Molecular Ecology (2000) **9**, 1911–1914

SHORT COMMUNICATION

Molecular analysis of orchid pollinaria and pollinaria-remains found on insects

A. WIDMER,* S. COZZOLINO,† G. PELLEGRINO,† M. SOLIVA* and A. DAFNI‡
Geobotanisches Institut, ETH Zürich, Zollikerstr. 107, CH-8008 Zürich, Switzerland, †Dipartimento di Biologia Vegetale, Università di Napoli Federico II, Via Foria 223, I-80139 Naples, Italy, ‡Laboratory for Pollination Ecology, The Institute of Evolution, University of Haifa, Haifa, Israel

Abstract

Direct observations of pollinator visits to orchids are often difficult and time consuming, especially in orchids with a deceptive pollination system where seed set is typically pollinator-limited. This lack of direct observations greatly inhibits our understanding of orchid–pollinator relationships and especially the degree of pollinator-specificity. Here we describe a molecular approach to the study of orchid–pollinator relationships based on the analysis of DNA recovered from pollinaria found on insects. The insects were collected from nectar-rich plants flowering near natural orchid populations, or taken from museum collections. Sequence analysis of the nuclear ribosomal ITS region allowed the identification of the orchid species or species-group from which the pollinaria originated. Four out of eight orchid–pollinator relationships established with this approach have not been reported previously, which highlights the value of molecular tools for the study of orchid pollination biology.

Keywords: Hymenoptera, identification, nuclear rDNA internal transcribed spacer (ITS), Orchidinae, plant-pollinator interaction, pollination

Received 29 April 2000; revision received 18 July 2000; accepted 18 July 2000

Introduction

Orchids are renowned for their extraordinary floral diversity and often intricate adaptations to pollinators. Despite the great interest in orchid biology and diversity, little is known about their pollination biology. While some orchid species are visited by a wide variety of pollinators, e.g. *Gymnadenia conopsea* (van der Cingel 1995) or *Anacamptis coriophora* (Eberle 1974; Peisl & Forster 1975; Vöth 1975; Dafni & Ivri 1979), others are visited by one or a few insect species, such as members of the genus *Ophrys* (e.g. Kullenberg 1961; Borg-Karlson *et al*. 1993; Schiestl *et al*. 1999). The associations with particular insects and *Ophrys* species is so tight, that Paulus & Gack (1990) stated that speciation in *Ophrys* was always accompanied by a change in pollinator. This view was supported by the observation that even sympatric *Ophrys* taxa with very similar floral morphology were typically pollinated by different pollinators. However, evidence for this specificity is relatively sparse as only few pollinations have ever been observed under natural conditions for many taxa. To improve our understanding of the specificity of orchid–pollinator interactions it is, therefore, of prime importance to increase the number of observations of orchid pollinations.

Direct observations of orchid pollination events are notoriously difficult, especially in species with a deceptive pollination system. The proportion of flowers visited per flowering season is often low in such orchid species (Neiland & Wilcock 1995). The long-life span of individual flowers in the absence of pollination and the low population densities commonly observed make direct observations exceedingly time consuming and difficult. However, orchid pollinators may be found relatively easily while foraging for nectar or pollen on food plants in the vicinity of orchid populations, and some can be attracted to scented baits (such as the neotropical Euglossine bees). If these pollinators have previously visited orchids, they often carry pollinaria or the remains of pollinaria on their body, mostly on their head or abdomen. The shape of these pollinaria sometimes allows identification of the orchid genus from which the pollinaria were picked up, but almost

Correspondence: Alex Widmer. Fax:. + 41 (0)1 632 14 63; E-mail: alex.widmer@geobot.umnw.ethz.ch

never allow the identification of the orchid species (M. Barone-Lumaga, personal communication). However, this is the crucial information needed for our understanding of the specificity of orchid–pollinator interactions.

The aim of this study, therefore, was to establish a method that allows the identification of the orchid species from which the pollinaria found on a pollinators body originated. We analysed a nuclear gene marker based on polymerase chain reaction (PCR) amplified DNA extracted from single pollinaria, assessed the suitability of pollinaria recovered from bees that were either recently collected in the field or were stored under museum conditions, and discuss the strengths and weaknesses of this approach for the study of orchid pollination biology.

For an appropriate molecular marker, we suggest the use of nuclear ribosomal internal transcribed spacer (nrITS) sequences, because: (i) sequence information for most European members of the Orchidinae is available for the ITS1 and ITS2 regions (Pridgeon *et al*. 1997; Aceto *et al*. 1999; A. Widmer *et al*. unpublished results); (ii) they were found to be variable among closely related taxa; (iii) nrITS sequences are easily amplified even from small amounts of degraded DNA because of its presence in high-copy numbers; and (iv) because of the extremely conserved primers available.

Materials and methods

Sample collection and storage

To test whether DNA quality in pollinaria remained suitable for PCR amplification over approximately one month, pollinaria were collected from *Barlia robertiana*, *Dactylorhiza romana*, *Ophrys sphegodes* and *Orchis pauciflora*, all grown in the orchid collection of the Naples botanic garden, and DNA was extracted either immediately, or after 1, 2, 3 or 4 weeks, respectively. Prior to extraction, pollinaria were stored without further fixatives at room temperature.

Insects carrying orchid pollinaria were collected in Italy and on the island of Lesvos (Greece) between 1986 and 1999 (Table 1). Most insects were caught with a butterfly net, killed, mounted on insect needles and stored at room temperature in boxes with naphthalene, to prevent insect damage. No particular measures were taken to prevent DNA degradation in the pollinaria.

