doi: 10.1111/j.1420-9101.2009.01703.x

Coevolution between crossbills and black pine: the importance of competitors, forest area and resource stability

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Keywords:

coevolution;
geographic mosaic;
Loxia curvirostra;
phenotypic selection;
Pinus nigra;
predator-prey interactions;
resource variability;
Sciurus vulgaris;
seed predation.

Abstract

Studies of predator-prey interactions have found that geographically structured coevolution has played an important role in the adaptive diversification of crossbills (*Loxia* spp.). We extend those studies by considering common crossbills (*L. curvirostra*) in the Mediterranean where they rely on seeds in the cones of black pine (*Pinus nigra*). On the continent, where tree squirrels (*Sciurus vulgaris*) are present, enhanced defenses against crossbills were most evident in larger areas of pine forest. On islands in the absence of tree squirrels, crossbills and black pine have coevolved in a predator-prey arms race on Cyprus but not Corsica. In contrast to other conifers that island endemic crossbills rely upon, black pine does not hold seeds in its cones year round. Consequently, key to the strong crossbill–pine interaction on Cyprus is likely the presence of an alternative conifer that provides seeds during early summer when black pine seeds are scarce.

Introduction

Over the last decade interest has grown on how geographic variation in the form and outcome of species interactions contributes to ecological and evolutionary processes (Thompson, 2005). Moreover, an increasing number of studies have documented the existence of geographic mosaics of coevolution, where populations exhibit adaptive divergence as a result of geographic variability in the occurrence and intensity of coevolutionary interactions (Brodie *et al.*, 2002; Thompson & Cunningham, 2002; Toju & Sota, 2006; Anderson & Johnson, 2007; Siepielski & Benkman, 2007). Little attention, however, has been given to the role of such geographic mosaics in contributing to patterns of diversification across adaptive radiations, with the exception of studies on the interactions between crossbills (*Loxia* spp.) and conifers. These studies, spanning a variety of crossbill–conifer interactions, have indicated that divergent selection arising from geographically variable coevolution has been an important process in the ongoing adaptive radiation of crossbills (Benkman, 1999; Benkman *et al.*, 2001, 2003; Parchman & Benkman, 2002, 2008; Mezquida & Benkman, 2005; Edelaar & Benkman, 2006; Parchman *et al.*, 2007; Smith & Benkman, 2007). Because these studies have produced a relatively clear understanding of the phenotypic interface of coevolution, further studies of the interactions between crossbills and conifers offer promise in addressing the role of coevolution across an adaptive radiation and gaining understanding of the conditions influencing the coevolutionary process.

Crossbills have evolved a mandible crossing as an adaptation for spreading apart conifer cone scales to feed on the underlying seeds (Newton, 1972; Benkman & Lindholm, 1991), and have diversified into an array of morphologically and vocally differentiated taxa (species, subspecies and call or vocal ‘types’; Newton, 1972; Groth, 1993; Benkman, 1999; Robb, 2000; Edelaar *et al.*, 2008) specialized for feeding on the cones of different conifer species (Newton, 1972; Benkman, 1987a, 1993a, 2003). Crossbills are also important seed predators (Juutinen, 1953; Génard & Lescourret, 1987; Castro *et al.*, 1999; Benkman *et al.*, 2003; Smith & Benkman, 2007) and selective agents on the conifers on which they specialize (Benkman, 1999; Benkman *et al.*, 2001, 2003; Parchman & Benkman, 2002, 2008; Mezquida & Benkman, 2005;

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Parchman *et al.*, 2007; Clouet & Joachim, 2008; see also Summers & Proctor, 1999). In particular, in areas where tree squirrels (*Sciurus* and *Tamiasciurus*) are uncommon or absent as preemptive competitors and selective agents, selection by crossbills has repeatedly led to the evolution of cone traits that defend seeds against crossbills. In many such areas, conifers have evolved generally larger cones with thicker scales and crossbills have evolved deeper bills in response to reciprocal selection, leading to localized coevolutionary arms races (Benkman, 1999; Benkman *et al.*, 2001, 2003; Parchman & Benkman, 2002; Siepielski & Benkman, 2005; Edelaar & Benkman, 2006; Parchman *et al.*, 2007). In sum, these studies indicate that geographic variation in the occurrence and intensity of coevolution between crossbills and conifers has been an important source of divergent selection across the adaptive radiation of crossbills. Furthermore, because the traits mediating and responding to reciprocal selection were found to evolve in a generally consistent and replicated manner across multiple interactions, these studies form a solid foundation for framing and testing hypotheses about coevolution for other interactions spanning the adaptive radiation of crossbills.

In Eurasia, as in North America, common (red) crossbills (*L. curvirostra* L.) are comprised of a group of morphologically and vocally diverse forms (Newton, 1972; Cramp & Perrins, 1994; Clouet & Joachim, 1996; Robb, 2000; Summers & Jardine, 2005; Summers *et al.*, 2007; Edelaar *et al.*, 2008; Förschler & Kalko, 2008). However, the extent to which specialization on individual conifers and ensuing coevolution has contributed to this diversity is little known (but see Mezquida & Benkman, 2005). On the continent, common crossbills engage in irruptive, nomadic movements following fluctuations in cone crops (Newton, 1972, 2006; Cramp & Perrins, 1994; Marquiss *et al.*, 2008), which likely limit local adaptation to geographic variation in conifers (Edelaar & Terpstra, 2004; Parchman & Benkman, 2008). Furthermore, genetic differentiation is subtle or nonexistent for the different vocal types or even several species of crossbills in Europe (Questiau *et al.*, 1999; see also Piertney *et al.*, 2001). Because adaptation is impeded by gene flow (e.g. Hendry *et al.*, 2001) resulting from such nomadic movements, local adaptation and coevolution are more likely to contribute to divergence in crossbills isolated on virtual or real islands with consistently available seed and where tree squirrels are absent or weak preemptive competitors (Benkman *et al.*, 2001, 2003; Parchman & Benkman, 2002; Mezquida & Benkman, 2005; Parchman *et al.*, 2007). Nearly all islands in the Mediterranean lack tree squirrels (all lack fossil evidence of them; Masseti, 2005) and three islands have resident and endemic common crossbill subspecies, namely the Mallorcan (*L. c. balearica*), Cyprus (*L. c. guillemardi*) and Corsican crossbills (*L. c. corsicana*) (Massa, 1987; Cramp & Perrins, 1994). Some of these subspecies have differentiated morphologically, indicating local adaptation and perhaps coevolution. For example, a recent study suggested that coevolution has occurred on Mallorca between Aleppo pine (*P. halepensis*) and crossbills (Mezquida & Benkman, 2005; see also Altaba, 2001; Alonso *et al.*, 2006). Here we assess whether coevolution has been an important process on the islands of Cyprus and Corsica where crossbills rely on seeds in the cones of European black pine (*Pinus nigra* J. F. Arnold). The Cyprus crossbill is very large-billed, vocally distinct, and resides only in the Troodos Mountains (Griscom, 1937; Knox, 1976; Massa, 1987; Clouet, 2003; Summers & Jardine, 2005). Some authorities lump crossbills from Cyprus, Turkey and Crimea as *L. c. guillemardi* (Cramp & Perrins, 1994) in part because crossbills collected from these three areas are similar in plumage (C.K. Roselaar, personal communication). However, crossbills on Cyprus have bill depths and widths that average 0.3 and 0.4 mm larger, respectively, than those in Turkey (C.K. Roselaar, unpublished data); these differences are as large or larger than the differences between ecologically distinct vocal types in North America (Benkman, 1993a; see also Edelaar *et al.*, 2008) and we therefore recognize the Cyprus crossbill as a distinct taxon. The Corsican crossbill has also been recognized, however, it is uncertain whether these birds are morphologically or vocally distinct from common crossbills on the mainland (Griscom, 1937; Massa, 1987; Clouet & Joachim, 1996; Robb, 2000; but see Förschler & Kalko, 2008). Indeed, Robb (2000) suggests that vocal 'type B' crossbills are adapted for foraging on the cones of black pine and occur throughout much of the range of black pine including Corsica but not Cyprus.

