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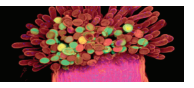

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RESEARCH PAPER

Spatial repartition and genetic relationship of green and albino individuals in mixed populations of *Cephalanthera* orchids

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Keywords

Albinos; amplified fragment length polymorphism; mixotrophy; mycoheterotrophy; Neottieae; photosynthesis; spatial repartition.

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ABSTRACT

Several green orchids of the Neottieae tribe acquire organic carbon both from their mycorrhizal fungi and from photosynthesis. This strategy may represent an intermediate evolutionary step towards mycoheterotrophy of some non-photosynthetic (albino) orchids. Mixed populations of green and albino individuals possibly represent a transient evolutionary stage offering opportunities to understand the evolution of mycoheterotrophy. In order to understand the emergence of albinos, we investigated patterns of spatial and genetic relationships among green and albino individuals in three mixed populations of *Cephalanthera damasonium* and one of *C. longifolia* using spatial repartition and Amplified fragment length polymorphism (AFLP) markers. Two of these populations were monitored over two consecutive flowering seasons. In spatial repartition analyses, albino individuals did not aggregate more than green individuals. Genetic analyses revealed that, in all sampled populations, albino individuals did not represent a unique lineage, and that albinos were often closer related to green individuals than to other albinos from the same population. Genetic and spatial comparison of genets from the 2-year monitoring revealed that: (i) albinos had lower survival than green individuals; (ii) accordingly, albinos detected in the first year did not correspond to the those sampled in the second year; and (iii) with one possible exception, all examined albinos did not belong to any green genet from the same and/or from the previous year, and *vice versa*. Our results support a scenario of repeated insurgence of the albino phenotypes within the populations, but unsuccessful transition between the two contrasting phenotypes. Future studies should try to unravel the genetic and ecological basis of the two phenotypes.

INTRODUCTION

Among plants, achlorophyllous species represent fascinating examples of nutritional adaptation because, by lacking photosynthetic pigments, they behave as heterotrophs and explore alternative strategies to acquire organic carbon (Graves 1995; Selosse & Roy 2009). To fulfil their carbon requirements, some plant taxa assimilate organic matter directly from a photosynthetic source (parasitic plants) or indirectly, with help of a fungal partner (Leake 1994). In the latter case, so-called 'mycoheterotrophic' plants have developed a dependence upon adjacent autotrophic plants from which they gain carbon indirectly by the way of shared mycorrhizal fungal partners (Leake 1994, 2004). This peculiar capacity of obtaining carbon heterotrophically is particularly relevant in orchids, which comprise approximately 35% of the fully heterotrophic angiosperms (Waterman & Bidartondo 2008). Among orchids, the high frequency of mycoheterotrophs is a convergent evolution that occurred more than 20 times (Molvray *et al.* 2000) and probably derived from a peculiarity of their seed germination and embryo development. In fact, one of the most distinctive orchid characteristics is that their minute seeds contain only minimal reserves of nutrients, and depend upon fungi for the resources necessary for germination and early seedling growth (Rasmussen 1995; Smith & Read 2008). Mycoheterotrophic orchid species retain this fungus-dependent nutrition at the adult stage and remain non-photosynthetic over their lifetime (Taylor *et al.* 2002).

Mycoheterotrophic orchids have been intensively investigated in the last decade and, more recently, interest has shifted to the intermediate condition in which fungi partly subsidise the nutrition of photosynthetic orchids. These plants can use fungal C in addition to their photosynthesis, a strategy called partial mycoheterotrophy or 'mixotrophy' (Selosse & Roy 2009). Although they are demonstrated to retain chlorophyll and photosynthesis (Julou *et al.* 2005;

Girlanda *et al.* 2006), mixotrophic orchids have an isotopic composition (^{13}C and ^{15}N) more or less close to that of mycoheterotrophic plants due to the use of fungal organic matter (Gebauer & Meyer 2003; Julou *et al.* 2005; Abadie *et al.* 2006). This occurs in a number of forest-dwelling species, especially orchids that were traditionally considered as obligate autotrophs (Gebauer & Meyer 2003; Bidartondo *et al.* 2004; Selosse *et al.* 2004; Julou *et al.* 2005). From an evolutionary point of view, mixotrophy is viewed as a predisposition to the evolution of mycoheterotrophy (Abadie *et al.* 2006; Selosse & Roy 2009), as it occurs in several orchid taxa where mycoheterotrophy secondarily evolved. Among those, the tribe Neottieae revealed to be especially pre-adapted to mycoheterotrophy by virtue of the association of green species with ectomycorrhizal fungi that link them to nearby trees (Bidartondo *et al.* 2004; Selosse *et al.* 2004; Julou *et al.* 2005).

