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POPULATION AND SUBSPECIFIC GENETIC DIFFERENTIATION IN THE FOXTAIL PINE (*PINUS BALFOURIANA*)

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Abstract.—We performed an allozyme survey of genetic differentiation in *Pinus balfouriana,* a subalpine conifer endemic to California that is comprised of two allopatric subspecies, one in the Klamath Mountains and the other in the southern Sierra Nevada. Although the two subspecies are morphologically distinct and gene flow between them is virtually nonexistent, we observed much higher levels of differentiation among populations within a subspecies than between the two subspecies. Differentiation is particularly strong in the Klamath populations (multilocus F_{ST} = 0.242), which are small, isolated, and ecologically marginal. We attribute this strong differentiation to the mountain island effect, in which populations restricted to high elevations become isolated from each other on different mountains separated by unsuitable intervening habitat, with consequent reduced gene flow allowing populations to evolve independently. Populations of *P. balfouriana* in the Klamath region only exist scattered on the few highest ridges and peaks that rise above 2000 m, which defines the lower limit of the species elevational distribution. This pattern of distribution has allowed genetic drift and allelic sorting through historical events to produce strong population-level differentiation, which was likely in place before the two subspecies were geographically separated. Because *P. balfouriana* occurs on both serpentine soils and nonserpentine soils in the Klamath Mountains, we tested for genetic differentiation between populations growing on serpentine versus nonserpentine soils and our results were equivocal. Our data, combined with several other studies of conifers, show that the mountain island effect can produce significant genetic differentiation in conifers whose life-history traits of widely dispersed pollen, long generation times, and high outcrossing rates would lead us to predict a more homogenous population genetic structure.

Key words.—Genetic drift, mountain islands, *Pinus balfouriana,* population structure, serpentine soil.

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The foxtail pine (*Pinus balfouriana* Grev. and Balf.), which is endemic to California, is a subalpine conifer that exists in two disjunct areas, one in the Klamath and Scott Mountains of northwestern California and the other in the southern Sierra Nevada (Fig. 1). *Pinus balfouriana,* the Great Basin bristlecone pine (*P. longaeva*), and the Rocky Mountain bristlecone pine (*P. aristata*) comprise the subsection Balfourianae, whose geographic range is restricted to western North America. Consistent differences for cone, needle, and bark characters between Klamath and Sierran *P. balfouriana* have led to their description as distinct allopatric subspecies: *P. b. balfouriana* in the north and *P. b. austrina* in the south (Mastrogiuseppe and Mastrogiuseppe 1980). In addition, Snajberk et al. (1979) found significant differences in terpenoid chemistry between the two subspecies. Each of the subspecies of *P. balfouriana* has a relatively restricted range compared to the large distance between them (~500 km minimum), precluding the possibility of significant gene flow. However, Critchfield (1977) measured an 84% success rate in crossing experiments between the northern and southern *P. balfouriana* relative to the within-subspecies crossing success. The separation between the two populations probably dates from the mid-Pleistocene (Bailey 1970).

Pinus balfouriana grows primarily on scattered peaks and ridges between 2000 m and 3000 m in the north and 2200 m to almost 4000 m in the south, where it frequently defines timberline. Both subspecies are more abundant on xeric south-facing slopes with well-drained soil, where the individual trees are widely spaced in open groves. However, local population characteristics differ greatly between the two subspecies. In the south, *P. balfouriana* grows most often in pure stands, with its only important associate being lodgepole pine (*P. contorta*), whereas, in the north, it frequently grows in mixed coniferous forests associated with shasta red fir (*Abies magnifica*), Jeffrey pine (*P. jeffreyi*), and lodgepole pine. The Klamath region is geologically complex, and the northern subspecies grows on soils derived from a variety of different parent rocks, including serpentine (ultramafic) soils. The southern subspecies grows almost exclusively on high-elevation, boulder-strewn, and poorly developed granitic soils derived from the Sierra Nevada batholith.

Marked differences in climatic regime also exist between the two subspecies, as previously described by Mastrogiuseppe and Mastrogiuseppe (1980). The northern population exists within the path of winter storms striking the Pacific Northwest and receives typical annual precipitation totals from 125 cm to 175 cm. The southern population is shielded from Pacific moisture by the rain shadow of the coastal ranges; typical annual precipitation in the region ranges from 50 cm to 75 cm. Temperature extremes in the north are moderated by the maritime influence from the Pacific Ocean, whereas a much wider range of temperature extremes is typical of the more continental climate in the southern Sierra.

Pinus balfouriana appears to be a textbook case of allopatric speciation—two geographically isolated populations with no gene flow between them, diverging independently while adapting to contrasting environments. The goal of this study was to use allozymes to investigate the effects of this isolation by determining the level of genetic differentiation between and within the two subspecies in the context of their

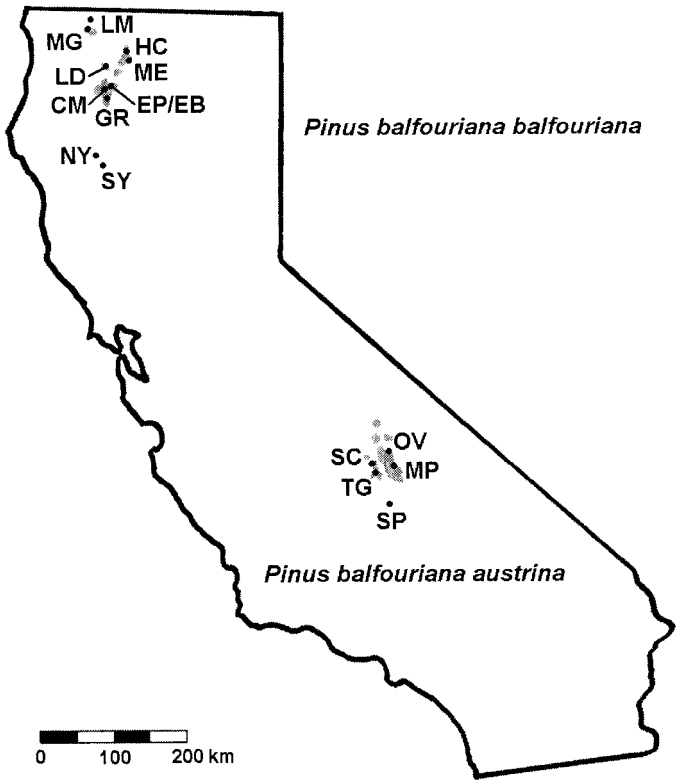

FIG. 1. Map of Calfornia showing the distribution of *Pinus balfouriana,* (from Little 1971) and the locations of sampled populations for this study. The abbreviations for populations are as in Table 1. Within the shaded areas, *P. balfouriana* grows primarily in scattered populations on high elevation peaks and ridges; in the Sierra Nevada it does occasionally form extensive monotypic subalpine forests. North and south Yolla Bolly (NY and SY) in the north and Sirretta Peak (SP) in the south are significant outliers from the core areas of their respective supspecies distribution.

degree of isolation and contrasting environments. Some of the differences between the northern and southern subspecies could be expected to have an effect on population structure through the mechanisms of genetic drift, competition, and selection. The greater diversity of competing coniferous species, greater diversity of geological substrates, and smaller population sizes in the north all predict a greater degree of population differentiation through these mechanisms in the north relative to the south.

Our initial data suggested that genetic differentiation exists between populations growing on serpentine and nonserpentine soils in the northern subspecies. Serpentine soils have a distinct chemistry consisting of high concentrations of heavy metals and low concentrations of plant-available nutrients. This creates challenging conditions for most plant species, and serpentine soils worldwide are commonly associated with high levels of endemism (Kruckeberg 1984; International Conference on Serpentine Ecology 1992). We tested our hypothesis of differentiation by soil type by making an additional set of collections on a wider variety of geological substrates and increasing our representation of nonserpentine populations in the northern subspecies.

