Explorative Genome Scan to Detect Candidate Loci for Adaptation Along a Gradient of Altitude in the Common Frog (*Rana temporaria*)

Aurélie Bonin,* Pierre Taberlet,* Claude Miaud,† and François Pompanon*

*Laboratoire d'Ecologie Alpine, CNRS-UMR 5553, Université Joseph Fourier, Grenoble, France; and
†Laboratoire d'Ecologie Alpine, CNRS-UMR 5553, Université de Savoie, Le Bourget du Lac, France

Today, with the rapid development of population genomics, the genetic basis of adaptation can be unraveled directly at the genome level, without any prerequisites about the selectively advantageous genes or traits. For nonmodel species, it is now possible to screen many markers randomly scattered across the genome and to distinguish between the neutral genetic background and outlier loci displaying an atypical behavior (e.g., a higher differentiation between populations). This study investigated the genetic frame of adaptation to a gradient of altitude in the common frog (*Rana temporaria*) by means of a genome scan based on 392 amplified fragment length polymorphism markers. Using two outlier detection methods never applied to dominant data so far, we sought for loci with a genetic differentiation diverging from neutral expectations when comparing populations from different altitudes. All the detected loci were sorted out according to their most probable cause for outlier behavior and classified as false positives, outliers due to local effects, or outliers associated with altitude. Altogether, eight good candidate loci were identified as potentially involved in adaptation to altitude because they were picked out in several independent interaltitude comparisons. This result illustrated the potential of genome-wide surveys to reveal selection signatures along selection gradients, where the association between environmental variables and fitness-related traits may be complex and/or cryptic. In this article, we also underlined the need for confirmation of the selection footprints for the outlier loci. Finally, we provided some preliminary insights into the genetic basis of adaptation along an altitudinal cline in the common frog.

Introduction

Unraveling the genetic framework of adaptive population divergence is a long-standing ambition for evolutionary biologists. Generally, adaptive phenotypes or traits are the convenient starting points of studies investigating adaptation at the genetic or genome level. One of the traditional approaches consists in surveying candidate genes potentially subject to natural selection (Akey et al. 2004; Ffrench-Constant, Daborn, and Le Goff 2004; Rosenblum, Hoekstra, and Nachman 2004), but it is reserved to a limited number of situations where the candidate gene sequence and function are well characterized. When the phenotypic trait of adaptive importance is easily observable and measurable, it is also possible to identify and map the underlying quantitative trait loci (QTL) with the help of experimental crosses (Colosimo et al. 2004; Erickson et al. 2004; Verhoeven et al. 2004). Now, with the outbreak of population genomics, the genetic basis of adaptation can be considered directly at the genome level without any prerequisites about the selectively advantageous genes or traits (Schlötterer 2003; Storz 2005).

Population genomics involves the genotyping of numerous random loci along large chromosomic regions or even entire genomes for many individuals in several populations (Black et al. 2001). This comprehensive assessment gives valuable clues to disentangle the evolutionary effects of forces acting on the whole genome (e.g., genetic drift, gene flow, or inbreeding) from the effects of forces influencing only particular loci (e.g., natural selection or recombination) (Luikart et al. 2003). All the loci across the genome are expected to respond similarly to demography and neutral history of populations, whereas a few loci only are imprinted an unusual schema of genetic differentiation by natural selection. Therefore, scanning the patterns of DNA polymorphisms at the genomic level enables to evaluate the amount of neutral genetic diversity and to identify "outlier" loci, i.e., loci that behave differently from the rest of the genome. In the context of local adaptation studies, such loci deserve particular attention because selection may be the underlying cause of their atypical behavior either because they are direct targets of selection or because they are genetically linked to a selected locus.

Within the last few years, with the technical improvements in DNA genotyping, multilocus scans tracked adaptive divergence in many organisms, ranging from humans (Payseur, Cutter, and Nachman 2002; Schlötterer 2002; Kayser, Brauer, and Stoneking 2003) to species of economical (Kohn, Pelz, and Wayne 2003) or agronomical interest (Vigouroux et al. 2002). They helped identify candidate genes for balancing (Polley, Chokejindachai, and Conway 2003) or directional selection (Wootton et al. 2002) and allowed the construction of selection maps for several organisms (Kohn, Pelz, and Wayne 2000; Akey et al. 2002; Harr, Kauer, and Schlötterer 2002). In order to resolve selection footprints properly, a multilocus screening has to be fine enough because a marker locus will display an outlier behavior only if it is in strong linkage disequilibrium with a selected region. Therefore, for a long time, the use of population genomics to investigate genetic support of local adaptation has been considerably hampered for nonmodel organisms where no or few molecular markers have been identified. Fortunately, this issue has been overcome with the breakthrough of the amplified fragment length polymorphism (AFLP) technique (Vos et al. 1995). This genotyping procedure is applicable to any species without any prior sequence knowledge and provides several hundred random markers for whole-genome coverage. With the recent technical improvements in data throughput and reliability, AFLP data sets are now relatively easy to produce. As a consequence, genomic surveys will probably soon grow in number for many organisms, urging the need for analysis

Table 1
Location and Altitude of the Studied Populations

Population name	Site	Altitude	Longitude	Latitude	Number of individuals genotyped
Low 1	Saint-Rémy-de-Maurienne	425 m	6.2775E	45.3697N	34
Low 2	Cognin	438 m	5.8730E	45.5635N	28
Inter 1	Col de Plainpalais	1,074 m	6.0207E	45.6475N	30
Inter 2	Tines	1,082 m	6.9022E	45.9522N	34
High 1	Lac des Aiguillettes	2,100 m	6.8106E	45.9249N	32
High 2	Lac des Tempêtes	2,130 m	6.5491E	45.6205N	32

methods designed to reveal outlier loci among numerous dominant markers.

So far, explorative genome scans aiming to track loci under selection have been applied to situations where individual adaptive traits segregate into two contrasted phenotypes. For example, such surveys opposed two distinct host races of the larch budmoth (Emelianov, Marec, and Mallet 2004), two sympatric morphotypes of periwinkle snail (Wilding, Butlin, and Grahame 2001), and two sympatric ecotypes of whitefish (Campbell and Bernatchez 2004). Yet, local adaptations arise often along environmental gradients (Endler 1977) along which many biotic and abiotic constraints vary, creating an adaptive phenotypic continuum instead of discrete phenotypic optima. Moreover, the genes underlying this adaptive continuum may interact in a complex way or respond to natural selection at various stages along the gradient (Sgro and Blows 2004). Fine selection footprints may thus be difficult to resolve by merely contrasting populations from extreme ends of the gradient and it may be more fruitful to sample populations that are evenly distributed along the gradient (Storz and Dubach 2004).

In this paper, we investigated local adaptation along an altitude gradient in the common frog (*Rana temporaria*). This species is the most abundant amphibian in Europe, occurring from northern Spain to subartic Scandinavia and from the sea level up to an altitude of 2,500 m in the Alps (Gasc et al. 1997). It is particularly prone to adaptive population divergence because environmental constraints and selection pressures vary substantially across its wide distribution range. Many local adaptation phenomena have been established along various selection gradients (see for example Merilä et al. 2000; Johansson, Räsänen, and Merilä 2001; Laugen et al. 2003; Palo et al. 2003). In particular, in the French Alps, quantitative analyses have shown that individuals from different altitudes display adaptive differences for a wide range of life-history traits, including larval development rate, age to maturity, clutch size, egg size, adult body size, etc. (Miaud, Guyétant, and Elmberg 1999; Miaud and Merilä 2001). As genome scans for divergent selection are expected to be more efficient when comparing closely related populations because selection signatures have to be extracted from a lower background neutral noise due to drift and mutation (Vasemägi, Nilsson, and Primmer 2005), we focused on a local geographical scale. Starting from a genome survey based on AFLP markers, we revealed outlier loci with the help of two outlier detection methods (Vitalis, Dawson, and Boursot 2001; Beaumont and Balding 2004) that had never been applied to dominant data such as AFLPs. We discussed the most probable causes underlying the outlier behavior of detected loci. Finally, we also provided some preliminary insights into the genetic frame of adaptation in the common frog along an altitudinal cline.