Black pine is a mid-elevation, montane conifer occurring in the northern Mediterranean (Fig. 1; Gaussen *et al.*, 1964). It is often a dominant conifer and an important food resource for crossbills and Eurasian red squirrels (*Sciurus vulgaris* L.) (Cagnin *et al.*, 2000; Molinari *et al.*, 2006). The regional taxonomy of black pine is unresolved with different authors giving different subspecies designations for the pines in different regions. We will adhere to Christensen (1997) who recognizes two subspecies: the western subspecies (*P. n. salzmannii*) that occurs from Italy westward and the eastern subspecies (*P. n. nigra*) that occurs east of the Adriatic Sea (see Fig. 1).

Tree squirrels have been repeatedly documented as important selective agents on the cone traits of conifers (Smith, 1970; Benkman *et al.*, 2001, 2003; Parchman & Benkman, 2002; Siepielski & Benkman, 2007), and geographic variation in the occurrence of these seed predators has played a large role in the geographic structuring of crossbill–conifer interactions as a result of both competitive and evolutionary effects (Benkman, 1999; Benkman *et al.*, 2001, 2003; Parchman & Benkman, 2002, 2008; Mezquida & Benkman, 2005). In North America, *Tamiasciurus*, which are among the most highly specialized conifer seed predators (Smith & Balda, 1979),

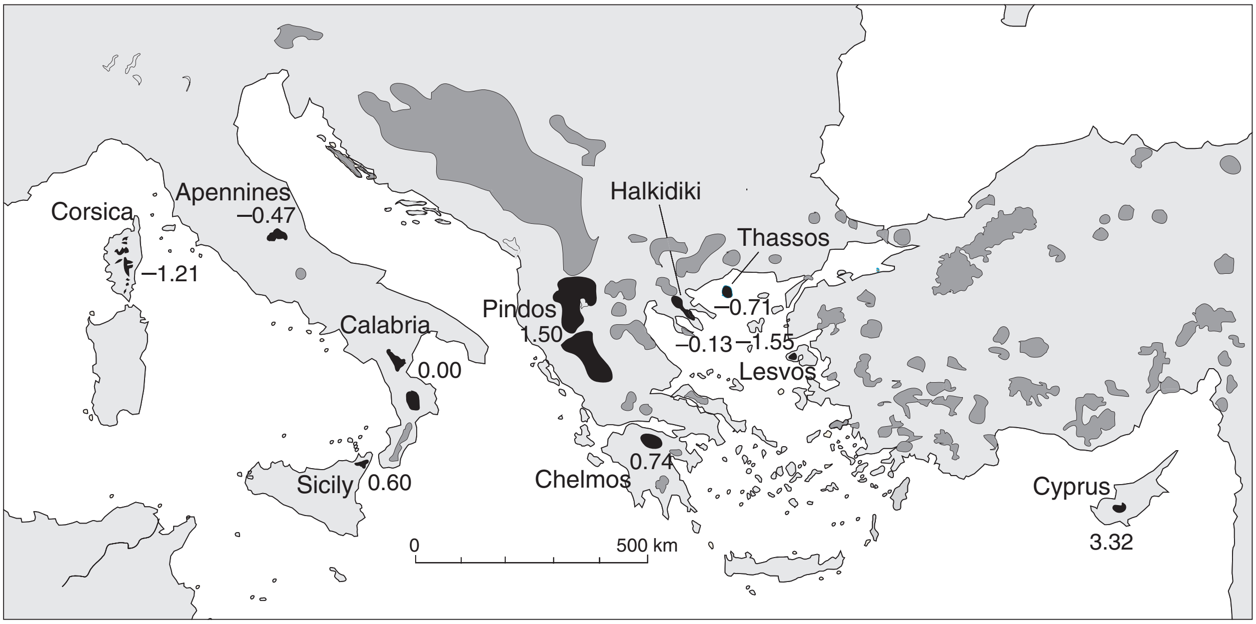

Fig. 1 The geographic distribution of black pine (*Pinus nigra*) in the northeastern Mediterranean (in grey) with the regions where cones were sampled highlighted in black. Located next to each of these regions is the mean value of the first principal component of eight cone traits (see Fig. 2), which is a composite trait representing the extent to which the cones of black pine are defended against crossbills. Red squirrels (*Sciurus vulgaris*) are present throughout the mainland but are absent from the islands. Crossbills occur in black pine forests on the continent, are resident on the Mediterranean islands of Corsica, Sicily and Cyprus, and are rare on Thassos and Lesvos. The distribution of black pine is based on maps in Isajev *et al.* (2004) and Thibault *et al.* (2006).

preferentially feed on trees having cones with a higher seed mass to cone mass ratio, and this has consistently caused evolutionary divergence of these traits between areas with and without *Tamiasciurus* in a variety of conifers, including lodgepole pine (*P. contorta latifolia*; Benkman *et al.*, 2001), black spruce (*Picea mariana*; Parchman & Benkman, 2002), limber pine (*P. flexilis*) and whitebark pine (*P. albicaulis*; Siepielski & Benkman, 2007). Tree squirrels in the genus *Sciurus* are less specialized than *Tamiasciurus* (Smith & Balda, 1979), but also have had important selective impacts on pines in both North America (Parchman & Benkman, 2008) and Europe (Mezquida & Benkman, 2005). *Tamiasciurus* are absent from the Old World, however throughout much of the distribution of black pine and other Eurasian conifers, red squirrels are an important seed predator, competitor and possibly selective agent (Moller, 1986; Summers & Proctor, 1999; Cagnin *et al.*, 2000; Mezquida & Benkman, 2005; Steele *et al.*, 2005; Molinari *et al.*, 2006).

