

Sex-ratio variation and spatial distribution of nuclear and cytoplasmic sex-determining genes in gynodioecious *Thymus praecox* across altitudinal gradients

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ABSTRACT

Background: Females and hermaphrodites co-exist in populations of gynodioecious plant species. Gynodioecy often depends on persistent polymorphisms for both cytoplasmic male-sterility (CMS) genes and nuclear restorers of male fertility.

Questions: How do ecological gradients affect population sex ratio? Is sex-ratio variation attributable to variation in the nuclear-cytoplasmic genetic composition of populations? Is there spatial covariation between CMS types and nuclear restorer alleles among populations?

Study system: Late-successional populations of gynodioecious *Thymus praecox*, a species widespread in the European Alps and distributed from subalpine to alpine altitudes.

Methods: We surveyed sex-ratio variation of adults along altitudinal gradients. We used offspring sex ratios from open pollination in natural populations and from controlled crosses within and among populations to estimate the diversity and spatial distribution of sex-determining alleles.

Results: The proportion of hermaphrodites decreased with increasing altitude. However, offspring sex ratios were constant across altitudes, indicating similar cytonuclear genetic diversity at contrasting adult sex ratios. Sex-determining alleles were geographically widespread, but significantly higher proportions of hermaphrodites produced by within-population crosses compared with those among populations indicated locally adjusted restorer frequencies. Balancing selection may act on sex-determining genes. Our findings further point to a role for environment-dependent selection via the relative maternal fitness of females and hermaphrodites in maintaining the altitudinal sex-ratio variation.

Keywords: cost of restoration, cytoplasmic male sterility, ecological gradient, gynodioecy, local adaptation, nuclear–cytoplasmic interactions, offspring sex ratio.

INTRODUCTION

Epistatic interactions between genes located in the cytoplasm and genes in the nucleus determine whether an individual is female or hermaphrodite in many gynodioecious plant species (Charlesworth and Laporte, 1998; Jacobs and Wade, 2003). In such species, the maintenance of gynodioecy requires persistent polymorphisms for both cytoplasmic and nuclear sex-determining genes (Charlesworth, 1981; Bailey *et al.*, 2003). Understanding the evolutionary dynamics of sex-determining genes is therefore basic to understanding the co-existence of female and hermaphrodite plants within gynodioecious populations (Frank and Barr, 2001; Charlesworth, 2002; Jacobs and Wade, 2003; Bailey and Delph, 2007a).

Cytoplasmic male-sterility (CMS) genes are mitochondrial mutants and, as such, predominantly maternally inherited (Couvet *et al.*, 1990; McCauley *et al.*, 2005). Several CMS types are usually found within natural populations (de Haan *et al.*, 1997c; Charlesworth and Laporte, 1998; Dudle *et al.*, 2001; Taylor *et al.*, 2001; van Damme *et al.*, 2004). Bi-parentally inherited nuclear genes act to restore male fertility when matching a given CMS type (Hanson and Bentolila, 2004). Hermaphrodites in gynodioecious populations are typically restored ones also carrying a CMS type, as inferred from the commonly observed segregation of females in seed-offspring of hermaphrodite mothers (Gouyon and Couvet, 1985; Charlesworth and Laporte, 1998; Dudle *et al.*, 2001; van Damme *et al.*, 2004). It appears that multiple restorer loci act epistatically or independently to restore particular CMS types (Koelewijn and van Damme, 1995; de Haan *et al.*, 1997a; Charlesworth and Laporte, 1998). Restoration may therefore best be viewed as a quantitative trait, with CMS types being restored when combined with sets comprising a sufficient number of appropriate restorers (Ehlers *et al.*, 2005; Bailey and Delph, 2007b). The presence of appropriate restorer alleles can be estimated from offspring sex ratios (Manicacci *et al.*, 1997; Taylor *et al.*, 2001; Koelewijn, 2003; Byers *et al.*, 2005). Theory predicts negative pleiotropic fitness effects of fertility restorers; without a cost of restoration, restorer alleles are expected to be driven to fixation (Charlesworth, 1981; Bailey *et al.*, 2003).

Several evolutionary forces may act simultaneously upon the complex nuclear-cytoplasmic sex system, hence sex-ratio variation among gynodioecious populations can have multiple causes (Frank and Barr, 2001; Charlesworth, 2002; Jacobs and Wade, 2003; Bailey and Delph, 2007a). In the following, we outline how sex-ratio data may provide insights into the relative importance of selection, stochasticity, and mutation for population sex-ratio variation.

Negative frequency-dependent selection (advantage of rare alleles) at sex-determining loci may cause cycling of different CMS types and their nuclear restorers if the cost of restoration is dominant (Gouyon *et al.*, 1991; Bailey *et al.*, 2003; Delph *et al.*, 2007). Sex-ratio variation among populations may then simply reflect different phases of a dynamic equilibrium (limit cycles) of nuclear and cytoplasmic sex-determining alleles that are largely independent of environmental conditions (Gouyon *et al.*, 1991). Empirical support for this hypothesis is however scarce (de Haan *et al.*, 1997b; Frank and Barr, 2001; Barr, 2008). In contrast, low population hermaphrodite frequencies have been found to be associated with harsh environmental conditions, such as drier sites in moisture gradients (Darwin, 1877; Alonso and Herrera, 2001; Delph, 2003; Barr, 2004b; Vaughton and Ramsey, 2004) and alpine sites in altitudinal gradients (Schrader, 1986; Delph, 1990; Alatalo and Molau, 1995; Puterbaugh *et al.*, 1997). Such patterns raise the question of how ecological context may affect population sex ratio: changes in environmental conditions may alter fitness differences between females and hermaphrodites (Delph and Carroll, 2001) or they may trigger stochastic processes influencing the cytonuclear composition of populations (Bailey and Delph, 2007a).