MATERIALS AND METHODS

We initially selected 10 sampling sites (five in the north and five in the south) based on published data (Mastrogiuseppe and Mastrogiuseppe 1980) and locality data from herbarium specimens at the University of Colorado Herbarium; we made our initial collections during July 1996. The choice of collection sites was designed to cover the full range of the species, including the core areas of *P. balfourianas* range in addition to several outlying populations. We made a second set of collections from six additional northern populations during August 1998 to expand our samples of trees growing in nonserpentine soils. We used additional locality data from the University of California and Jepson Herbaria (Berkeley, CA) and the Vascular Plant Herbarium at Humboldt State University (Arcata, CA). In an attempt to detect possible microgeographic variation based on soil type, we sampled from two populations, Eagle Peak and East Boulder Lake, which are immediately adjacent to each other on the same ridge ($<$1 km apart), but grow on soils derived from quartz diorite and serpentinized dunite, respectively. Details of the collection sites and sample sizes are in Table 1.

Previous studies have shown that conifers grow in spatially clustered family groups (Linhart et al. 1981; Latta et al. 1998); to reduce the probability of sampling closely related individuals, we maintained a distance of approximately 20 m between sampled trees. Because some of our sampled populations are very small, particularly those at Silliman Crest and Eagle Peak, we sampled a smaller number of trees to maintain the minimum distance between trees. From each tree sampled, we collected a small twig (roughly 10 cm length) with mature needle tissue; we placed the samples on ice until we returned them to the laboratory for electrophoresis. Because we sampled individual mature trees and not progeny arrays, we are unable to assess the mating system with the data from this study.

We prepared each sample by grinding 20–30 individual needles in liquid nitrogen using the buffer of Mitton et al. (1979). We stored the prepared samples at -80°C until they were thawed and absorbed onto filter paper wicks immediately prior to starch gel electrophoresis. The notation, buffer systems, and enzyme stains followed those of Murphy et al. (1996). We resolved fluorescent esterase (*Fest,* EC 3.1.1) and glutamate dehydrogenase (*Gtdh,* EC 1.4.1.2) on the lithium borate/tris-citrate system; malate dehydrogenase (*Mdh,* EC 1.1.1.37), glucose-6-phosphate isomerase (*Gpi,* EC 5.3.1.9), phosphogluconate dehydrogenase (*Pgdh,* EC 1.1.1.44), and shikimate dehydrogenase (*Skdh,* EC 1.1.1.25) on the histidine-citrate system; and glycerate dehydrogenase (*Glydh,* EC 1.1.1.29) and peptidase (*Pep,* EC 3.4.-.-) on the tris-citrate/borate system with pH 7.6. For multiple enzyme loci, we denoted the electrophoretically faster locus with a ''1'' and the slower locus with a ''2''; faster alleles at a locus were denoted by an ''A'' and slower alleles by a ''B.''

We calculated Wrights *F*-statistics from genotypic frequency data according to the method of Weir and Cockerham (1984). We performed 10,000 bootstrap replicates using the bias correction described by Dixon (1993) to determine confidence intervals for the F_{IS}-values and used the analysis of variance (ANOVA) procedure described by Weir (1996) to

TABLE 1. Collecting sites of *Pinus balfouriana* subspecies, with location, average elevation, geologic substrate, and sample size. All of the serpentinized rocks are classified as ultramafic.

Site	Location	Elevation (m)	Substrate	*n*
P. b. austrina				
Onion Valley (OV)	36°46.2′N 118°2′W	2900	granite	30
Mulkey Pass (MP)	36°26.0′N 118°1′W	3150	granite	25
Sirretta Peak (SP)	35°55.4′N 118°2′W	3000	granodiorite	20
Timber Gap (TG)	36°28.1′N 118°4′W	3100	granite	20
Silliman Crest (SC)	36°39.2′N 118°4′W	3150	granite	15
P. b. balfouriana				
Mount Eddy (ME)	41°19.1′N 122°3′W	2450	serpentinized peridotite	25
Grouse Ridge (GR)	41°3.1′N 122°6′W	2200	serpentinized peridotite	25
Lake Mountain (LM)	41°41.9′N 123°8′W	2050	serpentinized peridotite	20
High Camp Pass (HC)	41°21.2′N 122°4′W	2200	serpentinized peridotite	20
North Yolla Bolly Mtn. (NY)	40°11.6′N 122°6′W	2300	mica schist	26
Mount Linn (SY)	40°2.1′N 122°51′W	2400	granitic derived graywacke	23
Carter Meadows Summit (CM)	41°14.8′N 122°57′W	2250	micaceous phyllite	27
Little Duck Lake (LD)	41°18.3′N 122°57′W	2200	granodiorite	30
Marble Gap (MG)	41°34.4′N 123°13′W	2150	marble	30
East Boulder Lake (EB)	41°13.2′N 122°47′W	2250	serpentinized dunite	20
Eagle Peak (EP)	41°12.2′N 122°48′W	2350	quartz diorite	16

determine the significance levels of the F_{ST}-values. We analyzed all bootstrap distributions for normality and disregarded those cases in which low levels of diversity caused a failure of the bootstrap, as described by Van Dongen and Backeljau (1997). In the analysis of variance formulation of Weir and Cockerham (1984) for calculating *F*-statistics, the multilocus values are weighted by the levels of diversity at each individual locus; the multilocus values and confidence intervals thus provide a robust estimate of F_{IS} in cases where meaningful confidence intervals cannot be calculated for some of the individual loci due to their low diversity. We calculated G_{ST}-values according to Nei (1973) and similarly determined confidence intervals with 10,000 bootstrap replicates.

RESULTS

We resolved 11 loci from eight enzyme systems, three of which were polymorphic (*Fest*1, *Mdh*1, and *Gpi*) for a percent polymorphic loci of 27.3%, although not all populations were polymorphic for all three loci. Each of the polymorphic loci segregated only two alleles, for a mean number of alleles per locus of 1.27 and two alleles per polymorphic locus. We computed both H_S (genetic diversity, $H_S = 1 - \Sigma\ p_i^2$) and A_S (effective number of alleles, $A_S = 1/[1 - H_S]$) values as in Hamrick and Godt (1990); at the species level (using pooled data from both subspecies), H_S was 0.075 and A_S was 1.082. We also calculated H_S- and A_S-values at the subspecies level (northern and southern subspecies calculated separately) and at the population level within each subspecies; the results were similar (data not shown).

The genetic diversity statistics we report are all noticeably lower than those for other species of conifers (Hamrick et al. 1979) and pines in particular (Ledig 1998). The only other available allozyme data for *P. balfouriana* is that of Hiebert and Hamrick (unpubl. data cited in Hamrick et al. 1981), who report a P_S-value of 0.576, an A_S-value of 1.61, and an H_S-value of 0.208, all of which are notably higher than the values we report here ($P_S = 0.273$, $A_S = 1.273$, $H_S = 0.075$). Although Hiebert and Hamrick report data for more loci than we do (23 vs. 11), our sampling has been geographically more extensive (their four populations rangewide vs. 16 populations rangewide). An additional methodological difference that could explain the differences in these results is that Hiebert and Hamrick used seedlings grown from seeds returned from the field, whereas we used needles from mature adult trees and saplings.

In the south, two populations were fixed for *Gpi* and two different populations were fixed for *Fest*1; in the north, seven populations were fixed for *Fest*1. Each subspecies had a single, rare (not polymorphic at the 95% level), private allele at a different locus; however, we observed no fixed differences between subspecies. Chi-square tests of allelic and genotypic frequencies of the pooled northern ($n = 256$) versus pooled southern ($n = 106$) populations (data not shown) showed significant differentiation ($P < 0.001$) at two of the three loci (*Gpi* and *Fest*1).

Tests of Hardy-Weinberg equilibrium (data not shown) between the pooled southern and pooled northern populations demonstrated a strong Wahlund effect due to population-level differentiation within each subspecies. Hardy-Weinberg tests for individual populations in the south produced significant results in four of 11 tests (significantly more than expected by chance, $P < 0.01$), whereas in the north only two of 26 tests were significant ($P > 0.05$). In all cases, significant deviations from Hardy-Weinberg expectations were deficiencies of heterozygotes. In the south, the multilocus F_{IS}-value is 0.443 compared with 0.203 in the north (Table 2); the bootstrap distributions have nonoverlapping 95% confidence intervals and so are significantly different at the $P < 0.05$ level (south: 0.443, 95% confidence interval = 0.296–0.583; north: 0.203, 95% confidence interval = 0.124–0.281). These results suggest the possibility of higher levels of inbreeding within the southern subspecies than the northern subspecies.