Here we consider geographic variation in the cone morphology of black pine in the context of selection by crossbills to evaluate the hypothesis that coevolution has occurred between crossbills and black pine on Cyprus and Corsica. We first quantify variation in the cone traits among sites representing areas with and without crossbills and red squirrels. We measure the form of natural selection exerted by crossbills in both a natural population of black pine and through the use of foraging experiments with captive crossbills to determine the traits expected to evolve in response to crossbill predation. Cone traits of black pine are highly heritable (e.g. family heritabilities of 0.98 and 0.92 for seed mass and cone mass respectively; Kaya & Temerit, 1993), consequently evolutionary responses should be detectable if divergent selection experienced by black pine across geographic areas with different seed predators has been important. We then consider, based on previous studies, whether crossbills on islands have morphologies consistent with predator-prey coevolution. Bill size in crossbills is also highly heritable (bill depth, $h^2 = 0.58$–0.71; Summers *et al.*, 2007), so responses to selection should be evident. Finally, we consider variation in black pine in light of published foraging preferences of *Sciurus* spp. to determine if variation in cone traits are affected by selection exerted by red squirrels on the continent and evaluate the role of these competitors in structuring the interactions between crossbills and black pine. Even though cone- and seed-eating insects can also exert selection on cone traits (Siepielski & Benkman, 2004), geographic variation in black pine cone structure is unlikely the result of variation in selection exerted by insects because insect seed predation was consistently low in all our study areas (T.L. Parchman, personal observation).

Material and methods

Geographic variation in cone traits

To determine if geographic variation in the cone traits of black pine reflects the importance of selection by crossbills and red squirrels, we sampled and measured cones from 388 trees at 20 sites dominated by apparent natural stands of black pine in the Mediterranean region (Fig. 1; Appendix 1). We sampled cones from areas where crossbills and red squirrels are present as seed predators including sites in the central Apennine Mountains and southern or Calabrian Apennines, Italy, and from the Pindos Mountains, Mt. Chelmos and Halkidiki Peninsula, Greece. The breeding distribution of common crossbills (Cramp & Perrins, 1998) in particular coincides closely with the distribution of black pine in Italy, the Balkans and Turkey (Fig. 1). We sampled cones on islands where squirrels are absent and crossbills are either resident (Corsica, Cyprus and Sicily) or occur only rarely [Thassos and Lesvos; Persian squirrels (*S. anomalus*) occur on Lesvos, but are absent from the two small stands of black pine on the island (C. Thanos, personal communication; see also Matsinos & Papadopoulou, 2004)]. The following measurements were made on two cones collected from each tree: cone length, cone width at the widest point, cone mass, number of scales crossed by a transect along the long axis of the cone, thickness of five distal and five proximal scales, length of three scales from the middle third of the cone (measured from the scale tip to the distal end of the seed scars), number of full seeds, number of empty seeds (seed coat developed but lacking female gametophyte and embryo), and mass of five full seeds. Length measurements were made to the nearest 0.01 mm with digital calipers and mass measurements were made on oven-dried (60 °C for > 48 h) cones and seeds to the nearest 0.01 mg with a digital scale. The seed mass to cone mass ratio was calculated as the product of mean individual seed mass and the total number of seeds divided by cone mass. In these analyses and others comparing sites, we used the total number of seeds per cone instead of the number of full seeds because the number of full seeds is influenced by pollen availability (e.g. Smith *et al.*, 1988). A seed mass to cone mass ratio based on the total number of seeds therefore is more likely to reflect an evolutionary response to seed predation. We used principal components (PC) analysis based on eight cone traits [cone length, cone width, cone mass, number of scales, scale thickness (mean of distal and proximal scales), scale length, total number of seeds and seed mass] to display variation among regions sampled in a multivariate context and to guide subsequent comparisons. The mean values of cone traits measured for each tree were used in analyses because trees were the experimental units.

Studies of *Sciurus* show that they typically exert selection favouring fewer seeds per cone (Molinari *et al.*, 2006; Parchman & Benkman, 2008) and sometimes a smaller ratio of seed mass to cone mass (Parchman & Benkman, 2008). However, black pine cones approximate the size where *Sciurus* may favour the evolution of either smaller or larger cones (Mezquida & Benkman, 2005; Parchman & Benkman, 2008). If selection by red squirrels is important, we predict that the amount of seeds (number of full seeds and total number of seeds) and the ratio of seed mass to cone mass should be higher in areas where red squirrels are absent. We used nested ANOVA to test for such differences between areas with and without red squirrels, limiting comparisons to within subspecies of black pine (*P. n. salzmannii*: continental Italy vs. Corsica and Sicily; *P. n. nigra*: continental Greece vs. Thassos, Lesvos and Cyprus).

The form of phenotypic selection exerted by crossbills

To estimate the form of phenotypic selection exerted by crossbills on black pine, 65 mature black pines were sampled haphazardly in the Troodos Mountains, Cyprus (Fig. 1) between 10 and 16 January 2006 under the constraint that cones could be reached and cut with a 9-m extendable pruning tool. For each tree, we counted the number of cones on the ground that had been foraged on by crossbills (mean = 18.9; range = 0–160) as well the total number of cones remaining on the tree (mean = 163.8; range = 60–350). Cones preyed on by crossbills were easy to enumerate because foraging crossbills removed cones from branches, shredded and peeled apart scales to access seeds, and then dropped the cones to the ground. Assuming tree fitness is proportional to per cent of cones not preyed on by crossbills, relative fitness for a given tree was estimated as per cent un-preyed on cones divided by overall mean per cent for all trees. We measured the same suite of cone traits described above for two cones from each tree.