Materials and Methods
Sampling

The study was conducted at a local scale in the North French Alps in a geographical area covering approximately 7,000 km^2. Individuals were sampled in two low-altitude populations (around 400 m above sea level), in two intermediate-altitude populations (around 1,000 m), and in two high-altitude populations (around 2,000 m) (table 1) in different mountain massifs. For each population, 28–34 samples consisting of adult frog fingers and/or tadpoles were stored in silica gel until DNA extraction and genotyping.

AFLP and Microsatellite Genotyping

Total DNA was extracted using the DNeasy Tissue Kit (Qiagen, Valencia, Calif.) following the manufacturer's instructions, except for an extra centrifugation step after the digestion by proteinase K aiming at discarding residual pigments. Restriction enzymes *Eco*RI and *Taq*I were used for the AFLP procedure, according to the protocol indicated in Bonin, Pompanon, and Taberlet (2005). Selective amplifications were performed using 10 different primer pairs (table 2), and the fragments were separated by electrophoresis on an ABI Prism 3100 DNA sequencer (Applied Biosystems, Foster City, Calif.). The AFLP electrophoregrams were visualized with GeneScan Analysis version 3.7 (Applied Biosystems) and Genographer version 1.6.0 (Benham et al. 1999; available at http://hordeum.oscs. montana.edu/genographer/). AFLP markers were scored according to the absence/presence of peaks, i.e., as dominant markers. For a given marker, the scoring threshold was determined by scrutinizing a drop in intensity among the corresponding peaks after normalization of the profiles. This drop reveals the frontier between selective and nonselective amplification and usually shows up around 10% of the highest peak's intensity. Sampling variance is particularly high for markers presenting a low proportion of recessive phenotypes (band absence) in a given population; thus, loci with less than 3% of band absence for all individuals were not taken into consideration. In total, 190 individuals were scored for 392 AFLP markers, with each primer combination yielding between 19 and 51 polymorphic AFLP bands.

To test for Hardy-Weinberg equilibrium, three microsatellite loci (RtµE, RtµJ, and RtµP) were also amplified for a subset of the samples using the primers and polymerase

Table 2
Primer Pairs Used for the AFLP Procedure

Primer pair	Sequence primer *Eco*RI	Sequence primer *Taq*I
1	5′-GACTGCGTACCAATTC**ACG**-3′	5′-GATGAGTCCTGAGCGA**AAC**-3′
2	5′-GACTGCGTACCAATTC**ACG**-3′	5′-GATGAGTCCTGAGCGA**ACA**-3′
3	5′-GACTGCGTACCAATTC**ACG**-3′	5′-GATGAGTCCTGAGCGA**CAG**-3′
4	5′-GACTGCGTACCAATTC**ACT**-3′	5′-GATGAGTCCTGAGCGA**AAC**-3′
5	5′-GACTGCGTACCAATTC**ACT**-3′	5′-GATGAGTCCTGAGCGA**ACT**-3′
6	5′-GACTGCGTACCAATTC**ACT**-3′	5′-GATGAGTCCTGAGCGA**CAA**-3′
7	5′-GACTGCGTACCAATTC**ACT**-3′	5′-GATGAGTCCTGAGCGA**CAT**-3′
8	5′-GACTGCGTACCAATTC**AGA**-3′	5′-GATGAGTCCTGAGCGA**CAT**-3′
9	5′-GACTGCGTACCAATTC**AGA**-3′	5′-GATGAGTCCTGAGCGA**CAG**-3′
10	5′-GACTGCGTACCAATTC**ATC**-3′	5′-GATGAGTCCTGAGCGA**ACT**-3′

NOTE.—Selective bases are indicated in bold letters.

chain reaction (PCR) conditions described in the study of Pidancier et al. (2002). The PCR products were loaded on an ABI Prism 3100 DNA sequencer (Applied Biosystems) and analyzed using GeneMapper version 3.0 (Applied Biosystems). Overall, 101, 105, and 109 individuals were typed for RtμE, RtμJ, and RtμP, respectively, with 18–28 individuals genotyped for each population, leading to the identification of, respectively, 14, 4, and 3 alleles among the six populations.

Several precautions were taken to ensure reliability of the typings (see details in Bonin et al. 2004). Low-quality DNA samples were excluded, and negative controls were run at each step of the genotyping process. For the AFLP procedure, 12% of the samples were blindly genotyped twice. The data matrixes of the blind samples and the corresponding individuals were compared. Each band scored as present in one matrix and absent in the other one was counted as a difference. The markers cumulating too many differences were considered as prone to genotyping errors and were discarded from the data set (7 markers out of 399). The genotyping error rate per locus was then calculated as the ratio between observed number of differences and total number of comparisons (Pompanon et al. 2005). It was estimated at 2.0%, a value similar to those found in the literature (Ajmone-Marsan et al. 1997; Jones et al. 1997). The reproducibility of the microsatellite protocol was checked by replicating about 9% of the samples for each locus, and no difference was observed between the replicated genotypes.

Outlier Detection

Outlier loci were tracked in the AFLP data set using two different statistical methods, which have been adapted for the analysis of dominant data. Both of them are based on the straightforward principle that genetic differentiation between populations is expected to be higher for loci under divergent selection than for the rest of the genome. They use computer simulations to model the behavior of neutral loci under a defined evolutionary scenario, and loci lying outside the neutral distribution are detected as outliers.

The first program, DetSel, originally designed for codominant data (Vitalis, Dawson, and Boursot 2001) was slightly modified to process AFLPs (R. Vitalis, personal communication). DetSel relies on a model where a common ancestor population splits up into two populations, which afterward diverge only by random drift after a possible bot-

tleneck event (Vitalis, Dawson, and Boursot 2001; Vitalis et al. 2003). A generic parameter of population divergence F_i is then defined for each population i ($i = 1$ or 2), which is a function of the divergence time t and the population size N_i, that is $F_i \approx 1 - \exp\left(-t/N_i\right)$. Single-locus estimates of these parameters (conditioned on the number of alleles in the two populations) can be calculated for each locus using the nuisance parameters of the model: mutation rate (μ), ancestral population size before the bottleneck (N_e), ancestral population size during the bottleneck (N_0), and the number of generations during the bottleneck (τ_0). A joint distribution of F_1 and F_2 under neutral expectations can be generated using coalescent simulations, and every locus falling outside the resulting confidence envelope can be seen as potentially under selection. For our analyses, a null distribution was obtained based on a range of 20 different sets of nuisance parameters ($\theta = 2N_e\mu$ varying from 0.5 to 5, N_0 from 100 to 1,000, and τ_0 from 0 to 1,000). This procedure allowed for a maximum number of realistic demographic scenarios to get a more robust null confidence envelope. It was checked that (1) using these sets of parameters, the average F_i over 50,000 realizations of the coalescent process was equal to the parametric F_i (i.e., $1 - \exp(-t/N_i)$) used to generate the simulations and (2) these sets of parameters could be used to generate biallelic data sets. In total, 500 simulations were performed for each set of nuisance parameters. Loci that were monomorphic in only one of the two compared populations were systematically discarded from the simulations because confidence envelope appeared to vary greatly over different sets of nuisance parameters when such loci were used.