Although there is significant genetic differentiation be-

TABLE 2. *F*-statistics for the southern populations, northern populations, northern and southern subspecies, and northern serpentine populations only. We used the variance component formulation of Weir and Cockerham (1984) to calculate F_{IS} and F_{ST}. We tested the significance of F_{IS} with 10,000 bootstrap replicates using the bias correction as described by Dixon (1993) and the significance of F_{ST} with the ANOVA procedure described by Weir (1996).

	Southern populations		Northern populations	
Locus	F_{IS}	F_{ST}	F_{IS}	F_{ST}
Gpi	[0.656][1]	0.040	0.010	0.117****
*Mdh*1	0.536**	0.072	0.309****	0.311****
*Fest*1	[−0.036][1]	0.113**	0.242*	0.271****
Multilocus	0.443**	0.075	0.203****	0.242****

	North vs. south		Northern serpentine populations only	
Locus	F_{IS}	F_{ST}	F_{IS}	F_{ST}
Gpi	0.129**	0.066**	0.079	0.151***
*Mdh*1	0.372****	0.033*	0.394****	0.256****
*Fest*1	0.134*	−0.004[2]	0.225	0.255****
Multilocus	0.267****	0.038*	0.249***	0.221****

* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$.

[1] Brackets indicate values for which we are unable to provide bootstrap confidence intervals due to insufficient genotypic diversity among the compared populations, resulting in the failure of the bootstrap as described by Van Dongen and Backeljau (1997).

[2] In the formulation of Weir and Cockerham (1984), calculated F_{ST}-values are estimates, not parameters. Thus, small negative values may be calculated when F_{ST} is close to zero.

tween the two subspecies based on the three polymorphic loci, the level of differentiation is as high or higher among the populations of each subspecies, particularly in the north. χ^2 tests comparing the 11 northern populations showed significant differences ($P < 0.0001$) in both allelic and genotypic frequencies at all three polymorphic loci (data not shown). Using our multilocus F_{ST}-value in the north versus south comparison ($F_{ST} = 0.038$, Table 2), we can estimate the number of migrants per generation between the subspecies by $Nm = [(1/F_{ST}) - 1]/4 = 6.3$. Given the current distribution pattern of *P. balfouriana* (Fig. 1), however, we believe that gene flow between the subspecies is much less likely than this *Nm*-value would suggest, and that the value of 6.3 is representative of a relatively recent vicariant separation between the two subspecies.

A comparison of the multilocus F_{ST}-values of the among southern populations ($F_{ST} = 0.075$, 95% confidence interval = 0.031–0.195) and among northern populations ($F_{ST} = 0.242$, 95% confidence interval = 0.208–0.308) shows that interpopulation differentiation is significantly higher ($P < 0.05$) in the north. To confirm that our results for the northern populations were not biased by a larger sample size (11 populations vs. five populations in the south), we created a resampling procedure that randomly selected five of the 11 populations in the north and calculated F_{ST}; the average F_{ST} for five resampled populations was 0.239 (not significantly different from 0.242). We calculated multilocus G_{ST}-values to enable a comparison with data from Ledig's (1998) review of genetic diversity within *Pinus*: $G_{ST} = 0.078$ among southern populations and $G_{ST} = 0.227$ among northern populations. Whereas the southern value (0.078) is well within the typical range for *Pinus,* the northern value (0.227) is atypically high. (We tested for a sample size effect as we did for the F_{ST}-values above: the average G_{ST}-value for five randomly chosen northern populations was not significantly different at 0.182). Clearly, there are or have been forces acting to differentiate populations in the north that are not operating to the same degree in the south, and given the low differentiation between the subspecies as well as the 84% crossing rate between the subspecies found by Critchfield (1977), these forces are likely to have operated to produce this differentiation prior to the geographic separation between the two subspecies.

DISCUSSION

What could explain these strong differences in population structure and genetic processes? In the southern Sierra Nevada, there are large contiguous areas of potential *P. balfouriana* habitat between elevations of 2700 m and 3400 m, and west of the Sierra crest extensive subalpine forests exist that are monotypic stands of *P. balfouriana.* In the north, the mountain ranges are not as high or as contiguous—there are only occasional isolated ridges or peaks that rise above 2000 m to provide potential *P. balfouriana* habitat, and the populations sizes on these ridges and peaks are small. In terms of elevation, all of the northern populations are at the extreme edge of the species ecological limits. The evidence points to a strong mountain island effect, in which populations confined to high-elevation zones on separate ridges and peaks are effectively isolated by reduced levels of gene flow across intervening unsuitable habitat, thus allowing these ecologically marginal populations to evolve largely independently by the actions of drift and selection. In the closely related *P. longaeva,* a comparison of ''island'' populations on isolated ranges in the Great Basin to ''mainland'' populations in the contiguous plateaus of central Utah by Hamrick et al. (1994) showed a markedly higher G_{ST}-value for the island populations (0.169, the highest value they report for any of the nine Great Basin conifers in their study) than for the mainland populations (0.065). Ledig (1998) reports a G_{ST}-value of 0.212 for *P. ayacahuite,* which is similarly distributed in isolated mountain islands in central Mexico. These studies, combined with the data we report here, provide evidence that the mountain island effect is strong enough to produce significant genetic structure among populations in species whose life-history characteristics (which typically include wide dispersal, great longevity, high outcrossing rates, and inbreeding depression) would lead us to predict a much more homogenous genetic structure (Hamrick et al. 1979).

The primary factor driving the mountain island effect in the northern *P. balfouriana* is probably genetic drift. The high dispersal potential of wind-borne pollen as well as the extreme longevity of *P. balfouriana* (up to 1500 years in the north; Mastrogiuseppe and Mastrogiuseppe 1980) would be expected to retard genetic drift; however, the effects of small population sizes and historic bottlenecks have accelerated genetic drift through allelic sorting. Many of the populations of *P. balfouriana* that we sampled consist of 300–600 trees. Although these population sizes are not small when considering genetic drift, it is likely that previous bottlenecks and consequent allelic sorting have occurred during times of

changing climate. Coniferous species have been shown to migrate up and down available elevational gradients as a response to climate change (Wells 1983; Thompson 1988). In the case of the northern *P. balfouriana,* however, many populations currently exist at the very crests of ridges and mountains where they have no migratory path to a higher elevation refugium. Although populations may have survived by migrating to lower elevations during past times of cooler climate, during times of warmer climate than the present, many of these populations may have been greatly reduced and/or become locally extinct by being squeezed off the tops of mountains that are insufficiently high to provide suitable habitat. Genetic drift during times of reduced population size and the founder effect caused by the recolonization of sites where *P. balfouriana* had previously gone extinct locally may have enhanced genetic differentiation between such populations.

Because we made a specific effort to collect from populations on a wider variety of geological substrates in the northern subspecies, our measurements of population differentiation may be biased toward higher values of F_{ST} (and G_{ST}). Because *P. balfouriana* grows on a greater diversity of soil types (as defined by parent rock) in the Klamath region than in the Sierra Nevada (which only has granitic soils), we argue that the comparisons of population differentiation we have made above are valid in the context of a comparison between the two subspecies. But how much of the differentiation is due to soil type in the northern populations and how much is due to other factors such as the mountain island effect? To answer this question, we calculated F_{ST} among the five serpentine populations in the north and compared this value with that for the southern populations (Table 2). By making this comparison, we are controlling explicitly for soil type. The F_{ST}-value among the serpentine populations in the north is 0.221 (95% confidence interval = 0.166–0.316) and is not significantly different than the F_{ST}-value among the southern populations (F_{ST} = 0.075, 95% confidence interval = 0.031–0.195). Because the F_{ST}-value among all of the northern populations (0.242) was significantly different ($P < 0.05$) than the value among the southern populations (0.075), we conclude that some portion of the total population differentiation can be attributed to soil type.

Small populations existing near their ecological limits may also be expected to undergo stronger diversifying selection based on differences in local site characteristics. Our initial data suggested the possibility of genetic differentiation based on soil type (serpentine vs. nonserpentine) in populations of the northern subspecies. To test this hypothesis, we grouped the 11 northern populations into two groups, serpentine or nonserpentine, based on the geology of the parent rock at each site (Table 1). Allelic and genotypic frequencies did not differ significantly at the *Gpi* locus, but both *Mdh*1 and *Fest*1 had significantly different ($P < 0.0001$) allelic and genotypic frequencies (data not shown).

