

CONTRASTING PATTERNS OF QUANTITATIVE AND NEUTRAL GENETIC VARIATION IN LOCALLY ADAPTED POPULATIONS OF THE NATTERJACK TOAD, *BUFO CALAMITA*

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Abstract.—The relative importance of natural selection and genetic drift in determining patterns of phenotypic diversity observed in nature is still unclear. The natterjack toad (*Bufo calamita*) is one of a few amphibian species capable of breeding in saline ponds, even though water salinity represents a considerable stress for them. Results from two common-garden experiments showed a pattern of geographic variation in embryonic salinity tolerance among populations from either fresh or brackish environments, consistent with the hypothesis of local adaptation. Full-sib analysis showed increased variation in survival among sibships within population for all populations as osmotic stress was increased (broad-sense heritability increased as salinity raised). Nevertheless, toads native to the brackish water environment had the highest overall survival under brackish conditions. Levels of population genetic differentiation for salinity tolerance were higher than those of neutral genetic differentiation, the latter obtained through the analysis of eight microsatellite loci. Microsatellite markers also revealed little population differentiation, lack of an isolation-by-distance pattern, and moderate gene flow connecting the populations. Therefore, environmental stress tolerance appears to have evolved in absence of geographic isolation, and consequently we reject the null hypothesis of neutral differentiation.

Key words.—*Bufo*, F_{ST} , local adaptation, microsatellite loci, population differentiation, Q_{ST} , salinity tolerance.

Natural selection is a very powerful and widespread factor driving microevolutionary change (Endler 1986; Kingsolver et al. 2001). However, the extent to which natural selection and the adaptive responses of organisms should be held responsible for the patterns of biological diversity is far from settled. Because the molecular characterisation of genetic variation became technically feasible, a growing body of literature has been produced comparing this sort of genetic variation with estimates of quantitative genetic variation in morphological, behavioral, and life-history traits (for reviews, see Merilä and Crnokrak 2001; McKay and Latta 2002). Comparing patterns of population genetic differentiation in neutral marker loci with those of quantitative traits permits inferences about the relative role of selection in population divergence, by contrasting the degree of adaptive change with that of differentiation due solely to drift (Lande 1992; Lynch 1996; Merilä and Crnokrak 2001; Reed and Frankham 2001; McKay and Latta 2002). Thus, estimates of neutral genetic divergence provide a null hypothesis to the alternative hypothesis of adaptive divergence (Lynch 1990; Spitze 1993; Podolsky and Holtsford 1995; Schluter 2000). Comparisons of the degree of genetic population subdivision for quantitative traits (Q_{ST}) and neutral allelic divergence found between populations (F_{ST}) have showed that generally Q_{ST} is on average greater than F_{ST} (Merilä and Crnokrak 2001; McKay and Latta 2002). When population differentiation in neutral genetic markers equals that exhibited by quantitative traits ($Q_{ST} = F_{ST}$), the null hypothesis cannot be rejected and drift cannot be ruled out as the main causes of population divergence (Kuittinen et al. 1997; Waldmann and Andersson 1998; Lynch et al. 1999; Widén et al. 2002).

When Q_{ST} and F_{ST} differ significantly, selection is plausibly responsible for the pattern. Directional or disruptive selection would have promoted population divergence when $Q_{ST} > F_{ST}$, while stabilizing selection would have opposed divergence in quantitative traits despite drift if $Q_{ST} < F_{ST}$ (Prout and Barker 1993; Spitze 1993; Long and Singh 1995; Morgan et al. 2001; Petit et al. 2001; Steinger et al. 2002; Storz 2002).

It has been noted that quantitative trait variation would be a better indicator of the evolutionary potential of populations than neutral genetic variation and that conservation studies would benefit from incorporating this type of information (Lynch 1996; Storz 1996; Knapp and Rice 1998; Podolsky 2001). But the converse is also true. The joint analysis of quantitative traits and neutral marker loci in evolutionary biology not only provides a null expectation, but it also provides a quantitative description of the population structure in which population differentiation has taken place and the amount of gene flow that selection has opposed (if at all).

We compare the pattern of genetic variation at both microsatellite loci and osmotic stress tolerance in five populations of the natterjack toad (*Bufo calamita*) that inhabit either freshwater or brackish water ponds in southern Spain. Water salinity constitutes an environmental stress for amphibians due to their poor osmoregulatory capability (Balinsky 1981; Boutilier et al. 1992). Nevertheless, *B. calamita* is a moderate eurihaline toad, for it is associated with ponds and lakes that range from freshwater to brackish water (Balinsky 1981; Beebe 1985; Sinsch et al. 1992; Gomez-Mestre and Tejedo 2002, 2003) and occupies several inland brackish ponds in Spain. Evidence from field transplants and common-garden experiments aimed at comparing the osmotic tolerance of freshwater populations and that of brackish water ones, suggests that populations have diverged in osmotic tolerance (Gomez-Mestre and Tejedo 2003).