In order to detect outlier loci potentially selected along an altitude gradient, DetSel was run for every pairwise comparison involving populations from different altitude categories, i.e., 12 population pairs. The significance level was set at 95% because the Bonferroni correction and the 99% confidence level were too conservative, with no loci detected in most pairwise comparisons. For each marker, the number of alleles in each population, which was required as input data, was estimated directly from the AFLP matrix assuming Hardy-Weinberg equilibrium. This assumption was first checked in each population with Arlequin version 2.000 (Schneider, Roessli, and Excoffier 2000; available at http://lgb.unige.ch/arlequin/software/) using the microsatellite data set. For each AFLP marker

in each population, the number of alleles was then calculated from the phenotypic data as $n_0 = 2N\sqrt{(Q/N)}$ and $n_1 = 2N - n_0$, where n_0 and n_1 are the numbers of recessive and dominant alleles, respectively, N is the sample size, and Q is the number of individuals without the band (recessive phenotype) in the population for the marker under consideration.

The second program used to detect outlier loci, Dfdist, was recently modified from Beaumont and Balding (2004) to analyze dominant data. In particular, it implements the Bayesian method developed by Zhivotovsky (1999) to estimate allelic frequencies from the proportion of recessive phenotypes in the sample. Dfdist follows a hierarchical Bayesian approach to compute Fst values conditional on heterozygosity in a subdivided population under the symmetrical island model (Wright 1951). As a first step, Dfdist was run for each possible combination of populations of different altitude (12 single analyses in total) with two demes (i.e., populations) during the simulation step. A global analysis was also done for the three altitude categories using three demes. For each single analysis, a null distribution was generated based on 50,000 simulated loci, with a mean Fst similar to the trimmed mean Fst calculated from the empirical distribution. Computed by removing the 30% highest and lowest Fst values observed in the empirical data set, the trimmed mean Fst is an estimate of the average "neutral" Fst value uninfluenced by outlier loci (M. A. Beaumont, personal communication). Analyses were performed at the 95% and 99% confidence levels.

Genetic Diversity and Neutral Differentiation

After revealing selection signatures, loci were distributed in three AFLP sub–data sets according to their detection pattern: the Local Effects, Altitude, and Neutral sub–data sets (see *Results*). Genetic diversity was estimated by performing analyses of molecular variance (AMOVAs) on populations grouped according to their altitude category using Arlequin version 2.000 (Schneider, Roessli, and Excoffier 2000). The genetic differentiation between populations was calculated using AFLP-SURV version 1.0 (Vekemans et al. 2002; available at http://www.ulb.ac.be/sciences/lagev/). For each sub–data set, 1,000 bootstrapped Nei's distance matrices were generated in order to construct consensus trees with the programs NEIGHBOR and CONSENSE of the software package PHYLIP version 3.6 (Felsenstein 2004; available at http://evolution.genetics.washington.edu/phylip.html).

Results
Outlier Detection

The results of an interaltitude comparison performed with DetSel and highlighting three outliers are shown in figure 1. Across the 12 pairwise analyses performed with DetSel, 54 AFLP loci out of 392 fell outside the 95% confidence envelope (table 3). Among them, 35 loci were polymorphic in only one population involved in the pairwise comparison. They could not be considered as loci diverging undoubtedly from neutral expectations because such loci were excluded to build the confidence envelope. The 19 remaining outlier loci were polymorphic in both compared populations. Fourteen of them were detected in only one
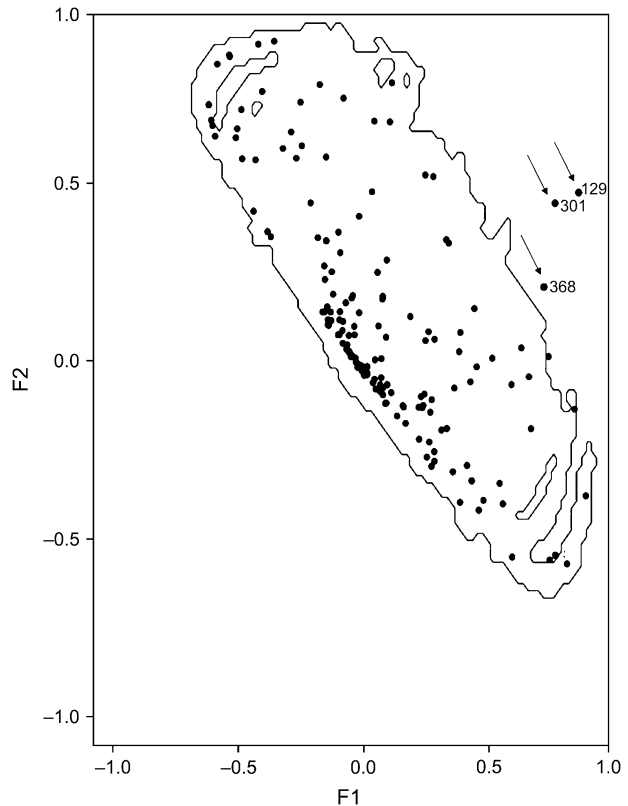

FIG. 1—Example of interaltitude pairwise comparison performed with DetSel: plot of F_2 against F_1 estimates for the population pair Low 1/High 1. Each dot indicates an AFLP marker, and the line represents the 95% confidence envelope. Outlier loci are pointed out by arrows and referred to by numbers.

single analysis and thus considered as false positives (i.e., loci detected because of the 5% type I error). The five remaining loci were tagged as outliers in several comparisons. Among them, three were associated to a particular population, and their outlier behavior was thus considered to be the result of local effects (i.e., not linked to altitude). Finally, two loci (loci 129 and 301) appeared in independent interaltitude comparisons. Only locus 301 was revealed at the 99% confidence level in one analysis.

An example of interaltitude comparison performed with Dfdist and revealing four outliers is shown in figure 2. The 12 interaltitude comparisons performed with Dfdist led to characterize 43 different loci at the 95% confidence level (table 3). Among them, 29 loci appeared in only one analysis (false positives). In total, 11 loci were attached to one population in particular and were thus classified into the category of outliers due to local effects. Finally, loci 84, 248, and 301 were highlighted in four, three, and four independent pairwise comparisons, respectively. A global analysis on the three altitude categories helped pick six loci lying above the 95% confidence line (table 3). One of these loci was locus 301, already pointed out by DetSel. Three of the five remaining loci (loci 84, 228, and 388) were picked out with DetSel too, but their outlier behavior was not considered as reliable because they were monomorphic in one population. Loci 84, 248, and 301 remained significant at the 99% level with Dfdist (table 3).