Investigation of those intermediate mixotrophic stages, particularly at population level, may provide a unique opportunity to understand the evolutionary transitions to mycoheterotrophy. In this regard, achlorophyllous (= 'albino') individuals that are observed in otherwise green species, such as *Epipactis* and *Cephalanthera* spp. (Salmia 1986, 1989), are of particular interest (Fig. 1). Being themselves mycoheterotrophic (Julou *et al.* 2005; Abadie *et al.* 2006), these albinos may document an evolutionary transition from mixotrophy to mycoheterotrophy. Studies on mixed populations of albino and green individuals have been particularly focused on the comparison of C and N sources, fungal specificity, photosynthetic capability and morphology, comparing albino and green individuals in order to detect pathways of this transition. In *Epipactis microphylla* as well as in *Cephalanthera damasonium* and *C. longifolia*, albino individuals do not differ in their community of fungal symbionts from green individuals (Selosse *et al.* 2004; Julou *et al.* 2005; Abadie *et al.* 2006), suggesting a potentially easy shift between the green

Fig. 1. Albino and green *C. damasonium* individuals from Bovino, Southern Italy.

and albino phenotype. The phenotype does not change in *Cephalanthera* spp. populations for up to 12 years (Julou *et al.* 2005; Abadie *et al.* 2006), which could reflect genetic, but also micro-environmental determinism of the phenotype.

Thus, spatial and genetic investigations may contribute to understanding the origin(s) of albino genotypes in mixed populations and their genetic correlation with other green and albino individuals. In particular, it would be interesting to gather information on the following questions:

Do albino individuals represent a unique lineage within each mixed population? Are the albino individuals more closely related to each other than to the green individuals?

Do some albino individuals belong to the same genet as green individuals? Are there temporal transitions of the same genet between green and albino phenotype or *vice versa*?

How are the albino individuals spatially distributed in populations? Are albino individuals spatially closer to each other than to the green individuals?

To answer these specific questions, we investigated the patterns of spatial and genetic relationships among green and albino individuals in three mixed populations of *Cephalanthera damasonium* and one of *C. longifolia* using spatial repartition and markers.

MATERIALS AND METHODS

Plant models and sampling

Cephalanthera damasonium (Miller) Druce and *C. longifolia* (L.) Fritsch are two closely related species belonging to the tribe Neottieae (Pridgeon *et al.* 2008). These species are widely distributed in the Eurasian temperate zone, and mainly grow on calcareous soil (Delforge 1995). We focused on three natural populations of *C. damasonium* and one of *C. longifolia* (see Table 1 for locations and abbreviations). The population of *C. longifolia* had previously been investigated by Abadie *et al.* (2006) for mycorrhizal associations and C metabolism. A subsample of each population was examined in genetic analyses. Healthy leaves were collected from a total of 155 individuals (115 green and 40 albinos) and quickly stored in silica gel or frozen at −80 °C. For the Bov *C. damasonium* population, we replicated the sampling for genetic analysis in two following years (May 2005 and 2006); here, all detected albino individuals were sampled and surrounding green individuals were randomly sampled on a spatial basis (see Table 1).

Spatial analysis

Spatial position and phenotype of each plant was recorded in July 2005 for *C. longifolia* at Est (precision ± 0.1 m) and in July 2007 and 2008 for *C. damasonium* at Mon (precision ± 0.2 m; positions were tagged *in situ* in 2007 and 2008 for phenotype monitoring). A database was generated for *C. damasonium* data using ArcGIS 9.2 (ESRI, Redland, CA, USA) to create maps of the populations. Minimum distances between two individuals were computed with ArcGIS 9.2 and analysed using Minitab statistical software 13.31 (Minitab Inc., State College, PA, USA). The matrix of distances was computed with ArcGIS 9.2 and its correlation with the matrix of genetic distances (see below) was tested using a Mantel test, with R 2.8.0 (http://cran.r-project.org/).

Table 1. Geographic location, population abbreviation, year of sampling for spatial and genetic analyses and sample size for genetic analysis of green and albino individuals in populations of *Cephalanthera longifolia* and *C. damsonium*.

species	location	population abbreviation	year(s) of spatial analysis	year(s) of genetic sampling	green individuals	albinos	N	E
C. longifolia	Estonia	Est	2005	2005	44	5	58°14′44″	22°00′41″
C. damasonium	Southern France	Mon	2007 and 2008	2006	27	18	43°39′27″	3°51′53″
C. damasonium	Southern Italy	Bov	–	2005 and 2006	12 and 17	6 and 7	41°14′49″	15°21′00″
C. damasonium	Northern Italy	Vic	–	2006	15	4	45°32′13″	11°32′25″
total		4 populations			115	40		

To analyse interplant distances, all data were introduced into R 2.8.0, and univariate spatial patterns (aggregation/regularity) of plants were analysed using Ripley's K(r) function (Ripley 1977). The K(r) function is defined as the expected number of plants within distance r from a randomly chosen plant. Assuming complete spatial randomness, $K(r) = pr^2$ (for detailed properties of the function K(r), see Diggle 1983). Assuming a complete spatial independence, the derived function $L(r) = [K(r)/p]^{0.5} - r$ has thus an expected value of zero. We tested whether observed distributions were more aggregated or more regular than random distributions expected from the null hypothesis. For each value of r, a 5% confidence interval was obtained after 10000 repetitions of random distributions. L(r) was calculated for r up to 40 m for *C. damasonium* and up to 4 m for *C. longifolia*. These scales were chosen in agreement with the population area (Fig. S1). As the *C. damasonium* population more widely spread than the *C. longifolia* population, the range of variation of r was larger for *C. damasonium* than for *C. longifolia*.