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Estimates of population differentiation in quantitative traits obtained from wild-caught animals can be inflated by environmental effects (Lee and Frost 2002; Palo et al. 2003). To avoid this effect, measurements of quantitative genetic variation were based on common-garden experiments involving animals in the embryonic stage (Gomez-Mestre and Tejedo 2003; M. Tejedo, I. Gomez-Mestre, and R. Reques, unpubl. data). Genetic variability in quantitative traits is known to vary depending on environmental conditions (Mitchell-Olds and Rutledge 1986; Jenkins et al. 1997; Hoffmann and Merilä 1999), although no universal pattern of increased or decreased genetic variance with increased environmental stress has been observed (Hoffmann and Parsons 1991; Bennington and McGraw 1996; Hoffmann and Merilä 1999). Consequently, estimates of population differentiation are likely to be affected by the environmental conditions under which they are being measured (Merilä and Crnokrak 2001), although this phenomenon has been addressed seldomly (but see Palo et al. 2003). In the common-garden experiments that we conducted, we reared either full-sibs or half-sibs from five populations (two populations from brackish ponds and three from freshwater ponds) under different water salinity concentrations. Thus, it was possible to study quantitative genetic variation within and among populations under different levels of water salinity.

We present an analysis of population differentiation in quantitative traits (as measured by Q_{ST}) under experimentally controlled varying environmental conditions. Furthermore, we tested whether local adaptation in this system has evolved in isolation or in spite of gene flow from nonadapted populations. We address the following questions: (1) Is population differentiation in salinity tolerance greater than expected solely as a result of isolation and drift (neutral evolution)? (2) Has population differentiation occurred in geographic isolation? (3) Does quantitative trait variation within and among populations change under different environmental conditions?

MATERIALS AND METHODS

Populations

The embryonic common-garden experiments from which the measurements of quantitative genetic variation were derived have been described in detail elsewhere and will only be described briefly here. One of the experiments included four populations (Fresh 1–3 and Saline 1) and involved comparisons among full-siblings within populations (Gomez-Mestre and Tejedo 2003), while the other included only two populations (Fresh 2 and Saline 2), but compared half-sibling variation within populations (M. Tejedo, I. Gomez-Mestre, and R. Reques, unpubl. data). Three of the populations studied, Fresh 1, 2, and 3, breed in different kinds of freshwater environments, either in shallow ponds on granitic soil in different regions of Sierra Morena (Fresh 2 and 3) or in ephemeral ponds on sandy soil of Parque Natural de Doñana (Fresh 1). Two populations naturally breeding in brackish water (Saline 1 and 2) were sampled in Laguna del Taraje and Laguna de Jarales, two temporary lagoons on haline sedimentary basins in Córdoba province, 3 km apart from one another.

Description of the Experiments

In the full-sib/four-population experiments, between six and nine clutches from each population were sampled in early spring 2000. Embryos from each clutch were taken to the laboratory and strings of 20 embryos were randomly distributed into petri dishes assigned to either one of three water salinity levels (freshwater, 6 g/L, and 8 g/L). Brackish water was obtained from Saline 2 original pond and diluted with fresh water to the targeted concentrations. Each treatment (full-sib X population X salinity) was replicated six times and the replicates were randomized in spatially distributed blocks along shelves in the laboratory.

For the half-sib/two-population experiment, adult *B. calamita* were randomly sampled and crossed, yielding 30 half-sibships from Saline 2 and 14 half-sibships from Fresh 2 in winter 1998 (M. Tejedo, I. Gomez-Mestre, and R. Reques, unpubl. data). Batches of five eggs from each sibling were randomly assigned to 20-ml wells on ice-cube racks, filled with either freshwater, 6 g/L, or 8 g/L. Each half-sib X population X salinity combination was replicated six times. In both experiments room temperature was kept roughly constant (range = 20–24°C) and photoperiod followed a 12:12 cycle.

Quantitative Variation Analysis

The trait considered in both experiments was survival to 25 stage Gosner (Gosner 1960), when tadpoles begin free feeding. Survival in this context reflects osmotic stress tolerance and it has a direct link to fitness. In all cases, we used the proportion of surviving embryos in each dish or well as the response variable, instead of the survival of each individual embryo. Using a single observation per experimental container helped prevent both pseudoreplication and potential common-environment effects. However, each observation averaged over a group of siblings approximates the breeding value of the two parents (Simons et al. 1998; D. A. Roff, pers. comm.), and therefore heritability estimates are obtained from the intraclass correlation itself in the case of full-sibs and twice (not four times) the value in the case of half-sibs.

