MOLECULAR ECOLOGY

Molecular Ecology (2013) **22**, 1431–1446 doi: 10.1111/mec.12169

Morphological, ecological and genetic aspects associated with endemism in the Fly Orchid group

YANN TRIPONEZ,*†[1] NILS ARRIGO,‡§[1] LOÏC PELLISSIER,§[1] BERTRAND SCHATZ¶[2] and NADIR ALVAREZ§[2]

*Department of Evolution, Ecology and Genetics, Research School of Biology, The Australian National University, Canberra ACT 0200, Australia, †Laboratory of Evolutionary Entomology, University of Neuchâtel, Emile-Argand 11, CH-2000 Neuchâtel, Switzerland, ‡Department of Ecology and Evolutionary Biology, University of Arizona, Tucson, AZ, 85721, USA, §Department of Ecology and Evolution, Biophore Dorigny, University of Lausanne, CH-1015 Lausanne, Switzerland, ¶Centre d'Ecologie Fonctionnelle et Evolutive, CNRS Montpellier, UMR 5175, 1919 Route de Mende, F-34293 Montpellier Cedex 5, France

Abstract

The European genus *Ophrys* (Orchidaceae) is famous for its insect-like floral morphology, an adaptation for a pseudocopulatory pollination strategy involving Hymenoptera males. A large number of endemic *Ophrys* species have recently been described, especially within the Mediterranean Basin, which is one of the major species diversity hotspots. Subtle morphological variation and specific pollinator dependence are the two main perceptible criteria for describing numerous endemic taxa. However, the degree to which endemics differ genetically remains a challenging question. Additionally, knowledge regarding the factors underlying the emergence of such endemic entities is limited. To achieve new insights regarding speciation processes in *Ophrys*, we have investigated species boundaries in the Fly Orchid group (*Ophrys insectifera* sensu lato) by examining morphological, ecological and genetic evidence. Classically, authors have recognized one widespread taxon (*O. insectifera*) and two endemics (*O. aymoninii* from France and *O. subinsectifera* from Spain). Our research has identified clear morphological and ecological factors segregating among these taxa; however, genetic differences were more ambiguous. Insights from cpDNA sequencing and amplified fragment length polymorphisms genotyping indicated a recent diversification in the three extant Fly Orchid species, which may have been further obscured by active migration and admixture across the European continent. Our genetic results still indicate weak but noticeable phylogeographic clustering that partially correlates with the described species. Particularly, we report several isolated haplotypes and genetic clusters in central and southeastern Europe. With regard to the morphological, ecological and genetic aspects, we discuss the endemism status within the Fly Orchid group from evolutionary, taxonomical and conservation perspectives.

Keywords: amplified fragment length polymorphisms, ecological differentiation, endemism, *Ophrys insectifera*, spatial genetic structure, speciation

Received 10 September 2011; revision received 11 October 2012; accepted 19 October 2012

Correspondence: Yann Triponez and Nadir Alvarez, Fax: +41 216924265; E-mails: yann.triponez@bluewin.ch and nadir.alvarez@unil.ch

[1]These authors contributed equally to this work and are considered as joint first authors.

[2]These authors supervised this work together and are considered as joined last authors.

Introduction

The identification of historical and ecological factors that have shaped the spatial patterns of species diversity is of long-standing interest in ecology and evolution (Wiens 2011). For example, past climatic oscillations have been shown to exert a major influence on the

current patterns of species diversity by causing shifts in species distributions (Tribsch & Schonswetter 2003; Médail & Diadema 2009; Triponez *et al.* 2011; Espíndola *et al.* 2012). While climatic oscillations have reduced the diversity of plant communities, such as the depauperate European tree flora during the Plio-Pleistocene (Svenning 2003), they can also be an important diversification force, as they have durably imprinted the genetic differentiation patterns of many extant species (e.g. Hewitt 1999; Schmitt 2009). When such differentiation leads to substantial phenotypic differences and occurs in a spatially limited range, it produces endemic taxa (Walters 1976).

Because of its intricate biogeographic history through the Tertiary and Quaternary periods (Thompson 2005), the Mediterranean region has been home to numerous diversification processes resulting in a very high number of endemic species (Blondel & Aronson 1999). With 25 000 vascular plant species listed in the area, the Mediterranean region is currently considered to be a major hotspot of species diversity (Heywood 1995; Médail & Quézel 1999). From an evolutionary perspective, the origin of floral endemism can be placed along a temporal continuum ranging from the Tertiary to the postglacial period and may have involved polyploid hybrids (Thompson 2005). The large diversity within the Orchidaceae family observed in the Mediterranean region (as well as in several other regions of the western Palearctic) is hypothesized to have mostly emerged from the ecological innovation of deceptive pollination ecology (Cozzolino & Widmer 2005; Delforge 2005; Schiestl 2005). Pollination by sexual deception, predominantly observed among orchids, ordinarily requires the presence of a specific pollinator—a male insect—that is attracted via female-pheromone-mimicking volatile compounds that are emitted by the flower (Kullenberg 1961; Borg-Karlson *et al.* 1993; Schiestl & Ayasse 2002; Ayasse *et al.* 2011). Such a reproductive strategy has evolved independently on at least four continents (particularly in Europe and Australia). It is predicted that new species can evolve quickly when only a few loci exert a large phenotypic effect (e.g. by causing shifts in attractive chemicals and floral morphology; Schlüter *et al.* 2011). This phenomenon can cause floral isolation and speciation on a rapid timescale due to shifts in pollinator attraction (e.g. Schiestl & Ayasse 2002; Schlüter *et al.* 2009; Peakall *et al.* 2010; Xu *et al.* 2011).

Pollination by sexual deception is a notable characteristic of the genus *Ophrys*. This western Palearctic genus is comprised of as many as ~250 described species according to some authors (Delforge 2005) and thus accounts for a large proportion of all European orchid species. Most of the recognized taxa are considered to be endemic species generally associated with particularly narrow climatic and ecological conditions (Delforge 2005; Bateman *et al.* 2009). Pillon & Chase (2007) noted that a taxonomic bias, which occurred due to the strong interest in this emblematic genus, has potentially led to numerous and sometimes confusing descriptions of endemic taxa. Adding to the confusion, the genus includes species groups encompassing widespread taxa and local endemics that are often poorly (or even not) genetically differentiated (Bateman *et al.* 2003). A recent controversy opposed the 'morpho-genetically' and 'etho-ecologically' based circumscriptions of species in *Ophrys* (Bateman *et al.* 2011; Vereecken *et al.* 2011). However, most investigators still suggest a recent (and sometimes ongoing) diversification of the species groups within the genus (e.g. Schlüter *et al.* 2007; Devey *et al.* 2008, 2009). Irrespective of the actual *Ophrys* species number, an understanding of the historical and ecological mechanisms that might generate diversification in *Ophrys* will also improve our general understanding of the diversification of endemics in the Mediterranean. From a conservation perspective, it is also worth defining how endemic species segregate in environmental space and determining whether their phenotypic differences are associated with genetic divergence. For this purpose, it is necessary to simultaneously investigate how species differ on a genetic, morphological and ecological basis. However, there is a lack of studies that combine these three types of information to understand diversification in *Ophrys* (Pillon & Chase 2007).

The present study investigates the evolution of Fly Orchids, a group representing a basal divergence within *Ophrys* (Devey *et al.* 2008). From a morphological and functional perspective, the group splits into three taxa including the representative and widespread *O. insectifera* and two endemic variants: *O. aymoninii*, from the karstic region of the southern French Massif Central (known as the 'Grands Causses' or 'Causses' region), and *O. subinsectifera*, a Spanish vicariant that grows in the southern foothills of the Pyrenees. All three taxa have distinctive morphologies and are associated with specific pollinators: the wasps *Argogorytes mystaceus* and *Argogorytes fargeii* are associated with *O. insectifera*, the bee *Andrena combinata* is associated with *O. aymoninii* and the sawfly *Sterictiphora gastrica* is associated with *O. subinsectifera* (Borg-Karlson *et al.* 1993; Hermosilla *et al.* 1999; Vereecken 2009).

This study is the first large-scale genetic investigation of the Fly Orchid group and the first to evaluate morphological, ecological and genetic differentiation among *Ophrys* taxa based on extensive sampling across most of the species' ranges. By analysing morphological and ecological data and inferring the spatial genetic structure of the species group, we herein establish the potential role of ecology vs. history in the evolution

and localization of endemism of the Fly Orchid group in Europe, and we answer the following questions: (i) Do the described species differ morphologically and ecologically? (ii) Are the recognized endemics genetically distinct from *O. insectifera*? and (iii) What are the diversification drivers of the Fly Orchid group?

Methods

Sampling, plant material and DNA extraction

Sampling was performed during flowering periods from April to June of 2007 and 2008. Samples were identified to the species level following Delforge (2005; see general habitus in Fig. 1). Two taxa (i.e. *Ophrys insectifera* and one endemic) occasionally co-occurred in sympatric populations (Fig. 2A, Table S1, Supporting information). In each population examined, the leaves from three to ten individuals were sampled and desiccated in silica gel (Chase & Hills 1991). The leaves of 158 *O. insectifera*, 98 *O. aymoninii* and 31 *O. subinsectifera* individuals were collected from 64 populations (see Table S1, Supporting information) covering most of their respective distributions. The outgroups for phylogenetic analyses (*O. araneola*, *O. holoserica*, *O. lutea* and *O. fusca*) were also collected and determined following Delforge (2005) when they were encountered in the vicinity of a Fly Orchid population. Total genomic DNA was extracted from 10 mg of dried leaf fragments using the DNeasy Plant Kit (Qiagen, Hilden, Germany).