Stochasticity associated with the history, spatial isolation or size of populations may govern sex-ratio variation by affecting the diversity of sex-determining alleles or by temporarily disrupting CMS types from matching restorers (Couvet *et al.*, 1986; Cuevas *et al.*, 2006; Nilsson and Agren, 2006). In gynodioecious *Thymus vulgaris* showing patchy metapopulations with frequent extinction and recolonization events in early-successional habitats, females attain highest frequencies in the youngest patches due to a temporary lack of appropriate restorer alleles as a result of a founder effect during population establishment (Manicacci *et al.*, 1996). In *Plantago maritima*, female frequencies and their variances have been found to increase with decreasing population size (Nilsson and Agren, 2006), and in *Lobelia spicata*, offspring sex ratios have revealed reduced restoration of male fertility in smaller populations (Byers *et al.*, 2005), which has been attributed to genetic drift impairing restorer diversity more severely in small populations than in larger ones. If differences in the cytonuclear genetic composition of populations underlie sex-ratio variation, one would expect a positive correlation between the restoration ability of hermaphrodites and their frequency (Couvet *et al.*, 1986; McCauley *et al.*, 2000; Byers *et al.*, 2005).

Covariation between CMS types and restorer frequencies at different spatial scales may finally provide information on the relative importance of mutation and selection for the maintenance of the joint nuclear-cytoplasmic polymorphism. Frequent formation of new CMS types could maintain gynodioecy even if restorers of existing CMS types become fixed within populations (Frank, 1989; Charlesworth, 2002; Fénart *et al.*, 2006). As a consequence, reproductively isolated populations should accumulate different CMS types together with sets of associated restorers, and crosses among divergent populations should result in female-biased progenies because of a general mismatch between CMS types and restorers. Accordingly, high female frequencies have been reported in hybrid crosses between different colour morphs in *Nemophila menziesii* (Barr, 2004a). Alternatively, particular CMS types together with their associated restorers may be maintained for long periods of time by balancing selection (Charlesworth, 2002; Städler and Delph, 2002; Houliston and Olson, 2006), which may result from a cost of restoration (Bailey *et al.*, 2003; Charlesworth, 2006). Because costs of mismatched restorer alleles are no longer offset by the benefits of restoration (Jacobs and Wade, 2003), the frequency of particular restorer alleles would then be expected to covary with the frequency of corresponding CMS types (Bailey and McCauley, 2005). Accordingly, the frequency of an otherwise rare restorer allele increases in patches of the corresponding CMS type in *Plantago lanceolata* (van Damme, 1986). However, studies testing for increased restoration ability in within- versus among-population crosses in *T. vulgaris* (Gigord *et al.*, 1998) and *Silene vulgaris* (Bailey and McCauley, 2005) detected no evidence for locally adjusted restorer frequencies.

In this study, we explored sex-ratio variation and the cytonuclear genetic composition of late-successional populations of gynodioecious *Thymus praecox* in the Swiss Alps. We indirectly estimated the diversity and spatial distribution of cytoplasmic and nuclear sex-determining alleles from offspring sex ratios obtained from open pollination in natural populations as well as from controlled crosses within populations, among spatially close populations and among geographically remote populations to address three questions: (1) Do altitudinal gradients affect population sex ratio? (2) Is sex-ratio variation attributable to differences in cytonuclear genetic diversity among populations? (3) Is there evidence of covariation between CMS types and nuclear restorer alleles at different spatial scales?

MATERIALS AND METHODS

Study species

The gynodioecious *Thymus praecox* agg. Opiz ampl. Jalas (Lamiaceae) is widespread in the European Alps at subalpine to alpine altitudes and is found on rocky surfaces and in pastures (Jalas, 1970). It is a long-lived perennial forming carpets with numerous inflorescences and with creeping sterile shoots at the margin (Jalas, 1970). Plants flower from June to August in populations of the Swiss Alps. Thyme produces four ovules per flower. The expression of sexual phenotype is stable across years and independent of altitude as shown by common garden experiments on *T. praecox* (Landergott, 2007).

Thymus praecox is tetraploid with tetrasomic inheritance (Landergott *et al.*, 2006). A high morphological, biochemical, and genetic diversity point to a reticulate evolutionary history of this aggregate species (Jalas and Kaleva, 1970; Bischof-Deichnik *et al.*, 2000; Landergott *et al.*, 2006). Our study populations are best assigned to *T. praecox* Opiz ssp. *polytrichus* (A. Kerner ex Borbás) Jalas (Tutin *et al.*, 1972). However, as the subspecific boundaries are vague (Jalas, 1970), we simply refer to *T. praecox* in the present study.

Natural populations: altitudinal gradients

To test for ecological correlates of sex-ratio variation, we chose study areas replicated in different regions of the Swiss Alps according to the following two criteria: (1) *Thymus praecox* had to be abundant and widely distributed within study regions from subalpine to alpine altitudes; and (2) study populations within regions had to be spatially non-isolated and located in late-successional habitats (pasture, rock steppe or alpine grassland) at sites characterized by similar slopes and southern exposition. These criteria were met in the five study regions of Flimserstein (F), Langwies (L), Piora (P), Säntis (S), and Zwinglipass (Z) (see Online Appendix 1 at evolutionary-ecology.com/data/2386appendix1.pdf). The study regions of Säntis and Zwinglipass were located in two valleys separated from each other by 4 km beeline; all other regions were geographically remote from one another with the greatest distance of 90 km between Säntis and Piora. In the five study regions, we estimated population sex ratios as well as ecological variables at a total of 30 study sites during peak flowering in the years 2000 to 2002. At each site and on an area of 300–400 m², we recorded the frequency of females, hermaphrodites, and intermediate sexual phenotypes (i.e. individuals showing flowers with both sterile and fertile anthers or inflorescences with both female and hermaphrodite flowers) along 9–17 randomly placed line transects (5-m measuring tape), so that on average 85 individuals were sampled per site (see Online Appendix 1 at evolutionary-ecology.com/data/2386appendix1.pdf). For each transect, the proportion covered with thyme was recorded to estimate the density of individuals within populations. For each population, we estimated the patchiness in the spatial distribution of females and hermaphrodites by calculating the average absolute deviation of sex ratios per transect from the mean sex ratio per population (intermediates were designated hermaphrodite to calculate sex ratios). Furthermore, we classified the cover of the vegetation around each of the recorded individuals of *T. praecox* into five ranks and calculated the mean vegetation cover in the vicinity of thyme plants per study site. Finally, we recorded plant species (on average 30 per site) co-occurring with thyme along transects to calculate site means of Landolt's ecological indicator values [equivalent to Ellenberg indicator values (Diekmann,

2003)]. These values indicate plant preferences for levels of soil moisture (f), soil pH (r), soil nitrogen (n), soil humus content (h), soil structure (air and water permeability; d), light (l), temperature (t), and continentality (k) (Landolt, 1977).