For the full-sib experiment in 2000, we estimated broad-sense heritability (H^2) for each population and each salinity treatment. Variance components due to sibship and experimental block were estimated using restricted maximum-likelihood analysis with the MIXED procedure of SAS (SAS Institute 1999). Broad-sense heritability was calculated as $H^2 = (ci_S^2)/(ci_S^2 + ci_B^2 + ci^2)$, where ci_S^2 , ci_B^2 , and ci^2 were the variance components associated with differences among full-sibs within population, experimental block effect, and the error term, respectively (Falconer and Mackay 1996). In the half-sib experiment of 1998, the additive variance component (ci_A^2) was calculated for each population at each salinity level using the software Quercus (Shaw 1987; Shaw and Shaw 1994). Both broad-sense and narrow-sense heritability estimates obtained from laboratory-reared progeny may be an imperfect reflection of heritability in nature, although estimates obtained in the laboratory are often strongly correlated with, and good indicators of, estimates obtained in the field (Roff 1997).

TABLE 1. Summary of results from the two experiments reported. The 2000 experiment included full-sibs. Thus, ci_s^2 is the variance component associated to full-sibs, and h^2 is only broad-sense heritability. The 1998 experiment analysed half siblings, and therefore additive genetic variance (V_A) could be computed. h^2 in this second case is narrow-sense heritability. In both cases variance components significantly different from zero are marked with an asterisk. The statistical probability for the underlined value was $0.05 < P < 0.10$.

Salinity (g/l)	Fresh 1				Fresh 2				Fresh 3				Saline 1				Saline 2					
	ci_s^2	H^2	Survival	SE	ci_s^2	H^2	Survival	SE	ci_s^2	H^2	Survival	SE	ci_s^2	H^2	Survival	SE	ci_s^2	H^2	Survival	SE	Population X salt	
2000	0	0	1	0	0	0	0.98	0.012	0.0005	0.06	1	0.003	0.009*	0.20	0.95	0.017	0.009*	0.20	0.95	0.017	Z = 2.02	
0	0	0	0.98	0.017	0.0004	0.09	0.99	0.002	0.0006	0.07	1	0.01	0.006*	0.09	0.97	0.01	0.006*	0.09	0.97	0.01	P = 0.023	
6	0.079*	0.45	0.38	0.04	0.032*	0.16	0.23	0.04	0.051*	0.23	0.37	0.04	0.040*	0.50	0.80	0.02	0.040*	0.50	0.80	0.02		
1998																						
					V_A	h^2	Survival	SE									V_A	h^2	Survival	SE	Population X salt	
0					0.011	0.25	0.93	0.02					0.031*	0.38	0.96	0.008	0.031*	0.38	0.96	0.008	F = 21.96	
6					<u>0.022</u>	0.09	0.88	0.02					0.037*	0.38	0.96	0.008	0.037*	0.38	0.96	0.008	df = 2,739	
8					0.035	0.13	0.59	0.05					0.045	0.28	0.81	0.02	0.045	0.28	0.81	0.02	P < 0.0001	

The proportion of between-population genetic variance (Q_{ST}) in salinity tolerance was calculated under the different salinity conditions as $Q_{ST} = ci_{GB}^2 / (ci_{GB}^2 + 2ci_{GW}^2)$, where ci_{GB}^2 and ci_{GW}^2 are the genetic variances between and within populations, respectively (Wright 1951; Lande 1992; Spitze 1993). The different variance components were estimated in both experiments by fitting a mixed linear model through restricted maximum likelihood for each salinity level and population pair using the MIXED procedure of SAS (SAS Institute 1999). For the 2000 full-sib experiment the model fitted was survival = block + population + sibship (population) + residual. The average genetic variance within populations (ci_{GW}^2) was estimated as the observational variance component for sibships within population. The average genetic variance between populations (ci_{GB}^2) was estimated indirectly by equating it to the observational component for populations. This procedure could result in a Q_{ST} estimate biased upward if there was significant environmentally induced phenotypic variation among populations. In this case, we assumed that the standardized conditions of the common-garden experiments rendered environmentally induced variation negligible. In the 1998 half-sib experiment, the model fitted for each salinity level was survival = population + sire (population) + dam (population) + sire X dam (population) + block + residual. The average variance between populations (ci_{GB}^2) was equated to the observational component for populations, while the variance within population (ci_{GW}^2) was equated to twice the sire nested within population component. In both experiments, standard errors of the genetic parameter estimates were obtained using the delta method (Podolsky and Holtsford 1995; Lynch and Walsh 1998).

Molecular Genetic Analysis

Eight microsatellite loci were analyzed in the five populations. The markers used were *Bcal 1*, *Bcal 2*, *Bcal 3*, *Bcal 4*, *Bcal 5*, *Bcal 7* (Rowe et al. 1997), *Bcal 10*, and *Bcal 11* (Rowe et al. 2000). Tissue samples were obtained by toe-clipping of adults during the breeding season at four of the five populations, randomly sampling 23–25 individuals per population. This procedure does not compromise the survival of adult toads (as revealed by subsequent recaptures of marked individuals; I. Gomez-Mestre and M. Tejedo, pers. obs.), and it is preferred over using tadpole tail tissue because the latter increases the probability of sampling siblings, and therefore inflating the inbreeding estimates (Hansen et al. 1997). However, samples had to be derived from tadpoles in Saline 2, so we sampled from disparate parts of the lagoon to maximize the chances of sampling unrelated animals.