A bivariate analysis was then performed in each population, using R 2.8.0, to test the correlation between the repartitions of green and albino individuals. The null hypothesis in this case was that green and albino sub-populations were independent from each other, *i.e.*, a random distribution of the phenotype (green or albino) among individuals.

Plant genotyping

For genetic analyses, total DNA was extracted from frozen or silica gel-dried leaves using a modified version of the cetyltrimethyl ammonium bromide (CTAB) method (Doyle & Doyle 1987). Approximately 100 mg of each sample were incubated in 800 μl of standard CTAB buffer and 1.8 μl beta-mercaptoethanol, and incubated at 60 °C for 30 min. Subsequently, the mixture was extracted twice with an equal volume of chloroform:isoamylic alcohol (24:1) and centrifuged at 6000 *g* for 10 min. DNA was isopropanol precipitated at 10000 *g* and 4 °C for 20 min, washed with 70% cold ethanol (4 °C) for 5 min and air dried for 15–30 min. DNA pellets were resuspended in 30 μl distilled water and quality of DNA was examined electrophoretically on 0.8% agarose gels.

Amplified fragment length polymorphism (AFLP) procedure was performed as in Vos *et al.* (1995) with minor modifications, as reported in Moccia *et al.* (2007) and using fluorescent dye-labelled primers. A preliminary test was conducted with 11 primer pairs on a sample subset of 10 individuals for each population. The size of AFLP fragments was determined with the software GENEMAPPER v3.0 (Applied Biosystems, Foster City, CA, USA) and amplified traces were scored as present or absent in a binary data matrix. In order to test the reproducibility of AFLP profiles, we replicated the DNA extraction procedures and amplification protocols on 22 randomly selected individuals (four out of 49 from Est, eight out of 45 from Mon, four out of 42 from Bov and six out of 19 from Vic). Only fragments with homogeneous, strong intensity were included in the data matrix; loci that did not give clear (*i.e.*, reproducible and easily scored) signals were excluded from the analysis. Loci accumulating too many differences (*i.e.*, 5% between two replicates) were considered as prone to genotyping errors and were discarded.

AFLP data analysis

Cephalanthera damasonium is a self-pollinating plant, while *C. longifolia* is a facultative allogamous plant (Scacchi & de Angelis 1991). Consequently, a low proportion of heterozygosity is expected and analysis of dominant markers like AFLP becomes less problematic (Lynch & Milligan 1994). Each peak in the AFLP fingerprint pattern was considered as a separate putative locus; all genotypes were scored for the presence (1) and absence (0) of polymorphic AFLP fragments as dominant markers (Pompanon *et al.* 2005), and the data were considered as haploid and entered into a binary matrix. The binary matrices of AFLP phenotypes were then assembled for statistical and genetic analyses. We calculated the total number of scored fragments, number of fixed fragments and percentage of polymorphic fragments. For each locus, we calculated the genotyping error rate by numbering the allelic differences between genotypes obtained from the same sample after separate DNA extraction and amplification. The error rate per locus was then calculated as the allelic differences between the genotypes obtained for each of the two replicates divided by the total number of fragments per profile (Bonin *et al.* 2004). All screenings were performed twice in two independent projects created with software GENEMAPPER 3.0 (Applied Biosystems) and results were compared as suggested by Bonin *et al.* (2004).

Similarity of AFLP profiles between pairs of individuals in each population was calculated using the similarity index (Lynch 1988, 1990) as: $S_{xy} = 2n_{xy}/(n_x + n_y)$, where n_x and n_y are the number of fragments present only in sample x and y, respectively, and n_{xy} is the number of fragments common for samples x and y. The same analysis was also conducted between all replicates of the 22 randomly selected individuals to obtain the mean value of similarity between replicates (*i.e.*, clones) of the same accession.

When applied to predominantly self-pollinating plant species where heterozygotes are rare, the gene diversity index

should yield relatively accurate estimations also for dominant markers (Lynch & Milligan 1994). To analyse the within-population gene diversity between subsets of albino and of green individuals, we used: (a) the mean of Nei's gene diversity ($He = 1 - \sum pi$) (Nei 1973) computed as the expected heterozygosity and based on allele frequencies for each locus and for all loci, both polymorphic and non-polymorphic (Ozbek *et al.* 2007); and (b) the mean of Shannon's information index ($I = \sum pi \ln pi$) (Lewontin 1972), indicating the degree of marker polymorphism within populations.

The significance of the mean differences between He and I values between albino and green individuals was assessed by paired sample *t*-tests carried out using SPSS 13.0 statistical package (SPSS Inc., Chicago, IL, USA). Furthermore, using the same parameters, for the Bov population in which two consecutive samplings were available, we also analysed the spatio-temporal genetic diversity across 2005 and 2006 sampling using the POPGENE version 1.31 program (Yeh *et al.* 1999).

