microsatellite
CA058128										0.53*	**		*Danio rerio* Atp6v1g1 protein
CA041953										0.23	**	1.96*	—
MHCI										0.17	**	2.0*	Major histocompatibility complex class I
SSF43										0.46	**		Anonymous microsatellite

NOTE.—Loci identified as outliers for all three tests are highlighted in bold, and nonsignificant results are left blank. F_{ST}, Genetic differentiation and corresponding significance value calculated according to Beaumont and Nichols (1996); *F*, significance value from a two-population split model (Vitalis, Dawson, and Boursot 2001); *lnRH*, standardized ratio of gene diversity and corresponding significance value from a Gaussian distribution (Kauer, Dieringer, and Schlötterer 2003); $*P < 0.05$, $**P < 0.01$, and $***P < 0.001$.

(*CA058586*) exhibited a considerable loss of genetic diversity in the Baltic Sea populations (gene diversity 0.02; number of alleles 2) compared to saltwater (gene diversity 0.82; number of alleles 21) and freshwater populations (gene diversity 0.37; number of alleles 5) (Supplementary Material Appendix 1). The departure from neutral expectations at this locus remained significant in the majority of single population comparisons showing similar trends in separate populations (table 3). The EST locus *CA060208* was an extreme outlier in both comparisons involving landlocked populations (freshwater habitat). Hence, we consider these five loci as the most promising candidates affected by divergent selection at a large geographical scale.

Outliers Among EST-Associated and Genomic Microsatellites

Contrary to the expectations, anonymous genomic microsatellites were not less frequently classified as outliers compared to gene-associated loci (χ^2, all neutrality tests, $P > 0.05$). Identification of two genomic microsatellites (*Ssa14*, *Ssa171*) as outliers simultaneously with two neutrality tests out of three at the local scale suggests that these loci might have been influenced by divergent selection (table 2 and fig. 2C). Additional genotyping of 24 and 20 individuals from the R. Vindelälven and Torne/Tornionjoki population, respectively, even further increased F_{ST} estimates between these samples (*Ssa14*, $F_{ST} = 0.43$; *Ssa171*, $F_{ST} = 0.188$).

Discussion

In this study 17 genomic and 78 EST-associated mini- and microsatellites were screened for the footprints of divergent selection among eight Atlantic salmon populations at different geographical scales occupying either relatively similar or contrasting habitats with the aim of identifying genes and genomic regions potentially important for adaptation. Several genes were identified which serve as promising candidates for adaptive divergence, and hence, ''local'' adaptation among wild Atlantic salmon populations at different spatial scales and environments.

Anonymous Versus EST-Targeted Polymorphism Screens for Selection

Two recent studies which utilized AFLP scans to search for footprints of divergent selection in sympatric ecotypes (dwarf and normal) of lake whitefish (*Coregonus clupeaformis*) and in snail (*Littorina saxatilis*) populations that differ in shell shape have identified that ca. 1%–5% of screened loci are likely influenced by directional selection (Wilding, Butlin, and Grahame 2001; Campbell and Bernatchez 2004). In the current study the proportion of