The allelic frequencies at the *Mdh*1 locus for the northern populations were extremely disparate, with frequencies for the electrophoretically fast allele (the one more prevalent on serpentine soils) ranging from 0.196 to 0.975. As a result of the nonnormal distribution of these allelic frequencies, the χ^2 distribution based on a pooled population comparison deviates markedly from the true χ^2 distribution. To provide a more robust evaluation of our hypothesis, we used a bootstrapping procedure in which five of the 11 populations were randomly chosen and assigned to one group, and their pooled allelic frequencies were compared by a χ^2 test to the pooled allelic frequencies of the other six populations. What this procedure allowed us to do is answer the following question: How often does a random grouping of populations produce a greater degree of allelic differentiation than our a priori grouping by soil type? When we performed this test with the *Mdh*1 frequencies, the χ^2-value for a random grouping was less than our observed value for the serpentine versus nonserpentine comparison ($\chi^2 = 35.52$, df = 1, $P = 0.13$) only 86.6% of the time. When we repeated the random-grouping resampling procedure using the *Fest*1 allelic frequencies, the χ^2-values were smaller than our serpentine versus nonserpentine comparison value ($\chi^2 = 33.79$, df = 1, $P = 0.03$) 96.7% of the time. We did not detect any microgeographic differentiation in our comparison between the adjacent East Boulder Lake (serpentine) and Eagle Peak (granite) populations: A χ^2 test of *Mdh*1 allelic frequencies did not produce a significant result (0.563 vs. 0.475, $P = 0.46$), although our sample sizes were small (EB, $n = 20$; EP, $n = 16$). Both of these populations were fixed for the same *Fest*1 allele.

Serpentine-derived soils are known to be a potent evolutionary force. Kruckeberg (1984) identified 152 species and 64 subspecies that are endemic to serpentine in northern California alone, as well as numerous other serpentine ecotypes from more widely distributed species. Similarly high levels of endemism occur in other serpentine belts worldwide (International Conference on Serpentine Ecology 1992). There are clear physiological reasons for this high level of endemism based on the chemistry of serpentine soils, such as high levels of heavy metals, including many that can be phytotoxic, such as cadmium, nickel, and chromium, the low availability of N, K, and P; and the high Mg^{++}/Ca^{++} ratio.

Previous studies of three other species in the genus *Pinus* have shown genetic adaptation to serpentine soils. Jenkinson (1966) reported superior growth of ponderosa pine (*P. ponderosa*) from seeds originating from serpentine soils in northern California. When grown in a common-garden experiment using serpentine soils, the growth advantage of the serpentine soil–derived seeds continued for 20 years, at which point their average tree volume was 50% greater than from those trees originating from granitic soil–derived seeds (Jenkinson, unpubl. data cited in Ledig 1998). Kruckeberg (1967) also reported a growth-rate difference between ecotypes of lodgepole pine (*P. contorta*) native to serpentine soils and to granitic soils in a similar common-garden experiment. Furnier and Adams (1986) compared allozymes from populations of Jeffrey pine (*P. jeffreyi*) in the Klamath Mountains and the central Sierra Nevada and found evidence for differentiation based on soil type. Of particular interest is their observation of significant differentiation of allelic frequencies at a malate dehydrogenase locus, as we found for *P. balfouriana.* However, they do not report any significant deviation at any of three fluorescent esterase loci. We believe that our study was more precise in attempting to detect genetic differentiation based on serpentine soils than that of Furnier and Adams (1986) because our study sites with dif-

ferent soil types are interspersed with each other in the Klamath region, where other environmental factors such as climate, vegetation history, and potential competitors are more similar among sites. The absence of Jeffrey pine from nonultramafic substrates in the Klamath region forced Furnier and Adams (1986) to make a comparison with geographically more distant populations in the Sierra Nevada.

In the context of these other studies, we suggest that natural selection for adaptation to serpentine soils may be occurring in the northern subspecies. Strong differentiation of allelic frequencies at the *Mdh*1 locus ($\chi^2 = 35.52$, df = 1, $P < 0.0001$) may be obscured by equally strong differentiation due to genetic drift and other factors, so much so that significant differentiation was not maintained under our population resampling procedure. The differentiation at the *Fest*1 locus, although it remains significant under the resampling procedure, must be interpreted with caution, because seven of 11 northern populations were fixed for the same allele at that locus. In addition, our comparison of immediately adjacent populations at East Boulder Lake (serpentine) and Eagle Peak (quartz diorite) did not show significant differentiation, perhaps because high gene flow has prevented differentiation between serpentine and nonserpentine populations, but also perhaps because our sample sizes from these populations were small (EB, $n = 20$; EP, $n = 16$). We predict that common garden experiments similar to those of Jenkinson (1966) and Kruckeberg (1967) conducted on *P. balfouriana* would demonstrate local adaptation to soil type by differences in growth rate. As mentioned previously, our results of differentiation at a malate dehydrogenase locus between serpentine and nonserpentine populations mirror those of Furnier and Adams (1986) in *P. jeffreyi*.

In the course of our field collections, we observed that northern *P. balfouriana* forms large monotypic stands only on serpentine soils; in addition, on serpentine soils it can be found on a greater diversity of aspects, including valley bottoms and along lake shores, such as at Mount Eddy and East Boulder Lake. On other soil types, *P. balfouriana* typically grows at only the very crests of ridges and mountains in tiny populations of several hundreds of individuals that give way to other competing conifers a short distance below the crest. These ridgetop habitats have more total solar exposure and also are subject to more desiccating winds, which we interpret as microhabitat selection for dry conditions in an otherwise generally mesic region. It is only in these microhabitats that *P. balfouriana* is able to outcompete other conifers on nonserpentine soils. It is also interesting to note that the northernmost occurrence of *P. balfouriana* at Lake Mountain occurs on a northwest facing slope at an atypically low elevation. The existence of serpentine talus on this slope has enabled a population of *P. balfouriana* to survive in what would otherwise be particularly challenging conditions.

Pinus balfouriana shows a high degree of interpopulation differentiation in its northern subspecies, a pattern that is at odds with the more homogenous pattern predicted by the life-history traits of conifers. However, the data we report here, along with that for *P. longaeva* (Hamrick et al. 1994) and *P. ayacahuite* (Ledig 1998), show that the mountain island effect can become strong enough to overcome homogenizing factors. In addition, *P. balfouriana* is not the only pine for which there is evidence suggesting genetic adaptation to serpentine soils, which has also been shown in *P. ponderosa* (Jenkinson 1966; Ledig 1998), *P. contorta* (Kruckeberg 1967), and *P. jeffreyi* (Furnier and Adams 1986). If natural selection is acting on the *Mdh*1 or *Fest*1 loci in *P. balfouriana*, it is heavily obscured by strong population differentiation due to genetic drift, historic bottlenecks, and consequent allelic sorting in the northern subspecies.

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STARVATION RESISTANCE AND ADULT BODY COMPOSITION IN A LATITUDINAL CLINE OF *DROSOPHILA MELANOGASTER*

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Abstract.—Latitudinal geographic variation in *Drosophila melanogaster* is pervasive. Parallel clines in traits such as body size, egg size, ovariole number, and development time have been found on several continents throughout the world. However, a cline in starvation resistance and fat content in *D. melanogaster* has so far been found only in India. Here we investigate starvation resistance and fat content in 10 populations from South America, in which clines in body size, egg size, and development time have previously been found. We find no evidence for a cline in starvation resistance or fat content in South America. We therefore suggest that the cline in starvation resistance in India may have evolved in response to specific climatic variation found only in India.

Key words.—*Drosophila melanogaster*, fat content, latitudinal cline, starvation resistance.

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Many traits of *Drosophila melanogaster* vary clinally with latitude on multiple continents. Flies from relatively high latitudes have larger body size (Stalker and Carson 1947; David and Bocquet 1975; Watada et al. 1986; Coyne and Beecham 1987; Imasheva et al. 1993; James et al. 1995; van 't Land et al. 1999), shorter development time (James and Partridge 1995; van 't Land et al. 1999), larger egg size (Azevedo et al. 1996), and higher ovariole number (Watada et al. 1986; Capy et al. 1993; Azevedo et al. 1996). The repeatability of the clines in different continents suggests that they are the result of natural selection (Endler 1977). However, it is unclear what selective agent is responsible for these clines or which traits are the targets of selection. Laboratory studies have indicated that temperature plays a significant role in the evolution of these characters. Flies that evolve at lower temperatures in the laboratory are larger (Anderson 1966; Cavicchi et al. 1985; Partridge et al. 1994), have a shorter development time (Anderson 1966; James and Partridge 1995), and lay larger eggs (Azevedo et al. 1996). As well as the evolutionary effect, temperature has a direct environmental effect on these traits; at lower temperature, body size and egg size are greater and development time longer (Azevedo et al. 1996; James et al. 1997).