For all populations, the genetic relationships among individuals was examined with a UPGMA dendrogram based on the Jaccard similarity calculated as $J = a/(n-d)$, where a is the number of positive matches (*i.e.*, the presence of a fragment in all samples), d is the number of negative matches (*i.e.*, the absence of a fragment from all samples), and n is the total sample size, including both matches and mismatches. Dendrograms were obtained with the FAMD software (Schlüter & Harris 2006).

RESULTS

Spatial distribution and survival of green and albino individuals

The Est *C. longifolia* population had eight albinos and 237 green individuals in an area of 585 m^2 in 2004 (Fig S1). The Mon *C. damasonium* population had 50 albinos and 592 green individuals in an area of 5096 m^2 in 2007, and 44 albinos and 825 green individuals in 2008 (Supporting Information). The percentage survival from 2007 to 2008 was 86% for green individuals and 60% for albino individuals. We also found 316 new green individuals and 14 new albinos in 2008. Production of new shoots in two successive years at Mon was thus higher for green (+233) than for albino (−6) plants. No phenotype shift was observed in any individual at Mon.

In the Mon *C. damasonium* population, the L(r) function of green individuals revealed two scales of aggregation at 10 and 40 m (Fig. 2a), and the L(r) function of albinos revealed a single scale of aggregation at 12.5 m (Fig. 2b). Bivariate analysis of the repartition of green *versus* albino individuals revealed no aggregation between the two phenotypes (Fig. 2 c). For the Est *C. longifolia* population, the L(r) functions revealed aggregations for both phenotypes, around 1.5 m for green individuals and 1.1 and 1.5 m for albino plants (Fig. 2d and e). The bivariate analysis failed to show any correlation between green and albino individuals of *C. longifolia* (Fig. 2f). However, the low number of albinos in Est limits the power of these analyses, as reflected in the large 5% confidence interval for L(r). We therefore also investigated minimal spatial distances between phenotypes. In both Mon and Est populations, the mean values significantly decreased in the order: green to nearest albino > albino to nearest albino > albino to nearest green > green to nearest green (Table 2). Thus, albinos were less spatially aggregated than green individuals, and were closer to green than to albino individuals.

Genetic variability and marker reproducibility

Among the 11 *Eco*RI/*Mse*I primer pairs tested, six highly polymorphic combinations with a large number of clear fragments were selected for the analysis. Using these six AFLP primer combinations, the 155 individuals produced a varying

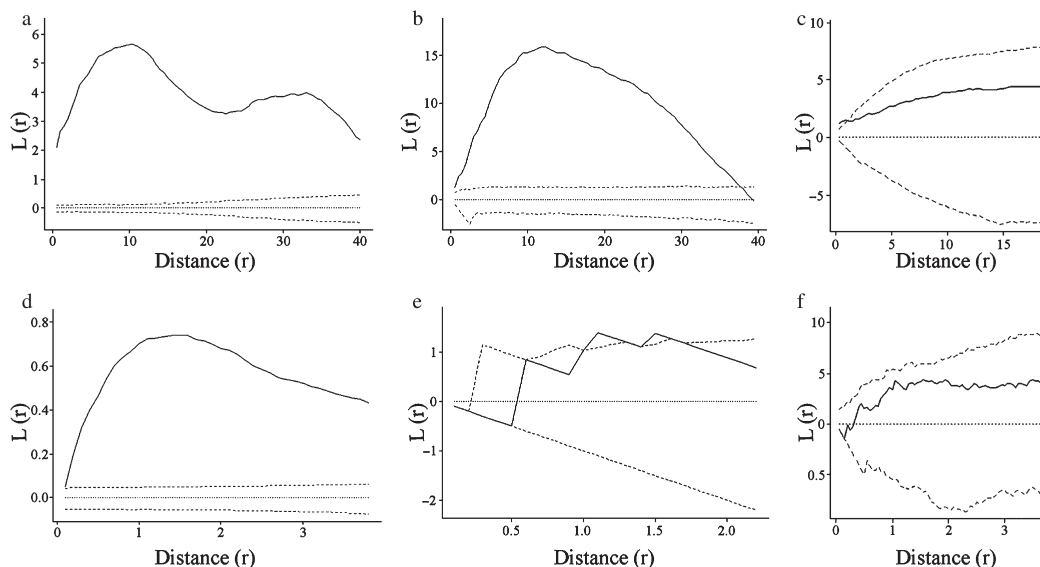

Fig. 2. Uni- and bivariate spatial patterns of individuals using the L(r) function. Assuming complete spatial independence between individuals, L(r) has an expected value of zero; the two dotted lines around zero indicate the 5% confidence interval. (a–e), univariate analyses: L(r) calculated for Mon (a, green individuals and b, albino individuals) and Est (d, green individuals and e, albino individuals) populations. (c–f), bivariate analyses: L(r) calculated for Mon (c) and Est (f).