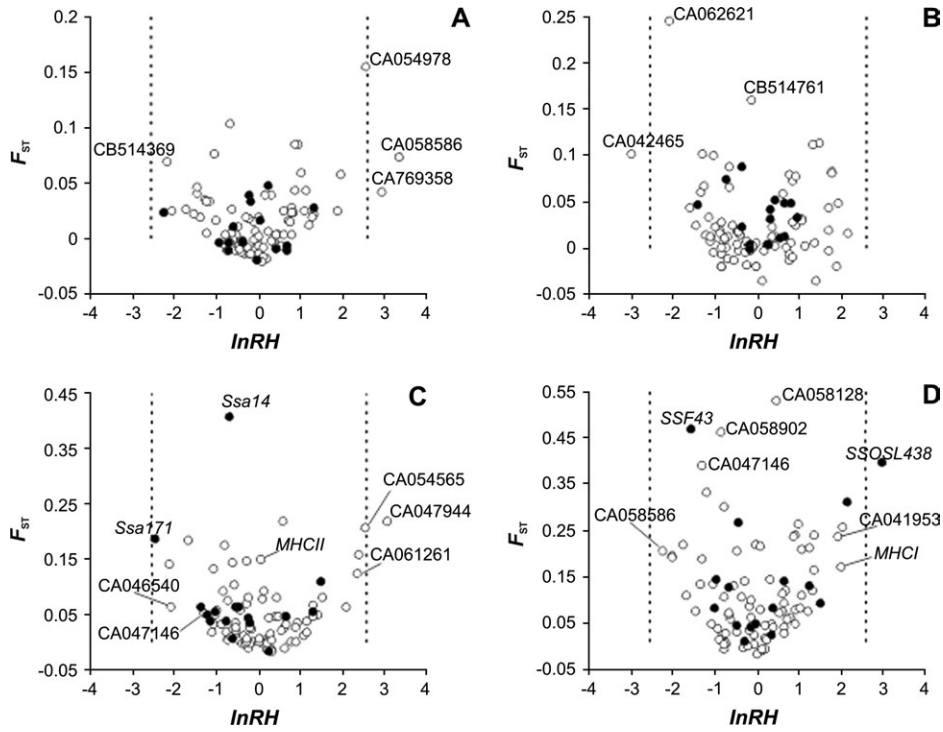

FIG. 2.—Plot of F_{ST} values against standardized *lnRH* estimates for 78 EST-associated (empty bullets) and 17 genomic (black bullets) tandem repeat markers. (*A*) R. Kitsa versus R. Varzuga. (*B*) R. Teno/Tana versus R. Tuloma. (*C*) R. Vindelälven versus R. Torne/Tornionjoki. (*D*) R. Taipale versus R. Syskynjoki. Dashed lines indicate the 99% CI (−2.58, +2.58) of standardized *lnRH* estimates. Accession numbers or locus names of putative candidate loci potentially affected by selection (see *Results*) are indicated.

outlier loci identified was considerably higher (9 of 78 EST-linked loci [12%]). This implies that application of EST-associated microsatellite loci could improve the efficiency of genome screens, especially in species with (1) low genome densities where anonymous loci may not be tightly linked with selected loci and/or (2) high recombination rates, as the signature of selection, may be lost rapidly due to recombination. Concordantly, recent genome scan in closely related oak (*Quercus*) species (Scotti-Saintagne et al. 2004) identified substantially higher frequency of outliers (21%) among gene-associated loci than among anonymous markers (9%; genomic microsatellites, AFLPs). In addition, as a number of the markers applied in this study are also polymorphic in other salmonid species (Vasemägi, Nilsson, and Primmer in press), the strategy will be useful in a broad range of salmonids for identifying candidate loci for further sequence analysis in order to further validate the footprints of selection.

To our knowledge, evidence of divergent selection among contemporary wild Atlantic salmon populations has been reported only at two genes (*MEP-2*, Verspoor and Jordan 1989; *MHCIIβ*, Landry and Bernatchez 2001). However, both studies have used a limited number of loci as a neutral baseline without applying simulations to further test whether the observed pattern deviates from the neutral expectations.

In the light of encouraging simulations of Beaumont and Balding (2004), who demonstrated a reasonable power of genome scans to identify loci under divergent selection, EST scans may provide suitable strategy to discover functionally important genetic variation both in model and nonmodel organisms and present a viable alternative to genome scans which utilize anonymous genetic markers such as AFLPs. Also, given the relative ease of conducting large-scale multi-locus screens for natural selection (Wilding, Butlin, and Grahame 2001; Campbell and Bernatchez 2004) it is likely that more emphasis will be directed to outlier verification and characterization in the future.

Performance of Neutrality Tests

The population-specific divergence (*F*) method of Vitalis, Dawson and Boursot (2001) revealed a much higher number of outlier loci than the other tests (tables 2 and 3). The explanations for such discrepancy might be that (1) identified outliers from the *F*-test are real and other methods have failed to detect the signatures of selection at these loci and (2) most of the detected outliers are false positives (type I error). Closer examination of the identified outliers at different spatial scales revealed a striking difference in a number of cases when the population-specific divergence test was the only method showing the deviations from neutrality. Particularly, the *F*-test identified only two additional outliers not supported by F_{ST}- or *lnRH* test at a local scale, while even 16 outliers from *F*-test were not supported by any other method at a broad scale (tables 2 and 3). Such apparent discrepancy between the population-specific divergence test and other methods at a large spatial scale suggests that the candidate status of these 16 loci must be taken with considerable caution.