Latitudinal clines in starvation resistance have been found

in *D. melanogaster, D. ananassae, D. kikkawai,* and *Zaprionus indianus* in India, with higher starvation resistance occurring at lower latitudes (Karan et al. 1998; Karan and Parkash 1998). Other studies in India have also found latitudinal trends in starvation resistance in smaller numbers of populations of *D. melanogaster* (Shamina et al. 1993), *D. kikkawai* (Parkash and Vandna 1994) *D. bipectinata,* and *D. malerkotliana* (Parkash et al. 1994). In Europe and Africa, differences in starvation resistance have been found between populations of *D. melanogaster,* but these differences were not related to latitude (Da Lage et al. 1990). The latitudinal differences in starvation resistance in India may be explained by ecological and/or climatic factors other than temperature (Karan et al. 1998). Starvation resistance has often been used as an indirect measure of fat content, because these two characters are highly correlated (David et al. 1975; Zwaan et al. 1991, 1995a, b). However, studying fat content directly would eliminate other factors, such as metabolic rate, that may affect starvation resistance (Hoffmann and Parsons 1989). It should also be noted that some of the latitudinal ranges studied may not have been wide enough to detect correlations with latitude.

In the present study, starvation resistance and fat content were measured in 10 populations from a transect along a wide latitudinal range in South America. Temperature varies with latitude over this transect, whereas a number of other climatic factors, such as humidity and amount of sun hours do not (van 't Land 1997). The collection sites for these populations were low-elevation, coastal sites to minimize variation caused by factors other than latitude. These populations have been maintained at 25°C since collection in 1995 and the latitudinal variation in body size, egg size, and development time is still present in the populations (Azevedo et al. 1996; van 't Land et al. 1999).

The aims of the present study were to establish whether starvation resistance and fat content are among the suite of traits that vary with latitude and for which temperature is implicated as the selective agent and to establish whether there is clinal variation in body composition in South America. Because these populations are known to increase in body size with latitude, we investigate whether fat content also increases with latitude, which could indicate an ability of high-latitude flies to acquire more food or to utilize food more efficiently. Alternatively, increased body size may be achieved by using the fat reserves for extra growth. Larval crowding has been shown to influence fat content and starvation resistance in temperate (Zwaan et al. 1991) and tropical (van 't Land 1997) populations of *D. melanogaster.* Therefore, this experiment was performed using adult flies that had been kept at either high or low density as larvae to assess whether differing larval density affects fat content, starvation resistance, or the relationship of fat content and starvation resistance with latitude.

Materials and Methods

Fly Populations

The 10 South American populations have been described elsewhere (Azevedo et al. 1996; van 't Land et al. 1999). They were collected in 1995 along a latitudinal range from 2.22°S to 41.5°S and have been maintained since collection in bottle culture at 25°C. The South American populations have previously been found to exhibit clinal variation in body size, development time, and egg size (Azevedo et al. 1996; van 't Land et al. 1999). Starvation resistance, wet weight, dry weight, water content, and fat content were measured in these populations. All experiments were carried out at 25°C with constant day length (12 h light, 12 h dark).

Production of Experimental Flies

To collect eggs, flies from each of the populations were transferred to laying pots and allowed an acclimatisation period of 12 h and a 4-h period on fresh food to encourage the laying of retained eggs. They were then allowed to lay on fresh medium for two consecutive periods of 4-h. First-instar larvae were picked from the surface of the medium and transferred to vials containing 7 ml of standard medium (composition: 1.0% [w/v] agar, 8.5% sugar, 6.0% maize meal, 2.0% dried yeast extract, 2.5% [v/v] of a 10% Nipagin solution in ethanol). To test whether crowding conditions had any effect on the characters measured or on their relationship to latitude, two larval densities, 50 larvae per vial and 200 larvae per vial, were used. For each population, three vials were set up at each larval density. Eclosing adults were collected as virgins and were transferred in single-sex groups of five to vials containing fresh medium, which were renewed regularly. The flies were kept for 15 days before measurements were made, because the starvation resistance of female flies changes rapidly in the first two weeks of adulthood (Fairbanks and Birch 1970; Service et al. 1985; Zwaan et al. 1991), which could lead to spurious differences when studying younger flies. The flies were kept unmated because there may be a trade-off between starvation and egg-laying (Chippindale et al. 1993), thus, any between-line differences in fecundity could confound the effects of our study if mated flies were used. The flies were then anaesthetized using carbon dioxide and removed from the food.

Experimental Measurements

Wet weight, dry weight, water content, and fat content

Wet weight, dry weight, water content, and fat content were determined for 15 flies of each sex from each population and each larval density. The flies used were kept in single-sex groups of five for weighing. An equal number of flies was taken from each of the original culture vials to equalize any effects caused by between-vial variation. After removal from food, the flies were anaesthetized on ice and were weighed to the nearest 0.002 mg using a Sartorius (Gottingen, Germany) microbalance to determine wet weight. The flies were dried in an oven at 60°C for 24 h and were then reweighed to determine dry weight. To remove fat from the flies, they were placed in sealed tubes with 1 ml of diethyl ether for 24 h with occasional light shaking. They were then removed from the ether and allowed to dry at room temperature for another 24 h, and then reweighed to determine the fat-free dry weight. From the weights obtained, it was possible to calculate water content (wet weight—dry weight), fat content (dry weight—fat-free dry weight), relative water content (wa-

ter content/wet weight) and relative fat content (fat content/dry weight; David et al. 1975).

Starvation resistance

Starvation resistance was measured for 15 flies of each sex from each population and each larval density, with an equal number of flies taken from each of the original culture vials. The flies were placed individually in vials containing 1 ml of autoclaved agar (5 g/L) to prevent desiccation. The vials were observed for dead flies after 24 h had elapsed and then at 3-h intervals. The frequency of observations was decreased as the number of deaths per observation decreased. Death was deemed to have occurred when a fly showed no sign of movement even after the vial was tapped lightly. Time of death was assigned to the midpoint between observations.

Statistical Analysis

All statistical analysis was performed using JMP 3.2.2 for the Macintosh (SAS Institute 1997).

Wet weight, dry weight, water content, fat content, and starvation resistance

Analysis of covariance was performed on each of the traits, with latitude as the covariate and sex and density as independent variables. A (quasi) minimum adequate model (MAM) was found by a stepwise backward-deletion procedure (Crawley 1993). The highest-order interaction was removed from the full model if it did not explain a significant proportion of the residual variance ($P > 0.05$), and a reduced model was fitted. The next-highest-order nonsignificant interaction with the highest P-value was then removed and a new reduced model was fitted. Nonsignificant factors or interactions were maintained in the model when any higher-order interactions including the factor or interaction were significant ($P < 0.05$).

Relationship between fat content and starvation resistance

To determine whether fat content and starvation resistance have a direct relationship and whether there are factors other than fat content that affect starvation resistance, we performed additional tests. To test whether the relationship between fat content and starvation resistance differed linearly with latitude, regression analyses were performed on population means of male and female starvation resistance and on population means of male and female relative fat content at each density. A multiple regression model was fitted using starvation resistance as the dependent variable with sex, crowding, relative fat, and latitude as factors. All interactions were included in the model, and a minimum adequate model was then found using a stepwise backward-deletion procedure (as above).

RESULTS

Wet weight, dry weight, water content, fat content, and starvation resistance

Mean values for wet weight, relative water content, relative fat content, and starvation resistance are shown in Table 1.

TABLE 1. Mean values for (a) wet weight, (b) relative water content, (c) relative fat content, and (d) starvation time.