Chloroplast DNA sequencing

In a subset of 170 individuals from 50 populations (spanning the complete distribution of the Fly Orchid group), we sequenced two noncoding and fast-evolving cpDNA regions (Shaw *et al.* 2007): the *ndhA* intron (primers ndhAx1 and ndhAx2) and the *psbJ-petA* intergenic spacer (primers psbJ and petA). We performed amplification using a standard PCR protocol by mixing 1.5 μL of extracted DNA, 3 μL of 10X PCR buffer (Promega, Madison, WI, USA), 3 μL of 25 mM $MgCl_2$ solution (Promega), 3 μL (1.5 mM each) of dNTPs (Promega), 1.5 μL of 10 mM forward and reverse primers (Microsynth AG, Balgach, Switzerland) and 0.3 μL (two units) of Taq DNA polymerase (Promega). The reactions were made up to a final volume of 30 μL with purified water. PCRs were performed in a TGradient thermocycler (Biometra, Goettingen, Germany) using the following program for both cpDNA regions: an initial denaturation of 1 min 30 s at 94 °C, 35 cycles of 35 s at 94 °C, 1 min at 52 °C and 45 s at 72 °C with a final elongation of 8 min at 72 °C.

The sequencing was performed on an ABI 3730XL (Applied Biosystems, Foster City, CA, USA; service provided by Macrogen Inc., Seoul, South Korea), and the base calling was checked using ChromasPro (version 1.34; Technelysium Ltd., Helensvale, Qld, Australia). The sequences were deposited in GenBank.

Sequences alignment, phylogenetic reconstruction, haplotype network analysis

Each chloroplast region was aligned using the Clustal–Wallis algorithm (Thompson *et al.* 1997) as implemented in BIOEDIT 7.0.5.3 (Hall 1999) with minor manual corrections. Gaps were coded using FASTGAP 1.2 (Borchsenius 2009) following the method of Simmons & Ochoterena (2000) and appended to the DNA matrix as a supplementary partition.

Bayesian analyses were performed on the two cpDNA regions (considered as separate partitions) in a supermatrix approach using MrBayes version 3.1 (Huelsenbeck & Ronquist 2001) with substitution models estimated by MrAIC.pl 1.4.3 (Nylander *et al.* 2004) and four alpha categories for the gamma shape (Yang 1994). Four simultaneous Monte Carlo Markov Chains were run for 5×10^7 generations in four independent runs, and a

Fig. 1 Flower habitus of the French endemic *Ophrys aymoninii* (left), the widespread *Ophrys insectifera* (middle) and the Spanish endemic *Ophrys subinsectifera* (right).

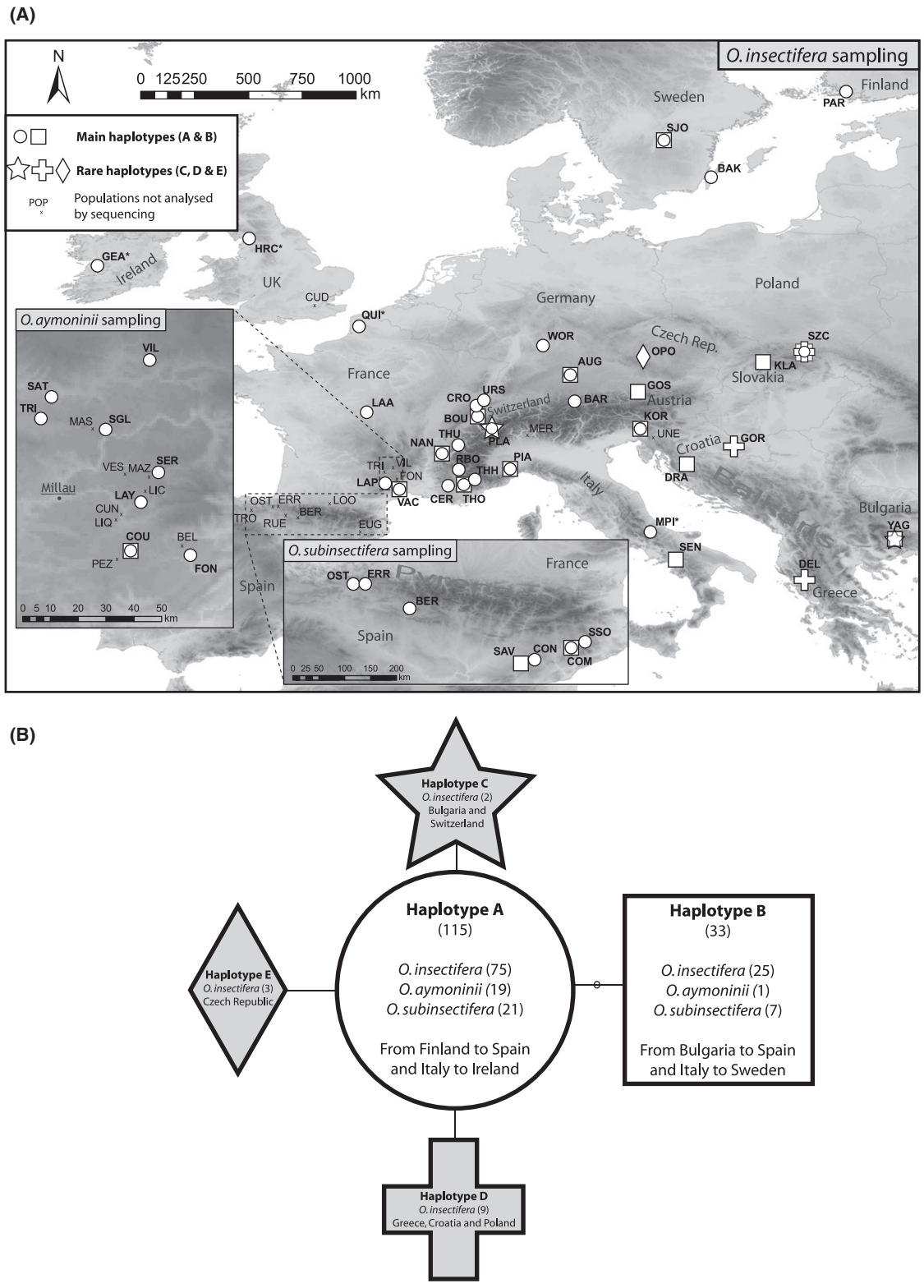

Fig. 2 (A) Map of the populations sampled for genetic analyses, showing the geographical location of each haplotype. Both main and rare haplotypes are represented with similar symbols in Figs 2B and S3 (Supporting information). Close-ups are provided for regions hosting endemic taxa. Populations represented by one single sequenced individual are marked with an asterisk. (B) Schematized results of the statistical parsimony network analysis. Each haplotype is represented by a different shape (reported in A). The total number of samples (detailed for each phenotypic species) and the general distribution of the corresponding haplotype are given.

tree was saved every 1000 generations. Convergence of the MCMC runs was tested by computing the potential scale reduction factor (Gelman & Rubin 1992) as implemented in MrBayes and by determining the effective sample size using Tracer 1.4.1 (Rambaut & Drummond 2008). Accordingly, the burn-in period was set to 10^7 generations until stationarity in the likelihood value was established among the runs, and 10 000 sample points were discarded. The last 40 000 trees were used to calculate the half-compatible topology (i.e. majority-rule) and the Bayesian posterior probability at each node.

A haplotype network was also performed on the combined data set of both regions using the statistical parsimony network as implemented in TCS 1.21 (Clement *et al.* 2000). The analysis was carried out by applying a 95% connection limit with gaps treated as missing data.

AFLP genotyping and scoring

Amplified fragment length polymorphisms (AFLP) reactions were performed following the method of Gugerli *et al.* (2008), which is similar to the protocol of Vos *et al.* (1995) with minor modifications. The reactions were conducted in 96-well plates in which individuals were distributed randomly. From a pilot study, two primer combinations (*Eco*RI-ACAG/*Mse*I-CAA and *Eco*RI-ACAG/*Mse*I-CTG with FAM-labelled EcoRI primers) were selected based on their suitability with respect to number of bands, level of variation among loci and reproducibility. The final selective PCR products were mixed with a 500 LIZ size ladder and analysed using an ABI 3730XL capillary sequencer (service provided by Macrogen Inc.). The raw electropherograms were analysed with PeakScanner V1.0 (ABI, using default peak detection parameters, light smoothing and a 100-rfu fluorescence threshold) to detect and calculate the sizes of the AFLP bands. The scoring was performed using RawGeno, an automated scoring R CRAN package (Arrigo *et al.* 2009). The library was developed following recommendations previously described by Arrigo *et al.* (2012): scoring range = 100–400 bp, minimum bin width = 1 bp, maximum bin width = 1.5 bp, minimum bin fluorescence = 150 rfu. The AFLP reactions were independently replicated with ten to 15 individuals chosen randomly from each plate (i.e. 15% of the final data set) to eliminate bands with low reproducibility.

