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Systematic position of *Ophrys gresivaudanica* Gerbaud

Abstract

Ophrys gresivaudanica is a late flowering species observed in south-eastern France. It grows in sympatry with some other orchid species including *O. fuciflora*. Plants are taller than those of *O. fuciflora* and flower one month later with smaller flowers. The relationships with other *Ophrys* are not yet known. Other late flowering *Ophrys* like *O. santonica* are described in stands distant of hundreds of kilometres. Is there a single late flowering taxon? Three stands of *O. gresivaudanica* were collected and analysed. A biometric approach and DNA markers were combined to determine the taxon status. Measures confirmed the higher plant size and the smaller flower size of *O. gresivaudanica*. ITS sequences were obtained and integrated in available published ones; they revealed a close relationship with *O. fuciflora* while *O. santonica* was related to *O. scolopax*, a close species to *O. fuciflora*. Variation in the ITS sequences was very low and the investigated taxa are not really differentiated. In *Ophrys*, many taxa are described as species, in spite of their low differentiation. This suggests that morphological variations are more relevant to varieties or subspecies than to species. Evolutive selection pressures on flower morphology in *Ophrys* limit utilisation of these traits in systematics.

Introduction

Several late flowering *Ophrys* species have been described like *Ophrys gresivaudanica and O. santonica* in France, they grows in sympatry with some other orchid species including *Ophrys sp. O. gresivaudanica* plants are taller and flower one month later with smaller flowers than *O. fuciflora* plants observed in the same stands (Gerbaud 2002). Are these late flowering orchids isolated from sympatric species or local varieties? We focus the study on *O. gresivaudanica* and *O. fuciflora* in order to compare plant morphology and genetics.

Materials and methods

Three stands located at different elevations (350 m, A, 650 m, B, and 730 m, C) in the French Isère department were sampled in 2003. Populations of *O. fuciflora* and *O. gresivaudanica* are there usually observed. *O. fuciflora* plants were measured and collected in May (9th - 16th) and *O. gresivaudanica* one month later, June (18th - 24th).

Plant height, leave width and length were measured. Various quantitative parameters were recorded on flowers (sepal size, petal size, lip size, column size). Ratios of some parameters were included in the study to represent organ shapes. Due to the possible effect of phenology on trait values, size of plant, leaves and flower could be influenced by different environmental conditions. Variables representing organ size were thus directly analysed or reported on one hand on the height of plant (height of first flower to avoid possible further growth effect) to represent the vegetative investment of plant and on the other hand, on the ovary length, to represent the reproductive investment of plants. Differentiations among stands and species were assessed from analysis of variance and shown by principle component analyses, using Statgraphics software.

DNA was extracted from bracts. Microsatellites loci according to Soliva et al. (2000) and ITS sequences were analysed to determine genetic differentiation of *O. gresivaudanica* and *O. fuciflora*. Data were completed with sequences found in genbank. A phylogenetic analysis was performed using PAUP software (Swofford, 1998) in order to obtain the parsimonious trees and then the strict consensus tree.

Results

The stand located at the lowest elevation consisted of very few plants, probably because of effects of the long and strong drought expressed in 2003. Only a single plant of *O.*

gresivaudanica was suitable for measurements. Plants of the two other stands exhibited the typical morphology and were probably not affected by drought.

Analyses of morphological traits confirmed differences between *O. gresivaudanica* and *O. fuciflora*: *O. gresivaudanica* plants are taller and bear smaller flowers than *O. fuciflora* ones. Most significant differences were noticed for sepal width, lip width and length, speculum length, stigmatic cavity width and length, distance between lateral protuberances and protuberance width. These traits show smaller values in *O. gresivaudanica*. It showed a higher ratio length/ width of lip and a more developed basal field than *O. fuciflora*. Variation among stands was noticed mostly for total plant height and stigmatic cavity size. When data were reported on height of the first flower, most recorded parameters on flower morphology showed significant reduced size in *O. gresivaudanica*. When they were reported on ovary length, almost variable became similar in both taxa. The complete flower was reduced with a similar shape in *O. gresivaudanica*.

Only two of the four microsatellites loci tested produced amplified DNA fragments, they revealed at least 11 alleles per locus. A high level of heterozygosity was noticed (0.584). The *O. gresivaudanica* sample did exhibit specific alleles.

ITS sequences were obtained in plants of *O. fuciflora*, *O. gresivaudanica* and *O. santonica*. The sequence consisted of 645 base pairs of ITS1, 5.8S rRNA and ITS2; 218 sites were polymorphic (mostly because of the outgroup species: only 107 sites were variable in *Ophrys*, indels exluded). Finally 135 sites were informative. No variation within species was detected for the analysed plants (2-3 per taxon). *O. santonica* exhibited a sequence identical to O. scolopax, *O. gresivaudanica* was very close to *O. fuciflora* (2 different sites) and seemed derived from this later (Figure 1). *O. fuciflora* and *O. scolopax* were also very close (1 different site). These four taxa expressed very low polymorphisms.

Discussion and Conclusion

The morphological analysis confirmed differences between *O. gresivaudanica* and *O. fuciflora*. Most of these differences could be due to environmental effects related to the phenology of the taxa. Microsatellite analysis did confirm a differentiation between both taxa. The recent development of molecular tools and of DNA sequencing provides new insights in plant systematics, including orchids (Bateman et al, 2003). ITS sequences that were analysed in the present study are largely used plant phylogeny. The differentiation of *Ophrys* species remains very low (Soliva et al., 2001; Bateman et al, 2003) as observed in the present study. Species differ by few nucleotides although the sequence is not coding for a protein and presents a high rate of evolution, suitable for phylogenetic studies on related sepecies.

Two late flowering taxa were investigated and remain very close to a respective sympatric species. *O. santonica* with a sequence identical to *O. scolopax* should be considered as a variant of it. *O. gresivaudanica* was little distant to *O. fuciflora* with differences at 2 base pairs, and appeared derived from it. Many plant species exhibit a larger variation within species. *O. gresivaudanica* should be considered as a variety rather as a subspecies of *O. fuciflora* and partly isolated from it because of the small phonological overlap.

Systematics of *Ophrys* is based on flower variation which probably reflects poorly taxon relationships due to selection pressure applied on flower morphology, colour and chemistry by pollinator insects. Unfortunately few vegetative traits are available for systematics in this genus. Systematics of this genus remains thus unclear. The number of species in the genus *Ophrys* increased recently and probably some described taxa are in fact just variations of pure species or hybrid forms. New investigations based on morphology and genetics are required to confirm their status.

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Figure 1. Strict consensus tree based on ITS sequences of *Ophrys* species, *Orchis militaris* was included as outgroup, numbers above branches are bootstrap value higher than 50%