	Female		Male	
Latitude (°S)	Low density	High density	Low density	High density
(a) Mean wet weight (mg)				
2.22	5.373	4.701	3.669	3.080
18.47	6.023	6.263	4.209	3.641
20.22	6.325	5.804	4.617	3.651
23.63	6.748	5.678	4.398	3.620
27.33	6.982	5.950	4.658	3.620
29.93	6.734	5.842	4.526	3.816
33.08	6.392	5.319	4.210	3.505
35.8	7.596	6.477	4.489	3.769
39.8	6.857	6.238	4.578	3.663
41.5	7.373	6.290	4.671	3.968
(b) Mean relative water content				
2.22	0.667	0.656	0.674	0.673
18.47	0.599	0.658	0.658	0.671
20.22	0.650	0.636	0.686	0.683
23.63	0.649	0.665	0.673	0.680
27.33	0.645	0.653	0.683	0.679
29.93	0.632	0.632	0.670	0.665
33.08	0.603	0.650	0.667	0.687
35.8	0.687	0.671	0.687	0.689
39.8	0.653	0.637	0.682	0.678
41.5	0.621	0.632	0.668	0.669
(c) Mean relative fat content				
2.22	0.057	0.070	0.043	0.036
18.47	0.136	0.062	0.059	0.044
20.22	0.090	0.092	0.041	0.039
23.63	0.068	0.056	0.041	0.032
27.33	0.077	0.074	0.026	0.033
29.93	0.092	0.087	0.036	0.044
33.08	0.130	0.082	0.058	0.029
35.8	0.038	0.058	0.034	0.027
39.8	0.073	0.087	0.036	0.031
41.5	0.092	0.085	0.045	0.042
(d) Mean starvation time (h)				
2.22	60.100	62.283	28.892	32.608
18.47	104.383	68.083	33.617	32.783
20.22	80.107	48.768	31.920	26.821
23.63	77.033	65.767	30.575	32.217
27.33	75.267	61.367	25.533	26.767
29.93	55.667	70.258	30.642	29.408
33.08	102.383	70.600	33.917	33.483
35.8	48.308	39.750	23.933	26.442
39.8	68.217	66.083	28.717	27.917
41.5	79.817	79.550	36.017	36.417

The results of the MAM analyses of covariance are summarized in Table 2. For wet weight and dry weight, latitude, density, and sex were all significant main effects with a significant latitude-by-sex interaction. Wet and dry weight were larger at high latitude, low density, and in females. The interaction results from different responses of males and females to increasing latitude. Table 1 shows mean values for wet weight for each sex; inspection of this data indicates that increasing latitude had a greater effect on females than it did on males. For water content, latitude, density, and sex were all significant with no significant interaction. Water content was greater at high latitudes, low density, and in females. For fat content, sex and density were significant main effects. Fat content was higher in females and at low density. For relative water content, relative fat content, and starvation resistance, only sex was significant, with males having a high-

TABLE 2. Minimum adequate model analysis of covariance with (a) wet weight, (b) dry weight, (c) water content, (d) relative water content, (e) fat content, (f) relative fat content, and (g) starvation resistance as dependent variables, latitude as covariate, and sex and density as independent variables.

Source	df	Mean square	*F*-ratio
(a) Wet weight			
Latitude	1	4.1156	48.5867***
Density	1	6.0321	71.2119***
Sex	1	3.8403	45.3363***
Latitude × sex	1	0.5944	7.0173*
Error	35	0.0847	
(b) Dry weight			
Latitude	1	0.5555	43.7765***
Density	1	0.8550	67.3765***
Sex	1	0.5842	46.0365***
Latitude × sex	1	0.1322	10.4216**
Error	35	0.0127	
(c) Water content			
Latitude	1	1.6471	24.9101***
Density	1	2.3451	35.468***
Sex	1	17.2082	260.2572***
Error	36	0.0661	
(d) Relative water content			
Sex	1	0.0098	36.2976***
Error	38	0.0003	
(e) Fat content			
Sex	1	1.1753	105.1813***
Density	1	0.0754	6.7475*
Error	37	0.0112	
(f) Relative fat content			
Sex	1	0.0172	55.7984***
Error	38	0.0003	
(g) Starvation resistance			
Sex	1	15022.1070	111.1708***
Error	38	135.1000	

*** $P < 0.001$; ** $P < 0.01$; * $P < 0.05$.

er relative water content and females having a higher relative fat content and a higher starvation resistance. In summary, only wet weight, dry weight, and water content showed clinal variation.

Relationship between fat content and starvation resistance

Regression analyses of male starvation resistance against female starvation resistance at low and high larval density were significant (both $P < 0.05$). Regression analysis of male relative fat against female relative fat was significant only at low larval density ($P < 0.05$). These results suggest differences between fat content and starvation resistance. The MAM multiple regression analysis is shown in Table 3. A number of significant interactions were found. The interaction between relative fat and latitude was significant (parameter estimate = 0.41312). This interaction indicates that the relationship between starvation resistance and relative fat varies with increasing latitude. The sign of the parameter estimate is positive, indicating that starvation resistance and relative fat are more closely correlated with increasing latitude. The interaction between relative fat and density was also significant (parameter estimate = 13.18336). This interaction indicates that larval density has an effect on the relationship between fat content, and starvation resistance. The sign of the parameter estimate indicates that at high density, fat explains more of the variation in starvation resistance than it does at low density.

TABLE 3. Minimum adequate multiple regression model with starvation resistance as the dependent variable, latitude and relative fat content as continuous factors, and sex and density as discrete factors.

Source	df	Mean square	*F*-ratio
Sex	1	620.8157	14.8234***
Density	1	747.5490	17.8494***
Latitude	1	650.7022	15.537***
Relative fat	1	347.1335	8.2886**
Sex × density	1	490.9332	11.7221**
Latitude × sex	1	611.9400	14.6114***
Sex × relative fat	1	65.0617	1.5535
Latitude × density	1	655.9321	15.6618***
Density × relative fat	1	814.1069	19.4386***
Latitude × relative fat	1	685.7342	16.3734***
Latitude × sex × density	1	495.9863	11.8428**
Latitude × density × relative fat	1	696.0447	16.6196***
Error	27	41.8800	

*** $P < 0.001$; ** $P < 0.01$.

DISCUSSION

The results of our analysis showed that relative water content, fat content, relative fat content, and starvation resistance did not vary clinally. Overall water content increased with increasing latitude, but this was paralleled by the increase in dry body weight; flies from each latitude contained the same amount of water per unit body weight. The multiple regression model for starvation resistance found a significant latitude-by-relative fat interaction, indicating that latitude affected the relationship between relative fat content and starvation resistance. Similarly, the density-by-relative fat interaction indicated that the relationship between fat and starvation resistance was affected by larval density. Factors other than fat content must therefore have partly determined starvation resistance. Previous studies have shown that starvation resistance is directly correlated with fat content (David et al. 1975; Service 1987; Zwaan et al. 1991). However, other factors (e.g., metabolic rate; Hoffmann and Parsons 1989) may also play a role, as has been suggested by our study. Previous studies of clines have concentrated on starvation resistance alone and concluded that it reflected differences in fat content (Da Lage et al. 1990; Shamina et al. 1993; Parkash et al. 1994; Parkash and Vandna 1994). However, studies that do not measure fat content may miss effects due to other factors. In studying both fat content and starvation resistance directly, we have been able to establish that there is no clinal variation in either and that other factors such as metabolic rate differences may explain the apparent variation in starvation resistance, independent of fat content.

Other studies of starvation resistance in *D. melanogaster* have found clinal variation in the Indian subcontinent (Karan et al. 1998), but not in Europe and Africa (Da Lage et al. 1990). The South American populations that we studied showed no evidence of clinal variation in starvation resistance. The starvation resistance of the South American populations may be a consequence of adaptation to laboratory conditions. However, this is unlikely because other geograph-

ically varying traits such as body size have not undergone laboratory adaptation since collection (Azevedo et al. 1996; van 't Land et al. 1999). Another possible reason for the difference in results between our study and that of Karan et al. (1998) is the differences in methods used. Karan et al. (1998) studied only males and tested starvation resistance at 3–4 days of age. Our study examined starvation resistance of both males and females. Incorporating females gives a more reliable estimate because they live longer. The females we used were kept as virgins, because there is a trade-off between egg production and starvation resistance (Chippindale et al. 1993). The flies used were also kept for 15 days before starvation resistance was measured because female starvation resistance increases steeply early in life, before reaching a plateau. Starvation resistance was measured at 15 days to avoid any confounding effects of between-population differences in the time taken to reach this plateau. The flies used in our study remained healthy until tested for starvation resistance and the differences between the methods would not be likely to explain the difference in results. It should also be noted that the study of Da Lage et al. (1990) used very similar methods to that of Karan et al. (1998) and also failed to find a latitudinal cline in starvation resistance. However, they only studied three populations.