Genotypic differentiation among species and phylogeographic pattern

The extent of the genetic structure was further evaluated using Analyses of Molecular Variance (AMOVA) as implemented in GenAlEx 6.5 (Peakall & Smouse 2006). Two different a priori criteria were used as subpopulation groups (i.e. morphological taxa and genetic clusters obtained by *K*-means; see below) to calculate Φ_{pt} estimates (analog of Fst for binary data such as that provided by AFLP markers). Statistical testing of Φ_{pt} was performed by random permutation (999 permutations).

Subsequently, the detailed phylogeographical patterns were investigated with nonhierarchical *K*-means clustering (Hartigan & Wong 1979), which is a method that has already been successfully applied in a phylogeographical framework based on AFLP markers (Burnier *et al.* 2009; Arrigo *et al.* 2010). The analyses were performed using R (package 'stats'; R Development Core Team, 2009) with custom R scripts (available from the authors upon request). Individuals were assigned to a defined number of genetic groups (hereafter, *K*) following an iterative process to maximize the intergroup variance (measured here as the inertia; Legendre & Legendre 1998). We performed 100 000 independent runs (i.e. starting from random points) for each assumed value of *K* (ranging between 1 and 10) and recorded the intergroup inertia of each run (following Kergoat & Alvarez 2008). We adapted the strategy proposed by Evanno *et al.* (2005) to select the most likely number of groups using intergroup inertia as a proxy of clustering accuracy (see details in Fig. S2, Supporting information). As a complementary representation of genetic structure, a principal coordinate analysis (PCoA) was computed between individuals on a Jaccard distance matrix and labelled with the *K*-means clusters. The level of congruence between *K*-means clusters and species identification was assessed using a χ^2 test, and all computations were performed using methods implemented in R CRAN.

Morphological and ecological differentiation among taxa

From 2005 to 2008, within the sympatric ranges between *O. insectifera* and the endemics (i.e. from the Spanish Pyrenees and the Causses region in France), we examined in many populations covering the full range of the two endemics (see Fig. S1, Supporting information, populations encircled in white) a large number of individuals (*O. insectifera:* $N = 230$, *O. aymoninii*: $N = 131$, *O. subinsectifera*: $N = 84$) for which we measured the following morphological traits: (i) average labellum length, (ii) average distance between subsequent flowers and (iii) number of flowers. The length of the labellum (measured from the point of contact with the base of the column to its tip) can be matched with the body length of the pollinator (van der

Cingel 1995; Delforge 2005; Vereecken 2009). The number of flowers and the distance between them (measured from the base of one peduncle to the adjacent peduncle) are essential components of floral display and attractiveness to pollinators. Still, both of these traits are subject to wide environmental plasticity reflecting habitat quality. These three morphological characteristics are quickly measured in the field and are crucial species-specific traits of the pollination syndrome. All measurements were performed on fresh material using only blooming flowers from living plants. We then used a logistic regression model with a binomial distribution (i.e. a model that assesses whether an explanatory variable can discriminate between two states of a response variable, which, in our case, is a variable belonging to one species or the other) to discriminate the taxa based on these three morphological traits. To complement this analysis, we reviewed all the previously described biological differences between these three morphologically recognized species from the available literature (see Table 1).

Within the sympatric ranges (see Fig. S1, Supporting information), we also compared the ecological characteristics of species distribution. In addition to the populations from our own sampling that were used for morphological measurements and/or for genetic analyses, we collected GPS occurrences of the three species from reliable data (i.e. adequately precise and obtained using a modern GPS system) that were obtained by amateur members of the botanical network of the French Society of Orchidophily (http://www.sfo-asso.com). We compiled two digital elevation models (DEM), one for each region, at a resolution of 30 m using the *ASTER* Global DEM obtained from NASA. From the DEM, we obtained three ecological descriptors of the stations occupied by the species: elevation (a good proxy for temperature, Körner 2007), slope (important for water drainage) and curvature (an estimate of soil nutrient accumulation, Körner 2003). The latter descriptor defined whether the station lies on a convex (positive values) or concave (negative values) surface; lower values of curvature indicate a surface with greater concavity that is thus likely to accumulate nutrients from neighbouring slopes. Again we used a logistic regression model with a binomial distribution to discriminate the ecological conditions associated with *O. insectifera* and *O. subinsectifera* in the Pyrenees (Spain) as well as *O. insectifera* and *O. aymoninii* in the Causses region (France).

Results

Chloroplast data

Amplification of the chloroplastic *ndhA* intron and the *psbJ-petA* intergenic spacer was successful for 169 and

Table 1 Morphological and ecological differences between the three Fly Orchid species compiled from the literature

	Ophrys aymoninii	*Ophrys insectifera*	*Ophrys subinsectifera*
Petal color	Green	Dark brown	Dark brown with green edges
Petal size	4–7 mm	4–7 mm	2–4 mm
Labellum length	9–12 × 8–12 mm	(8–) 9–12 × (5–) 6–10 mm	6–10 × 5–8.5 mm
Labellum yellow border	1–2.5 mm	0 mm	1–1.5 mm
Plant height	15–60 (–80) cm	15–60 (–80) cm	9.5–30 (–45) cm
Ploidy	$2n = 36$	$2n = 36$	Unknown
Pollinator	*Andrena combinata* (Hymenoptera, Apidae)	*Argogorytes mystaceus* & *Argogorytes fargeii* (Hymenoptera, Crabronidae)	*Sterictiphora gastrica* (Hymenoptera, Argidae)
Pollinator body length	8.5–9.5 mm	9.5–12 mm	6.8–7.2 mm
Scent composition	1-pentanol, 1-hexanol, 2-hexanol, 1-heptanol, 1-octanol, 1-nonanol, 1-dodecanol, 1-tetradecanol, 1-octadecanol, methyl pentadecanoate, methyl hexadecenoate, methyl octadecanoate, linalool, oxygenated monoterpene, *trans*-thujanol	Tridecene, tetradecene, pentadecene (a and b), hexadecene, nonadecene, 2-nonanol, *trans*-furanoid linalool oxide,	Unknown
Phenology	V–VI	(IV–) V–VII	V–VI
Climate	Mild and dry	From warm and dry to cold and moist	Warm and dry

Delforge (1983, 2005), Borg-Karlson *et al.* (1993), Amardeilh (1996) and Vereecken (2009).

166 individuals, respectively. Both regions were amplified for 162 individuals. An alignment yielded a total of 1582 bp for the two cpDNA regions: 821 bp for the *ndhA* intron (one parsimony-informative site among 11 polymorphic sites in the ingroup) and 761 bp for the *psbJ-petA* intergenic spacer (two parsimony-informative sites among two polymorphic sites in the ingroup). The phylogenetic analysis confirmed the monophyly of the Fly Orchid group with high support, indicating that this group was well segregated from all outgroup species (see Fig. S3, Supporting information). Genetic variation was very low among the 162 individuals of the *Fly Orchid* group because only three parsimony-informative sites were observed (see above). Figure 2B shows the SNP haplotype network analysis of the five distinct haplotypes detected (two frequent and three rare). Among the 45 populations represented by at least two sequenced individuals (out of 50 analysed in total), 37 had a single haplotype (82%), 12 comprised two haplotypes (27%) and one (the Polish population SZC) was composed of three haplotypes. The two main haplotypes (A and B) were connected by two mutation steps and included more than 90% of all the individuals analysed. However, they could not discriminate between *O. insectifera*, *O. aymoninii* and *O. subinsectifera*, indicating clear haplotype sharing among taxa. Both of the main haplotypes were widely distributed across Europe (Fig. 2A). While haplotype A occurred more frequently in Western Europe (and was absent from the Balkans), haplotype B was mostly limited to middle and lower latitudes (with the notable exception of the Swedish population SJO). Only 14 individuals (9%) possessed rare haplotypes (C, D and E), and each of these was connected by one single mutation step to haplotype A (Fig. 2B). These rare haplotypes occurred mainly in Eastern Europe (Czech Republic, Poland, Croatia, Bulgaria and Greece; Fig. 2A) as well as in a Swiss population (PLA). The rare haplotypes were found either in spatially distant populations (haplotype C was found in Bulgaria and Switzerland) or in a restricted part of the distribution (haplotype D was found in the Balkans and Carpathians). Finally, haplotype E was specific to a Czech population (OPO). Among the six populations presenting rare haplotypes, three (PLA, SZC and YAG) also comprised frequent haplotypes.

AFLP fingerprinting and genetic differentiation among species

All individuals from the three species of the *O. insectifera* group were scored together, resulting in a total of 167 AFLP markers with 95% reproducibility. Each individual (287 in total from 61 populations; see Table S1, Supporting information) produced between 88 and 107 fragments. As presented in Table 2, some genetic differentiation was detected among the three Fly Orchid species (global $\Phi_{pt} = 0.065$, P value <0.001, with 7% estimated variance among species). The PCoA analysis indicated a trend towards structuring by species (see Fig. S4A, Supporting information). The two endemics showed distinct levels of genetic differentiation with the widespread species *O. insectifera* being more distant from *O. aymoninii* than from *O. subinsectifera*. Finally, the largest genetic distance was observed between *O. aymoninii* and *O. subinsectifera*.