We used least squares linear regressions between relative tree fitness and various cone traits of each tree including the first two PC of eight traits, each standardized to zero mean and unit variance, to estimate regression coefficients and to determine which traits were under selection because of predation by crossbills (Lande & Arnold, 1983). We used the regression bootstrap technique of Efron & Tibshirani (1993) to test for significance of regression coefficients and based these tests on 1000 replicates. We checked for nonlinear selection in all traits by examining regression models with quadratic terms, and by using cubic splines (Schluter, 1988) to visualize the form of selection.

To further analyse the form of selection exerted by crossbills and to examine the consequences for crossbills of geographic variation in black pine, we used foraging experiments with captive crossbills. We used call type 9 crossbills (Benkman, 1999) captured in Idaho. They are smaller than European crossbills and we discuss the potential impact on the results of this size difference in the Discussion. Crossbills were housed in six aviaries (3 m × 1 m × 2 m) each containing seven to 10 crossbills at the University of Wyoming. In the afternoon prior to trials, other food sources were removed, but grit, charcoal and water were available. The following morning we placed four cones in each aviary and after two hours recorded the per cent of each cone that had been foraged on (0%, 25%, 50%, 75% or 100%). Cones were from trees used in analyses to characterize geographic variation in cone traits, so that mean values for all measured cone traits could be estimated based on the tree means. We randomly selected cones from our samples of *P. n. salzmannii* (mainland Italy, Sicily and Corsica), and used a minimum total of two cones from each tree. To determine which cone traits were associated with variation in foraging propensity, we used univariate least square regression analyses based on the relationship between relative tree fitness (mean per cent of cones not preyed upon for a given tree divided by the overall mean value for all trees) and the individual traits, each standardized to zero mean and unit variance.

To visually examine selection exerted by crossbills on black pine, we used a nonparametric multivariate cubic spline procedure based on the relationship between relative tree fitness estimated both in the wild and in foraging experiments and the first two PC derived from the trees that were used in the analyses to characterize cone variation. We produced these surfaces by running projection pursuit approximation with λ corresponding to the lowest Generalized Cross-Validation (GCV) Score (Schluter & Nychka, 1994), where the GCV Score is a metric of the prediction error associated with a particular value of λ (Schluter, 1988). We then drew the surfaces using a running average smoothing function in Sigma Plot (Systat Inc., Evanston, IL, USA). We used these surfaces to qualitatively depict how crossbill feeding rates and tree fitness resulting from crossbill seed predation vary as a result of cone morphological variation.

Results

Geographic variation in the cone traits

Relative to cones from continental Greece, cones from Cyprus had large PC1 scores [loaded positively by cone, scale and seed sizes (Table 1); nested ANOVA: $F_{1,4} = 54.73$, $P < 0.0001$], whereas cones from the Greek islands (Lesvos and Thassos) had small PC1 scores (Figs 1 and 2a; $F_{1,5} = 35.71$, $P < 0.0001$). The traits that were especially large on Cyprus relative to continental Greece were overall cone size (length and mass), scale size (thickness and length) and seed mass (Table 2; $F_{1,4} \geq 10.62$, $P < 0.002$ for all five comparisons). We did not sample black pine from mainland Turkey (Fig. 1), however, other studies indicate that seed and cone sizes there [mean seed mass = 20.0 mg, mean cone mass = 13.6 g (Kaya & Temerit, 1993; see also Turna

Table 1 Loadings of eight black pine (*Pinus nigra*) cone traits on the first two principal components of the principal components (PC) analysis.

Cone trait	PC1	PC2
Cone length	0.426	0.044
Cone width	0.419	0.060
Cone mass	0.451	0.021
No. scales	0.241	0.513
Scale thickness	0.320	−0.298
Scale length	0.371	−0.185
No. seeds	0.171	0.678
Seed mass	0.335	−0.384
Variation explained (%)	56.7	16.9
Eigenvalues	4.53	1.35

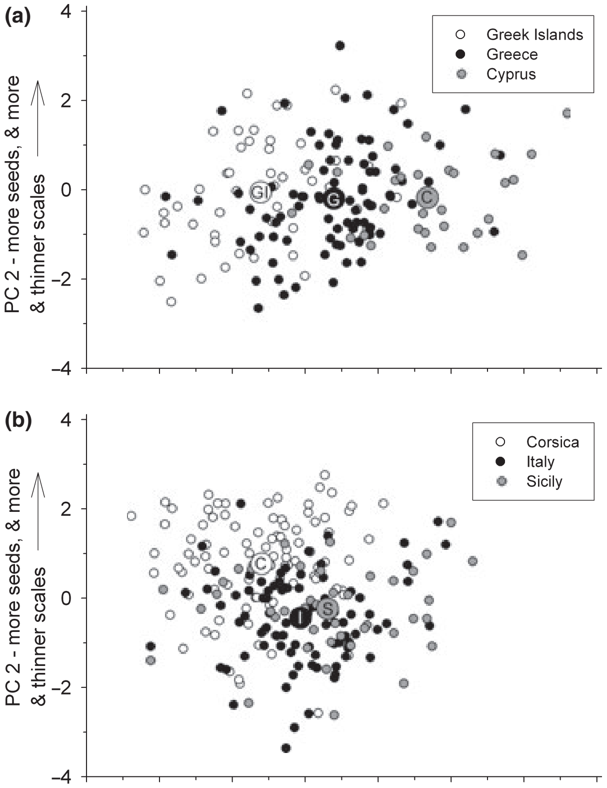

Fig. 2 Variation in black pine cone structure in relation to the first two principal components from an analysis of eight cone traits. (a) Variation in cones among Greek islands (Lesvos and Thassos; crossbills rare and red squirrels absent), continental Greece (Pindos, Mt. Chelmos and Halkidiki; crossbills and red squirrels present) and Cyprus (an endemic crossbill is abundant and red squirrels are absent). (b) Variation in cones between Corsica and Sicily (crossbills are resident and red squirrels absent) and continental Italy (Apennines and Calabria; crossbills and red squirrels present). The larger symbols represent the centroids for each of the regions.

Table 2 Mean cone measurements for black pine.