DNA was obtained following a phenol-chloroform extraction protocol and was stocked in concentrations of 60–100 ng/jil. The 5' to 3'-primers were labeled with a color fluorophore, either HEX, TET, or FAM. Amplification of the loci was conducted by polymerase chain reactions (PCRs) of 15 jil total volume with 5 jil of DNA. The PCR amplifications followed a 66–50°C touchdown procedure. PCR products of each of the eight loci for each individual sampled were aliquoted and mixed according to their abundance. An aliquot of 1.5 jil of the resulting mix was added to 13 jil of formamide plus 0.3 jil of Tamra 500 (Applied Biosystems, Foster City,

TABLE 2. Pairwise estimates of population quantitative genetic differentiation (Q_{ST}) among the four populations considered in the full-sib experiment (A) and half-sib experiment (B). The estimates were obtained for three different water salinity levels. Mantel tests used to test for correlation between Q_{ST} estimates for each environment and F_{ST} estimates are also given.

A						
	Saline 1 vs. Fresh 1	Saline 1 vs. Fresh 2	Saline 1 vs. Fresh 3	Fresh 1 vs. Fresh 2	Fresh 1 vs. Fresh 3	Fresh 2 vs. Fresh 3
Fresh water						
Pop	0.005	0.002	0.004	0.001	<0.001	<0.001
Fam (Pop)	0.004	0.004	0.004	0.001	<0.001	0.001
Q_{ST}	0.391	0.165	0.319	0.154	<0.001	0.065
2 SD CI	0.389 – 0.393	0.164 – 0.167	0.318 – 0.320	0.141 – 0.167		0.056 – 0.074
Mantel Q_{ST} vs. F_{ST}	$Z = 0.051, P = 0.584, r = -0.30$					
6 g/l						
Pop	0.001	0.002	0.002	<0.001	<0.001	<0.001
Fam (Pop)	0.001	0.002	0.002	<0.001	0.003	<0.001
Q_{ST}	0.315	0.293	0.349	<0.001	0.008	<0.001
2 SD CI	0.314 – 0.315	0.291 – 0.295	0.348 – 0.349		0.008 – 0.008	
Mantel Q_{ST} vs. F_{ST}	$Z = 0.048, P = 0.413, r = -0.15$					
8 g/l						
Pop	0.244	0.446	0.292	0.018	<0.001	0.010
Fam (Pop)	0.061	0.032	0.039	0.057	0.057	0.037
Q_{ST}	0.667	0.875	0.790	0.138	<0.001	0.115
2 SD CI	0.521 – 0.813	0.578 – 1.172	0.659 – 0.921	0.114 – 0.162		0.109 – 0.121
Mantel Q_{ST} vs. F_{ST}	$Z = 0.531, P = 0.252, r = -0.35$					
B						
Saline 2 vs. Fresh 2	ci_{Pop}^2	$ci_{Sire(Pop)}^2$	Q_{ST}	2 SD CI		
Fresh	<0.001	0.028	0.014	0.007 – 0.021		
6 g/L	0.014	0.005	0.211	0.188 – 0.233		
8 g/L	0.071	0.020	0.497	0.483 – 0.510		

CA) standard. The samples were then vortexed, heated at 90°C for 5 min, and then analyzed in a fluorescence-based automatic fragment analyzer (ABI-PRISM 310 Genetic Analyzer, Applied Biosystems). Allele sizes for each locus were resolved by comparison of the peaks obtained to those yielded

by the Tamra 500 standard using the GeneScan 3.1.2 version (Applied Biosystems). Allele frequencies, mean number of alleles per locus, and observed and expected heterozygosities were analyzed using Microsatellite Analyzer (MSA; Dieringer and Schlotterer 2003) and Arlequin (Schneider et al.