Low- versus high-altitude populations

To gain insight into the cytonuclear genetic composition of populations from contrasting altitudes, we chose both a low (L) and high (H) altitudinal population of *T. praecox* from each of the five study regions F, L, P, S, and Z (abbreviation ZH thus refers to the high-altitude population from region Z) (see Online Appendix 1 at evolutionary-ecology.com/data/2386appendix1.pdf). Within each of these ten populations, 15–20 individuals displaying a minimum of five inflorescences were chosen at random, as well as their nearest neighbour of the complementary sex type, and permanently marked as focal plants. In the years 2001–2003, the local neighbourhood sex ratio of each focal plant was determined by recording the sex types of their six nearest conspecifics. Average neighbourhood sex ratios per population were calculated for focal females and hermaphrodites separately to obtain a further estimate of the patchiness of the two sex types within populations.

The average of female and hermaphrodite neighbourhoods per population also provided another overall estimate of adult sex ratios appropriate for comparison with offspring sex ratios from focal plants. To this end, mature fruits were collected from permanently marked open-pollinated focal plants. In August 2003 (populations LH, LL, PH, PL, SL, and ZL) and in April 2004 (SH and ZH), up to 66 seeds per family were sown in a greenhouse at the Botanical Garden of Zürich in plastic multi-pot plates (up to 33 seeds per 4.5-cm diameter pot containing a standard potting mixture). For an average of 12 seed-parents per sex type per population from each of the four regions L, P, S, and Z, up to five offspring were pricked out and individually grown outdoors in 6-cm diameter biodegradable pots containing a mixture of topsoil and sand (2:1). In October 2003 and July 2004 (for populations SH and ZH), 923 offspring were bedded out to an experimental field near Arosa (Maran, 1850 m above sea level; latitude: 46°47'34.0", longitude: 9°41'15.2"). In summer 2005, 97.3% of these offspring plants survived and 96% from populations LH, LL, PH, PL, SL, and ZL and 84% from populations SH and ZH flowered. On average, 53 offspring per maternal sex type per population were sexed. Intermediates were designated hermaphrodite to calculate offspring sex ratios.

Crossing experiment

To further explore the genetic diversity and spatial distribution of sex-determining genes in *T. praecox*, we set up a crossing experiment using parental individuals from the low- and high-altitude populations from the two geographically remote regions P and Z (populations PL, PH, ZL, and ZH). Within these regions, the study populations were separated from one another by 1.5–3.0 km, but the contrasting altitudes represented contrasting population sex ratios (see Online Appendix 1 at evolutionary-ecology.com/data/2386appendix1.pdf). Three crossing treatments were applied to each of 32 females (eight per population): a within-population cross (FP), a cross among altitudes within region (FA), and a cross among regions but within altitude (FR). Correspondingly, 32 hermaphrodites were used as pollen donors once per treatment. The fully balanced crossing scheme resulted in a total of

96 cross families also comprising 32 maternal half-sib and 32 paternal half-sib families (see Online Appendix 2 at evolutionary-ecology.com/data/2386appendix2.pdf), which allowed us to control for effects of dams and sires (Taylor *et al.*, 2001).

Hand pollinations were performed in summer 2003 using parental individuals that had been transplanted to a low-altitude experimental field in Zürich (Burghölzli, 460 m a.s.l., latitude: 47°21'11.7", longitude: 8°33'56.7") and to the high-altitude experimental field near Arosa (see above; Landergott, 2007). Pollinators were excluded from cross-parents by cages (30 × 30 × 15 cm) made from a wire netting frame and covered with fly screen. The cages reduced light levels to some extent, but did not affect flowering compared with uncaged inflorescences (U. Landergott, personal observation). Groups of 6–10 inflorescences per treatment were cut free prior to anthesis on females. Pollen was collected on small pieces of nylon thread and transferred to receptive stigmas. Hand pollinations were carried out every 2–3 days per cross family during the entire flowering season (Zürich: May to June; Arosa: June to July). Fruits were collected from June to July at the low- and from July to August at the high-altitude field. Mean seed set per fruit was not affected by cross treatment (U. Landergott, unpublished data).

Seeds were sorted according to the number of developed seeds per fruit (influencing seed weight) and standardized mixtures of seed classes were used for sowings. Two pots per cross family were germinated in April 2004 following the procedure described above. Germination success did not differ between the three cross treatments (U. Landergott, unpublished data). For each cross family, up to 15 offspring were pricked out and grown individually outdoors in biodegradable pots. In mid-June 2004, 794 offspring were bedded out to the low-altitude experimental field. Another 637 offspring were bedded out by the end of July 2004 to the high-altitude experimental field. Survival rate was similarly high among the three cross treatments (FP: 0.95; FA: 0.93; FR: 0.96; some offspring that were lost were replaced for sex-ratio estimates). In summer 2005, 1219 offspring from the three cross treatments flowered (FP: 89%; FA: 95%; FR: 92%). Intermediate sexual phenotypes with predominantly hermaphrodite flowers were designated hermaphrodite; those with predominantly female flowers were designated female in the calculation of family sex ratios.

Statistical analyses

We analysed sex-ratio data (proportion of hermaphrodites) by means of generalized linear models (GLMs) with a binomial error term and logit link function (Wilson and Hardy, 2002) using JMP 6.0 (SAS Institute Inc., Cary, NC, USA). Over- or under-dispersion was estimated by dividing the Pearson's χ^2 -value by the residual degrees of freedom, and the likelihood ratio χ^2 -statistics for effect tests were adjusted by rescaling. When random factors were involved, we constructed *F*-tests for affected fixed effects using the likelihood ratio χ^2 -statistics analogous to ANOVA sums of squares, with the appropriate denominator term specified according to the ANOVA method (Taylor *et al.*, 2001; Quinn and Keough, 2002). Models were checked by inspection of goodness-of-fit, by the magnitude of overdispersion, and by residual analysis (Quinn and Keough, 2002; Wilson and Hardy, 2002).

To identify ecological gradients potentially affecting population sex ratio in *T. praecox*, we performed a principal component analysis (PCA) on the 30 study sites (Legendre and Legendre, 1998). Principal components were extracted from the correlation matrix of 12 ecological descriptors (altitude, density of individuals, patchiness of sex types, vegetation cover, and

eight ecological indicator values), and the factor scores for each study site were recorded using SPSS 11.0.4 for Macintosh (SPSS Inc., Chicago, IL, USA). Axes with eigenvalues larger than 1.0 were used for further analyses. A GLM with the factor scores of the principal components as continuous predictors and region as a categorical predictor was performed to test for effects of ecological factors on population sex ratio. As explorative analyses revealed no principal component \times region interactions ($P > 0.35$ in all cases), interaction terms were omitted from the analyses.