Table 3
Results of the Outlier Detection Among the 392 AFLP Markers at the 95% Confidence Level

Outlier detection program	Analysis procedure	Number of loci detected		Most probable cause of outlier behavior	Good candidates
DetSel	Interaltitude analyses (12 possible pairwise comparisons)	54	• 35 loci monomorphic in one of the two populations	Poorly reliable outliers	—
			• 14 loci appearing in only one comparison	False positives	—
			• 3 loci attached to one population in particular	Local effects	—
			• 2 loci appearing in at least two independent comparisons	Adaptation to altitude	Locus 129 (3/0) Locus 301 (3/1)
Dfdist	Interaltitude analyses (12 possible pairwise comparisons)	43	• 29 loci appearing in only one analysis	False positives	—
			• 11 loci attached to one population in particular	Local effects	—
			• 3 loci appearing in at least two independent comparisons	Adaptation to altitude	Locus 84 (4/3) Locus 248 (3/3) Locus 301 (4/4)
	Global analysis on the three altitude categories	6		Adaptation to altitude	Locus 84 (1/1) Locus 97 (1/0) Locus 228 (1/0) Locus 250 (1/0) Locus 301 (1/1) Locus 388 (1/0)

NOTE.—The number of significant single analyses at the 95% and 99% levels are indicated in parentheses (left and right, respectively).

Overall, one locus (locus 301) is strongly revealed by the two statistical tests, which gave good support to its status of outlier because of adaptation to altitude. Loci 84, 228, and 248 were detected by Dfdist only but were monomorphic in some populations and could therefore not be considered as reliable outliers by DetSel. Finally, four other loci can be seen as good candidates too (loci 97, 129, 250, and 388), but they were revealed either by DetSel or by Dfdist but not by the two tests.

Genetic Diversity and Population Differentiation

From the original AFLP data set (392 loci), three sub–data sets were constructed: (1) the "Local Effects" data set, which included 12 loci attached to one population with DetSel or/and Dfdist, (2) the "Altitude" data set, which included the eight loci considered as good candidates for an outlier behavior due to altitude, and (3) the "Neutral" data set, bringing together the 343 loci that never appeared in the detection tests. False positives were discarded from the rest of the analyses.

Analyses on the Neutral data set revealed that 81.1% of the neutral genetic diversity was observed within populations (fig. 3A), but the genetic variance among populations was high enough to structure populations, with neutral pairwise Fst values ranging from 0.02 to 0.20 (table 4). The global Fst was 0.11 versus 0.15 for the initial AFLP data set with all 392 markers. When considering the Altitude data set, the percentage of genetic diversity measured within populations was much lower (31.1%, fig. 3B), whereas altitude was responsible for 35.5% of the molecular variance. As expected, in the Altitude data set, genetic differentiation (based on Fst indexes) between populations from different altitudes was higher than in the Neutral data set, with interaltitude Fst values ranging from 0.10 to 0.76 (table 4) and a global Fst of 0.31.

Two different topologies emerged from the consensus trees constructed with Nei's genetic distances calculated from the three different data sets (fig. 4). Neutral and Local Effects data sets led to comparable topologies, whereas populations from extreme altitudes (low or high) gathered when using the Altitude data set.
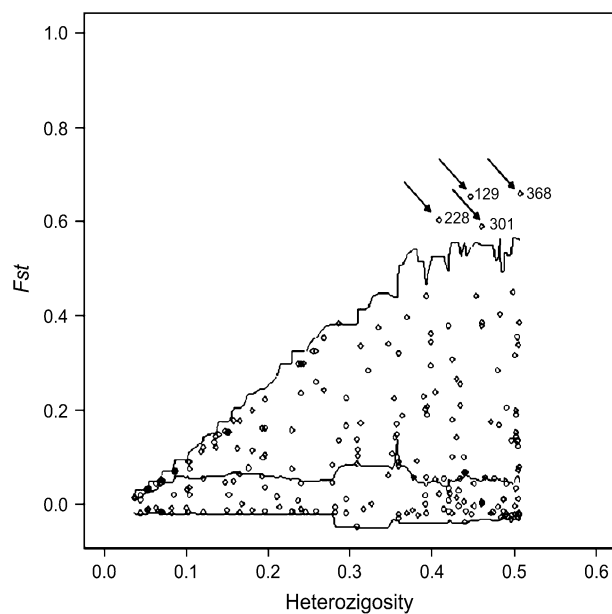
FIG. 2—Example of an analysis performed with Dfdist: plot of Fst values against heterozygosity estimates for the population pair Low 1/High 1. Each dot indicates an AFLP marker. The lower, intermediate, and higher lines represent the 5%, 50%, and 95% confidence intervals, respectively. Outlier loci are pointed out by arrows and referred to by numbers.
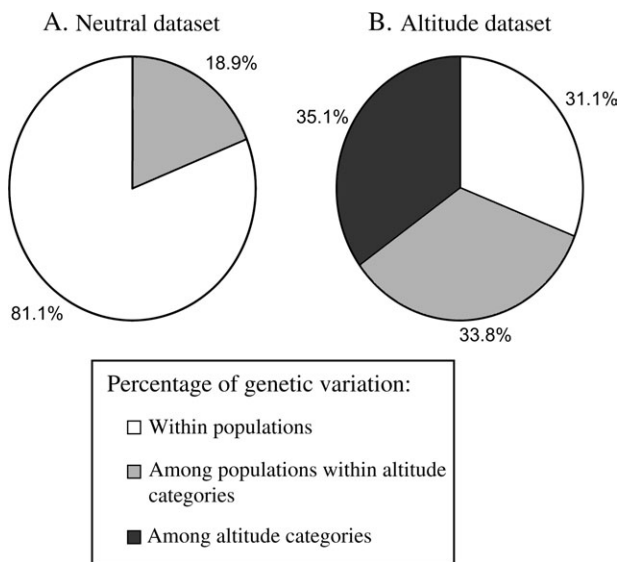
A. Neutral dataset

B. Altitude dataset

Percentage of genetic variation:

☐ Within populations

☐ Among populations within altitude categories

■ Among altitude categories

FIG. 3—Distribution of the genetic variation calculated from AMO-VAs performed on the (A) Neutral and (B) Altitude data sets.

Discussion

Detecting Outlier Loci

Across the genome, loci are influenced similarly by random genetic drift, whereas the effect of natural selection can be null to predominant, creating a variation continuum between purely neutral loci and loci directly under selection. In practice, this continuum has to be simplified and transformed into discrete categories (neutral vs. selected loci). The detection of outlier loci thus consists in screening many loci scattered in the genome to bring out the few ones diverging from empirical or simulated neutral expectations (Luikart et al. 2003). Compared with the rest of the genome, these outlier loci have an atypical behavior, which can range from an excess or a deficit of rare alleles in a given population to aberrant patterns of genetic variability within or between populations (Luikart et al. 2003; Schlötterer 2003; Nielsen 2005). Diversifying selection shapes the genetic variation of selected loci in two opposite directions, depending on the analysis level. At the intrapopulation level, the variability is reduced as a beneficial allele spreads in the population, whereas the interpopulation differentiation increases with the establishment of different phenotypic optima (Storz 2005). Many outlier detection methods developed so far were thus designed to monitor either a reduction

Table 4
Pairwise Population Differentiation Based on Wright's F_{st} Index

	Low 1	Low 2	Inter 1	Inter 2	High 1	High 2
Low 1	—	0.32	0.40	0.41	0.69	0.76
Low 2	0.18	—	0.10	0.18	0.24	0.24
Inter 1	0.15	0.07	—	0.06	0.22	0.20
Inter 2	0.19	0.03	0.09	—	0.26	0.44
High 1	0.20	0.09	0.12	0.11	—	0.20
High 2	0.15	0.07	0.02	0.09	0.13	—

NOTE.—Lower diagonal, results obtained with the Neutral data set (343 markers); upper diagonal, results obtained with the Altitude data set (8 markers).