Phylogeography of the Fly Orchid group

Based on inertia values (see Fig. S2, Supporting information), the best *K*-means results were obtained when

Table 2 Pairwise Φ_{pt} values from AMOVAS performed on the amplified fragment length polymorphisms data set with (A) the taxonomy or (B) the *K*-means genetic clustering used as grouping criterion. Φ_{pt} values are shown below the diagonal, with corresponding *P*-values shown above the diagonal (***$P < 0.001$)

A	*Ophrys aymoninii*	*Ophrys insectifera*	*Ophrys subinsectifera*
O. aymoninii	0.000	***	***
O. insectifera	0.068	0.000	***
O. subinsectifera	0.079	0.047	0.000

B	K1	K2	K3	K4	K5	K6	Average pairwise Φ_{pt} value
K1	0.000	***	***	***	***	***	0.221
K2	0.189	0.000	***	***	***	***	0.137
K3	0.293	0.141	0.000	***	***	***	0.165
K4	0.200	0.121	0.130	0.000	***	***	0.122
K5	0.198	0.095	0.147	0.091	0.000	***	0.124
K6	0.254	0.145	0.105	0.087	0.128	0.000	0.144
K7	0.194	0.127	0.174	0.100	0.087	0.144	0.138

considering $K = 4$ or $K = 7$ clusters. The PCoA analysis showed a weak cluster-effect (see Fig. S4B, Supporting information). The genetic clusters showed a slightly higher level of genetic structure than previously obtained considering the interspecific level (global $\Phi_{pt} = 0.129$*** with 13% estimated variance among clusters). The geographic distribution of the $K = 7$ genetic clusters (hereafter K1–K7; results for $K = 4$ are provided in Fig. S5, Supporting information) appeared fuzzy (Fig. 4) because the majority of populations (52/61) included individuals from more than one single cluster. Even if most genetic clusters were widely distributed across Europe and showed no clear geographic structure, our results outlined an eastern-western gradient of genetic differentiation. For example, the K2 genetic cluster (Fig. 4, green) was frequent in western populations (except within the *O. aymoninii* endemic area; see below). In contrast, the K7 cluster (white) occurred mostly in southern and eastern European populations but was missing in the British Isles and southeastern Balkans. Corresponding results were obtained with $K = 4$ clusters (see Fig. S5, Supporting information). Finally, the K5 genetic cluster (Fig. 4, yellow) was dominant in central Europe. The following three genetic clusters were more restricted to particular regions of Europe: The K1 cluster (Fig. 4, pink) was almost exclusively present in the southwestern Balkans, forming homogeneous populations in Greece and Bulgaria. The K3 and K6 clusters (Fig. 4, purple and red, respectively) were highly prevalent in many populations from the southwestern distribution (France and Spain); however, these clusters were rare elsewhere in Europe.

Some degree of overlap occurred between the *K*-means clusters and the taxonomic species boundaries (Fig. 5), which was confirmed by the χ^2 test performed on a matrix with clusters and species in columns and lines, respectively ($\chi^2 = 199.09$, d.f. = 12, P value <0.0001). The widespread *O. insectifera* included a homogenous set of individuals from several genetic clusters. The homogeneity in the proportion of genetic clusters seems to vary regionally though (see below). Two genetic clusters were strongly representative of *O. insectifera*: the restricted Balkan K1 cluster (exclusive to this species) and the K7 cluster (except for one *O. subinsectifera* individual). The Spanish endemic *O. subinsectifera* was clearly dominated by the K4 genetic group (14 of 20 samples). A similar pattern was observed for the sympatric *O. insectifera* individuals collected in the southern Pyrenees (top left pie chart in Fig. 5). The French endemic *O. aymoninii* was composed of two dominant clusters, K3 and K6, which together represented 83 of 98 individuals. With respect to the sympatric *O. insectifera* individuals collected in the Causses region (top right pie chart in Fig. 5), relative proportions of genetic clusters did not particularly match those found in *O. aymoninii*.

Morphological and ecological differentiation among taxa

Significant morphological differences (Fig. 3A) were observed between *O. insectifera* and *O. aymoninii* (average labellum length: $z = -6.75$, P value <0.0001; average distance between flowers: $z = -3.57$, P value = 0.0003; number of flowers: $z = -11.56$, P value <0.0001) as well as between *O. insectifera* and *O. subinsectifera* (average labellum length: $z = 15.02$, P value <0.0001; average distance between flowers: $z = -7.27$, P value <0.0001; number of flowers: $z = -5.47$, P value <0.0001). The variance in labellum length was larger in *O. insectifera* than in the two endemics. We did not investigate variation on an interpopulation level given that our sampling was highly variable across populations (ranging, for example, from one to seven measures made for some variables in *O. aymoninii* and *O. subinsectifera*).

In the Causses region, significant ecological differentiation (Fig. 3B) was detected between *O. insectifera* and *O. aymoninii* with respect to elevation ($z = 4.36$, P value <0.0001) and slope ($z = -2.48$, P value <0.0001) but not curvature ($z = -0.60$, P value = 0.56). Additionally, a large variance difference was observed between ecological factors when comparing *O. insectifera* and *O. aymoninii* in their sympatric range, attesting to the narrower ecological niche of the latter. In contrast, we found no significant ecological differentiation in the Pyrenees between *O. insectifera* and *O. subinsectifera* (elevation: $z = 0.55$, P value = 0.56; slope: $z = -0.22$, P value = 0.58; curvature: $z = 0.74$, P value = 0.46).

Discussion

Fly Orchid species differ morphologically and ecologically

Our study established that *Ophrys insectifera* and its endemics differ morphologically (Fig. 3A). These results corroborate the current taxonomic treatment of the group, in which a total of three species are described based on habitus evidence (Breistroffer 1981; Delforge 1983, 2005; Amardeilh 1996; Hermosilla *et al.* 1999). Accordingly, the two endemics differ significantly from *O. insectifera* with respect to every floral trait investigated, with the largest differences observed for labellum length (Fig. 3A). Differences among taxa were further confirmed by our review of morphological and functional characteristics, which highlighted marked differences in terms of scent composition and pollinators (Table 1). Flower morphology is expected to primarily

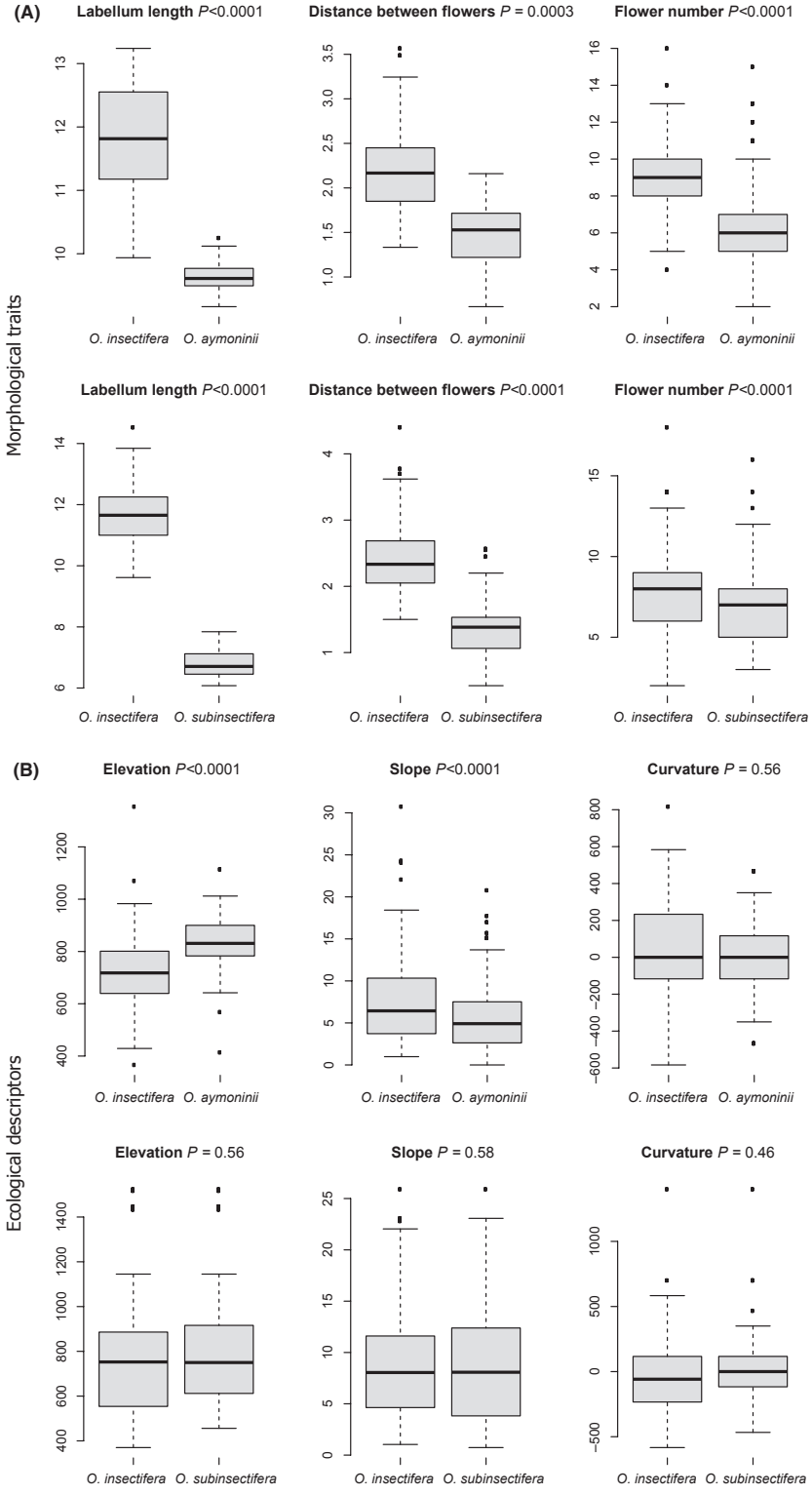

Fig. 3 Boxplots displaying the differences in morphological (A) and ecological (B) traits investigated in individuals of *Ophrys insectifera* and *Ophrys aymoninii* in the Causses region and *O. subinsectifera* in the Pyrenees. For each boxplot, the centre line represents the median, the hinges mark the first and third quartiles and the whiskers extend to the lowest and highest non-outliers. The *P*-values obtained from the logistic regression analyses are also provided.