	Continental Italy		Continental Greece			Mediterranean Islands				
Measurement	Apennines	Calabria	Pindos	Chelmos	Halkidiki	Corsica	Sicily	Thassos	Lesvos	Cyprus
Cone length, mm	61.1	63.5	66.1	65.4	61.2	60.0	64.4	58.4	57.0	77.3
Cone width, mm	30.1	31.6	34.4	33.1	31.1	30.1	32.9	30.0	29.3	35.6
Cone mass, g	12.9	14.2	17.5	15.3	14.1	11.4	15.2	13.4	12.1	22.8
No. scales	11.6	11.3	12.0	12.6	12.1	11.9	11.8	11.7	11.6	12.8
Scale thickness, mm	3.35	3.20	3.57	3.23	3.29	2.85	3.31	3.17	2.96	3.65
Scale length, mm	17.4	18.2	18.8	17.3	17.3	16.9	18.2	17.7	15.5	20.9
No. full seeds	26.8	26.7	37.7	23.6	20.1	36.1	32.5	27.3	27.8	30.1
No. empty seeds	20.1	18.7	19.9	21.6	20.4	18.9	18.6	17.1	18.9	26.5
Total no. seeds	46.9	45.4	57.6	45.2	40.5	55.0	51.0	44.3	46.7	56.6
Seed mass, mg	18.3	19.6	20.9	21.6	20.3	16.4	21.1	18.2	17.7	26.3
Seed mass/cone mass	0.068	0.062	0.069	0.064	0.057	0.079	0.072	0.061	0.066	0.066
No. trees sampled	29	58	36	20	29	95	42	18	31	30
No. sites sampled	2	3	2	1	2	5	2	1	1	1

Scale thickness is the mean of the distal and proximal scale thicknesses. Red squirrels (*Sciurus vulgaris*) are present at all the continental sites, but absent from the islands. Common crossbills (*Loxia curvirostra*) are found in all areas but occur only rarely on Thassos and Lesvos, and are resident on Corsica, Sicily and Cyprus.

et al., 2006)] are much smaller than on Cyprus and in the range of our continental sites (Table 2).

In contrast to Cyprus, cones from Corsica had smaller PC1 values than cones from the corresponding continental Italy (nested ANOVA: $F_{1,8} = 19.02$, $P < 0.0001$) and larger values of PC2 (more seeds with more and thinner scales; Figs 1 and 2b; Table 2; PC2: $F_{1,8} = 63.14$, $P < 0.0001$). As in continental Greece, PC1 scores varied among sites in continental Italy and on average their scores were smaller than in continental Greece (Fig. 1). Sicily, which lacks red squirrels, had relatively large cones compared with continental Italy (PC1: $F_{1,5} = 5.13$, $P = 0.025$) and to the Greek islands (Thassos and Lesvos) also lacking red squirrels (Fig. 1; Table 2; $F_{1,2} = 17.42$, $P < 0.0001$).

We found that the number of seeds (full and total) and the ratio of seed mass to cone mass were significantly greater on Corsica and Sicily in the absence of red squirrels than in continental Italy (nested ANOVA s: $F_{1,10} > 14.45$, $P < 0.0002$) as expected if red squirrels were important selective agents. However, we did not detect differences in these traits between continental Greece and Thassos, Lesvos and Cyprus ($F_{1,6} < 0.43$, $P > 0.50$; excluding Cyprus similar results hold: $F_{1,5} < 1.44$, $P > 0.23$).

The form of phenotypic selection exerted by crossbills

Crossbills preferentially foraged on trees having smaller cones with smaller seeds and fewer, shorter and thinner scales resulting in selection favouring the opposite values of these traits (Table 3). Each univariate selection gradient estimated in the wild and from foraging experiments with captive crossbills was in the same direction and the two sets of selection gradients were highly correlated ($r = 0.81$, $P < 0.001$; Table 3). We detected evidence of nonlinear selection on cone length and mass, and the number of scales in the wild, and for seed mass in the foraging experiments (quadratic terms: $P = 0.02$–0.04), but in all cases cubic splines showed that selection was directional rather than stabilizing (not shown). Scale thickness was detected as experiencing selection only in the experiments, perhaps because cones in these experiments represent a wider range of phenotypic variation than that existing in the single natural population, which should facilitate the detection of selection (Schluter, 1988). Why selection was detected on the number of scales and the number of empty seeds in the wild but not in the experiments is less clear (Table 3). There was a strong positive relationship between PC1 and relative tree fitness, whereas PC2 was unrelated to relative tree fitness (Table 3). Fitness surfaces produced from the two measurements of selection were nearly identical, except for the greater range of relative fitness values for the captive studies than in the wild, and further illustrate that selection exerted by crossbills favours increases in PC1 (Fig. 3). Taken together, these analyses suggest that selection from crossbills should favour the evolution of larger cones with larger scales and seeds, and that PC1 can be viewed as a composite trait representing the extent of seed defenses against crossbills.

Nearly all the traits detected as being under selection by crossbills (Table 3) differed on Cyprus in the direction predicted if crossbills were important selective agents driving cone evolution (Fig. 3; Table 2). In addition, the selection gradients in the wild (Table 3) are good predictors of the differences between cones from Cyprus and continental Greece (examining only

Table 3 Estimates of univariate selection gradients using regression analyses between relative tree fitness and cone traits of black pine in the Troodos Mountains, Cyprus, and from experiments in aviaries.

	Wild		Aviary	
Cone trait	β' ± SE	*P*-value	β' ± SE	*P*-value
Cone length	**0.095 ± 0.019**	**< 0.001**	**0.322 ± 0.073**	**< 0.001**
Cone width	**0.078 ± 0.020**	**< 0.001**	**0.260 ± 0.075**	**< 0.001**
Cone mass	**0.094 ± 0.019**	**< 0.001**	**0.285 ± 0.074**	**< 0.001**
No. scales	**0.082 ± 0.020**	**< 0.001**	0.072 ± 0.078	0.359
Distal scale thickness	0.023 ± 0.022	0.313	**0.259 ± 0.075**	**< 0.001**
Proximal scale thickness	0.016 ± 0.023	0.479	**0.217 ± 0.077**	**0.005**
Scale length	**0.054 ± 0.021**	**0.014**	**0.273 ± 0.075**	**< 0.001**
No. full seeds	−0.011 ± 0.022	0.621	−0.021 ± 0.079	0.787
No. empty seeds	**0.053 ± 0.021**	**0.018**	0.051 ± 0.079	0.514
Total no. seeds	0.028 ± 0.022	0.219	0.017 ± 0.079	0.835
Individual seed mass	**0.064 ± 0.021**	**0.003**	**0.285 ± 0.074**	**< 0.001**
Seed mass/cone mass	**−0.064 ± 0.021**	**0.003**	**−0.159 ± 0.077**	**0.042**
PC1	**0.038 ± 0.009**	**< 0.001**	**0.146 ± 0.047**	**< 0.001**
PC2	−0.033 ± 0.019	0.087	−0.004 ± 0.058	0.951

Thousand bootstrap replicates were used to establish the significance of β'. Significant parameter estimates are in bold.