In our low- and high-altitude study populations, we checked for non-random spatial distribution of the sex types and for variation in the extent of possible patchiness across altitudes using a GLM on the sex ratios derived from local neighbourhoods, with focal plant gender, altitude, and their interaction as fixed factors and testing the effect of altitude over the effect of population nested within altitude (F -test). A structurally analogous GLM was applied to test for a difference in sex ratios of open-pollinated offspring between altitudes (with the effects of altitude, maternal sex and their interaction, and population nested within altitude). Finally, to determine whether hermaphrodites may contribute offspring to the next generation via their female reproductive function, we tested for a difference between the sex ratios of adult populations and of the offspring of females as well as for an interaction between this difference and altitude (in a GLM also accounting for the effects of altitude and population within altitude).

The first part of the analysis of progeny sex ratios from controlled crosses aimed to test for variation in the genetic diversity of sex-determining genes among populations. We assumed the effects of dams to mainly reflect variation among CMS types and the effects of sires to reflect variation among nuclear restorers (Taylor *et al.*, 2001). Therefore, separate models were constructed including the altitudinal and regional provenance of either dams or sires. Full-factorial GLMs were first fitted with the three factors cross treatment, altitude, and region to check for significant interactions of altitude and region with cross treatment. As no such interactions were detected ($P > 0.35$ in all cases), they were excluded, and two models were fitted including dams and sires as blocking factors, respectively.

Subsequently, to test for covariation of nuclear and cytoplasmic sex-determining alleles at different spatial scales, a sequence of planned contrasts of cross treatments was performed based on the above model accounting for the effects of individual dams. First, the two among-population cross treatments (FA vs. FR) were compared to determine whether the restoration ability of sires depended on the geographic distance between populations. Depending on the outcome of this test, we contrasted the within-population crosses (FP) with either the FA-crosses or the pooled among-population crosses (FA and FR) to test for enhanced restoration ability of sires within populations. The latter analysis was complemented by pairwise Pearson correlations between sex ratios of maternal half-sib families, to determine whether the restoration of individual mothers in one cross treatment is a good predictor of their restoration in another treatment (Gigord *et al.*, 1998).

Finally, we wished to estimate the relative contribution of nuclear and cytoplasmic factors and of cytonuclear interactions to variance in sex expression in our crossing experiment. To this end, we performed a general linear model analysis on arcsine-transformed family sex ratios to estimate the variance components (REML method) of dams, sires, and their interactions (residual).

RESULTS

Adult populations

Intermediate sexual phenotypes were rarely observed in natural populations of *T. praecox* (proportion of intermediates: mean = 0.005, maximum = 0.047) (see Online Appendix 1 at evolutionary-ecology.com/data/2386appendix1.pdf). Population sex ratios (proportions of hermaphrodites) of 30 late-successional populations of *T. praecox* from five regions in the Swiss Alps ranged from 0.40 to 0.73 (see Online Appendix 1 at evolutionary-ecology.com/data/2386appendix1.pdf).

Four main principal components were extracted from the set of 12 ecological variables characterizing the 30 study sites (Table 1). PC1 represented the altitudinal gradient, with decreased average temperatures during the vegetation period and with increased soil moisture at higher compared with lower altitudes. PC2 corresponded to a gradient in resources, with less light being available at more nutrient-rich sites. PC3 corresponded to soil types, indicating that *T. praecox* grew in denser vegetation on calcareous soils than on siliceous soils. PC4 represented structural characteristics of populations of *T. praecox*, with the extent of patchiness in the spatial distribution of females and hermaphrodites being negatively correlated with the density of individuals (Table 1). None of the latter three ecological gradients significantly affected population sex ratio, and there was no significant variation among regions (Table 2). In contrast, the altitudinal gradient was a highly significant predictor of population sex ratio (Table 2): the proportion of hermaphrodites decreased with increasing altitude (Fig. 1).

Sex ratios in the local neighbourhood of females and hermaphrodites revealed significant patchiness in the distribution of the two sex types within populations (focal plant sex: $\chi^2_1 = 33.06$, $P < 0.001$). The latter GLM analysis further confirmed the significant effect of altitude on adult sex ratio (altitude: $F_{1,8} = 25.23$, $P = 0.001$) and that the extent of patchiness

Table 1. Factor loadings from principal components analysis on 12 ecological descriptors recorded in 30 late-successional populations of *Thymus praecox* from the Swiss Alps

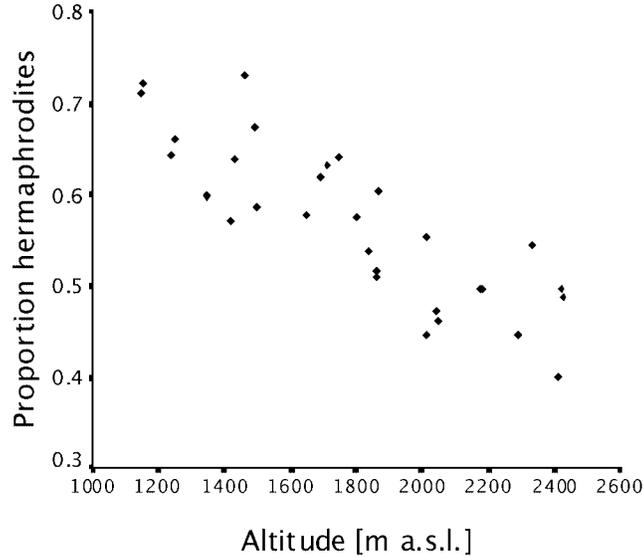
Variable	PC1	PC2	PC3	PC4
Temperature (Landolt's t)	-0.96	0.16	0.10	0.12
Altitude	0.95	-0.19	0.08	-0.08
Soil moisture (Landolt's f)	0.83	0.49	0.13	-0.11
Soil nitrogen (Landolt's n)	-0.14	0.89	-0.02	-0.07
Continentality (Landolt's k)	-0.36	-0.83	-0.26	0.06
Light (Landolt's l)	0.60	-0.72	-0.21	0.03
Soil pH (Landolt's r)	-0.15	0.13	-0.80	0.10
Vegetation cover	-0.20	0.22	0.75	0.08
Soil humus content (Landolt's h)	0.49	0.32	0.69	0.09
Soil structure (Landolt's d)	-0.32	0.57	0.62	0.02
Patchiness of sex types	-0.03	0.21	-0.04	-0.83
Density of individuals	-0.21	0.10	-0.03	0.82

Note: PC1 explained 30.85% of the total variance (eigenvalue 3.70), PC2 28.87% (3.46), PC3 13.17% (1.58), and PC4 9.84% (1.18).