Interestingly, the consistency with which the same outlier loci were identified using different tests at the large

Table 3
Candidate Loci for Adaptive Genetic Divergence Between Atlantic Salmon Populations from Different Habitats (salt-, brackish, and freshwater) and Geographic Areas (Barents vs. White Sea)

Acc. no/	Brackish Water Versus Saltwater						Brackish Water Versus Freshwater						Saltwater Versus Freshwater						Barents Sea Versus White Sea						
Locus Name	*B*	F_{ST}	*F*	#	*lnRH*	#	*B*	F_{ST}	*F*	#	*lnRH*	#	*B*	F_{ST}	*F*	#	*lnRH*	#	*B*	F_{ST}	*F*	#	*lnRH*	#	Homology/Gene
CA058586	**	0.45**	***	(8/8)	−4.44***	(7/8)		0.12	**	(3/4)	−2.78**	(2/4)													*Oryctolagus cuniculus* glycogen debranching enzyme
CA048136	*	0.46*	**	(6/8)		(0/8)								0.46	**	(4/8)		(0/8)							—
CA064333		0.40**	**	(8/8)		(0/8)		0.24	**	(4/4)	−2.46*	(2/4)													—
CA056586	**	0.25	*	(3/8)		(0/8)								0.30	***	(4/8)		(0/8)							*Oncorhynchus gorbuscha* microsatellite locus Ogo2
CA038562		0.26	**	(5/8)		(1/8)								0.24	***	(5/8)		(2/8)		0.08	**	(2/4)		(0/4)	*Danio rerio* proto galectin (Gal1-L2)
CA054957		0.22	**	(5/8)		(2/8)								0.29	***	(6/8)		(0/8)							*Oncorhynchus mykiss* Cu/Zn-superoxide dismutase (SOD1)
SSF43		0.07	**	(2/8)		(0/8)																			Anonymous microsatellite
CA039588	**	0.11		(1/8)		(2/8)							**	0.14		(0/8)		(0/8)	**	0.19*	*	(1/4)		(0/4)	—
CA060208							**	0.69**	***	(4/4)		(1/4)		0.46*	***	(8/8)	1.98*	(5/8)							—
CA054538								0.52*	***	(2/4)		(0/4)		0.39	***	(4/8)		(0/8)							*Danio rerio* cyclin E
CA769358								0.17	***	(3/4)	2.2*	(2/4)		0.26	***	(8/8)	2.25*	(4/8)							*Oncorhynchus mykiss* viral haemorrhagic septicaemia virus-induced protein-5
CA055420								0.21	***	(4/4)	2.44*	(2/4)		0.24	***	(8/8)	2.7**	(6/8)							*Homo sapiens* KIAA0587 protein
MHCI								0.22	***	(3/4)	2.13*	(2/4)		0.29	***	(8/8)		(4/8)							Major histocompatibility complex class I
Ssa14								0.40**		(1/4)	2.45*	(0/4)		0.45*	**	(3/8)		(0/8)							Anonymous microsatellite
CB515794								0.30	**	(4/4)	−2.14*	(1/4)													—
Ssa289							**	0.30	*	(0/4)		(0/4)													Anonymous microsatellite
SSD30								0.46	***	(2/4)		(2/4)		0.42	***	(7/8)		(0/8)							Anonymous microsatellite
CB512797								0.17	**	(2/4)		(0/4)													*Oncorhynchus mykiss* carbonyl reductase/20 beta-hydroxysteroid dehydrogenase B
CA050376								0.11	**	(2/4)		(1/4)													—
CA058128								0.27	**	(2/4)		(0/4)													*Xenopus laevis* Atp6v1g1-prov protein
CA042465														0.38	***	(8/8)	−2.09*	(4/8)							—
CA047944														0.18	**	(8/8)		(0/8)							—
CA062621																			*	0.18**	**	(3/4)		(0/4)	—
CA054978																			*	0.08		** (3/4)		(2/4)	—
CA053162																				0.14		** (4/4)	−2.06*	(0/4)	—