be associated with pollinator characteristics (see Table 1). Several authors have reported a significant correlation between labellum length and pollinator body size (Benitez-Vieyra *et al.* 2009; Xu *et al.* 2012), which is a pattern usually interpreted as a signature of pollinator-driven selection because the labellum length constrains the removal of pollinia by the pollinator (Xu *et al.* 2012). Accordingly, the three Fly Orchid species

investigated here are pollinated by Hymenoptera that belong to phylogenetically distant families (see Table 1) and differ in their body size (this is particularly true for *O. subinsectifera*, which is pollinated by a sawfly substantially smaller than the other Fly Orchid pollinators; Vereecken 2009).

Furthermore, our study shows that pollinator shifts are correlated with ecological differences (see Fig. 3B) because simple abiotic factors were observed to segregate among species within sympatric areas. This is illustrated by the case of *O. insectifera* and *O. aymoninii*, the latter of which grows at higher elevations and on more gentle slopes, and the two species are found in sympatry only in particular conditions (i.e. along forest edges or in open woodlands; B. Schatz, personal observation). No such ecological trends could be identified between *O. insectifera* and *O. subinsectifera*. Thus, other less obvious abiotic (e.g. soil chemical composition or humidity) or biotic (e.g. associated mycorrhizal strains) factors should be tested to further understand discriminating ecological factors.

Hence, in addition to the correlation between pollinator shifts and morphological variation, which might incur strong reproductive isolation that could potentially lead to progenitor-derivative speciation in sexually deceptive orchids (Schlüter *et al.* 2011), our results suggest that ecological features could also contribute to the differentiation processes on a local scale. The Fly Orchid group represents a well-suited system in which to examine this hypothesis. For example, strong morphological differences (extreme values for labellum length and distance between flowers, Fig. 3A) and morphologically distinct pollinators (Table 1) might keep *O. subinsectifera* and *O. insectifera* reproductively isolated even if they are growing in similar ecological conditions. In contrast, *O. insectifera* and *O. aymoninii*, which were shown to be more similar morphologically, might need additional ecological isolation to efficiently avoid cross-breeding, a phenomenon revealed here by differences in elevation and slope of locations where the two species are found (see Fig. 3B). Thus, the strength of the reproductive barriers between these two species deserves further attention via experimental hybridization.

Finally, the mismatch between the distributions of the endemic taxa and that of their pollinators is surprising. Cozzolino & Widmer (2005) demonstrated that the spatial limitation in dispersal abilities of orchids is strongly related to the presence of highly specialized pollinator species. However, *A. combinata* and *S. gastrica* are widely distributed across Europe (source: Fauna Europaea, http://www.faunaeur.org), while the two *Ophrys* endemics only appear in specific areas of *O. insectifera*'s widespread distribution (i.e. at its southern margin and mostly in mountainous areas, Fig. 2A and S1, Supporting information). This pattern might suggest that the endemic species resulted from a recent diversification (see below for further arguments in that respect) and have not yet reached the limits of their ecological distribution. Alternatively, specific ecological conditions, such as those in which the pollinator of *O. insectifera* is locally less abundant, might favour the emergence of endemic taxa specialized for pollination by common local Hymenoptera species.

Fly Orchid lineages are weakly differentiated genetically

Our study detected limited levels of genetic differentiation between the two endemics *O. aymoninii* and *O. subinsectifera* and the widely distributed *O. insectifera* (but see below). Indeed, although DNA sequencing of highly variable chloroplast regions consistently identified the four outgroups, only five haplotypes were detected within the Fly Orchid group. These haplotypes were not well differentiated (i.e. two step mutations at most) and the three investigated species could not be discriminated (see Figs. 2B and S3, Supporting information). Moreover, the relationship between the three rare and the two more frequent haplotypes could not be unravelled based on phylogenetic analysis, given the limited resolution of the topology. The inclusion of additional plastid markers might have depicted a clearer picture of differentiation. Such haplotype sharing suggests that the extant diversity of the Fly Orchid group results from a recent diversification process despite a Pliocene origin (based on the stem age of the group), which has also been recently inferred for *O. insectifera* s.l. (Inda *et al.* 2012). This pattern is consistent with conclusions drawn in other orchids, where the majority of extant diversity resulted from recent diversifications (e.g. Soliva *et al.* 2001; Devey *et al.* 2009; Stahlberg & Hedrén 2010). Accordingly, AFLP fingerprinting revealed only slight genetic differentiation between *O. aymoninii, O. subinsectifera* and the widespread *O. insectifera* (Table 2 and Fig. S4, Supporting information). Here, we suggest that the divergence between the species of the Fly Orchid group might be so recent that neutral differentiation did not have time to accumulate, which would explain the low level of discrimination using AFLP. Although somewhat unexpected (in general, AFLP markers adequately identified genetic structures in taxonomically challenging European orchid genera, for example, Hedrén *et al.* 2001; Schlüter *et al.* 2007; Devey *et al.* 2009; Gögler *et al.* 2009; Pfeifer *et al.* 2009), these results were in agreement with insights from chloroplast sequences and further suggested a recent diversification in the Fly Orchid group (Table 2 and Fig. S4, Supporting information).

We further examined how broad-scale spatial patterns shaped the genetic diversity of the Fly Orchid group irrespective of taxonomic considerations. This analysis, based on *K*-means clustering of AFLP fingerprints, detected seven distinct clusters (Fig. 4) with little genetic differentiation observable between the clusters (pairwise Φ_{pt} values ranging between 0.087 and 0.293; see Table 2). Fuzzy spatial patterns were outlined because several clusters, such as K2 and K5, were widespread across Europe, and more than 80% of the populations were characterized by cluster admixture. These results could reflect incomplete lineage sorting of ancestral alleles, owing to a potentially recent diversification of the group. Alternatively, long-distance dispersal and admixture all over Europe might account for the observed pattern. Indeed, the dispersion of most orchids is based on the outstanding flight abilities of the dust-like seeds (Squirrell *et al.* 2001). This biological feature could have maintained a low level of genetic differentiation by homogenizing populations over large distances (e.g. Alexandersson & Ågren 2000; Devey *et al.* 2009; Fay *et al.* 2009). In this context, any historical pattern in the extant genetic diversity of central European populations is likely to have been largely blurred. Yet, this scenario is mitigated by significant levels of spatial autocorrelation observed in the genetic diversity of several orchid species; a pattern suggesting that seed dispersal may predominantly occur locally (Chung *et al.* 2004; Jacquemyn *et al.* 2006).

Cryptic diversity and endemism in the Fly Orchid group

Identifying the patterns and processes of endemism is of major interest from a conservation perspective. Although low levels of genetic differentiation were observed between Fly Orchid species, our phylogeographic survey detected faint but consistent spatial patterns in several geographical areas. For instance, our phylogeographic results outlined the southeastern Balkans region (Fig. 4) as comparatively highly differentiated, with a unique cluster (K1) showing the highest genetic distance from all other clusters (the average pairwise Φ_{pt} value was 0.221, see Table 2). These results corroborate the remote nature of the southeastern Balkans, as reported in previous studies examining other species, which might be notably due to the orientation of the prevailing winds in Europe (Devey *et al.* 2009). The probable lack of optimal environmental connections with northwestern European areas during the last glacial maximum (Triponez 2010) is another result to further investigate with respect to the isolation of