A. Neutral dataset

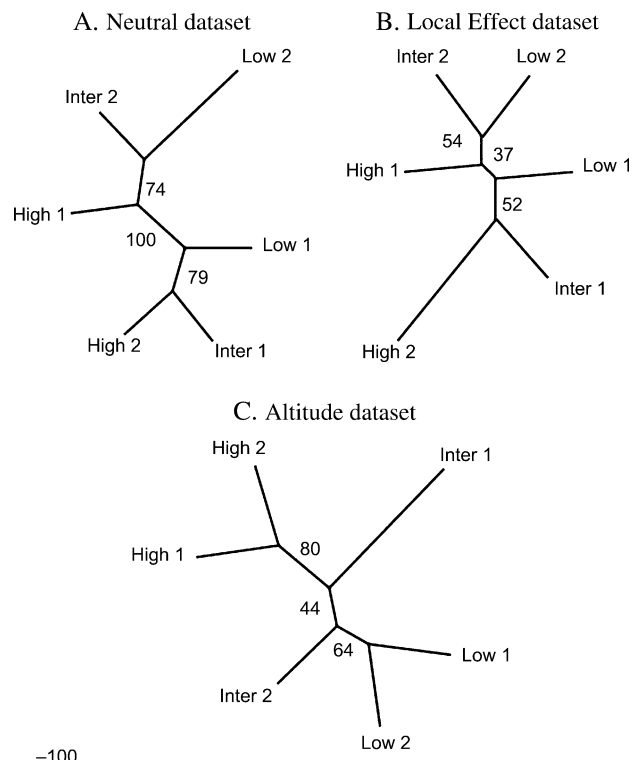## B. Local Effect dataset

C. Altitude dataset

FIG. 4—Neighbor-joining trees based on Nei's genetic distance obtained with (A) the Neutral data set, (B) the Local Effects data set, and (C) the Altitude data set. Bootstrap values are indicated in percentage.

of within-population diversity (Schlötterer 2002; Kauer, Dieringer, and Schlötterer 2003) or a particularly high differentiation between populations (Beaumont and Nichols 1996; Vitalis, Dawson, and Boursot 2001; Beaumont and Balding 2004).

Estimators of population differentiation, especially the F_{st} index, have the great advantage to be robust to many demographic scenarios (Beaumont 2005; Nielsen 2005). They also keep the selection signature for a longer period of time compared with intrapopulation estimators (Storz 2005). The population differentiation approach for outlier detection is thus worthwhile. In particular, estimators of population divergence are central in the two statistical tests for biallelic dominant data described in this article and in the only other one reported in the literature (Wilding, Butlin, and Grahame 2001; Campbell and Bernatchez 2004). Derived from Beaumont and Nichols (1996), this last method is very similar to the Dfdist test used here, except that it computes the F_{st} coefficient under an island model of migration between only two populations.

Because revealing outlier loci in genome scans currently depends on statistical tests, one of the main concerns is to highlight truly significant loci while avoiding the detection of false positives as much as possible. The detection methods used here lack power for biallelic data, especially when examining populations by pairs (Beaumont 2005; R. Vitalis, personal communication). Therefore, they might not be sensitive enough to disentangle weak selection signatures. This was especially true for DetSel, where no loci but one (locus 301) was significant at the 99% level. As a

result, we did not try to adjust the significance level, as recommended when using multiple tests (Schlötterer 2003), and did not apply the conservative Bonferroni correction. Instead, we kept the standard 95% significance level and focused our efforts on discarding as many false positives as possible among loci detected as outliers. This is why we excluded all the loci appearing only once among several analyses, which was a conservative criterion because it led to the exclusion of two third to three quarters of the detected loci.

Overall, the results obtained by the two methods are not totally consistent, probably because of a lack of statistical power of DetSel. Both methods only agree on locus 301 as a good candidate for selection due to altitude. However, three other significant loci (loci 84, 228, and 248) detected by Dfdist also appeared out of the confidence envelope several times but could not be accepted as trustworthy outliers with DetSel due to technical constraints. Interestingly, the particular pattern that made them incompatible for analyses with DetSel (monomorphism in one population of the pair) is also a good clue for selection due to altitude because it is observed repeatedly in different populations of the same altitude. Therefore, there is reliable evidence that these three loci are also involved into adaptation to altitude, as also illustrated by the different topologies of the Altitude versus Neutral trees. The goal of this study was to pick up the best candidate loci for selection but clearly not to compare the performance of the two detection methods in terms of power, false-positive discovery rate, and robustness to violation of assumptions. Our data did not offer such an opportunity because the true loci under selection are not known. It is also impossible to determine if loci picked out by only one method are false positives or real outliers that the other method failed to detect.

Seeking the Cause for Outlier Behavior

Today, with the improvement of genotyping throughputs and the development of new statistical tools, the challenge does not lie anymore in isolating outlier loci but in accounting for them afterward in the analyses. Loci tagged as outliers can be dealt with in two ways depending on the primary objectives of the genetic study (Luikart et al. 2003). If the multilocus sampling focuses on neutral genetic variation across the genome to infer classical population parameters (e.g., gene flow, population structure or size, migration rate, genetic distances), any loci diverging from neutral expectations should be simply excluded from the data set to get better parameter estimates (Wilding, Butlin, and Grahame 2001). Here, cleaning the total data set (392 loci) from all the detected loci (49 loci in total) led to a decrease of almost 25% in the value of the Fst estimate. On the other hand, if the purpose is to examine locus-specific evolutionary processes by tracking loci with an atypical pattern of polymorphism, then the exact cause of the outlier behavior has to be dug out before any further analyses (Luikart et al. 2003; Storz 2005).

Outlier loci detected in genome scans are of two kinds. The first one corresponds to loci with an atypical behavior for reasons other than selection. Some of them may display a variation pattern in the tail of the neutral distribution simply by chance (type I error) because of some statistical bias or some degree of departure between the simulated and the empirical data (Akey et al. 2004; Beaumont 2005). Outlier loci due to chance are not expected to exhibit parallel trends in several comparisons (Campbell and Bernatchez 2004; Storz 2005; Vasemägi, Nilsson, and Primmer 2005). In the common frog genome scan, we kept only the significant loci appearing in at least two analyses for each detection method, assuming that loci detected only once might be false positives (14 and 29 loci revealed by DetSel and Dfdist, respectively). Doing multiple tests with methods assuming different demographic scenarios is also a good, even if conservative, way to point out loci diverging artificially from the rest of the genome because the simulated neutral data are not realistic (Nielsen 2005). Here, we used two simulation models to analyze our data, which agreed on locus 301 and on loci 84, 228, and 248 in some extent. The demographic hypotheses are yet not fundamentally different in both tests, limiting the relevance of such a verification. Moreover, we discarded loci attached to one population in particular (3 and 11 loci for DetSel and Dfdist, respectively). Indeed, the similar topologies of trees constructed from the Neutral and Local Effects data sets may well indicate that such loci are influenced by local neutral effects such as genetic drift (Nielsen 2005). Likewise, Akey et al. (2004) have shown that many apparent selection signatures can actually be attributed to demographic events like bottlenecks. Finally, genome scans based on dominant data face a major but currently unavoidable statistical pitfall, which has never been taken into account or even discussed in the literature. All the outlier detection methods developed so far indeed rely on the estimation of allelic frequencies assuming Hardy-Weinberg proportions. Even if this strong hypothesis turns out to be true overall, natural selection might unfortunately create locus-specific deviations from the Hardy-Weinberg equilibrium and therefore unobtrusively bias the results of outlier detection in an unpredictable way.