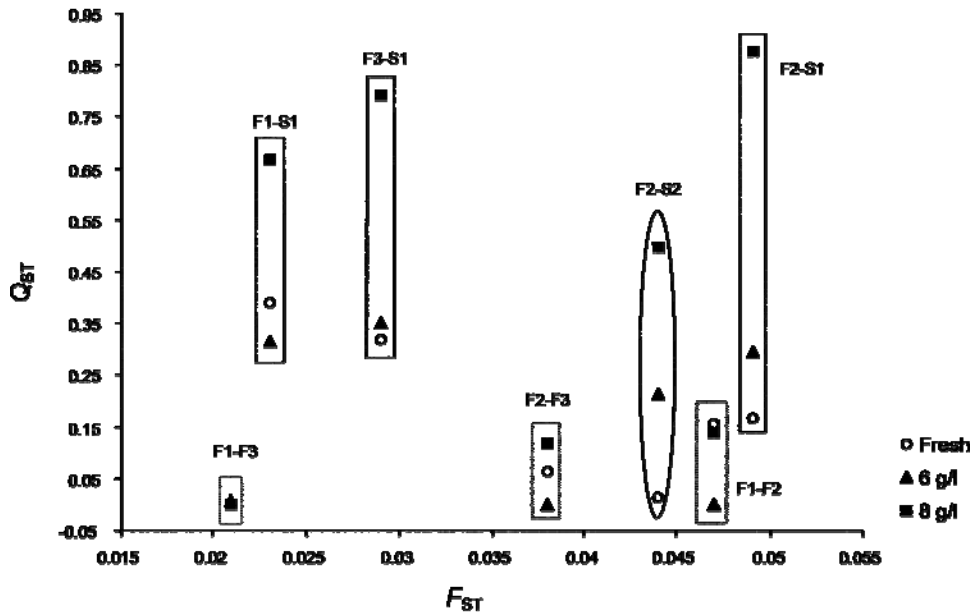


FIG. 1. Q_{ST} versus F_{ST} scatterplot. Each pairwise population comparison is associated with one F_{ST} -value and three Q_{ST} -values, one per experimental salinity level applied to the embryos. Ranges of Q_{ST} -values between any two freshwater populations ($F_x - F_y$) are framed with dashed rectangles, while solid line rectangles frame fresh-brackish population pairs ($F_x - S_y$). The oval encircles the distances estimated from the half-sib experiment.

TABLE 3. Number of alleles (NA), expected (H_e), observed (H_o), heterozygosity, and null alleles estimated frequency (Null) Significant deviations from Hardy-Weinberg equilibrium are marked with an asterisk.

Locus	Fresh 1				Fresh 2				Fresh 3				Saline 1				Saline 2				Total							
	NA	H_e	H_o	Null	NA	H_e	H_o	Null	NA	H_e	H_o	Null	NA	H_e	H_o	Null	NA	H_e	H_o	Null	NA	H_e	H_o	Null	NA	H_e	H_o	Null
<i>Bcal 1</i>	13	0.92	0.77	n.s.	13	0.91	0.65*	0.13	15	0.93	0.77*	0.09	13	0.91	0.74*	0.09	10	0.88	0.76	n.s.	20	0.91	0.74	n.s.	20	0.91	0.74	n.s.
<i>Bcal 2</i>	7	0.61	0.37*	0.15	3	0.41	0.07*	0.24	8	0.81	0.50*	0.17	5	0.64	0.33*	0.19	7	0.59	0.40*	0.12	11	0.64	0.46	0.12	11	0.64	0.46	0.12
<i>Bcal 3</i>	16	0.92	0.76*	0.08	9	0.86	0.44*	0.23	15	0.87	0.79	n.s.	11	0.88	0.88	n.s.	15	0.93	0.82	n.s.	21	0.91	0.74	n.s.	21	0.91	0.74	n.s.
<i>Bcal 4</i>	11	0.82	0.32*	0.27	8	0.79	0.40	n.s.	13	0.90	0.68	n.s.	13	0.88	0.52*	0.19	14	0.90	0.59*	0.16	23	0.88	0.51	0.16	23	0.88	0.51	0.16
<i>Bcal 5</i>	10	0.83	0.91	n.s.	6	0.81	0.79	n.s.	7	0.82	0.71	n.s.	7	0.82	0.72	n.s.	12	0.88	0.76	n.s.	15	0.88	0.78	n.s.	15	0.88	0.78	n.s.
<i>Bcal 7</i>	7	0.56	0.35*	0.13	8	0.81	0.37*	0.24	8	0.73	0.59	n.s.	7	0.80	0.72	n.s.	13	0.79	0.76	n.s.	16	0.76	0.56	n.s.	16	0.76	0.56	n.s.
<i>Bcal 10</i>	6	0.82	0.64	n.s.	5	0.67	0.90	n.s.	8	0.85	0.91	n.s.	8	0.82	0.70	n.s.	11	0.83	0.61*	0.12	13	0.83	0.75	0.12	13	0.83	0.75	0.12
<i>Bcal 11</i>	14	0.91	0.55*	0.19	9	0.86	0.67	n.s.	16	0.78	0.46*	0.18	11	0.84	0.39*	0.25	14	0.92	0.78*	0.07	23	0.93	0.57	0.07	23	0.93	0.57	0.07

2000) software packages. Tests for linkage disequilibrium and departures from Hardy-Weinberg expectations were performed using the Markov chain method, with 10,000 permutations. Population differentiation was analyzed computing F_{ST} estimators (Weir and Cockerham 1984) and their confidence intervals through 10,000 parametric bootstrapping with both MSA and Arlequin software packages. When multiple comparisons were made, a sequential Bonferroni correction of the significance level was applied using a global significance level of 0.05 (Rice 1989). A nested molecular analysis of variance (nested AMOVA) was carried out using Arlequin to compare genetic diversity between and within populations, as well as between ecological environments (fresh and brackish), grouping populations in either freshwater or brackish water populations. The existence of a geographic pattern of molecular genetic variation was analyzed through Mantel tests carried out between matrices of log-transformed geographic distances and odds-transformed genetic distances ($F_{ST}/[1 - F_{ST}]$; Rousset 1997) using isolation-by-distance (IBD) software (Bohonak 2002). The same software and statistical technique were also used to test for a geographic pattern of quantitative variation and correlation between molecular and quantitative distances. Odds from Q_{ST} estimates were used instead of the original values for these analyses.