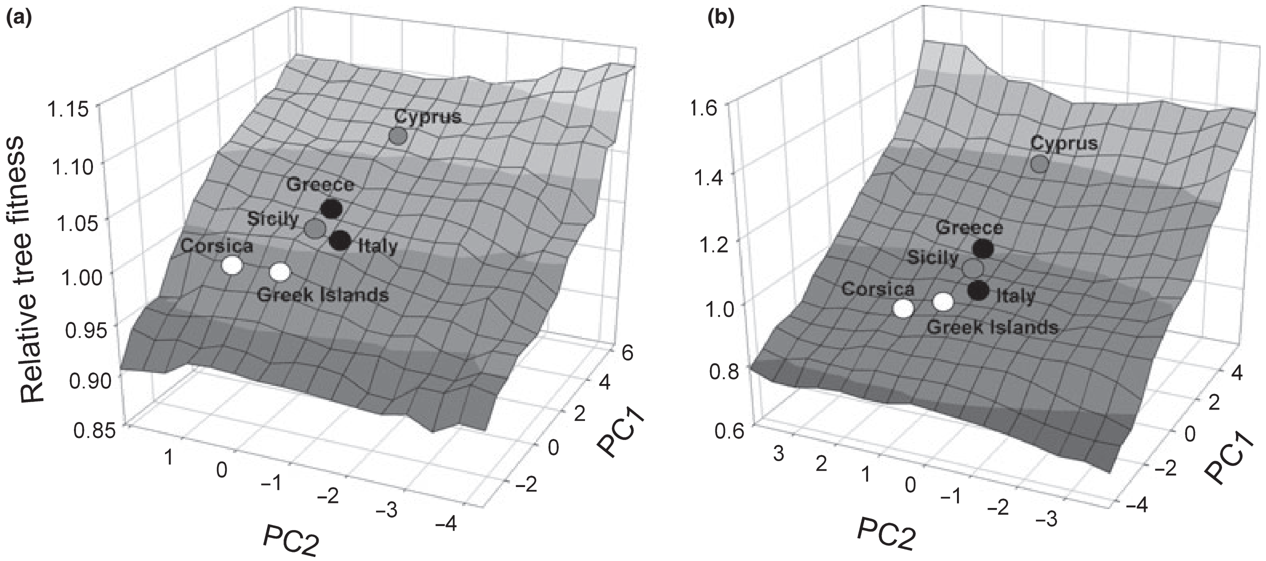

Fig. 3 Fitness surfaces generated from projection pursuit approximation for relative tree fitness in Cyprus (a) and in aviary experiments (b) in relation to the first two principal components (PC) representing eight cone traits. PC1 increases with increasing cone size and larger scales, and PC2 increases with cones having more seeds and more and thinner scales. The mean values of the first two PC for the different study regions in Fig. 2 are plotted on the surface. Note the different ranges of relative tree fitness in (a) and (b).

statistically significant selection gradients: ln (trait difference/SD of trait on Greece) = −1.1 + 14.7 (selection gradient), $r^2 = 0.69$, $F_{1,6} = 13.43$, $P = 0.01$; if all traits considered: $r^2 = 0.78$, $F_{1,10} = 31.32$, $P = 0.0003$). In contrast, cones from Corsica differed in the opposite direction from that expected if crossbills have been important selective agents (Fig. 3; Table 2). For the other islands (all lacking red squirrels), the PC1 values were negative (Lesvos and Thassos) except for Sicily (Fig. 1). Although crossbills are rare on Lesvos and Thassos, crossbills are common on Sicily (Massa, 1987). The relatively high PC1 values on Sicily suggest that cones here have evolved in response to selection exerted by crossbills. Crossbill abundances are known to be higher in larger areas of forest (Siepielski & Benkman, 2005), and variation in PC1 values among continental sites is consistent with variation in the area of black pine in the region (Fig. 1). The highest PC1 values occur in the Pindos Mountains where there are extensive areas of black pine, whereas PC1 values are low in the smallest and most isolated areas of black pine (Apennines, Calabria and Halkidiki; Fig. 1).

Discussion

The geographic variation in the structure of bills and cones indicate that coevolution has driven the divergence of crossbills and black pine on Cyprus, but has not played such a role on Corsica. Similarly, geographic variation in the cone traits of black pine indicates that red squirrels have had an inconsistent selective impact on black pine, whereas the evolutionary impact of crossbills increases with forest area on the continent. Below we discuss these findings in light of previous studies on the influence of competitors, forest area, and resource stability on coevolution and the evolution of crossbill diversity.

The form of phenotypic selection exerted by crossbills

The overall form of phenotypic selection exerted by crossbills on black pine in the wild and during foraging experiments was strikingly similar (Fig. 3). These analyses indicate that selection exerted by crossbills favoured increases in cone and scale sizes (Table 3), consistent with other studies of crossbills and conifers (Summers & Proctor, 1999; Benkman *et al.*, 2001, 2003; Parchman & Benkman, 2002, 2008; Parchman *et al.*, 2007; Clouet & Joachim, 2008). Selection favoured increases in PC1 (Table 3; Fig. 3), which therefore can be viewed as a composite variable reflecting overall crossbill defense. The similarity between our estimates of selection in the wild and in aviaries along with the consistent evolutionary divergence of these traits in our studies (Benkman *et al.*, 2001, 2003; Parchman & Benkman, 2002, 2008; Mezquida & Benkman, 2005; Edelaar & Benkman, 2006; Parchman *et al.*, 2007) suggests that environmentally induced variation (e.g. Scheiner *et al.*, 2002; Stinchcombe *et al.*, 2002; Kruuk *et al.*, 2003) has neither confounded our estimates of the form of selection in the wild nor interfered with our ability to predict evolutionary responses. The former is consistent with the foraging behaviour of crossbills (Benkman, 1987b).

One difference between our two sets of analyses was the greater range of relative fitness values for crossbills in captivity than for crossbills in the wild (Fig. 3). This difference likely reflects the different crossbills used in the two analyses. Captive crossbills had bill depths that averaged < 10 mm whereas those on Cyprus average over 12 mm (Massa, 1987). Smaller billed crossbills will be more challenged by the large cones of black pine so that variation among cones will lead to greater variation in feeding rates and greater avoidance of the most difficult cones than found in large-billed crossbills presumably adapted to the average (and large) cones on Cyprus (see Benkman, 1987a, 1993a).