A fake outlier behavior can also be created by a susceptibility to genotyping errors. It is thus important to ensure the reliability of the data and to estimate the error rate. In particular, one must avoid population-specific errors, which have a special bearing on outlier loci, by following a careful experimental protocol where samples are for example treated in a random order. In this study, the error rate was 2.0%, and errors were distributed randomly across markers, individuals, and populations. As a result, we considered that they only add some arbitrary noise in the parameter estimations, potentially alleviating the selection signature. However, some genotyping errors can occur, which are not detectable and can be concentrated on a locus in particular (Pompanon et al. 2005). Because the number of recessive phenotypes (band absence) in a population is crucial to estimate allelic frequencies, genotyping errors have a greater impact when the proportion of recessive phenotypes is low. It was not the case for the strong candidate loci for selection. Likewise, loci with an aberrant mutation or recombination rate can inconveniently happen to be significant in statistical tests for selection. This category of loci is a plague because it is impossible to eliminate them a priori without any further investigations.

The second kind of outlier loci included those displaying a real footprint of selection. In practice, their identification is the ultimate goal of most, if not all, genomic scans conducted so far. Therefore, it is important to validate selection as the cause of the deviant variation pattern of detected loci or alternatively to rule out other reasons creating similar and thus misleading polymorphism patterns. This confirmatory step is as crucial as the outlier detection procedure, if not more, but it is beyond the scope of this article to discuss all the possible evidences for selection and how to collect them because it would ultimately lead to functional genomics (Nielsen 2005; Storz 2005). Local adaptation is a global phenomenon resulting from many selection pressures, possibly interacting, and whose individual effects can be difficult to characterize and even to work out. Thereby, defining population replicates at each altitude level is a tricky step because selection pressures might not be totally identical in apparently homogeneous environments. In the common frog genome scan, the 12 outlier loci attached to a population in particular (Local Effects data set) might also be the target of a past or current selection process at a local scale (Vitalis, Dawson, and Boursot 2001; Vasemägi, Nilsson, and Primmer 2005). On the contrary, based on the assumption that selection pressures due to altitude were similar in populations with the same elevation, eight loci appearing repeatedly in independent interaltitude comparisons were considered as involved in local adaptation to altitude (Altitude data set). Nonetheless, this procedure implicitly assumed that selection targets the same genes across all the replicates, a prerequisite that was shown not to be respected in some cases (Nachman, Hoekstra, and D'Agostino 2003).

Insights into Adaptation Along a Gradient of Altitude in the Common Frog

The survey of neutral genetic diversity in the six common frog populations revealed two major features about the genetic background concomitant with local adaptation to altitude. First, genetic diversity within populations is globally high (data not shown), informing to some extent about an existing genetic potential of the sampled populations to undergo future adaptations (Waples 1995; Miaud and Merilä 2001). Moreover, the proportion of intrapopulation genetic variation appeared to be lower for loci identified as outliers compared with neutral loci (31% vs. 82%). This offers some attractive outlooks as regards the development of new outlier detection tests focusing on levels of diversity within populations (Schlötterer 2002; Kauer, Dieringer, and Schlötterer 2003).

At the interpopulation level, the sampled populations are highly structured at a local scale independently from altitude, with a mean Fst value of 0.11. This is consistent with other studies carried out at the same scale in the Pyrenean and Cantabrian mountains in Spain (Veith et al. 2002) and in the French Alps (A. Cercueil, personal communication). Such a population subdivision is regularly observed in amphibian species because their dispersion abilities are poor, especially in fragmented landscapes (Hitchings and Beebee 1997; Joly, Morand, and Cohas 2003) and also because amphibian adults are highly phylopatric (Smith and Green 2005). Even if some examples of adaptive divergence have been described in the face of gene flow for several organisms (Saint-Laurent, Legault, and Bernatchez 2003; Gomez-Mestre and Tejedo 2004), many local adaptation events are documented together with a reduced gene flow (see for example Hendry et al. 2004). However, the cause to consequence relationships between reduced gene flow and adaptive divergence are still a subject of intense debate in the literature (Hendry and Taylor 2004; Nosil and Crespi 2004).

To our knowledge, our Fst-based survey is the first one espousing a population genomics perspective to reveal genetic adaptation along an environmental gradient. Detection of outlier loci was done both globally (Dfdist) and with pairwise analyses (Dfdist and DetSel). This helped reveal loci with a major overall effect as well as loci responding at different stages along the altitude gradient. A similar procedure had already been successful to underline the role of the albumin locus in adaptation along an altitudinal transect in the deer mice *Peromyscus maniculatus* (Storz and Dubach 2004). Likewise, our results illustrate the potential of genome-wide surveys to reveal selection signatures in subtle cases of adaptation, where the association between environmental variables and fitness-related traits may be complex and/or cryptic. Given that many traits may give an adaptive response to this many-sided selection gradient (Sgro and Blows 2004), it is reasonable to expect that several genomic regions are the final target of selection in relation with altitude, contrary to selection mechanisms such as insecticide or drug resistance known to affect a single metabolic pathway (Polley, Chokejindachai, and Conway 2003; Ffrench-Constant, Daborn, and Le Goff 2004). However, it remains unclear whether (1) our sampling across the genome was truly representative and screened all the genomic regions equally (Young, Schupp, and Keim 1999) and (2) the statistical tests we used were sensitive enough to detect weak selection signatures. It is thus too ambitious to answer one of the fundamental questions concerning genetic adaptation, i.e., the number and relative effect of genes involved in adaptation processes (Nielsen 2005; Storz 2005). Our genome scan pointed out eight outlier loci potentially involved in adaptation to altitude, that is about 2% of the loci inspected, a value similar to those found for explorative genome-wide surveys performed in periwinkle (5%; Wilding, Butlin, and Grahame 2001) or in lake whitefish (between 1% and 3%; Campbell and Bernatchez 2004). This rather low yield yet reached 12% when focusing on Atlantic salmon expressed sequence tag–-linked microsatellites (Vasemägi, Nilsson, and Primmer 2005), which underlines the fact that coding sequences are better sources of adaptive polymorphisms.

Perspectives and Conclusions

This study was a first step toward characterizing the selection marks imprinted by a gradient of selection pressures on the common frog genome. Its immediate objective was to isolate loci with a deviant level of variation relative to the rest of the genome and then to have a closer look on these atypical loci in order to bring out the most promising candidates for directional selection. However, these supposedly selected loci will have to be considered with caution until corroborating evidence is acquired that selection is really

responsible of their outlier nature. For instance, it has still to be proved whether they display a significant interaltitude differentiation along other altitudinal transects. The sampling has also to be broadened to include populations distributed uniformly along the same altitudinal transect in order to give a finer representation of the evolution of selection pressures with altitude and possibly to draw attention on loci with smaller or spatially localized adaptive effects.

Once the selection footprint is attested for some outlier loci, one may choose to move toward a functional approach of adaptation by dissecting the adaptive mechanisms at the nucleotide level, with the ultimate intent to characterize the underlying selected mutations or genes (see for example Wootton et al. 2002; Kohn, Pelz, and Wayne 2003; Turner, Hahn, and Nuzhdin 2005). However, because genome scans have a resolution power of several kilobases in general, crossing the threshold between the genomic and the nucleotide level is currently strenuous, even for model species (Flint and Mott 2001; Erickson et al. 2004). The increased number of fully sequenced genomes promises yet to give valuable information for this purpose in the future. Alternatively, combining information provided by genome scans with QTL mapping may enlighten the genetic architecture and functional organization hidden behind more complex adaptive traits. Such strategies helped establish, for instance, that loci with significantly higher differentiation tend to gather in the same chromosomic locations (Emelianov, Marec, and Mallet 2004; Rogers and Bernatchez 2005). This underpins the emerging concept of a mosaic genome, where natural selection maintains islets with a reduced gene flow (Wu 2001; Beaumont 2005).