Table 2. Relationship between four principal components and sex ratio of 30 late-successional populations of *Thymus praecox* from the Swiss Alps

Source	Parameter estimate		Effect test		
	β	SE	d.f.	χ^2	<i>P</i>
PC1: altitudinal gradient	-0.28	0.04	1	44.20	0.000
PC2: resource availability	0.07	0.05	1	2.02	0.155
PC3: soil type	-0.01	0.07	1	0.01	0.914
PC4: population attributes	0.06	0.06	1	1.09	0.297
Region			4	1.80	0.773

Note: Study region ($n = 5$) was included as a blocking factor in generalized linear model analysis. SE = standard error.

**Fig. 1.** Negative relationship between altitude and sex ratio (proportion of hermaphrodites) of 30 late-successional populations of *Thymus praecox* in the Swiss Alps ($r = -0.84$, $P < 0.001$).

of sex types was independent of altitude (interaction altitude \times focal plant sex: $\chi^2_1 = 0.17$, $P = 0.680$).

Offspring from open pollination

Intermediate sexual phenotypes were also rarely observed in open-pollinated offspring from natural populations of *T. praecox* (proportion of intermediates: 0.013, $n = 845$). In contrast to adult populations, however, the proportion of hermaphrodites was not significantly different between offspring from contrasting altitudes ($F_{1,6} = 0.21$, $P = 0.661$). Irrespective of altitude, hermaphrodites were more frequent than females in the offspring of hermaphrodite mothers and, similarly, females were more frequent than hermaphrodites in

Table 3. Adult and offspring sex ratios (proportion of hermaphrodites) of open-pollinated females and hermaphrodites from low- and high-altitude, late-successional populations of *Thymus praecox*

Population	Proportion of hermaphrodites		
	Adults	Offspring of females	Offspring of hermaphrodites
FL	0.71	—	—
FH	0.54	—	—
LL	0.63	0.47	0.67
LH	0.57	0.47	0.79
PL	0.62	0.34	0.52
PH	0.46	0.36	0.87
SL	0.61	0.44	0.89
SH	0.52	0.52	0.78
ZL	0.67	0.47	0.79
ZH	0.48	0.31	0.65

Note: Adult sex ratios were derived from neighbourhood sex ratios of focal plants. Offspring sex ratios were estimated from a total of 42–62 seed-offspring from 10 to 13 focal plants per sex type and population. For populations, see text.

the offspring of females (maternal sex: $\chi^2_1 = 47.93$, $P < 0.001$; altitude \times maternal sex: $\chi^2_1 = 0.83$, $P = 0.363$; Table 3). The proportion of hermaphrodites in the offspring of females was lower than in adult populations (generation: $\chi^2_1 = 44.29$, $P < 0.001$), and this difference was more pronounced at lower altitudes (altitude \times generation: $\chi^2_1 = 5.49$, $P = 0.019$; Table 3).

Progenies from controlled crosses

The frequency of intermediate sexual phenotypes in the progenies from controlled crosses on females was low and in the order of magnitude of that found in the offspring from natural populations, but it increased slightly with increasing geographic distance between cross parents (FP: 0.018; FA: 0.022; FR: 0.031). The progeny sex ratios were female biased, and the within-population crosses yielded similar proportions of hermaphrodites as found in the offspring of open-pollinated females in the corresponding natural populations (Table 3; Fig. 2). The female seed-parents (dams) accounted for 22% of the variance in sex ratio among the 96 crosses, the hermaphrodite pollen-parents (sires) for 4%, and the residual 74% was attributable to sire \times dam interactions (variance component \pm standard error; dams: 0.029 ± 0.019 ; sires: 0.005 ± 0.014 ; residual: 0.097 ± 0.019). Three of the 32 females, one each from populations PH, ZL, and ZH, did not segregate any hermaphrodite offspring in any of the three cross treatments (Fig. 3). Two of the 32 hermaphrodites, one each from populations PH and ZH, failed to restore male fertility in all crosses in which they were involved (see Online Appendix 2 at evolutionary-ecology.com/data/2386appendix2.pdf).

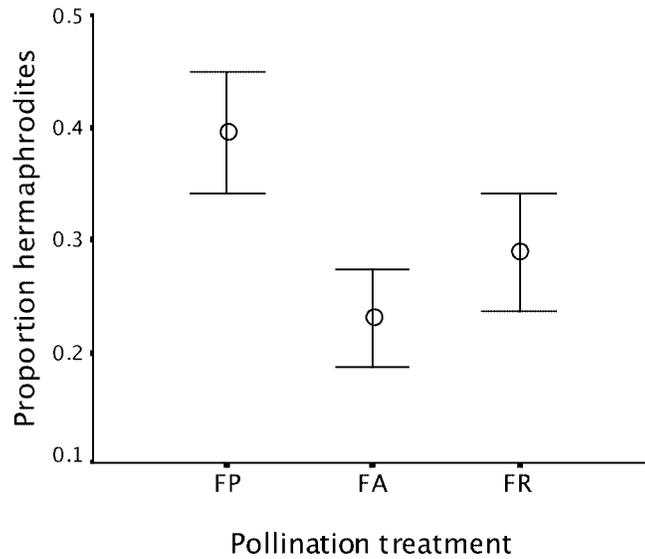


Fig. 2. Effect of cross type (FP: within population; FA: among altitudes within region; FR: among regions within altitude) on the proportion of hermaphrodites in the offspring of 96 crosses on females of *Thymus praecox* from the Swiss Alps. Data are means \pm standard errors ($n = 32$ families per treatment).