RESULTS

Quantitative Genetic Variation

As reported elsewhere (Gomez-Mestre and Tejedo 2003; M. Tejedo, I. Gomez-Mestre, and R. Reques, unpubl. data), survival differed significantly across salinity levels in both experiments. There were significant differences in mean population survival across salinity levels: those populations native to brackish ponds experienced higher survival under brackish water conditions, resulting in significant population X salinity interactions (Table 1). In the half-sib experiment (1998), Saline 2 seemed to have a larger additive genetic component than Fresh 2, although this may just reflect differences in our statistical power between populations (twice as many siblings analyzed in Saline 2). Although often not significantly different from zero, additive genetic variances seemed to increase with increasing osmotic stress (Table 1), while narrow-sense heritability decreased or remained zero. The full-sib experiment (2000), in which additive and non-additive effects were undistinguishable, showed increased broad-sense heritability with increased salinity. In this case the statistical power was not greater for the brackish water population than for the freshwater ones, but differences among siblings were more pronounced in Saline 1 (Table 1).

Most estimates of quantitative genetic distances between population pairs (Q_{ST}) increased as salinity increased (see Table 2), showing that differences in salinity tolerance among populations broadened with increased osmotic stress. Furthermore, Q_{ST} estimates measured between any two freshwater populations were consistently smaller than freshwater versus brackish water population pairs, suggesting local adaptation of both Saline 1 and Saline 2 (Fig. 1). There is a significant positive correlation between Q_{ST} and heritability (including narrow- and broad-sense) estimates for the brack-

TABLE 4. Nested molecular analysis of variance, where df stands for degrees of freedom, SS for sum of squares, Varcomp for variance components, and %Var for proportion of total variance accounted for by each source.

Source	df	SS	Varcomp	% Var
Fresh vs. brackish	1	7.78	0.018	0.64
Among populations within pond type	3	17.40	0.070	2.45
Within populations	215	591.31	2.750	96.91
Total	219	616.48	2.838	100

ish-fresh pairwise population comparisons ($r_s = 0.599$, $P = 0.039$, $N = 12$), whereas the correlation for the pairwise fresh-fresh population comparisons was nonsignificant ($r_s = -0.383$, $P = 0.308$, $N = 9$). Tests for correlation between matrices of quantitative and neutral genetic distances were performed at each salinity level for the full-sib experiment using Mantel tests, and were all found to be nonsignificant (see Table 2A).

Molecular Variation and Population Differentiation

Pooled gene diversity was high, ranging between 0.64 and 0.93 among populations (Table 3). All loci were polymorphic, and the mean number of alleles per locus ranged between 11 and 23. Significant departures from Hardy-Weinberg expectations (considering heterozygote deficit the alternative hypothesis) were detected in different loci across all populations. Because allelic diversity was considerable and the primers used to amplify the microsatellite loci were originally designed for populations much less diverse (Beebe and Rowe 2000), nonamplifying alleles were assumed to be the cause of the heterozygote deficiencies observed. Estimated null allele frequency for each locus and population was calculated following Brookfield (1996) and is shown in Table 3. Analyses ran excluding the two loci with higher presumed incidence of null alleles (loci *Bcal 2* and *Bcal 11*) were not qualitatively different from those ran on the entire dataset, and therefore all loci were included in the final analyses.

Results from the nested AMOVA indicated a lack of population substructuring such that 96.9% of overall variation was held within populations (Table 4). The ecological distinction between brackish and freshwater populations was not supported by the analysis of microsatellite markers, since only 0.6% of the total variance could be attributed to differences between these two groups.

Fixation indices (F_{ST}) were generally low, ranging from 0.0109 to 0.0486, and four out of 10 pairwise comparisons gave fixation indices not significantly different from zero. Fresh 1 was undifferentiated from Fresh 3 and from the two brackish populations, which in turn did not significantly dif-

fer from one another (Table 5). The fixation index over all loci and all populations was low ($F_{ST} = 0.029$) but significantly different from zero. The pattern of neutral genetic distances among populations showed no correlation with that of geographical distances (Mantel test, $F_{ST}/[1 - F_{ST}]$ vs. $\log[\text{geographic distance}]$; $Z = 0.624$, $P = 0.29$), rejecting the hypothesis of isolation by distance. The average level of gene flow estimated in the system was $Nm = 4.3$.