In addition to differences in methods, our study covered a broader range of latitudes than that of Karan et al. (1998). The broader latitudinal range means that we also covered a wider range of temperature than the study of Karan et al. (1998) and this is further evidence to suggest that temperature variation is not solely responsible for the variation in starvation resistance found in Indian populations.

The apparent inconsistency of the results of Karan et al. (1998) and the ones we report here can be accounted for if differences in starvation resistance are a result of adaptation to climatic factors other than temperature, which differ between continents. Karan et al. (1998) suggested that the clinal variation in starvation resistance found in India was due to either average yearly temperature or average winter temperature. However, average yearly temperature in India is not well correlated with latitude. It is therefore unlikely that average yearly temperature is responsible for clinal variation of a trait. Similarly, although winter temperature in India is negatively correlated with latitude, summer temperature is positively correlated with latitude, and it is not clear why only winter temperature should affect this trait. In our study, average yearly temperature was highly correlated with latitude and we found no significant clinal variation in starvation resistance. Thus, it seems likely that the variation in starvation resistance in India is actually due to climatic factors other than temperature or a combination of factors (which may include temperature) that differ between South America and India. Sampling populations along latitudinal clines with varying climatic factors could pinpoint the key parameters in the evolution of these and other traits. In addition, specific hypotheses can be tested using laboratory evolution experiments.

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EVALUATION OF SELECTION ON CLIFF SWALLOWS

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Abstract.—Estimates of the intensity of selection based on measurements of the living and the dead require knowledge of the fraction of the original population dying. We apply recently developed methods (Blanckenhorn et al. 1999) to estimate the intensity of selection in a population of cliff swallows. In this population the fraction of individuals dying was unknown, but certainly high. The inferred selection is very strong and impossible to achieve if the original population is assumed to have followed a normal distribution. We consider several alternative explanations for this result including measurement biases, undetected immigration, and sampling biases. Of these, sampling biases are perhaps the most likely. We conclude that the intensity of selection on the swallows was probably strong, but its absolute magnitude is unknown.

Key words.—Cliff swallows, mortality, natural selection, selection index, selection intensity.

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Over six days in May 1996, severe weather in southwestern Nebraska resulted in massive mortality of cliff swallows, *Petrochelidon pyrrhonota* (Brown and Brown 1998). After the storms, a total of 1856 dead swallows were collected and 1028 living swallows were captured and released. Five traits were measured on each individual (wing length, tail length, tarsus length, culmen length, and culmen width). Morphological differences between the living and the dead were large. The most striking comparison was in tarsus length, which averaged 10.0 mm in the dead and 11.5 mm in the living, a difference of about three standard deviations (Table 1). The differences between the dead and the living suggest very strong selection (for further details, see Brown and Brown 1998). We use recently developed methods (Blanckenhorn et al. 1999) in an attempt to evaluate the strength of that selection.

The measurement of selection on a single trait starts with the selection differential, s, which is the difference in the mean value of the trait in the population before and after selection. The standardized version of the selection differential is the selection intensity, i ($i = s/\sigma$), where σ is the standard deviation of the trait in the population before selection. A quantitative measure of the selection intensity thus requires estimation of both the population mean and population variance before selection. The mean and variance before selection are not directly observed in samples of the living and the dead. However, they can be estimated as a weighted sum of the means and variances of the living and the dead samples provided the fraction of individuals dying is known (Blanckenhorn et al. 1999).

In the swallow population the mortality rate was unknown, but was clearly high. Based on the number of active nests in 1995 and 1996 the population was reduced by 53% (Brown and Brown 1998). A mark-recapture study of 512 birds caught

TABLE 1. Means and standard deviations for the living and dead swallows. Sample sizes are >1026 for the living and >1794 for the dead (for complete details of sample sizes, see Brown and Brown 1998). All measurements are in millimeters.

	Living		Dead	
	Mean	SD	Mean	SD
Wing length	106.87	2.31	107.53	2.73
Tail length	46.13	1.83	46.65	1.97
Tarsus length	11.52	0.59	10.00	0.47
Culmen length	6.94	0.31	6.33	0.39
Culmen width	6.27	0.37	5.54	0.37

TABLE 2. Correlation matrices of traits. Values for dead swallows are above the diagonal and for living swallows are below the diagonal.

	Wing length	Tail length	Tarsus length	Culmen length	Culmen width
Wing length		0.375	0.232	0.108	0.122
Tail length	0.301		0.023	0.027	0.052
Tarsus length	0.155	0.058		0.231	0.237
Culmen length	0.056	0.024	0.044		0.579
Culmen width	0.086	−0.007	0.004	0.630	

before the event (but not measured) led to an estimate of mortality of 73% (C. R. Brown and M. Bomberger Brown, unpubl. obs.). Because of the uncertainty surrounding the mortality rate, we estimated the selection intensity over a wide range of mortality values using equations (2–4) in Blanckenhorn et al. (1999).

We estimated selection intensity for tarsus length and also for a selection index based on all five traits. The selection index, I, is the linear combination of traits such that if selection acted directly on the index alone the five traits would evolve in the observed directions, with the observed relative magnitudes (see Lande and Arnold 1983, p. 1214). The construction of the index requires an estimate of the covariances between traits before selection (Lande and Arnold 1983), which can be obtained from the covariances and means of the living and dead groups using a modification of equation (3b) in Blanckenhorn et al. (1999). The correlation matrices for the living and dead are given in Table 2. These were used along with the means and standard deviations in Table 1 to reconstruct estimates of the covariances in the population before selection. The variances, covariances, and selection differentials were then combined to calculate the selection intensity on the index, as derived in the Appendix.

The estimated selection intensities on tarsus length and the index as a function of the amount of assumed mortality are shown in Figure 1a. If mortality is assumed to be low, the selection intensity is low because the population before selection is very similar to the living sample. However, if the mortality is assumed to have been high (as appears probable in the cliff swallows), the selection intensity is inferred to have been very high because the population before selection is reconstructed to be very similar to the characteristics of the dead sample.

A useful measure of the strength of selection is the amount of mortality needed to produce an observed selection differential (Lande 1976). The *minimum required mortality* arises when selection is truncation (all individuals one side of the truncation point die, and all individuals on the other side survive). Provided the distribution before selection is approximately normal, the minimum required mortality can be calculated from the selection intensity and tables of the cumulative normal distribution (e.g., Becker 1967, pp. 123–124). The minimum required mortality when selection is acting on more than one trait is calculated using the intensity of selection on the index, and this was our reasoning behind the construction of the index. We also illustrate the consequence of relaxing the assumption of truncation selection, which is unlikely to ever apply in nature, by fitting a Gaussian fitness function to the data. The Gaussian function has two parameters, the optimum and width of the function, which can be estimated from the change in mean and variance of the distribution, assuming the original population is normally distributed (e.g., Bulmer 1980, p. 151). If fitness at the optimum is scaled to be 1.0, the mean fitness based on this function is an estimate of the mortality required to produce the observed selection intensity. The required mortality estimates for truncation selection on the index and for truncation and Gaussian selection on tarsus length are plotted in Figure 1b.