Table 4. Generalized linear model (GLM) analysis on progeny sex ratios of 96 controlled crosses on females in *Thymus praecox* from the Swiss Alps testing for effects of the geographic source of hermaphrodite sires (altitude and region) and the geographic distance between parents (cross treatment; FP: within population; FA: among altitudes within region; FR: among regions within altitude) on the restoration of male function

Source	d.f.	χ^2	<i>P</i>
Sire altitude	1	1.20	0.274
Sire region	1	2.70	0.100
Sire altitude \times sire region	1	2.39	0.122
Cross treatment	2	5.83	0.054
FA vs. FR	1	0.24	0.627
FP vs. (FA and FR)	1	5.64	0.018
Dam	31	69.77	<0.001

Note: Dam was included as a blocking factor to account for differences in restorability of females.

The geographic source of dams had no significant effect on progeny sex ratio (dam altitude: $\chi^2_1 = 0.01$, $P = 0.915$; dam region: $\chi^2_1 = 0.50$, $P = 0.477$; dam altitude \times region: $\chi^2_1 = 0.82$, $P = 0.364$; in a GLM also accounting for effects of cross treatment and of individual sires). Similarly, the altitude from which the sires were sampled had no significant effect on their restoration ability (Table 4), and the effects of sire region and its interaction

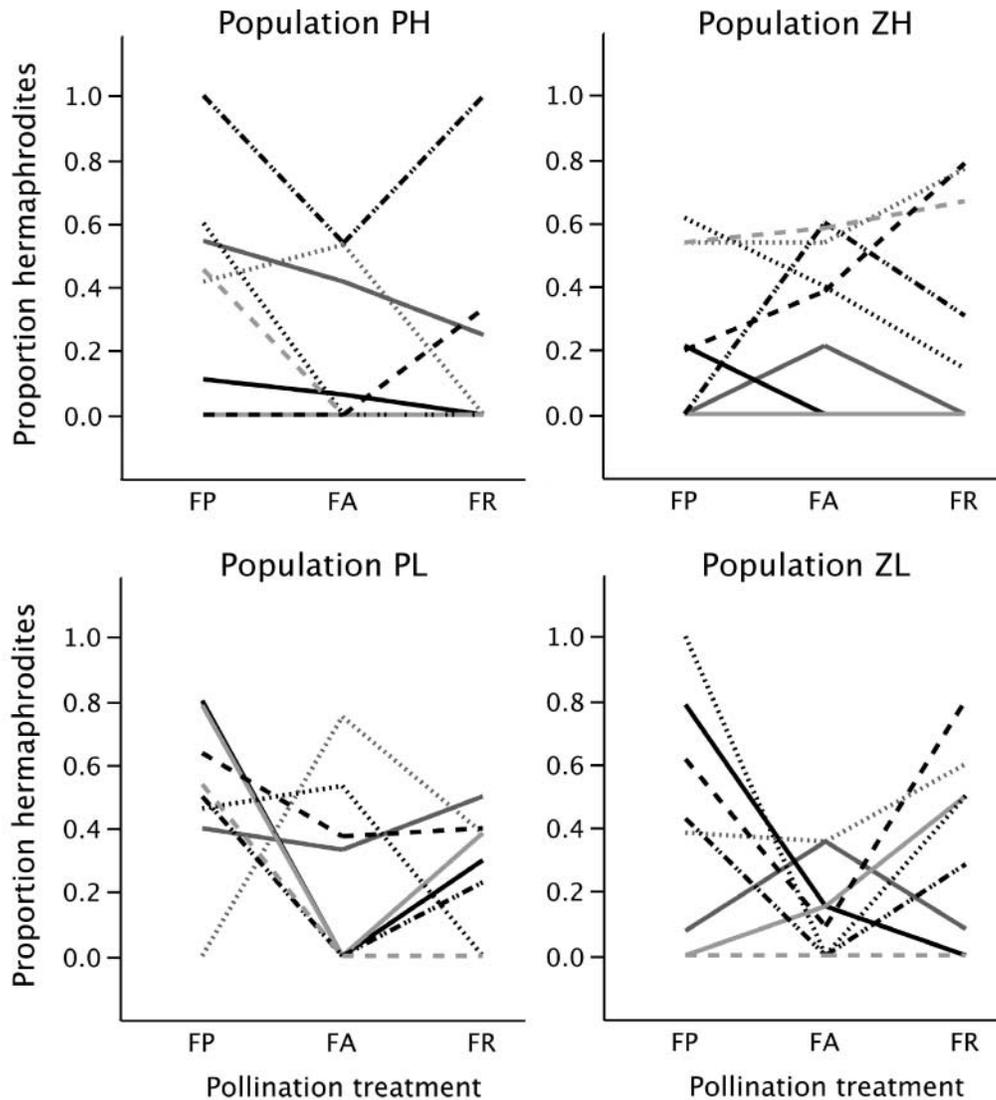


Fig. 3. Interaction plots for 32 females of *Thymus praecox* from the Swiss Alps grouped according to four populations and showing their restorability (expressed as proportion of hermaphrodites) in three different cross treatments (FP: within population; FA: among altitudes within region; FR: among regions within altitude).

with altitude were not statistically significant either (Table 4). However, removing these latter effects from the analysis would have increased the deviance and overdispersion of the GLM. In fact, in region Z, the hermaphrodites from the high-altitude population (ZH) showed considerably lower restoration ability than those from the corresponding low-altitude population (ZL) in all cross treatments (Fig. 4). In accordance with this finding, lower proportions of hermaphrodites were observed in the open-pollinated offspring of

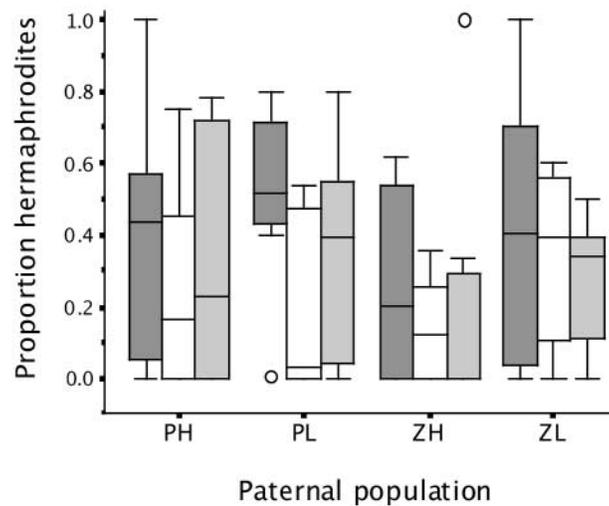


Fig. 4. Box-plots illustrating the restoration ability (expressed as proportion of hermaphrodites) of four populations of sires when crossed with females from within the same population (FP, dark grey), from a different altitude within the same region (FA, white), and from a different region but similar altitude (FR, light grey) in *Thymus praecox* from the Swiss Alps.

both females and hermaphrodites from population ZH when compared with population ZL (Table 3).