DISCUSSION

In agreement with previous studies (Gomez-Mestre and Tejedo 2003; M. Tejedo, I. Gomez-Mestre, and R. Reques, unpubl. data) salinity tolerance in *B. calamita* seemed to have a genetic basis, and significant variation within populations was found with both the full-sib and the half-sib approach. However, the analysis of variation among full-sibs confounds additive, dominance, and common environment effects (such as maternal effects) into the term ‘‘genetic variance’’ (Falconer and MacKay 1996). Results of this study indicated that heritability of the trait increased as the osmotic stress increased, as differences among full-sibs became accentuated (Table 1). However, the more informative analysis of half-sibs revealed that narrow-sense heritability may actually be decreasing as salinity increases. This apparent contradiction is most likely caused by the presence of nonadditive components (dominance and epistasis) and maternal effects, that may be sensitive to the level of stress (M. Tejedo, I. Gomez-Mestre, and R. Reques, unpubl. data).

The *B. calamita* populations studied vary in their degree of salinity tolerance (Gomez-Mestre and Tejedo 2003). These differences are reflected in different population pairs showing very different ranges of Q_{ST} -values under the different salinity conditions. However, the emergent pattern was not random, as higher Q_{ST} -values were found for brackish-fresh population pairs than for fresh-fresh population pairs (Figs. 1, 2) indicating stronger quantitative differentiation between brackish and freshwater populations than among freshwater populations. Nevertheless, as indicated above for the case of within population variation, Q_{ST} -values computed from full-sibs designs are likely to be biasing the estimate of population

TABLE 5. Pairwise fixation indices (F_{ST} , lower diagonal) and geographic distances (km, upper diagonal). F_{ST} estimates marked with an asterisk were not significantly different from zero.

	Fresh 1	Fresh 2	Fresh 3	Saline 1	Saline 2
Fresh 1	—	190	114	195	200
Fresh 2	0.047 (0.019–0.070)	—	85	80	85
Fresh 3	0.021*(–0.003–0.055)	0.038 (0.016–0.055)	—	130	135
Saline 1	0.023* (0.005–0.041)	0.049 (0.019–0.078)	0.029 (0.001–0.072)	—	3
Saline 2	0.012* (0.002–0.020)	0.044 (0.19–0.070)	0.024 (0.001–0.056)	0.011* (0.000–0.023)	—

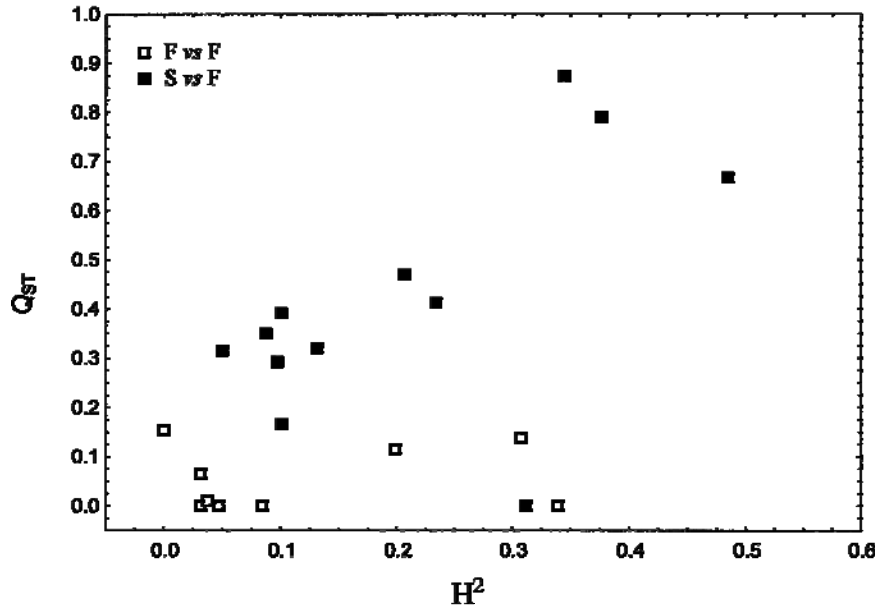


FIG. 2. Relationships between estimates of pairwise populational subdivision (Q_{ST}) and the average heritability within populations for embryo survival at the different treatments (see Table 1). Filled squares are pairwise comparisons between brackish and freshwater populations ($N = 12$, including 1998, half-sibs experiment, $r_s = 0.599$, $P = 0.039$), whereas empty squares are between freshwater populations ($r_s = -0.383$, $P = 0.308$, $N = 9$).

divergence, since Cl_{GW}^2 then includes portions of nonadditive variance (dominance plus epistasis) and variance due to maternal effects in addition to purely additive genetic variance. The effect of dominance variance on Q_{ST} estimates is unclear and could cause both an increase or decrease with neutral differentiation (Whitlock 1999). In our system, Q_{ST} increased with salinity even when only additive genetic variance was considered (half-sib analysis, oval in Fig. 1). The range of Q_{ST} values between Saline 2 and Fresh 2 (half-sib) was shorter than the ranges shown by brackish-fresh population pairs in the full-sib design. This result is not expected considering that only additive variance was considered in the half-sib experiment. However, the within-population component for the highest salinity treatment in the half-sib experiment was similar to that observed for the full-sib one, whereas the between-population component was proportionally lower for the half-sib, thus resulting in a lower Q_{ST} (Table 2).