NOTE.—Loci identified as outliers in three or four tests in a single comparison are highlighted in bold, and nonsignificant results are left blank. Significance value calculated using hierarchical-Bayesian method, *B*, of Beaumont and Balding (2004); F_{ST}, genetic differentiation and corresponding significance value calculated according to Beaumont and Nichols (1996); *F*, significance value from a two-population split model (Vitalis, Dawson, and Boursot 2001); *lnRH*, standardized ratio of gene diversity and corresponding significance value from a Gaussian distribution (Kauer, Dieringer, and Schlötterer 2003); #, numbers in parentheses indicate significant pairwise population comparisons from all possible population combinations; $*P < 0.05$, $**P < 0.01$, and $***P < 0.001$.

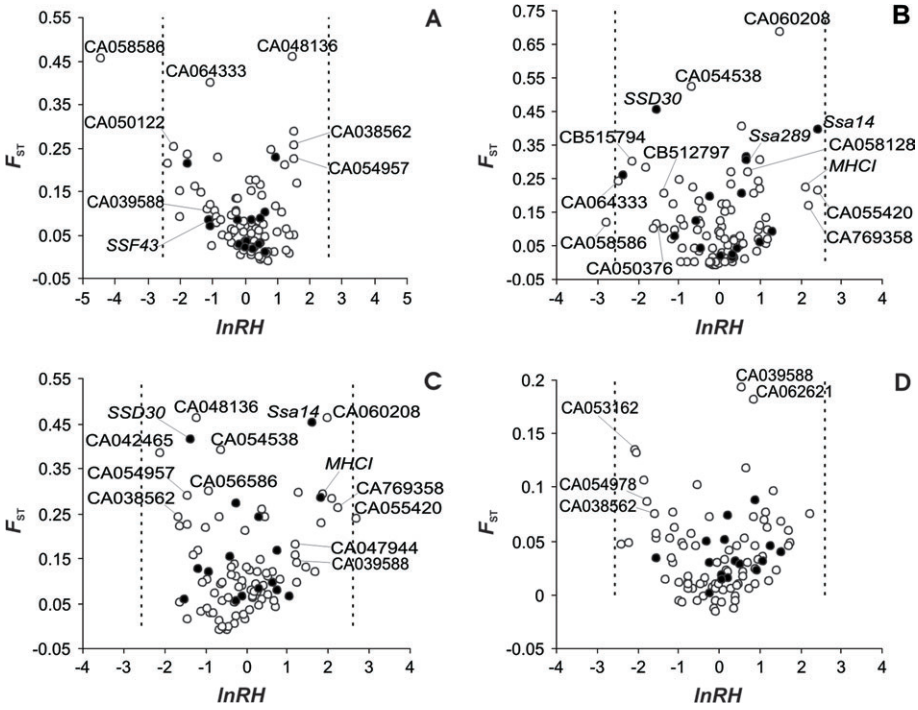

FIG. 3.—Plot of F_{ST} values against standardized *lnRH* estimates for 78 EST-associated (empty bullets) and 17 genomic (black bullets) tandem repeat markers. (*A*) Brackish versus saltwater. (*B*) Brackish versus freshwater. (*C*) Salt- versus freshwater. (*D*) Barents versus White Sea. Dashed lines indicate the 99% CI (−2.58, +2.58) of standardized *lnRH* estimates. Accession numbers or locus names of putative candidate loci potentially affected by selection (see *Results*) are indicated.

spatial scale was lower for other methods as well (outlier overlap: *F*-test vs. F_{ST}-test, small scale 48%, large scale 23%; *F*-test vs. *lnRH* test, small scale 65%, large scale 31%; F_{ST}-test vs. *lnRH* test, small scale 33%, large scale 17%). Outliers from the hierarchical-Bayesian method, which treated each population in a particular comparison separately, showed the most congruent results with the F_{ST}-test (outlier overlap: 36%) while only a single deviation from neutral expectations was supported simultaneously by the Bayes and *lnRH* tests at the large scale (outlier overlap: 5%). High frequency of simultaneous identification of the same loci as outliers with several methods at the local scale supports the prediction that comparison of closely related populations is expected to enhance the efficiency of genome scans for divergent selection because (1) potential selective footprints are likely not obscured by mutations and (2) random drift has a reduced effect on the genetic parameters used to infer the outlier loci (Beaumont and Nichols 1996; Vitalis, Dawson, and Boursot 2001; Schlötterer 2002a).