Acknowledgments

Literature Cited

Ajmone-Marsan, P., A. Valentini, M. Cassandro, G. Vecchiotti-Antaldi, G. Bertoni, and M. Kuiper. 1997. AFLP markers for DNA fingerprinting in cattle. Anim. Genet. **28**:418–426.

Akey, J. M., M. A. Eberle, M. J. Rieder, C. S. Carlson, M. D. Shriver, D. A. Nickerson, and L. Kruglyak. 2004. Population history and natural selection shape patterns of genetic variation in 132 genes. PLoS Biol. **2**:1591–1599.

Akey, J. M., G. Zhang, K. Zhang, L. Jin, and M. D. Shriver. 2002. Interrogating a high-density SNP map for signatures of natural selection. Genome Res. **12**:1805–1814.

Beaumont, M. A. 2005. Adaptation and speciation: what can FST tell us? Trends Ecol. Evol. **20**:435–440.

Beaumont, M. A., and D. J. Balding. 2004. Identifying adaptive genetic divergence among populations from genome scans. Mol. Ecol. **13**:969–980.

Beaumont, M. A., and R. A. Nichols. 1996. Evaluating loci for use in the genetic analysis of population structure. Proc. R. Soc. Lond. B Biol. Sci. **263**:1619–1626.

Benham, J., J. U. Jeung, M. Jasieniuk, V. Kanazin, and T. Blake. 1999. Genographer: a graphical tool for automated fluorescent AFLP and microsatellite analysis. J. Agric. Genom. **4**:399.

Black, W. C., C. F. Baer, M. F. Antolin, and N. M. DuTeau. 2001. Population genomics: genome-wide sampling of insect populations. Annu. Rev. Entomol. **46**:441–469.

Bonin, A., E. Bellemain, P. Bronken Eidesen, F. Pompanon, C. Brochmann, and P. Taberlet. 2004. How to track and assess genotyping errors in population genetics studies. Mol. Ecol. **13**:3261–3273.

Bonin, A., F. Pompanon, and P. Taberlet. 2005. Use of amplified fragment length polymorphism (AFLP) markers in surveys of vertebrate diversity. Pp. 145–161 in E. A. Zimmer and E. Roalson, eds. Molecular evolution: producing the biochemical data, Part B Methods in Enzymology, Vol. 395. Academic Press, New York.

Campbell, D., and L. Bernatchez. 2004. Generic scan using AFLP markers as a means to assess the role of directional selection in the divergence of sympatric whitefish ecotypes. Mol. Biol. Evol. **21**:945–956.

Colosimo, P. F., C. L. Peichel, K. Nereng, B. K. Blackman, M. D. Shapiro, D. Schluter, and D. M. Kingsley. 2004. The genetic architecture of parallel armor plate reduction in threespine sticklebacks. PLoS Biol. **2**:E109.

Emelianov, I., F. Marec, and J. Mallet. 2004. Genomic evidence for divergence with gene flow in host races of the larch budmoth. Proc. R. Soc. Lond. B Biol. Sci. **271**:97–105.

Endler, J. A. 1977. Geographic variation, speciation, and clines. Princeton University Press, Princeton, NJ.

Erickson, D. L., C. B. Fenster, H. K. Stenoien, and D. Price. 2004. Quantitative trait locus analyses and the study of evolutionary process. Mol. Ecol. **13**:2505–2522.

Felsenstein, J. 2004. PHYLIP (phylogeny inference package). Distributed by the author, Department of Genome Sciences, University of Washington, Seattle.

Ffrench-Constant, R. H., P. J. Daborn, and G. Le Goff. 2004. The genetics and genomics of insecticide resistance. Trends Genet. **20**:163–170.

Flint, J., and R. Mott. 2001. Finding the molecular basis of quantitative traits: successes and pitfalls. Nat. Rev. Genet. **2**:437–445.

Gasc, J. P., A. Cabela, J. Crnobrnja-Isailovic et al. (14 co-authors). 1997. Atlas of amphibians and reptiles in Europe. Societas Europaea Herpetologica & Muséum National d'Histoire Naturelle (IEGB/SPN), Paris.

Gomez-Mestre, I., and M. Tejedo. 2004. Contrasting patterns of quantitative and neutral genetic variation in locally adapted populations of the natterjack toad, *Bufo calamita*. Evolution **58**:2343–2352.

Harr, B., M. Kauer, and C. Schlötterer. 2002. Hitchhiking mapping: a population-based fine-mapping strategy for adaptive mutations in *Drosophila melanogaster*. Proc. Natl. Acad. Sci. USA **99**:12949–12954.

Hendry, A. P., Y. E. Morbey, O. K. Berg, and J. K. Wenburg. 2004. Adaptive variation in senescence: reproductive lifespan in a wild salmon population. Proc. R. Soc. Lond. B Biol. Sci. **271**:259–266.

Hendry, A. P., and E. B. Taylor. 2004. How much of the variation in adaptive divergence can be explained by gene flow? An evaluation using lake-stream stickleback pairs. Evolution **58**:2319–2331.

Hitchings, S. P., and T. J. C. Beebee. 1997. Genetic substructuring as a result of barriers to gene flow in urban *Rana temporaria* (common frog) populations: implications for biodiversity conservation. Heredity **79**:117–127.

Johansson, M., K. Räsänen, and J. Merila. 2001. Comparison of nitrate tolerance between different populations of the common frog, *Rana temporaria*. Aquat. Toxicol. **54**:1–14.

Joly, P., C. Morand, and A. Cohas. 2003. Habitat fragmentation and amphibian conservation: building a tool for assessing landscape matrix connectivity. C R Biol. **326**:132–139.

Jones, C. J., K. J. Edwards, S. Castaglione et al.. 1997. Reproducibility testing of RAPD, AFLP and SSR markers in plants by a network of European laboratories. Mol. Breed. **3**:381–390.

Kauer, M., D. Dieringer, and C. Schlötterer. 2003. A microsatellite variability screen for positive selection associated with the "Out of Africa" habitat expansion of *Drosophila melanogaster*. Genetics **165**:1137–1148.

Kayser, M., S. Brauer, and M. Stoneking. 2003. A genome scan to detect candidate regions influenced by local natural selection in human populations. Mol. Biol. Evol. **20**:893–900.

Kohn, M. H., H. J. Pelz, and R. K. Wayne. 2000. Natural selection mapping of the warfarin-resistance gene. Proc. Natl. Acad. Sci. USA **97**:7911–7915.

———. 2003. Locus-specific genetic differentiation at Rw among warfarin-resistant rat (*Rattus norvegicus*) populations. Genetics **164**:1055–1070.

Laugen, A. T., A. Laurila, K. Rasanen, and J. Merila. 2003. Latitudinal countergradient variation in the common frog (*Rana temporaria*) development rates—evidence for local adaptation. J. Evol. Biol. **16**:996–1005.

Luikart, G., P. R. England, D. Tallmon, S. Jordan, and P. Taberlet. 2003. The power and promise of population genomics: from genotyping to genome typing. Nat. Rev. Genet. **4**:981–994.

Merilä, J., A. Laurila, A. T. Laugen, K. Räsänen, and M. Pahkala. 2000. Plasticity in age and size at metamorphosis in *Rana temporaria*—comparison of high and low latitude populations. Ecography **23**:457–465.