Cross treatment showed a marginally significant overall effect on progeny sex ratio (Table 4). However, in the two among-population cross types, the geographic distance (within vs. among regions) did not have a significant effect on progeny sex ratio (Table 4; Fig. 2). A significant positive correlation between the sex ratios of maternal half-sib families from the two among-population cross treatments FA and FR ($r = 0.39$, $P = 0.028$; Fig. 3) further identified that the restorability of individual females in crosses among regions was related to their restorability in crosses among populations within regions. In contrast, restorability in crosses among populations did less well at predicting restorability in within-population crosses (correlation between sex ratios of maternal half-sib families from treatments FP and FA: $r = -0.01$, $P = 0.936$; FP and FR: $r = 0.34$, $P = 0.057$), but the within-population crosses produced significantly higher proportions of hermaphrodites than the pooled among-population crosses (Table 4; Figs. 2, 4). Thus, sets of nuclear restorers (inherited by hermaphrodites) were most efficient in restoring male fertility when interacting with those CMS-restorer combinations (females) present in the same population.

DISCUSSION

Altitudinal gradients showed a clear pattern of sex-ratio variation in gynodioecious *T. praecox* in the Swiss Alps: the proportion of hermaphrodites decreased with increasing altitude (Fig. 1). This consistent pattern stresses a role of ecology for sex-ratio variation (Gouyon *et al.*, 1991; Bailey and Delph, 2007a). Furthermore, offspring sex ratios were not generally related to adult sex ratios (Table 3), indicating that lower hermaphrodite frequencies at

higher altitudes were not attributable to reduced restorer frequencies or increased mismatches between restorers and CMS types in *T. praecox*. There was thus no evidence of an effect of stochastic processes on sex ratio at higher altitudes. Rather, our late-successional study populations apparently exhibited the cytonuclear-genetic profiles of unrestricted populations, suggesting that they would provide favourable conditions for detecting the signature of selective forces acting upon the cytonuclear sex system in *T. praecox*. Accordingly, the wide geographical distribution of sex-determining alleles (Fig. 3) and the increased restoration of male fertility in within- versus among-population crosses in *T. praecox* (Fig. 2) may be indicative of balancing selection acting upon the nuclear-cytoplasmic polymorphism (Charlesworth, 2002; Jacobs and Wade, 2003).

Characteristics of nuclear-cytoplasmic inheritance of sex

Thymus praecox showed several characteristics of nuclear-cytoplasmic inheritance of sex. First, a major component of variance in progeny sex ratio from controlled crosses was attributable to dam \times sire interactions or cytonuclear epistasis (Gigord *et al.*, 1998; Taylor *et al.*, 2001). This implies that a single-family sex ratio is indicative of the match between the particular CMS type and the set of nuclear restorers involved. Accordingly, variation in sex ratios among half-sib families of individual cross parents pointed to the occurrence of different CMS types and corresponding sets of nuclear restorers in *T. praecox* (Fig. 3; see Online Appendix 2 at evolutionary-ecology.com/data/2386appendix2.pdf) (Manicacci *et al.*, 1997; Dudle *et al.*, 2001). Differences in average progeny sex ratio of individual cross parents will then indicate variation in general restorability or restoration ability among individual females and hermaphrodites, respectively. A small variance component attributable to individual sires suggested only minor variation in general restoration ability among different sets of restorers. As a consequence, the considerable variance in progeny sex ratio attributed to dam effects arguably mainly reflected variation in the likelihood of restoration among CMS types in *T. praecox*.

The bias in offspring sex ratios towards the sex of the maternal parent in *T. praecox* represents another typical feature of nuclear-cytoplasmic gynodioecy (Table 3) (Charlesworth and Laporte, 1998). This implies that factors affecting the relative maternal fitness of females and hermaphrodites may influence population sex ratio (Charlesworth, 1999). Furthermore, under predominantly local seed dispersal, the above bias may induce a patchy distribution of sex types within populations (van Damme, 1986; Graff, 1999; Olson *et al.*, 2006). Accordingly, in *T. praecox*, patchiness of sex types was related to the density of individuals within populations (Table 1). Patchiness of sex types calls attention to frequency-dependent processes acting within populations (Frank and Barr, 2001), such as small-scale spatial dynamics of sex-determining genes (van Damme, 1986) or effects of local neighbourhood on pollen availability (Graff, 1999) and selfing rate (Landergott, 2007). However, the degree of patchiness of sex types was not related to population sex ratio in *T. praecox*.

Diversity of sex-determining genes at different adult sex ratios

Sex-ratio variation among populations may result from variation in the genetic diversity of nuclear and cytoplasmic sex-determining genes (Couvet *et al.*, 1986; McCauley *et al.*, 2000; Byers *et al.*, 2005). One of our study populations, ZH, exemplified how a lack of appropriate restorer alleles may affect sex ratio. Hermaphrodites from population ZH showed lower average

restoration ability than those from the other three populations included in the controlled crosses, irrespective of cross type (Fig. 4), suggesting a reduced frequency or diversity of restorer alleles. Accordingly, five of the eight females from population ZH segregated more hermaphrodites in at least one of the among-population crosses than in a within-population cross (Fig. 3), indicating that appropriate restorers were indeed missing in population ZH. Hence, stochastic processes in conjunction with restricted gene flow may have disrupted CMS–restorer combinations and impaired restorer diversity in population ZH. In line with this interpretation, the ZH population also displayed lower genetic diversity at nuclear microsatellite loci than the corresponding low-altitude population ZL (Landergott, 2007). The resulting difference in segregation ratios between populations ZH and ZL (Table 3) would be sufficient to explain the difference in adult sex ratios between these two populations (see Online Appendix 3 at evolutionary-ecology.com/data/2386appendix3.pdf).