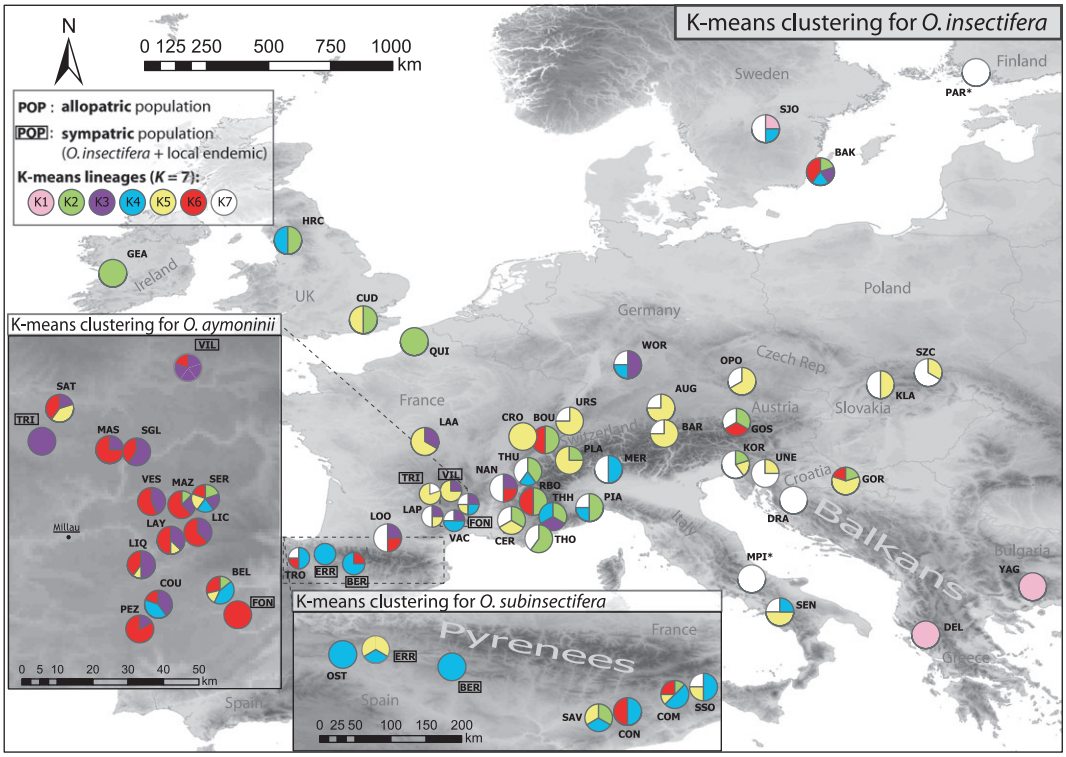

Fig. 4 Phylogeographical pattern of the *Ophrys insectifera* group using nonhierarchical *K*-means clustering (showing $K = 7$). The seven genetic clusters obtained are represented by different colours. For each population, represented as a pie chart, the average proportion assigned to each genetic group is indicated. Populations indicated with an asterisk were represented by a single individual.

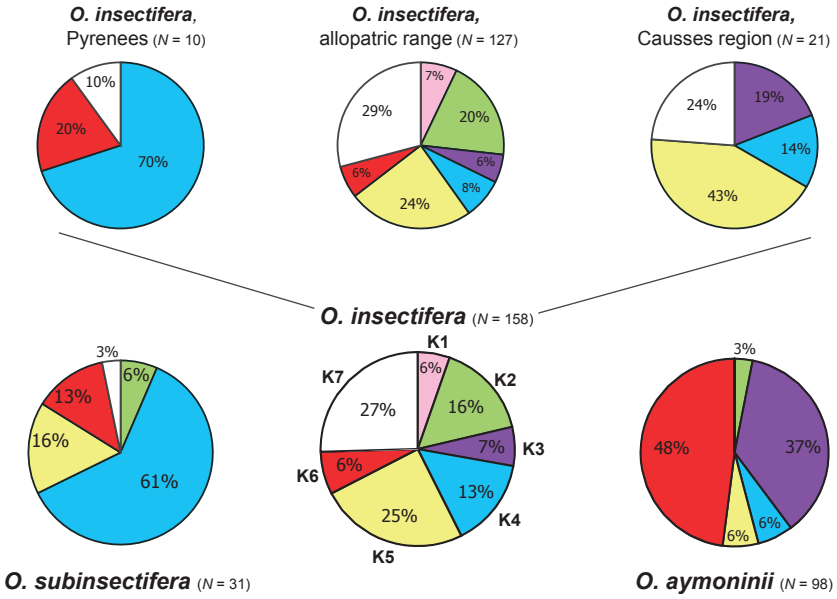

Fig. 5 Respective proportion of the seven genetic clusters obtained using nonhierarchical *K*-means clustering ($K = 7$) for each of the three species of the Fly Orchid group (bottom three pie charts). In *Ophrys insectifera*, patterns for both the two sympatric regions and the allopatric range are provided (top three pie charts).

Balkans region. Accordingly, this region could have been a Pleistocene refuge for several plant species (Thompson 2005; Fady & Conord 2009) including orchids (Hedrén *et al.* 2007). From a taxonomic perspective, this Balkan cluster is not currently recognized as an endemic species (Antonopoulos 2009; Tsvetanov et al. 2005), owing to its limited morphological distinctiveness (Y. Triponez, pers. obs.). A detailed morphological investigation would be required to better assess the endemism and the taxonomic status of these populations. Such cryptic diversity, if detected in some other parts of the Fly Orchid distribution, should be taken into account for optimizing conservation perspectives.

Three clusters defining as well consistent spatial patterns (Fig. 4) were detected in central France (K3 and K6) and Spain (K4). Remarkably, these three AFLP clusters overlapped with the endemics described by taxonomists: K3 and K6 included 85% of the specimens attributed to *O. aymoninii*, while K4 matched with 61% of the *O. subinsectifera* specimens (Fig. 5). An initial hypothesis on the processes of endemism would consider that the 'original' widespread species *O. insectifera* is currently differentiating into *O. aymoninii* and *O. subinsectifera*, two local entities corresponding to clearly distinctive morphotypes (Breistroffer 1981; Delforge 1983, 2005; Borg-Karlson *et al.* 1993; Amardeilh 1996; Hermosilla *et al.* 1999; Vereecken 2009). Additionally, differentiation between endemic taxa is mostly achieved via phenotypic signals, suggesting that these adaptive processes might be ongoing. For example, selection on these morphotypes (linked to the specialization of different pollinators as evidenced by the highly specific profile of chemical substances found in the floral scents of the different endemics; Vereecken *et al.*, in preparation) could be a possible evolutionary factor driving the emergence of new species. Independent ecological factors, such as those acting to isolate *O. aymoninii* and *O. insectifera* in the Causses, might also contribute to the general isolation process. The pattern of the genetic cluster proportion of each species (see Fig. 5) might give some credit to the hypothesis of current differentiation of *O. insectifera* into locally adapted entities. In particular, similar genetic composition were retrieved when comparing *O. insectifera* from the southern Pyrenees and *O. subinsectifera*, which indicates that differentiation associated with adaptation to a new type of pollinator might be at work here. Conversely for *O. aymoninii*, the high proportion of the K6 cluster (i.e. 48%) was not at all retrieved in *O. insectifera* from the Causses region. The French endemic should then have a more ancient origin, so that the particular K6 genetic cluster had time to emerge. Alternatively, *O. aymoninii* could be the result of a hybridization event with another *Ophrys* taxon, responsible for the large proportion of the K6 genetic cluster. Finally, with respect to such a hybrid origin of taxa, the hypothesis that *O. insectifera* is a more recent entity with a hybrid origin from several endemic parents is also possible. For example, one could imagine a scenario of secondary contact after a phase of allopatric separation, for example, after the ice age (Comes & Kadereit 1998; Widmer *et al.* 2009).

Conclusions

Our investigation highlighted a situation in which endemics were consistently distinct on a morphological, ecological and geographical level but did not exhibit comparable genetic differentiation. Similarly, previous

studies on orchids have identified very subtle changes in a few genes that result in a pattern of well-differentiated species with little detectable genetic differentiation (Schlüter *et al*. 2011). This paradoxical situation is also well-illustrated by the role of developmental plasticity in the emergence of new species. It has become increasingly clear that speciation can be initiated by phenotypic differences upon which local environmental selection acts, and this is a process that can ultimately lead to genetic differentiation and complete speciation (West-Eberhard 2003). The role of epigenetic factors also deserves further investigation, as it will be important to assess whether phenotypes and methylation patterns are related (e.g. Paun *et al*. 2010). As a final word considering species biodiversity and conservation, in view of our results, one should consider that morphology does not stand as an absolute indicator of underlying genetic diversity. To isolate particular areas of interest in which to explore the genetic diversity of the Fly Orchid group, most researchers would simply have agreed upon the notable importance of southwestern Europe due to the presence of endemic, morphologically distinct taxa. However, our large-scale phylogeographic study addresses the importance of hidden genetic diversity (i.e. diversity that is indiscernible based on morphological clues alone) in central and southeastern Europe that should still be taken into consideration from a conservation context.

Acknowledgements

Yann Triponez, Nils Arrigo and Nadir Alvarez received funding from the Swiss National Science Foundation (Prospective Researcher Fellowships PBNEP3-136536 and PBNEP3-132747 and Ambizione fellowship PZ00P3-126624, respectively). Yann Triponez received complementary funding for fieldwork from the Swiss Orchid Foundation. Loïc Pellissier was funded by the SNSF Bioassemble project (No 31003A-125145). Bertrand Schatz was funded by the 'Parc naturel regional des Grands Causses' (Ct no 040763) and by the 'Société Française d'Orchidophilie'. We thank Rod Peakall, Brigitte Marazzi, Mike Barker, Katrina Dlugosch and five anonymous reviewers for their insightful comments, as well as Anahí Espíndola and Nicolas Margraf for help with sampling. We thank all scientists and keen amateurs who permitted us to locate *Ophrys* populations across Europe: J.E. Arnold, A. Croce, C. Bernard, F. Dabonneville, B. Dolinar, P. Geniez, C.E. Hermosilla, L. Jacob, A. Jacquet, J. L. Menos, N. Petrou, D. Průša, G. Scopece, R. Souche, A. Soulié and numerous others for punctual information. This study was funded by the Swiss National Science Foundation (project no. 3100A0-116778).

References

Alexandersson R, Ågren J (2000) Genetic structure in the non-rewarding, bumblebee-pollinated orchid *Calypso bulbosa*. *Heredity*, **85**, 401–409.