Miaud, C., R. Guyétant, and J. Elmberg. 1999. Variations in life-history traits in the common frog *Rana temporaria* (Amphibia: Anura): a literature review and new data from the French Alps. J. Zool. **249**:61–73.

Miaud, C., and J. Merilä. 2001. Local adaptation or environmental induction? Causes of population differentiation in alpine amphibians. Biota **2**:31–50.

Nachman, M. W., H. E. Hoekstra, and S. L. D'Agostino. 2003. The genetic basis of adaptive melanism in pocket mice. Proc. Natl. Acad. Sci. USA **100**:5268–5273.

Nielsen, R. 2005. Molecular signatures of natural selection. Annu. Rev. Genet. **39**:197–218.

Nosil, P., and B. J. Crespi. 2004. Does gene flow constrain adaptive divergence or vice versa? A test using ecomorphology and sexual isolation in *Timema cristinae* walking-sticks. Evolution **58**:102–112.

Palo, J. U., R. B. O'Hara, A. T. Laugen, A. Laurila, C. R. Primmer, and J. Merila. 2003. Latitudinal divergence of common frog (*Rana temporaria*) life history traits by natural selection: evidence from a comparison of molecular and quantitative genetic data. Mol. Ecol. **12**:1963–1978.

Payseur, B. A., A. D. Cutter, and M. W. Nachman. 2002. Searching for evidence of positive selection in the human genome using patterns of microsatellite variability. Mol. Biol. Evol. **19**:1143–1153.

Pidancier, N., P. Gauthier, C. Miquel, and F. Pompanon. 2002. Polymorphic microsatellite DNA loci identified in the common frog (*Rana temporaria*, Amphibia, Ranidae). Mol. Ecol. Notes **2**:304–305.

Polley, S. D., W. Chokejindachai, and D. J. Conway. 2003. Allele frequency-based analyses robustly map sequence sites under balancing selection in a malaria vaccine candidate antigen. Genetics **165**:555–561.

Pompanon, F., A. Bonin, E. Bellemain, and P. Taberlet. 2005. Genotyping errors: causes, consequences and solutions. Nat. Rev. Genet. **6**:847–859.

Rogers, S. M., and L. Bernatchez. 2005. Integrating QTL mapping and genome scans towards the characterization of candidate loci under parallel selection in the lake whitefish (*Coregonus clupeaformis*). Mol. Ecol. **14**:351–361.

Rosenblum, E. B., H. E. Hoekstra, and M. W. Nachman. 2004. Adaptive reptile color variation and the evolution of the Mc1r gene. Evolution **58**:1794–1808.

Saint-Laurent, R., M. Legault, and L. Bernatchez. 2003. Divergent selection maintains adaptive differentiation despite high gene flow between sympatric rainbow smelt ecotypes (*Osmerus mordax* Mitchill). Mol. Ecol. **12**:315–330.

Schlötterer, C. 2002. A microsatellite-based multilocus screen for the identification of local selective sweeps. Genetics **160**: 753–763.

———. 2003. Hitchhiking mapping—functional genomics from the population genetics perspective. Trends Genet. **19**:32–38.

Schneider, S., D. Roessli, and L. Excoffier. 2000. Arlequin: a software for population genetics data analysis. Version 2.000. Distributed by the authors, Genetics and Biometry Laboratory, University of Geneva, Geneva, Switzerland.

Sgro, C. M., and M. W. Blows. 2004. The genetic covariance among clinal environments after adaptation to an environmental gradient in *Drosophila serrata*. Genetics **167**: 1281–1291.

Smith, M. A., and D. M. Green. 2005. Dispersal and the metapopulation paradigm in amphibian ecology and conservation: are all amphibian populations metapopulations? Ecography **28**: 110–128.

Storz, J. F. 2005. Using genome scans of DNA polymorphism to infer adaptive population divergence. Mol. Ecol. **14**:671–688.

Storz, J. F., and J. M. Dubach. 2004. Natural selection drives altitudinal divergence at the albumin locus in deer mice, *Peromyscus maniculatus*. Evolution **58**:1342–1352.

Turner, T. L., M. W. Hahn, and S. V. Nuzhdin. 2005. Genomic islands of speciation in *Anopheles gambiae*. PLoS Biol. **3**:1572–1578.

Vasemägi, A., J. Nilsson, and C. R. Primmer. 2005. Expressed sequence tag (EST) linked microsatellites as a source of gene associated polymorphisms for detecting signatures of divergent selection in Atlantic salmon (*Salmo salar* L.). Mol. Biol. Evol. **22**:1067–1076.

Veith, M., M. Vence, D. R. Vieites, S. Nieto-Roman, and A. Palanca. 2002. Genetic differentiation and population structure within Spanish common frogs (*Rana temporaria* complex; Ranidae, Amphibia). Folia Zool. **51**:307–318.

Vekemans, X., T. Beauwens, M. Lemaire, and I. Roldan-Ruiz. 2002. Data from amplified fragment length polymorphism (AFLP) markers show indication of size homoplasy and of a relationship between degree of homoplasy and fragment size. Mol. Ecol. **11**:139–151.

Verhoeven, K. J., T. K. Vanhala, A. Biere, E. Nevo, and J. M. van Damme. 2004. The genetic basis of adaptive population differentiation: a quantitative trait locus analysis of fitness traits in two wild barley populations from contrasting habitats. Evolution **58**:270–283.

Vigouroux, Y., M. McMullen, C. T. Hittinger, K. Houchins, L. Schulz, S. Kresovich, Y. Matsuoka, and J. Doebley. 2002. Identifying genes of agronomic importance in maize by screening microsatellites for evidence of selection during domestication. Proc. Natl. Acad. Sci. USA **99**:9650–9655.

Vitalis, R., K. Dawson, and P. Boursot. 2001. Interpretation of variation across marker loci as evidence of selection. Genetics **158**:1811–1823.

Vitalis, R., K. Dawson, P. Boursot, and K. Belkhir. 2003. DetSel 1.0: a computer program to detect markers responding to selection. J. Hered. **94**:429–431.

Vos, P., R. Hogers, M. Bleeker et al. (11 co-authors). 1995. AFLP: a new technique for DNA fingerprinting. Nucleic Acids Res. **23**:4407–4414.

Waples, R. S. 1995. Evolutionary significant units and the conservation of biological diversity under the endangered species act.

Pp. 8–27 *in* J. L. Nielsen and G. A. Powers, eds. Evolution and the aquatic ecosystem: defining units in population conservation. American Fisheries Society, Bethesda, Md.

Wilding, C. S., R. K. Butlin, and J. Grahame. 2001. Differential gene exchange between parapatric morphs of *Littorina saxatilis* detected using AFLP markers. J. Evol. Biol. **14**: 611–619.

Wootton, J. C., X. Feng, M. T. Ferdig, R. A. Cooper, J. Mu, D. I. Baruch, A. J. Magill, and X. Z. Su. 2002. Genetic diversity and chloroquine selective sweeps in *Plasmodium falciparum.* Nature **418**:320–323.

Wright, S. 1951. The genetical structure of populations. Ann. Eugen. **15**:323–354.

Wu, C. I. 2001. The genic view of the process of speciation. J. Evol. Biol. **14**:851–865.

Young, W. P., J. M. Schupp, and P. Keim. 1999. DNA methylation and AFLP marker distribution in the soybean genome. Theor. Appl. Genet. **99**:785–790.

Zhivotovsky, L. A. 1999. Estimating population structure in diploids with multilocus dominant DNA markers. Mol. Ecol. **8**:907–913.