Table 2. Analysis of minimal inter-plant distance between individuals at Mon and Est. For each population, values followed by different letters differ significantly according to an ANOVA ($P < 0.001$). A Tukey *post hoc* test was not applied because of samples of different size.

average minimal distance between	Mon	Est
green and nearest green individual	0.70 ± 0.87 a	0.30 ± 0.23 a
green and nearest albino individual	7.92 ± 5.58 b	3.03 ± 2.13 b
albino and nearest green individual	0.91 ± 0.74 c	0.40 ± 0.20 c
albino and nearest albino individual	2.35 ± 2.72 d	1.92 ± 1.51 d

number of fragments per combination (Table 3). The mean number of fragments in each primer combination ranged from 17.7 (*Eco*AGC-*Mse*ACAC) to 41.0 (*Eco*AGG-*Mse*AGAC) (mean 26.12, SD ± 8.52). The mean number of monomorphic (or non-polymorphic) fragments ranged from 3.25 (*Eco*AGG-*Mse*AGAC, *Eco*AGC-*Mse*ACAC) to 7.75 (*Eco*ACG-*Mse*CTG) (mean 5.20, SD ± 1.71). The percentage of polymorphic fragments considering all primer combinations ranged from 66.4% (Bov population) to 83.7% (Est population; see Table 3).

The reproducibility of scoring of the AFLP patterns was checked by analysing the genotyping error rate in repeated extractions and runs of 22 replicates (see Materials and Methods). The percentage error per duplicate sample, defined as the frequency of differences observed between two runs of the same sample (Bonin *et al.* 2004), varied from 1.5% in the primer combination *Eco*ACG-*Mse*AGAC to 4.33% in *Eco*AGC-*Mse*CAC; the mean percentage of errors for all the primer combinations was 2.93% (SD ± 0.95). The mean difference between the two independent scorings was 2.69% (SD ± 0.42) (Est: 3.10%; Mon: 2.95%; Bov: 2.18%; Vic: 2.52%), in agreement with previous reports (below 5%; Bonin *et al.* 2004). The mean similarity index in AFLP profiles between pairs of replicated individuals (equated to clones) was 0.98 (ranging from 0.97 to 1.0); the mean similarity index between individual pairs (resulting from dendrograms) in the examined populations was 0.93 (ranging from 0.85 to 0.98; Fig. 3).

Genetic analyses

Values of Nei's gene diversity (He) and Shannon's information index (I) of the population of *C. longifolia* (Est) were in the range of values found for the three populations of *C. damasonium* (Fig. 4). Nei's gene diversity (He) and Shannon's information index (I) did not differ significantly between albino and green individuals within populations (He: $t = 0.38$, $P = 0.72$. I: $t = 0.10$, $P = 0.92$) (Fig. 4), indicating that genetic diversity was similar for both phenotypes. This also appeared to be consistent in the Bov population across the two consecutive years 2005 and 2006 (Fig. 4).

The UPGMA dendrograms (Fig. 5) illustrating genetic similarity among individuals showed that the albino individuals formed neither a unique phyletic lineage nor a unique clone within each population. Even if, in a few circumstances, some albino individuals partially clustered together (*e.g.*, at Mon), generally they were scattered throughout the dendrograms and, as a result, seemed more genetically similar to some green individuals. Among paired individuals in each population, seven pairs displayed levels of similarity index ranging from 0.97 to 0.98 that may support either a clonal origin or a close kinship, given the percentage of error per duplicate sample (Fig. 3). No such pairs included two albinos, and among these, a single individual pair (at Bov) encompassed an albino and a green individual (sampled in 2005 and 2006, respectively; similarity index: 0.97). In the Bov population, the probability that two individuals have the same AFLP pattern by chance can be calculated by taking into account the frequency of absence/presence of each AFLP band. Assuming that bands are genetically independent, the probability of an AFLP pattern is $p = \Pi f_i$ where f_i is the phenotypic frequency for AFLP band i (presence or absence of band i) in the population considered. It ranged from 1^{-20} (most common AFLP phenotype, with most frequent alleles at each locus) to 1.66^{-65} (less common AFLP phenotype, with less frequent alleles at each locus). The probability that a green and an albino individual would share the same AFLP pattern by chance was 1.49^{-31}.

Spatial genetic structure at Est and Mon

According to a Mantel test, genetic distance between individuals (not considering their phenotype) was significantly correlated with geographic distance ($r = 0.149$, $P = 0.022$) in the Mon population, as was also the case for the subset of green individuals ($r = 0.173$, $P = 0.018$). However, the correlation

Table 3. Number of AFLP fragments and levels of polymorphism among all samples in each examined population (see Table 1 for population abbreviations).