Interpreting Departures from Neutrality

In the present study, EST-associated tandem repeat markers did not deviate more frequently from the neutral expectations than anonymous genomic microsatellite loci. Therefore, it is possible that (1) some of the genomic microsatellites are affected by selection; (2) a considerable number of the outliers are false positives; or (3) a combination of (1) and (2) can occur. It is likely that false positives (type I error) resulting from multiple testing, possible violations of test assumptions, and genome-wide heterogeneity in variability are responsible for some of the observed outliers. On the other hand, deviations from neutrality at genomic microsatellite *Ssa14* with several neutrality tests both at local (Baltic Sea: R. Vindelälven vs. Torne/Tornionjoki) and large geographical scales (brackish vs. freshwater; salt- vs. freshwater) suggest that *Ssa14* might have been influenced by divergent selection. The linkage of this locus to any functional gene is currently unknown (Gilbey et al. 2004).

It is important to note that, significant deviation from neutral expectations using one or multiple tests does not necessarily mean that a particular locus has been affected by selection. We applied four different neutrality tests in eight separate comparisons using 95 loci (local scale: $3 \times 4 \times 95 = 1{,}140$ separate tests; large scale: $4 \times 4 \times 95 = 1{,}520$ separate tests) which is expected to result in approximately 27 false positives at 99% *P* level. The fact that we found three times more deviations at 99% *P* level (altogether 82 deviations were observed) indicates that it is unlikely that all the outliers are false positives (type I error). As emphasized in earlier studies, significant results with more than one neutrality test only raise the candidate status of particular locus but does not demonstrate selection per se (e.g., Vigouroux et al. 2002; Schlötterer 2002a; Campbell and Bernatchez 2004). Therefore, the identified candidate EST loci will serve as a basis for further sequence analysis to validate the role of divergent selection in these genes because the violation of test assumptions is another factor potentially producing false positives. Particularly, F_{ST}-test of Beaumont and Nichols (1996) is based on a symmetrical

island model of population structure which is based on the assumptions of equal population sizes and migration rates between populations. It is likely that at least some comparisons within our data set (e.g., saltwater vs. freshwater) violate such assumptions, and outliers from F_{ST}-test alone should be therefore taken with caution. On the other hand, identification of the same outliers using the F_{ST}- and Bayes test of Beaumont and Balding (2004) which does not assume equal populations sizes and migration rates strengthens the candidate status of the five loci (*CA058586*, *CA048136*, *CA060208*, *CA062621*, *CA039588*). The inconsistent results of the *F*-test and other methods at a large spatial scale were probably largely caused by mutations at microsatellite loci which occurred after the population divergence, as indicated by the R_{ST} permutation test of Hardy et al. (2003). In addition, because the *F*-test is based on the joint distribution of the population-specific divergence estimates conditional on the number of alleles, it is possible that different within-locus mutation rates affect the results of *F*-test more severely than the *lnRH* test, which is based on gene diversity. Nevertheless, different within-locus mutation rates are likely affecting the outcome of the *lnRH* test as well. Therefore, when predominantly the shortest alleles are associated with the putative selective sweep, the outlier status of particular loci identified using the *lnRH* test should be taken with caution. Another potentially unrealistic assumption of *F*-test is that no migrants have been exchanged after the divergence of two populations. However, Vitalis, Dawson, and Boursot (2001) have shown that moderate levels of migration do not increase the false-positive results (type I error) of the *F*-test.

An important direction for future research is therefore the formal testing of the effect of the model assumptions on the identification of outlier loci. In the absence of such information, it has been suggested that a practical approach for strengthening the candidate status of identified outlier loci is to simultaneously apply two or more neutrality tests which are based on different assumptions and parameter estimation (e.g., Storz, Payseur, and Nachman 2004) and only consider outlier loci that are supported by several methods for subsequent validation steps (e.g., further sequence analysis of flanking regions).

Supplementary Material

Appendix 1 is available online at the MBE web site (http://www.molbiolevol.org).

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