The importance of forest area

Cone structure varied considerably among the mainland sites (Fig. 1; Table 1). A possible factor contributing to this variation is variation in the abundance of crossbills. We do not have measures of crossbill abundance in the different mainland areas, however in earlier studies we have found higher densities of crossbills, higher levels of seed predation by crossbills, and higher levels of seed defenses directed at crossbills with increases in area of pine forest. Crossbill population densities increase with the size of isolated forest islands of lodgepole pine, which influences the strength of selection exerted by crossbills as indicated by the positive correlation between crossbill densities and the extent of seed defenses (Siepielski & Benkman, 2005). A similar pattern is found on the Iberian Peninsula, where mountain pine (*P. uncinata*) has evolved greater seed defenses in response to selection exerted by crossbills in the extensive forests of the Pyrenees where seed predation levels are higher than in two small isolated forests (E.T. Mezquida & C.W. Benkman, unpublished data). Black pine forests are largest and most extensive in the Pindos Mountains of Greece where cone structure is consistent with enhanced defenses evolved in response to seed predation by crossbills (Fig. 1). In contrast, cones appear relatively poorly defended against crossbills in the smaller and much less extensive forests of black pine in the Apennine Mountains, Calabria and Halkidiki Peninsula (Fig. 1). Cones are even more poorly defended against crossbills in the very small isolated islands of Lesvos and Thassos (Fig. 1) where crossbills are rare.

Have red squirrels influenced geographic variation in the cone traits of black pine?

Studies of *Sciurus* show that they forage preferentially on trees with cones providing greater feeding rates (Moller, 1986; Steele & Weigl, 1992), and often exert selection favouring fewer seeds per cone (Moller, 1986; Molinari *et al.*, 2006; Parchman & Benkman, 2008) and sometimes a smaller ratio of seed mass to cone mass (Parchman & Benkman, 2008). However, geographic patterns of cone variation in our study suggest that red squirrels are not uniformly important selective agents on black pine. In the absence of red squirrels, black pine appears to have lost squirrel defenses on Corsica and Sicily but not on Thassos and Lesvos. Likewise, the patterns of selection have not been consistent among studies on *Sciurus* (e.g. Molinari *et al.*, 2006; Parchman & Benkman, 2008). Some of this variation appears related to cone size (Mezquida & Benkman, 2005; Parchman & Benkman, 2008) where *Sciurus* prefer smaller cones when average cone size is large but prefer larger cones when average cone size is small. For pines, cone lengths around 70–80 mm appear to be the threshold where preferences shift (Mezquida & Benkman, 2005; Parchman & Benkman, 2008); black pine cones average over 60 mm

(Table 2). In the case of Aleppo pine in Spain, which averages around 76 mm long, selection was not detected on either the number of seeds per cone or the ratio of seed mass to cone mass (Mezquida & Benkman, 2005). Other variation may be related to stand densities, where at lower stand densities the high risk of predation experienced by tree squirrels may be a more important determinant of tree preferences than cone traits (E.T. Mezquida & C.W. Benkman, unpublished data).

Although we could not detect a consistent evolutionary effect from red squirrels, they appear to have a competitive effect that limits crossbill abundance and thereby influences cone evolution. As an extreme case, crossbill population densities are about 20 times higher in areas of lodgepole pine lacking *Tamiasciurus* than in even larger areas with *Tamiasciurus* (Benkman, 1999; Siepielski & Benkman, 2005). Similarly, the absence of red squirrels on islands with abundant pine forests in the Mediterranean has allowed crossbills to become more abundant and for the interactions between crossbills and pines to have stronger evolutionary consequences (Mezquida & Benkman, 2005; this study). We suspect that the elevated PC1 Score on Sicily relative to that of nearby Calabria (Fig. 1) is related to the absence of red squirrels. However, the close proximity of Sicily to continental forests (Fig. 1) increases the opportunity for gene flow preventing divergence of especially the crossbills on Sicily.

Has black pine coevolved with crossbills on the islands?

Geographic variation in cone traits is consistent with the hypothesis that black pine on Cyprus has evolved defenses against crossbills. Cyprus black pine shows differentiation in nearly every trait on which selection by crossbills was detected (Tables 2 and 3), consistent with an evolutionary response to crossbill predation. Cones from Cyprus were larger, had larger scales and larger PC1 scores than any other area (Table 2; Fig. 1). In experiments, crossbills were least likely to forage on cones characteristic of Cyprus (Fig. 3). Based on other studies of the relationship between crossbill feeding performance and the morphologies of crossbills and conifer cones (Benkman, 1987a, 1993a, 1999; Benkman *et al.*, 2001, 2003; Parchman & Benkman, 2002), the larger cones with larger scales on Cyprus should cause selection favouring larger, deeper billed crossbills. Indeed, the Cyprus crossbill has evolved a substantially larger more strongly decurved bill than other crossbills in the region (Knox, 1976; Massa, 1987; Clouet, 2003) consistent with the hypothesis that crossbills have evolved in response to elevated seed defenses on Cyprus. This is also consistent with a predator-prey arms race driving the divergence of crossbills and pines on Cyprus, and similar to that documented for crossbills in lodgepole pine forest islands (Benkman, 1999; Benkman *et al.*, 2001, 2003; Siepielski & Benkman, 2005; Edelaar & Benkman, 2006), on the islands of Newfoundland (Parchman & Benkman, 2002) and Hispaniola (Parchman *et al.*, 2007), and in the Pyrenees (E.T. Mezquida & C.W. Benkman, unpublished data). Moreover, the Cyprus crossbill is the only island subspecies in the Mediterranean with distinct flight calls (Summers & Jardine, 2005). Because vocalizations are an important aspect of reproductive isolation in crossbills (Snowberg & Benkman, 2007), this suggests that coevolution is driving crossbill speciation on Cyprus, and along with previous studies, points to the potential of coevolution to cause speciation (Smith & Benkman, 2007).

In contrast, the cone morphology of black pine on Corsica is not consistent with coevolution driving divergence (Fig. 3). Cones on Corsica have the second lowest PC1 value of any region (Fig. 1), and cone traits have diverged in the direction opposite of that predicted if crossbills have had an important selective impact. In foraging experiments, crossbills also had among the highest feeding propensities on cones with morphologies typical of black pine from Corsica (Fig. 3) implying reduced crossbill defenses. In addition, the bill size of the Corsican crossbill is similar to crossbills occurring in mainland areas and crossbills on Corsica may not represent a distinct form (Massa, 1987; see also Robb, 2000; Clouet, 2003). However, the Corsican crossbill has a less decurved upper mandible than other crossbills in the Mediterranean (Clouet, 2003), which may reflect local adaptation to the thin-scaled cones on Corsica (Table 2).