	population											
	Est			Mon			Bov			Vic		
primer combinations	total no. of bands	no. of fixed bands	% polymorphic bands	total no. of bands	no. of fixed bands	% polymorphic bands	total no. of bands	no. of fixed bands	% polymorphic bands	total no. of bands	no. of fixed bands	% polymorphic bands
EACG-MCCAA	40	8	80.00	20	3	85.00	26	6	77.00	21	1	95.23
EACG-MAGAC	26	5	80.76	32	4	87.50	29	5	82.75	32	8	75.00
EACG-MCTG	25	6	76.00	14	7	50.00	29	11	62.06	16	7	56.00
EACG-MCAC	22	6	72.72	18	0	100	22	16	27.27	20	4	80.00
EAGC-MACAC	14	0	100	17	3	82.35	14	5	64.28	26	7	73.00
EAGG-MAGAC	41	3	92.68	38	2	94.73	48	7	85.41	37	1	97.00
total over 6 loci	168	28	83.69	139	19	83.26	168	50	66.46	152	28	79.37

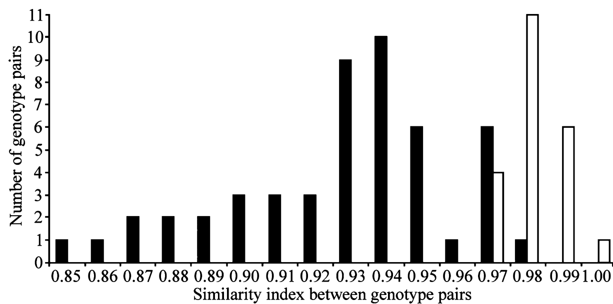

Fig. 3. Similarity index (horizontal axis) of AFLP profiles between replication pairs of the same samples (white) and between two individuals (black) of each terminal pair in UPGMA dendrograms (as displayed in Fig. 5) for all populations. Numbers of pairs are plotted on the vertical axis.

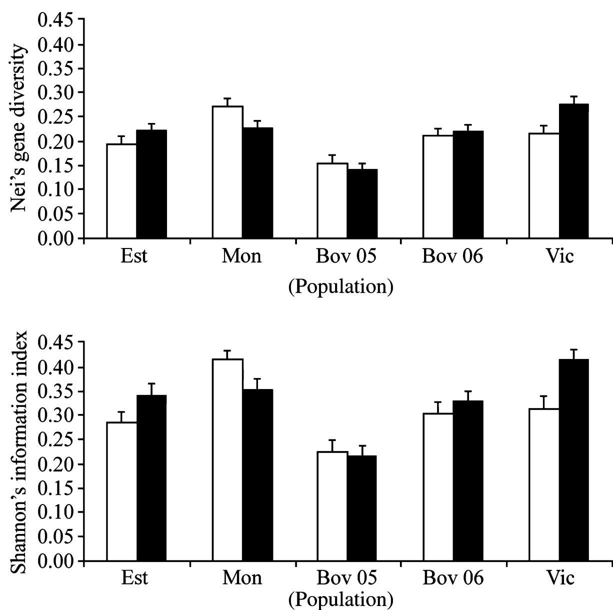

Fig. 4. Genetic diversity within-population between albino (white columns) and green individuals (black columns) based on Nei's gene diversity (He) values and Shannon's information index (I).

was not significant for the subset of albinos in the Mon populations ($r = 0.095$, $P = 0.228$). For the Est population, the results were similar; the correlation between genetic and geographic distance was significant when all individuals were included ($r = 0.619$, $P = 0.001$) and for the subset of green individuals ($r = 0.683$, $P = 0.001$), but not for the subset of albinos ($r = 0.726$, $P = 0.071$). In all, although more similar genotypes tended to group together spatially in populations, we did not find clear genetic aggregation on the whole population in Mon ($r = 0.142$, $P = 0.006$). Even when only investigating albino to green individual distance, no strong correlation was detected between genetic and geographic distance in Mon ($r = 0.022$, $P = 0.001$). However, the opposite result was found in Est, albino and green individuals closely genetically related were also geographically closer ($r = 0.741$, $P < 0.0001$) but the correlation was not significant when considering the whole population ($r = 0.0079$, $P = 0.051$). Such results underline the absence of a genetic structure for both albino and green individuals.

DISCUSSION

Possible origin of albinos

Albino individuals in populations of usually green orchids represent a hitherto poorly analysed phenomenon that could be an intermediate step in the evolutionary emergence of mycoheterotrophy. Previous studies failed to show any significant difference in mycorrhizal colonisation and morphology between albino and green phenotypes in mixed population of *C. longifolia* (Abadie *et al.* 2006) and of *C. damasonium* (Julou *et al.* 2005). However, the high variance in mycorrhizal fungi and individual development may obscure differences. Without doubt, albinos belong to the same species as green individuals, based on ribosomal internal transcribed sequence identification, flower morphology and crossing experiments (Mélanie Roy & Marc-André Selosse, unpublished data). Although the mechanism responsible for the albino phenotype is unknown, our study shows that albino individuals do not constitute single isolated lineages within each population.