Amardeilh JP (1996) *Ophrys subinsectifera* Hermosilla & Sabando. Une nouvelle orchidée du nord de l'Espagne. *L'Orchidophile*, **123**, 149–154.

Antonopoulos Z (2009) *The Bee Orchids of Greece – The Genus Ophrys*. Mediterraneo Editions, Rethymno.

Arrigo N, Tuszynski JW, Ehrich D, Gerdes T, Alvarez N (2009) Evaluating the impact of scoring parameters on the structure of intra-specific genetic variation using RawGeno, an R package for automating AFLP scoring. *BMC Bioinformatics*, **10**, 33.

Arrigo N, Felber F, Parisod C *et al*. (2010) Origin and expansion of the allotetraploid *Aegilops geniculata*, a wild relative of wheat. *New Phytologist*, **187**, 1170–1180.

Arrigo N, Holderegger R, Alvarez N (2012) Automated scoring of AFLPs using RAWGENO v 2.0, a free R CRAN library. In: *Data Production and Analysis in Population Genomics* (eds Pompanon F, Bonin A), pp. 155–176. Humana Press, Berlin, Germany.

Ayasse M, Stoeckl J, Wittko F (2011) Chemical ecology and pollinator-driven speciation in sexually deceptive orchids. *Phytochemistry*, **72**, 1667–1677.

Bateman RM, Hollingsworth PM, Preston J, Yi-Bo L, Pridgeon AM, Chase MW (2003) Molecular phylogenetics and evolution of *Orchidinae* and selected *Habenariinae* (Orchidaceae). *Botanical Journal of the Linnean Society*, **142**, 1–40.

Bateman RM, Devey DS, Malmgren S, Bradshaw E, Rudall PJ (2009) Conflicting species concepts underlie perennial taxonomic controversies in *Ophrys*. In: *Actes of the SFO colloquium*, Montpellier.

Bateman RM, Bradshaw E, Devey DS *et al*. (2011) Species arguments: clarifying competing concepts of species delimitation in the pseudo-copulatory orchid genus *Ophrys*. *Botanical Journal of the Linnean Society*, **165**, 336–347.

Benitez-Vieyra S, Medina AM, Cocucci AA (2009) Variable selection patterns on the labellum shape of *Geoblasta pennicillata*, a sexually deceptive orchid. *Journal of Evolutionary Biology*, **22**, 2354–2362.

Blondel J, Aronson J (1999) *Biology and Wildlife of the Mediterranean Region*. Oxford University Press, Oxford.

Borchsenius F (2009) *FastGap 1.2*. Department of Biological Sciences, University of Aarhus, Aarhus, Denmark.

Borg-Karlson AK, Groth I, Agren L, Kullenberg B (1993) Form-specific fragances from *Ophrys insectifera* L. (Orchidaceae) attract species of different pollinator genera. Evidence of sympatric speciation? *Chemoecology*, **4**, 39–45.

Breistroffer M (1981) Notes succinctes sur quelques équivalences nomenclaturales d'espèces céveno-caussenardes et description d'une sous-espèce nouvelle d'Orchidée. *Bulletin de la société botanique de France. Lettres botaniques*, **128**, 69–72.

Burnier J, Buerki S, Arrigo N, Küpfer P, Alvarez N (2009) Genetic structure and evolution of Alpine polyploid complexes: *Ranunculus kuepferi* (Ranunculaceae) as a case study. *Molecular Ecology*, **18**, 3730–3744.

Chase MW, Hills HH (1991) Silica-Gel – an ideal material for field preservation of leaf samples for DNA studies. *Taxon*, **40**, 215–220.

Chung MY, Nason JD, Chung MG (2004) Spatial genetic structure in populations of the terrestrial orchid *Cephalanthera longibracteata* (Orchidaceae). *American Journal of Botany*, **91**, 52–57.

van der Cingel NA (1995) *An Atlas of Orchid Pollination: European Orchids*. Balkema, Rotterdam.

Clement M, Posada D, Crandall KA (2000) TCS: a computer program to estimate gene genealogies. *Molecular Ecology*, **9**, 1657–1659.

Comes HP, Kadereit JW (1998) The effect of quaternary climatic changes on plant distribution and evolution. *Trends in Plant Science*, **3**, 432–438.

Cozzolino S, Widmer A (2005) Orchid diversity: an evolutionary consequence of deception? *Trends in Ecology and Evolution*, **20**, 487–494.

Delforge P (1983) Remarques sur *Ophrys insectifera* subsp. *aymoninii* Breistroffer et description d'un hybride nouveau de cette sous-espèce: *Ophrys insectifera* nsubsp. *tytecaeana* Delforge. *L'Orchidophile*, **55**, 307–312.

Delforge P (2005) *Guide des orchidées d'Europe, d'Afrique du Nord et du Proche-Orient*, 3rd edn. Delachaux et Niestlé, Paris.

Devey DS, Bateman RM, Fay MF, Hawkins JA (2008) Friends or relatives? Phylogenetics and species delimitation in the controversial European orchid Genus *Ophrys*. *Annals of Botany*, **101**, 385–402.

Devey DS, Bateman RM, Fay MF, Hawkins JA (2009) Genetic structure and systematic relationships within the *Ophrys fuciflora* aggregate (Orchidaceae: Orchidinae): high diversity in Kent and a wind-induced discontinuity bisecting the Adriatic. *Annals of Botany*, **104**, 483–495.

Espíndola A, Pellissier L, Hordijk W, Maiorano L, Guisan A, Alvarez N (2012) Predicting present and future intra-specific genetic structure through niche hind casting across 24 millennia. *Ecology Letters*, **15**, 649–657.

Evanno G, Regnaut S, Goudet J (2005) Detecting the number of clusters of individuals using the software STRUCTURE: a simulation study. *Molecular Ecology*, **14**, 2611–2620.

Fady B, Conord C (2009) Macroecological patterns of species and genetic diversity in vascular plants of the Mediterranean basin. *Diversity and Distributions*, **16**, 53–64.

Fay MF, Bone R, Cook P *et al.* (2009) Genetic diversity in *Cypripedium calceolus* (Orchidaceae) with a focus on north-western Europe, as revealed by plastid DNA length polymorphisms. *Annals of Botany*, **104**, 517–525.

Gelman A, Rubin DB (1992) Inference from iterative simulation using multiple sequences. *Statistical Science*, **7**, 457–511.

Gögler J, Stokl J, Sramkova A *et al.* (2009) Ménage à trois – two endemic species of deceptive orchids and one pollinator species. *Evolution*, **63**, 2222–2234.

Gugerli F, Englisch T, Niklfeld H *et al.* (2008) Relationships among levels of biodiversity and the relevance of intraspecific diversity in conservation: a project synopsis. *Perspectives in Plant Ecology, Evolution and Systematics*, **10**, 259–281.

Hall TA (1999) BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucleic Acids Symposium Series*, **41**, 95–98.

Hartigan JA, Wong MA (1979) Algorithm AS 136: a k-means clustering algorithm. *Applied Statistics*, **28**, 100–108.

Hedrén M, Fay MF, Chase MW (2001) Amplified fragment length polymorphisms (AFLP) reveal details of polyploid evolution in *Dactylorhiza* (Orchidaceae). *American Journal of Botany*, **88**, 1868–1880.

Hedrén M, Nordström S, Persson Hovmalm HA, Pedersen HA, Hansson S (2007) Patterns of polypoloid evolution in Greek marsh orchids (Dactylorhiza; Orchidaceae) as revealed by allozymes, AFLPs, and plastid DNA data. *American Journal of Botany*, **94**, 1205–1218.

Hermosilla CE, Amardeilh JP, Soca R (1999) *Sterictiphora furcata* Villers, pollinisateur d'*Ophrys subinsectifera* Hermosilla & Sabando. *L'Orchidophile*, **139**, 247–254.

Hewitt GM (1999) Post-glacial re-colonization of European biota. *Biological Journal of the Linnean Society*, **68**, 87–112.

Heywood VH (1995) The Mediterranean flora in the context of world diversity. *Ecologia Mediterranea*, **21**, 11–18.

Huelsenbeck JP, Ronquist F (2001) MRBAYES: Bayesian inference of phylogenetic trees. *Bioinformatics*, **17**, 754–755.

Inda LA, Pimentel M, Chase MW (2012) Phylogenetics of tribe Orchideae (Orchidaceae: Orchidoideae) based on combined DNA matrices: inferences regarding timing of diversification and evolution of pollination syndromes. *Annals of Botany*, **110**, 71–90.

Jacquemyn H, Brys R, Vandepitte K, Honnay O, Roldán-Ruiz I (2006) Fine-scale genetic structure of life history stages in the food-deceptive orchid *Orchis purpurea*. *Molecular Ecology*, **15**, 2801–2808.

Kergoat G, Alvarez N (2008) Assessing the phylogenetic usefulness of a previously neglected morphological structure through elliptic Fourier analyses: a case study in *Bruchus* seed-beetles (Coleoptera: Chrysomelidae: Bruchinae). *Systematic Entomology*, **33**, 289–300.

Körner C (2003) *Alpine Plant Life*, 2nd edn. Springer, Heidelberg.

Körner C (2007) The use of 'altitude' in ecological research. *Trends in Ecology and Evolution*, **22**, 569–574.

Kullenberg B (1961) Studies on *Ophrys* pollination. *Zoologiska Bidrag Fran Uppsala*, **34**, 1–340.