The lack of divergence in the predicted direction of crossbill and cone traits on Corsica is surprising and suggests that coevolution has been limited in this interaction. Although black pine has been thought to occur on Corsica for a long time (Raffi *et al.*, 1996), this is uncertain. For instance, the pollen record suggests that black pine was not present on Corsica during the last glacial and that its arrival on the island did not occur until around 10 000 BP cal. yr (Reille *et al.*, 1997). However, coalescent analyses of cpDNA indicate that Corsican populations have diverged from those on the Italian mainland for over 50 000 years (Afzal-Rafii & Dodd, 2007). Regardless, 10 000 years has been sufficient for predator-prey coevolution to drive divergence in other isolated crossbills (Benkman *et al.*, 2001; Parchman & Benkman, 2002). One possibility is that black pine had a very restricted distribution until recently that prevented many crossbills from residing on Corsica. This explanation is doubtful however based on the fossil record of Corsican black pine, which indicates that it was much more widespread in the past, and the occurrence of an endemic species of nuthatch (*Sitta whiteheadi*) that relies on black pine (J.C. Thibault, personal communication).

Alternatively, seeds are scarcer for longer periods for Corsican than Cyprus crossbills, which would lower crossbill abundance and their selective impact on black pine. Unlike other conifers on islands supporting endemic crossbills (e.g. *Picea mariana* and *Pinus halepensis*), black pine does not hold seeds in its cones for extended periods. Crossbills require alternative seed sources after black pine seeds have been shed from cones in May (Skordilis & Thanos, 1997; C. Thanos and J. C. Thibault, personal communication) and before seeds become available in developing cones in July or August. Cyprus, although having only about one-fourth the area of black pine as Corsica (50 and 213 km^2 of black pine respectively; Hatzikyriakou & Christou, 2003; Thibault *et al.*, 2006), has extensive forests of Turkish pine (*Pinus brutia*: 1600 km^2; Hatzikyriakou & Christou, 2003) at and below the lower elevations of black pine. Although cones of Turkish pine are so robust that crossbills are likely unable to access seeds in the closed serotinous cones, many cones open in May and June (Skordilis & Thanos, 1997) and would provide an alternative seed resource for crossbills when black pine seeds are unavailable. In contrast, Corsica lacks Turkish pine. The one pine that might provide sufficient seeds during the early summer is maritime pine (*Pinus pinaster*), but it has become common in Corsica only recently having increased in the past several hundred years because of human-caused fires (Carcaillet *et al.*, 1997; J. C. Thibault, personal communication). Crossbills feed at least occasionally on maritime pine seeds (J.C. Thibault, personal communication), which may help crossbills persist on Corsica and result in a stronger interaction between crossbills and black pine. However, it may be centuries before an evolutionary effect from this interaction is detectable.

This illustrates the importance of conifers or groups of conifers that hold seeds for extended periods for local adaptation and crossbill–conifer coevolution on islands (Benkman, 1989, 1999). The absence of tree squirrels may also be important because selection exerted by tree squirrels appears to favour cone-crop fluctuations (Benkman *et al.*, 2003; see also Kelly & Sork, 2002). Greater stability of seed availability within and among years and reduced competition in the absence of tree squirrels presumably enables crossbills to be more abundant and exert stronger selection (Benkman *et al.*, 2001, 2003; Parchman & Benkman, 2008). Where cone crops fluctuate and seeds are seasonally scarce, crossbills are nomadic so that the strength of the crossbill–conifer interaction depends on the extent of forests. The more extensive the forests the more likely crossbills will be able to locate large cone crops (Benkman, 1993b). Larger forests should therefore permit higher abundances of crossbills and lead to stronger crossbill–pine interactions, which is what we find with mountain pine in Spain (E.T. Mezquida & C.W. Benkman, unpublished data) and may account for much of the cone variation in black pine cones in Italy and Greece (Fig. 1).

Acknowledgments

National Science Foundation grants (DEB-0212271 and DEB-0344503) to CWB, and an Environmental Protection Agency GRO Fellowship to TLP supported this work. We thank J. C. Thibault and C. Thanos for answering numerous queries and facilitating research in Corsica and Greece, respectively, M. Silva for help collecting cones, K. Larsen for measuring cones and M. Seymour for help with Fig. 1. We thank K. Roselaar for providing data on bill sizes of crossbills in Turkey and Cyprus. A. Caballero, P. Edelaar, A. Knox, J. C. Thibault and an anonymous reviewer provided helpful comments on the manuscript. J. C. Thibault deserves special thanks for suggesting the explanation for why the crossbill–pine interaction differs between Cyprus and Corsica.

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Appendix 1

Locations, sample sizes and dates where black pine cones were sampled.

Region	Site	Latitude (N)	Longitude (E)	No. trees	Date
Appenines	Falterano	43°47′58″	11°40′14″	15	January 2005
	Tajkep	42°26′30″	13°35′14″	14	January 2005
Calabria	Sila Grande N	39°40′22	16°67′72″	19	December 2004
	Sila Grande W	39°24′07″	16°58′38″	20	December 2004
	Sila Grande S	39°36′40″	16°70′47″	19	December 2004
Pindos	Pertouli	39°31′49″	23°28′02″	16	January 2006
	Katara	39°42′00″	21°16′59″	20	January 2006
Mt. Chelmos	Rizanorema	37°57′30″	21°16′26″	20	January 2006
Halkidiki	Flogita	40°26′59″	23°21′59″	15	January 2006
	Halkidiki	40°24′27″	23°29′35″	14	January 2006
Corsica	Casamaccioli	42°19′06″	9°02′67″	19	January 2005
	Valdu Niellu	42°01′32″	9°56′11″	21	January 2005
	La Foci	42°06′54″	9°07′06″	17	January 2005
	Restonica	42°17′30″	9°08′09″	20	January 2005
	Col de Vergio	42°17′96″	9°22′02″	18	January 2005
Sicily	Etna Nord	37°47′40″	15°03′03″	22	December 2004
	Adrano	37°44′06″	14°58′53″	20	December 2004
Thassos	Touba	40°39′10″	24°38′57″	18	January 2006
Lesvos	Agiasos	39°05′19″	26°17′47″	31	January 2006
Cyprus	Troodos	34°56′53″	32°50′39″	30	January 2006

Received 1 September 2008; revised 29 December 2008; accepted 11 January 2009