A paradox in our results is that even the minimum amount of required mortality (i.e. truncation selection on the index) is higher than postulated mortality across the whole range of values tabulated in Figure 1. A main assumption underlying the methods used to calculate the required mortality is that the population before selection is approximately normally distributed. In fact, the samples of the living and the dead themselves appear to be more or less normally distributed (e.g., Fig. 2). A mixture of two different normal distributions cannot itself be normal, and if the two distributions are more than two standard deviations apart (as in the case for tarsus length, see Fig. 2), the reconstructed population is not even unimodal. In this case, the reconstructed population is bimodal, with one mode corresponding to the dead and the other corresponding to the living

There are several possible explanations for these results. One is that measurement techniques differed between the living and the dead, with live birds consistently measured as larger due to effects of specimen shrinkage. Although this is a potential problem for any study of selection based on living birds and specimens (Jenni and Winkler 1989), in cliff swallows there was no difference in measurements for 29 birds processed both alive and dead (Brown and Brown 1998) and thus no apparent measurement bias. Another possible problem with selection studies based on separate samples of living and dead animals is immigration by individuals after the selection event. If the immigrants are morphologically different from the individuals exposed to the event, the intensity of selection will be incorrectly estimated. In the case of the cliff swallows, there clearly was immigration after the severe weather, because the number of breeding pairs later in the summer was greater than expected based on the estimated mortality during the storm. However, the measurements of survivors were taken immediately after the severe weather ended, thus minimizing the possibility of including immigrants among those measured (Brown and Brown 1998). In an explicit test of the immigration hypothesis, we compared live birds measured during the first two days after the storm

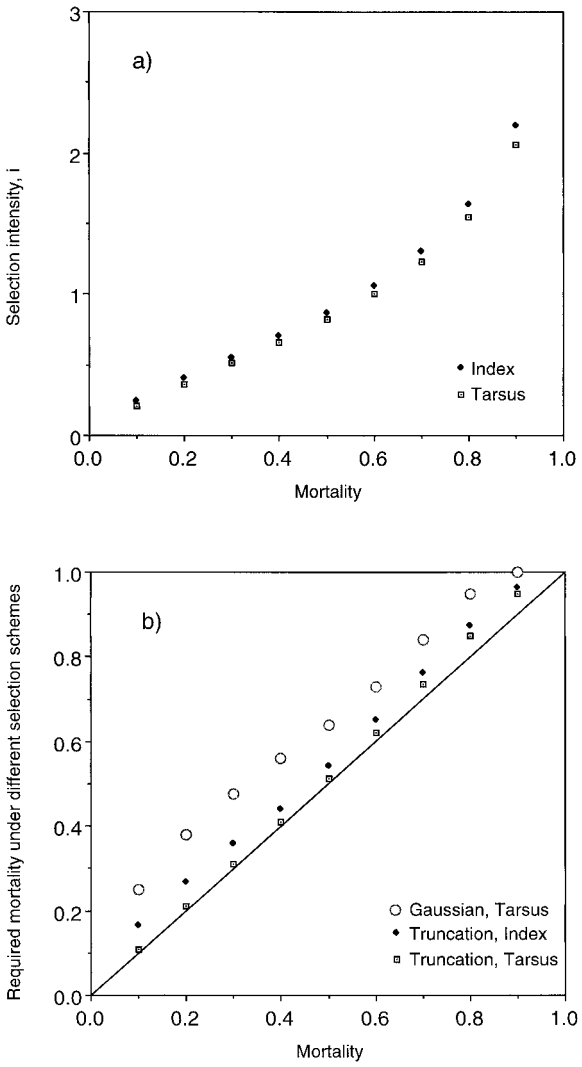

FIG. 1. (a) The selection intensity on tarsus length and an index based on all five traits (see Appendix for derivation) given different amounts of mortality in the swallow population. (b) The required amount of mortality, assuming a normal distribution in the original population, given the selection intensities in the top figure. The x-axis is the same in both graphs. Required mortality under truncation selection for tarsus length and the index is shown, as well as mortality under a Gaussian fitness function for tarsus length (the plotted function is $(\omega^2/(\omega^2 + \sigma^2))\ \exp[-(\bar{\chi} - \theta)^2/(2\omega^2 + 2\sigma^2)]$, where ω^2 and θ are the width and optimum of the fitness function respectively, estimated from the change in mean and variance due to selection). The line is the line of equality between the y and x values.

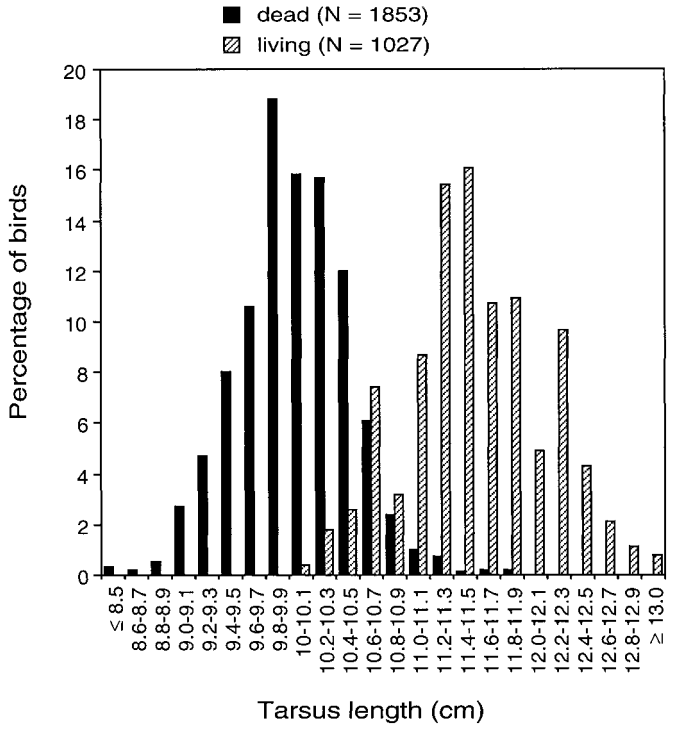

FIG. 2. Distribution of tarsus length among the dead ($N = 1853$) and living ($N = 1027$) swallows. The distributions are separately scaled to 100%.

(which were unlikely to have had time to immigrate to the study area) with birds measured eight days later (ones more likely to have had time to immigrate). We found that the birds measured earlier were actually larger than those measured later. For example, mean (± SD) tarsus length for birds on days 1–2 was 11.63 mm (± 0.58, $N = 176$) and on day 8 was 11.37 mm (± 0.58, $N = 107$) mm. (Wilcoxon test, $P < 0.001$). Thus, in this case, immigration during the time period that measurements were taken was unlikely to have contributed to the morphological patterns observed.

Perhaps the most likely possibility is that the dead birds salvaged for measurement may have been a nonrandom subset that included only the smallest. Most of the dead birds collected were ones found on the ground below nests or on nearby sandbars. These birds either fell out of nests or never had nests. Thousands more birds died inside nests, and we could not retrieve those. Perhaps only the smallest birds were apt to be found dead on the ground, only the largest ones survived, and the intermediate sized birds were those that died in nests. This might be the case if the smallest birds were unable to secure nesting sites prior to the severe weather and were thus more exposed to the elements. Their smaller legs also might have influenced their ability to cling to protected substrates (such as the eaves of bridges), causing them to be more likely to fall to the ground, die, and be represented in our measurements. While this does not change the conclusion that the population was subject to intense selection on body size, it prevents us from accurately quantifying the strength of that selection.

Our results highlight the difficulties of obtaining random samples in studies of selection. These difficulties are not restricted to methods based on comparisons of samples of individuals. Thus, although it is possible to apply regression methods to the well-known dataset of Bumpus (1899), the sample of house sparrows Bumpus studied across a storm is certainly a highly biased sample of all the sparrows that experienced the storm (Price and Yeh 1999). Any study of selection needs to be accompanied by a consideration of potential biases in the dataset (Blanckenhorn et al. 1999).

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Appendix

The numerical value of the index for an individual bird is defined as $I = (\mathbf{P}^{-1} s)^T z$, where $\mathbf{P}$ is the phenotypic variance-covariance matrix before selection, s is the vector of selection differentials for the traits, z is the vector of trait measurements for that individual, and superscript T indicates transpose (Lande and Arnold 1983, p. 1214). Using the result that the covariance of a character with relative fitness (w) is the selection differential on that character (Price's theorem) we calculate the selection differential on the index as

$$s_I = \text{cov}(I, w) = \text{cov}[(\mathbf{P}^{-1}s)^T z, w] = (\mathbf{P}^{-1}s)^T s$$
$$= s^T \mathbf{P}^{-1} s. \quad \text{(A1)}$$

The covariance of I with $\mathbf{z}$ is $\text{cov}(I, z) = (\mathbf{P}^{-1}s)^T \mathbf{P} = s$ (Lande and Arnold 1983). Substituting I for z in this formulation shows that the variance in I equals its selection differential, $\sigma_I^2 = s_I$. The definition of the selection intensity on I is the selection differential divided by the standard deviation $s_I/\sigma_I = \sigma_I = \sqrt{s_I}$. Using this and (A1), we find the selection intensity for I to be $\sqrt{(s^T \mathbf{P}^{-1} s)}$. It is this value that is plotted in Figure 1. The use of a similar formula to estimate minimum intensities of selection in retrospective studies of selection (based on the genetic variance-covariance matrix) is discussed briefly by Lande (1979, p. 408).