Legendre P, Legendre L (1998) *Numerical Ecology*. Elsevier Science B.V., Amsterdam.

Médail F, Diadema K (2009) Glacial refugia influence plant diversity patterns in the Mediterranean Basin. *Journal of Biogeography*, **36**, 1333–1345.

Médail F, Quézel P (1999) Biodiversity hotspots in the Mediterranean basin: setting global conservation priorities. *Conservation Biology*, **13**, 1510–1513.

Nylander JAA, Ronquist F, Huelsenbeck JP, Nieves-Aldrey JL (2004) Bayesian phylogenetic analysis of combined data. *Systematic Biology*, **53**, 47–67.

Paun O, Bateman RM, Fay MF, Hedrén M, Civeyrel L, Chase M (2010) Stable epigenetic effects impact adaptation in allopolyploid orchids (*Dactylorhiza*: Orchidaceae). *Molecular Biology and Evolution*, **27**, 2465–2473.

Peakall R, Smouse PE (2006) GENALEX 6: genetic analysis in Excel. Population genetic software for teaching and research. *Molecular Ecology Notes*, **6**, 288–295.

Peakall R, Ebert D, Poldy J *et al.* (2010) Pollinator specificity, floral odour chemistry and thephylogeny of Australian sexually deceptive *Chiloglottis* orchids: implications for pollinator-driven speciation. *New Phytologist*, **188**, 437–450.

Pfeifer M, Schatz B, Pico FX *et al.* (2009) Phylogeography and genetic structure of the orchid *Himantoglossum hircinum* (L.) Spreng. across its European central-marginal gradient. *Journal of Biogeography*, **36**, 2353–2365.

Pillon Y, Chase MW (2007) Taxonomic exaggeration and its effects on orchid conservation. *Conservation Biology*, **21**, 263–265.

R Development Core Team (2009) *R: A Language and Environment for Statistical Computing*. R Foundation for Statistical Computing, Vienna.

Rambaut A, Drummond AJ (2008) *Tracer v1.4.1*. Distributed by the authors. Institute of Evolutionary Biology, University of Edinburgh, Edinburgh, UK. Available at http://tree.bio.ed.ac.uk/software/tracer

Schiestl FP (2005) On the success of a swindle: pollination by deception in orchids. *Naturwissenschaften*, **92**, 255–264.

Schiestl FP, Ayasse M (2002) Do changes in floral odor cause speciation in sexually deceptive orchids? *Plant Systematics and Evolution*, **234**, 111–119.

Schlüter PM, Ruas PM, Kohl G, Ruas CF, Stuessy TF, Paulus HF (2007) Reproductive isolation in the Aegean *Ophrys omegaifera* complex (Orchidaceae). *Plant Systematics and Evolution*, **267**, 105–119.

Schlüter PM, Ruas PM, Kohl G, Ruas CF, Stuessy TF, Paulus HF (2009) Genetic patterns and pollination in *Ophrys iricolor* and *O. mesaritica* (Orchidaceae): sympatric evolution by pollinator shift. *Botanical Journal of the Linnean Society*, **159**, 583–598.

Schlüter PM, Ruas PM, Kohl G, Ruas CF, Stuessy TF, Paulus HF (2011) Evidence for progenitor-derivative speciation in sexually deceptive orchids. *Annals of Botany*, **108**, 895–906.

Schmitt T (2009) Biogeographical and evolutionary importance of the European high mountain systems. *Frontiers in Zoology*, **6**, 9.

Shaw J, Lickey EB, Schilling EE, Small RL (2007) Comparison of whole chloroplast genome sequences to choose noncoding regions for phylogenetic studies in angiosperms: the tortoise and the hare III. *American Journal of Botany*, **94**, 275–288.

Simmons MP, Ochoterena H (2000) Gaps as characters in sequence-based phylogenetic analyses. *Systematic Biology*, **49**, 369–381.

Soliva M, Kocyan A, Widmer A (2001) Molecular phylogenetics of the sexually deceptive orchid genus *Ophrys* (Orchidaceae) based on nuclear and chloroplast DNA sequences. *Molecular Phylogenetics and Evolution*, **20**, 78–88.

Squirrell J, Hollingsworth PM, Bateman RM *et al.* (2001) Partitioning and diversity of nuclear and organelle markers in native and introduced populations of *Epipactis helleborine* (Orchidaceae). *American Journal of Botany*, **88**, 1409–1418.

Stahlberg D, Hedrén M (2010) Evolutionary history of the *Dactylorhiza maculata* polyploid complex (Orchidaceae). *Biological Journal of the Linnean Society*, **101**, 503–525.

Svenning JC (2003) Deterministic Plio-Pleistocene extinctions in the European cool-temperate tree flora. *Ecology Letters*, **6**, 646–653.

Thompson JD (2005) *Plant Evolution in the Mediterranean*. Oxford University Press, New York.

Thompson JD, Gibson TJ, Plewniak F, Jeanmougin F, Higgins DG (1997) The clustalX windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Research*, **24**, 4876–4882.

Tribsch A, Schonswetter P (2003) Patterns of endemism and comparative phylogeography confirm palaeoenvironmental evidence for Pleistocene refugia in the Eastern Alps. *Taxon*, **52**, 477–497.

Triponez Y (2010) *Multi-species phylogeographic investigations in closely-related taxa and mutualistic plant-insect systems*. PhD thesis. Faculty of Sciences, Evolutionary Entomology, University of Neuchâtel, Neuchâtel, Switzerland. http://doc.rero.ch/record/20350

Triponez Y, Buerki S, Borer M, Naisbit RE, Rahier M, Alvarez N (2011) Discordances between phylogenetic and morphological patterns in alpine leaf beetles attest to an intricate biogeographic history of lineages in postglacial Europe. *Molecular Ecology*, **20**, 2442–2463.

Tsvetanov T, Vladimirov V, Petrova A (2005) New localities of *Ophrys insectifera* (Orchidaceae) in Bulgaria. In: *Proceedings of the Balkan Scientific Conference of Biology from 19th to 21st May 2005* (eds Gruev B, Nikolova M & Donev A), pp. 312–316. Plovdiv University, Plovdiv, Bulgaria.

Vereecken NJ (2009) *Pollinator-mediated selection, reproductive isolation and the evolution of floral traits in the genus Ophrys (Orchidaceae)*. PhD thesis. Faculty of Sciences, Behavioural and Evolutionary Ecology, Free University of Brussels, Brussels, Belgium.

Vereecken NJ, Streinzer M, Ayasse M *et al.* (2011) Integrating past and present studies on *Ophrys* pollination – a comment on Bradshaw *et al*.. *Botanical Journal of the Linnean Society*, **165**, 329–335.

Vos P, Hogers R, Bleeker M *et al.* (1995) AFLP- a new technique for DNA-fingerprinting. *Nucleic Acids Research*, **23**, 4407–4414.

Walters SM (1976) The conservation of threatened vascular plants in Europe. *Biological Conservation*, **10**, 31–41.

West-Eberhard MJ (2003) *Developmental Plasticity and Evolution*. Oxford University Press, New York.

Widmer A, Lexer C, Cozzolino S (2009) Evolution of reproductive isolation in plants. *Heredity*, **102**, 31–38.

Wiens JJ (2011) The niche, biogeography and species interactions. *Philosophical Transactions of the Royal Society B*, **366**, 2336–2350.

Xu SQ, Schluter PM, Scopece G *et al.* (2011) Floral isolation is the main reproductive barrier among closely related sexually deceptive orchids. *Evolution*, **65**, 2606–2620.

Xu SQ, Schluter PM, Schiestl FP (2012) Pollinator-driven speciation in sexually deceptive orchids. *International Journal of Ecology*, doi: 10.1155/2012/285081.

Yang ZH (1994) Maximum-Likelihood phylogenetic estimation from DNA-sequences with variable rates over sites-approximate methods. *Journal of Molecular Evolution*, **39**, 306–314.

Y.T. and N. Alvarez designed the study. Y.T., B.S. and N. Alvarez collected samples. Y.T. produced genetic data. B.S. provided morphological data. Y.T., N. Arrigo and L.P. performed data analysis. Y.T. drafted and edited the manuscript. All authors contributed to manuscript writing.

Data accessibility

DNA sequences: GenBank accession numbers KC120261–KC120595.

Final DNA sequence assembly, AFLP matrix, morphological and ecological data: DRYAD entry, doi:10.5061/dryad.16b00.

Supporting information

Additional supporting information may be found in the online version of this article.

Table S1 Detailed list of the populations sampled for DNA analyses.

Fig. S1 Close-ups of endemic regions (A: *O. subinsectifera*; B: *O. aymoninii*) showing on a layer consisting of an elevation model all populations from which ecological descriptors were obtained.

Fig. S2 The ΔK statistic, adapted from Evanno *et al.* (2005), was used to identify the optimal K number of groups from the K-means analysis.

Fig. S3 Phylogenetic analysis based on Bayesian inference showing the monophyly of the clade comprising specimens from *O. insectifera sensu lato*.

Fig. S4 PCoA plots of all genotyped specimens within the *O. insectifera* group with convex hulls encapsulating each group according to (A) the taxonomic identity of the species or (B) the seven K-means clusters.

Fig. S5 Phylogeographical pattern of the *O. insectifera* group using nonhierarchical K-means clustering (showing $K = 4$).