It is worth noting that much of the variation in Q_{ST} was associated with variation in combined heritability (Fig. 2). This relationship was more pronounced for the brackish-fresh population comparisons than for the comparisons between fresh populations. This similar association has been found in other organism (Lynch et al. 1999; but see Koskinen et al. 2002). Under the same population structure, the degree of genetic differentiation between populations for a trait under selection is a function of the magnitude of additive genetic variance (Falconer and Mackay 1996), but the degree of differentiation for a neutral trait is independent of its heritability (Lande 1992; Lynch 1994; Lynch et al. 1999). In our system, the lack of association between Q_{ST} and heritability in freshwater population pairs suggested that embryonic survival is driven by neutral forces in freshwater populations, whereas selection may be responsible for the brackish-freshwater divergence.

One potential source of uncertainty in our analysis concerns the uncontrolled nonadditive or maternal effects that may affect both within- and between-population components of variance. Results from the half-sib experiment revealed that this component is smaller in magnitude and probably does not bias our within-population estimates. However, between-population estimates of embryo survival were clearly affected by maternal effects as revealed by comparing reciprocal hybrids between Saline 2 and Fresh 2 populations (M. Tejedo, I. Gomez-Mestre, and R. Reques, unpubl. data). Consequently, it seems likely that the very high Q_{ST} estimates may be partly attributable to environmental variation.

The concurrent analysis of genetic variation at eight microsatellite loci in these populations of *B. calamita* provided a null hypothesis of neutral divergence to test against that of local adaptation (Lynch 1990; Schluter 2000). Q_{ST} -values were not markedly different from F_{ST} -values when comparing freshwater populations with each other, and, even at the highest salinity, Q_{ST} was only two-fold higher than F_{ST} . However, Q_{ST} was clearly higher (over one order of magnitude) than F_{ST} between brackish and freshwater populations. These results support the hypothesis of population differentiation under selection (local adaptation) over the null hypothesis of neutral divergence. In some cases quantitative and neutral molecular distances have been reported to show a significant positive correlation (Merilä 1997; Merilä and Crnokrak 2001; Steinger et al. 2002), in which case variation in molecular markers can be used as a predictor of the degree of quantitative differentiation. Some other systems, including the one described in this paper (Mantel tests, Fig. 1), do not show such correlation, and hence molecular markers become inaccurate predictors of variation in quantitative traits (Reed and Frankham 2001).

Genetic diversity at the eight microsatellite loci scored,

measured as either number of polymorphic loci, mean number of alleles per locus, or allelic richness (H_e), was higher for the populations studied here than that reported for other populations of *B. calamita* throughout its distribution (Rowe et al. 1998; Beebee and Rowe 2000). No evidence for an isolation-by-distance pattern of population differentiation was found, and F_{ST} estimates were lower than those among U.K. populations, even if some differed significantly from zero. This finding supports Beebee and Rowe's (2000) assertion that Iberia had been a Pleistocene refugium for *B. calamita*, from where it may have expanded northward in repeated events of recolonization. However, the small number of populations analyzed does not allow us to assume a lack of geographic substructure of neutral genetic variation in Iberian populations. The overall estimated level of differentiation among the five populations considered was relatively low ($F_{ST} = 0.029$), indicating that the populations have not been isolated for long periods of time. Specifically, estimated F_{ST} values between Fresh 1 and either Saline 1 or Saline 2 were not significantly different from zero, suggesting that either they currently exchange some gene flow or they have stopped doing so in the recent past.

The pond basins of both Saline 1 and Saline 2 were formed 5000 years ago on Quaternary saline sediments (Martín-Serrano García 1986). Assuming that these ponds were colonized by *B. calamita* when newly formed, that they have been successfully breeding in them ever since (they have not; M. Tejedo and I. Gomez-Mestre, pers. obs.), and a generation time of two years (Tejedo et al. 1997), 2000 is a rough estimate of the maximum number of generations that salinity tolerance has required to evolve in these populations. In principle, gene flow can prevent the differentiation of populations under different selection pressures (Hanks and Denno 1994; Storfer and Sih 1998; Lenormand 2002), but strong selection can counterbalance gene flow so that the population(s) under selection may remain genetically distinct in the face of migration (Mopper et al. 1995; Mopper 1996; Nagy and Rice 1997). Gene flow might have precluded strong differentiation at neutral loci of the *B. calamita* populations studied, while intense selection maintained differences in salinity tolerance, as found for other organisms and traits (Rice and Hostert 1993; Merilä 1997; Bensch et al. 1999; Schneider et al. 1999; Luttikhuisen et al. 2003). Alternatively, if populations have become isolated only in the recent past, selection may have acted on genetic variation underlying salinity tolerance more rapidly than drift could act on neutral genetic variation.

In summary, salinity tolerance in the brackish water populations studied seems to have been under strong selection, causing their quantitative genetic differentiation from freshwater populations, despite lack of geographic isolation.

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