Although parentage analysis are almost impossible to carry out using dominant markers, the genetic relationship among green and albino individuals within the four examined mixed populations shows that albinos do not represent a monophyletic lineage within each population (Fig. 5). Also, the absence of spatial aggregation of albinos at small scales (Fig. 2) suggests that we do not face the spread of a single clone (by vegetative propagation) in each population. The over-dispersion of albinos in dendrograms (Fig. 5) and spatial repartition (Table 2) suggest that the albino phenotype occurred repetitively within different progenies or different plants. This could explain why, in the analysed populations, albinos are more closely related to green individuals than to other albinos. This scenario is congruent with the fact that we recover similar levels of genetic diversity for albinos in *C. damasonium* and *C. longifolia*, in spite of the presumed differences in their mating system (mostly autogamous *versus* mostly allogamous; Scacchi & de Angelis 1991).

The genetic basis for albinism remains unknown. Albinos could be either permanent mutants, as suggested by phenotype stability over the years (see below), or transitory phenotypic stages, in which genes involved in the photosynthetic pathway can switch off depending on micro-environmental conditions (*e.g.*, the amount of C resources provided by the nearby fungal mycelia or tree roots) that prevent greening. The results of our study are congruent with both hypotheses. Noteworthy, under the hypothesis of environmental determinism, the spatial distribution of albinos would indicate that conditions enhancing this phenotype are very heterogeneously distributed, occurring in very small patches (Supporting Information), and are very stable over years, so that albinos do not group together and remain phenotypically stable (Table 2; Fig. 2).

The partial genetic clustering of albinos in the *C. damasonium* Mon population (although they remain genetically different from each other; Fig. 5) may indicate a closer relationship among some albinos than with green individuals. Because these genetically related albinos do not represent clones, their genetic proximity could be due to reproduction, perhaps autogamously, of an albino or a green individual with a mutation in a generative cell that generated progeny of closely related albino individuals. Since the levels of

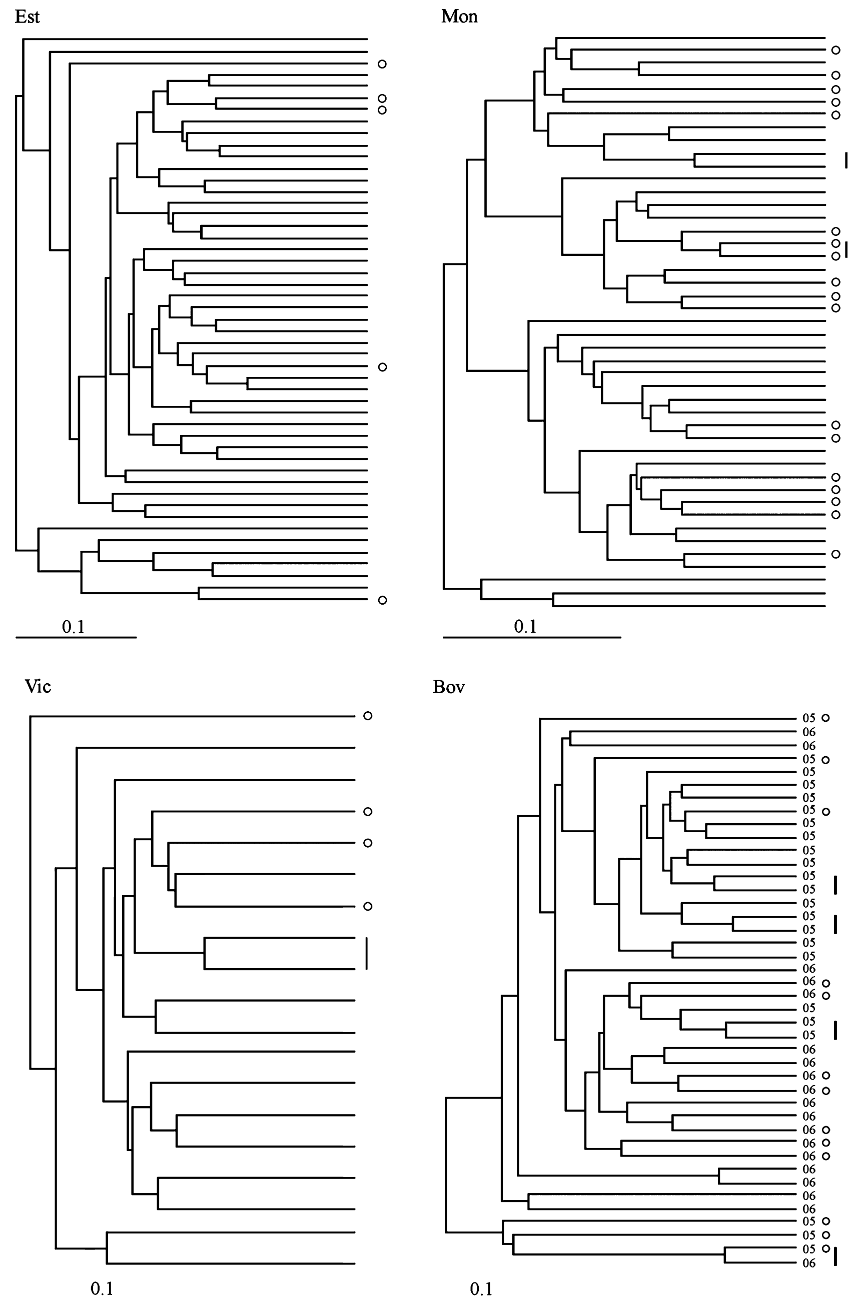

Fig. 5. UPGMA dendrograms based on Jaccard genetic distance among individuals in the four examined populations. Empty circles indicate albino individuals, and black vertical lines indicate individuals that potentially belong to the same clone.

genetic diversity in the subset of albino individuals are equivalent to those of corresponding green individuals within the same mixed population, this would indicate that the level of selfing or outbreeding reproduction in green and albino individuals are comparable and that non-assortative mating does not occur between the two different phenotypes. It is, however, not known whether albinos can reproduce.