In the remaining study regions, however, offspring sex ratios from controlled crosses (Figs. 3, 4) and open-pollination (Table 3) provided no evidence of a generally reduced diversity or an increased mismatch of nuclear and cytoplasmic sex-determining alleles in high-altitude populations of *T. praecox*: in contrast to adult sex ratios, segregation ratios were constant across altitudes within regions (except Z; Table 3). From this we conclude that the altitudinal sex-ratio variation in *T. praecox* was not generally governed by factors affecting the nuclear-cytoplasmic genetic diversity of populations.

Spatial covariation between CMS types and nuclear restorers

Progeny sex ratios of crosses within and among populations provide insights into the spatial distribution of nuclear and cytoplasmic sex-determining genes (Taylor *et al.*, 2001; Bailey and McCauley, 2005). We applied different types of crosses representing different geographic distances in an attempt to estimate the relative importance of two inherently different evolutionary scenarios – that is, frequent formation and turnover of CMS types versus long-term maintenance of CMS types and corresponding restorers (Charlesworth, 2002).

Evolutionary turnover of CMS types should lead to genetic differentiation, and thus to mismatches in the cytonuclear system among populations (Frank, 1989; Barr, 2004a). If this were the case in *T. praecox*, we would have expected pronounced mismatches between CMS types and restorers in our among-region crosses. Recall that the two study regions P and Z were located in remote areas of the Swiss Alps. In addition, morphological peculiarities of the populations from region Z suggest genetic differentiation from other study regions (Seitter, 1989). However, among-region crosses did not yield lower hermaphrodite frequencies than crosses among populations within regions in *T. praecox* (Table 4; Fig. 2). Instead, some of the crosses (i.e. particular CMS–restorer combinations) produced a high proportion of hermaphrodite offspring (Fig. 3). Moreover, the likelihood of CMS types being restored in an among-region cross was positively correlated with the likelihood of restoration in a cross among populations within the same region. CMS types and corresponding restorer alleles thus appeared to be geographically widespread in *T. praecox*. These findings indicate long-term maintenance of cytoplasmic and nuclear sex-determining alleles, in line with recent interpretations of molecular genetic analyses on gynodioecious *S. acaulis* (Städler and Delph, 2002; Klaas and Olson, 2006).

At a local scale, a positive correlation between allele frequencies of corresponding nuclear and cytoplasmic sex-determining genes was evident in *T. praecox*: within-population crosses yielded higher proportions of hermaphrodites than among-population crosses (Table 4;

Fig. 2). The restoration ability of particular sets of restorers, as represented by sires, was therefore highest in within-population crosses, even in the genetically depauperate ZH population (Fig. 4; see above). These results conform to theoretical predictions of local adaptation between antagonistically interacting systems (Gandon *et al.*, 1996). Cytonuclear gynodioecy has indeed been regarded as an example of genomic conflict between cytoplasmic and nuclear genes (Gouyon and Couvet, 1985; Couvet *et al.*, 1990; Frank and Barr, 2001). However, cytonuclear co-evolution is not consistent with simple antagonistic interactions, if there are negative pleiotropic fitness effects of nuclear restorers (Jacobs and Wade, 2003). Assuming variation in the frequency of CMS types among populations, locally adjusted frequencies of restorer alleles could then point to costs of restoration (Jacobs and Wade, 2003; Bailey and McCauley, 2005), which may, in turn, cause balancing selection on cytoplasmic and nuclear sex-determining alleles (Bailey *et al.*, 2003; Charlesworth, 2006). Understanding the costs of restoration in *T. praecox* would therefore be crucial for a conclusive interpretation of the increased restoration of male fertility in within-population crosses.

Our results contrast with those of similar studies on *T. vulgaris* and *S. vulgaris* where prevalence of stochastic processes associated with the dynamics and history of populations may have blurred signals of selective processes favouring spatial covariation of sex-determining alleles (Gigord *et al.*, 1998; Bailey and McCauley, 2005; but see Belhassen *et al.*, 1991; Manicacci *et al.*, 1997). Additionally, inbreeding effects (dominance effects of nuclear restorers) may down-bias sex ratios in within-population crosses (Bailey and McCauley, 2005). However, in the tetraploid *T. praecox*, self-fertilization versus outcrossing of hermaphrodites showed no effect on offspring sex ratio (U. Lander Gott, unpublished data), precluding a bias due to inbreeding in the present study.

Why fewer hermaphrodites at higher altitudes?

Our findings raise two potentially linked questions for future research: What maintains females at high frequencies in gynodioecious *T. praecox*? And why are females more frequent at higher altitudes?

If high frequencies of females do not simply result from a lack of appropriate restorers, theory predicts costs of restoration to explain female frequencies higher than 50% (Bailey *et al.*, 2003; Delph *et al.*, 2007). Our finding of high female frequencies that are largely independent of cytonuclear genetic diversity therefore stresses the need for empirical data on the costs of restoration and on their potential influence on sex-ratio variation in *T. praecox* (Bailey and Delph, 2007a).

The discrepancy between adult and offspring sex ratios in *T. praecox* draws attention to the potential contributions of females and hermaphrodites to the next generation, and thus to variation in their relative maternal fitness. A lower proportion of hermaphrodites in the offspring of females than in adult populations of *T. praecox* suggests that hermaphrodites contribute to the next generation via their female reproductive function, as in other *Thymus* species (Manicacci *et al.*, 1998). However, the maternal fitness of hermaphrodites relative to that of females should be reduced at higher altitudes in *T. praecox*, as inferred from adult and offspring sex ratios (except in population ZH) (see Online Appendix 3 at evolutionary-ecology.com/data/2386appendix3.pdf). Hence, environment-dependent selection via maternal fitness (quantity and/or quality of seed-offspring) could favour females at higher altitudes. Low temperatures and short growing seasons at high altitudes (Table 1) impose adverse environmental conditions that could impair seed production in

hermaphrodites more severely than in females if there is any sex-differential plasticity (Delph, 2003). Alternatively, increased habitat harshness at higher altitudes may enhance a difference in offspring performance between hermaphrodites and females as it may result from costs of restoration (Delph and Mutikainen, 2003).

Taken together, both the spatial distribution of sex-determining alleles and high population female frequencies may point to a role for costs of restoration of male fertility in the maintenance of nuclear-cytoplasmic gynodioecy in *T. praecox*. Testing for negative pleiotropic effects of restorers on the maternal fitness of hermaphrodites is a particularly interesting line of work since altitudinal variation in the expression of such costs could cause the observed sex-ratio gradient in *T. praecox*.

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