Spatial distribution

There is evidence that albinos do not necessarily occur near green individuals (Julou *et al.* 2005). The Bov and Vic populations also suggest that some albinos occurred at large distances from green and other albino individuals (data not shown). Our spatial analysis at Est and Mon suggest that, although albinos occur within dense patches of green individuals, they do not tend to be closer to other orchids (albino or green) than to green orchids (Table 2) and no correlation was revealed between albino and green individual distribution in Mon and Est (Fig. 2). These observations are relevant, since the C is conveyed to mycoheterotrophic orchids from surrounding green plants through shared mycelial partners. Assuming that roots donating C need to be spatially close (Selosse *et al.* 2002), these observations further corroborate that the ultimate C sources are not green conspecific individuals (Julou *et al.* 2005), but more likely other fully autotrophic plants, such as nearby trees, that supply C to the shared mycorrhizal fungi.

Temporal trends

Most of the time, albino orchids are rare and do not invade the populations where they occur (Renner 1938; Mairold & Weber 1950). Congruently, albinos were seen at constantly low frequency, with slower demography than green individuals. In Mon, twice as many albinos died or became dormant as compared to green individuals; albino number increased more slowly in 2008 (+28%) than the number of green individuals (+40%).

In the Bov population, where an exhaustive sampling of the albinos was performed over 2 years, genetic analyses demonstrated that albinos of two different years always showed divergent AFLP patterns. According to the efficiency of detecting clones in our AFLP approach (Fig. 3), we can confidently exclude that they belonged to the same genet (unfortunately, no spatial data are available for the Bov population). Congruent with the absence of shoots from the same genet over 2 years, the albinos collected in two different years at Bov were also genetically more closely related to green individuals than to each other. The observation that many albinos did not form a shoot in the next year may either suggest lower survival or that they remain dormant underground after a blooming season. The latter condition has been reported for green *Cephalanthera* sp. individuals (Rasmussen 1995; Shefferson *et al.* 2003) and was described in previous monitoring of albino individuals (Abadie *et al.* 2006).

As previously reported in the Est *C. longifolia* population (Abadie *et al.* 2006), the 2-year samplings in Bov and Mon populations confirmed the stability of the albino phenotype over years. At least, the transition between green and albino phenotypes within the same genet is an uncommon event (no change among the 509 green and 30 albino individuals observed in both 2007 and 2008 at Mon). In the 2-year exhaustive sampling of albinos in Bov population, we only found one case that could represent a temporal transition between phenotypes – *i.e.*, a green individual that could be part of an albino genet sampled the year before. However, due to the significant probability of obtaining a similar genotype only by chance, we cannot firmly reject the alternative hypothesis that the albino and the green individual of this pair represent two genetically closely related but different genets. Moreover, given the potential existence of a dormant stage and the lack of spatial data at Bov, it could also be that the albino is of recent origin, and that the green individual was not detected in 2006. With this potential exception, all examined albino shoots never belonged to green genets from the same or previous flowering year. In long-term investigations of Est (Abadie *et al.* 2006), as in our observations, the lack of intermediate phenotypes further corroborated the stability of the albino phenotype. A different situation was observed for *Epipactis helleborine*, where intermediate (yellowish-green or variegated) phenotypes occur (Salmia 1989), and for *E. neerlandensis* where albinos sometimes reverse to yellowish-green over a growing season (Lemagnen & Selosse, unpublished data).

CONCLUSIONS

In the investigated *Cephalanthera* populations, we found a limited spatial structure (with similar genotypes grouping together) and high level of genetic diversity within and among populations. Albinos were as genetically diverse as green individuals, and did not show any significant spatial structure. However, the low number of albinos limited our analyses in terms of statistical power. Two-year monitoring suggested lower survival or higher shift to dormancy of albinos as compared to green individuals. In our study, within the limits of our sampling, albinos do not represent a unique lineage within each examined populations and do not tend to cluster together either spatially or genetically. In conclusion, our results support a scenario of a repeated insurgence of the albino phenotypes within the populations, but unsuccessful transition between the two contrasting phenotypes. Although experimental work on mixotrophic orchids is challenging (Sadovsky 1965; Rasmussen 1995), future studies should try to unravel the genetic and ecological basis of the two phenotypes.

SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Figure S1. Map of the green (black circles) and albino (open circles) individuals of two investigated populations, Mon (a, *C. damasonium* in 2008) and Est (b, *C. longifolia* in 2004).

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