DNA extraction, PCR and sequencing

DNA from single pollinaria was extracted using a slight modification of the CTAB protocol of Doyle & Doyle (1987). A single pollinarium was crushed with a sterile pistil in 200 µL of standard CTAB buffer, incubated at 60 °C for 15 min, extracted twice with chloroform-isoamyl alcohol, precipitated with isopropanol and washed with 70% ethanol. DNA was resuspended in 50 µL of 1× TE buffer or water. Alternatively, small portions of pollinaria from the same plants and preserved under identical storage conditions were amplified directly, without prior isolation of DNA, by adding some massulae (pollen packages typical for orchids) directly to the PCR mixture.

ITS1 was amplified with a pair of primers which anneal in the 3′ region of the 18S (5′-GGA GAA GTC GTA ACA AGG TTT CCG-3′) and in the 5′ region of the 5.8S (5′-ATC CTG CAA TTC ACA CCA AGT ATC G-3′), respectively. PCRs were then carried out for 30–35 cycles in a Perkin-Elmer Cetus 9700 thermocycler. Initial conditions were as

Table 1 Insect males (m) and females (f) collected with pollinaria or pollinaria remains and information on collection date and locality

Locality	Date	Orchid species in flower	Insect species	Orchid identified
Monticchio, Italy	10/04/1986	—	*Xylocopa violacea* (f)	*Anacamptis laxiflora*
Loutra, Greece	05/05/1998	*Anacamptis sancta*, *A. papilionacea*	*Anthidium septemdentatum* (m)	*Anacamptis sancta*
Marina di Lesina, Italy	31/03/1999	*Ophrys garganica*	*Andrena flavipes* (m)	*Ophrys fusca-lutea* group
Mattinata, Italy	04/04/1999	*Anacamptis collina*, *A. papilionacea*, *Barlia robertiana*, *Ophrys archipelagi*, *O. fusca* s.l., *O. garganica*	*Colletes cunicularius* (m)	*Ophrys archipelagi*
Manfredonia, Italy	05/04/1999	*Barlia robertiana*, *Ophrys fusca* s.l., *O. lutea* s.l., *O. sipontensis*, *Serapias* sp.	*Bombus ruderatus*(f) *Xylocopa iris* (m) *Xylocopa iris* (f) *Xylocopa iris* (m)	*Barlia robertiana* *Ophrys sphegodes* group *Serapias lingua* *Serapias lingua*

follows: 1 min denaturation at 94 °C, 1 min annealing at 55 °C, 45 s extension at 72 °C. Samples were denatured for 3 min at 94 °C before the beginning of the first cycle; extension time was increased by 3 s/cycle; extension was further prolonged for 7 min at the end of the last cycle. Alternatively, the complete ITS region, including ITS1, 5.8S, and ITS2, were amplified using primers ITS4 and ITS5 (White *et al*. 1990) as described in Widmer & Baltisberger (1999). PCR products generated with primers '18S' and '5.8S' (see above) were sequenced according to Aceto *et al*. (1999), and those obtained with primers 'ITS4' and 'ITS5' were sequenced as described in Widmer & Baltisberger (1999).

To identify the origin of pollinaria, ITS sequences were subjected to a FASTA search against GenBank or compared to personal orchid sequences (A. Widmer *et al*., unpublished results).

Results and Discussion

PCR products were obtained from all pollinaria and pollinaria remains extracted, irrespective of their age. No visible reduction of PCR product quantity or quality was observed when DNA was extracted either from fresh or 1–4 week-old pollinaria of *Barlia robertiana*, *Dactylorhiza romana*, *Ophrys sphegodes* and *Orchis pauciflora*. This result is consistent with our observation that pollinaria collected from insects and extracted 2–8 weeks thereafter could be easily amplified. PCR products obtained directly from massulae, without prior DNA extraction, gave slightly inconsistent results, in that the PCR product quantity, as judged from the fluorescence intensity on an ethidium-bromide stained agarose gel, varied considerably. However, this is most likely due to differences in the amount of massulae added to the reactions. DNA extracted from pollinaria that were attached to the heads of insects stored in insect collections at room temperature between 1986 and 1998, also yielded good PCR products. These PCR products were successfully sequenced without prior cloning.

The relative ease with which PCR products could be obtained from a 12-year-old sample (Table 1) that was stored under room conditions is presumably due to the presence of high copy numbers of the nuclear ribosomal RNA to which the ITS region belongs, and to the highly conserved primers. In addition, it is well established that orchid pollen remains viable over several weeks, presumably as an adaptation to the rare pollination events (Neiland & Wilcock 1995).

ITS sequences were obtained from pollinaria of *Anacamptis laxiflora*, *A. sancta*, *B. robertiana*, *Ophrys archipelagi*, one member each of the *O. fusca-lutea* and *O. sphegodes* group, and *Serapias lingua* (Table 1). Despite the small number of pollinaria and pollinaria remains analysed from insects, we found some undescribed orchid–pollinator relationships and confirmed others, some of which were based on very few observations.

We identified *Xylocopa violacea* as a pollinator of *A. laxiflora*. To the best of our knowledge, this is the first pollinator record for this orchid species. The same is true for *A. sancta* for which we identified *Anthidium septemdentatum* as a pollinator. A *Bombus ruderatus* queen was found to carry pollinaria of *B. robertiana*. These orchid species differ considerably in pollination biology. *A. laxiflora* is a nectarless orchid and presumably deceives a variety of large-bodied Hymenoptera, such as other *Xylocopa* or *Bombus*. *A. sancta*, on the other hand, produces nectar as does the closely related *A. coriophora*. The presence of nectar in the spur of *B. robertiana* remains a matter of debate and needs to be tested in the field.

In contrast to most other orchids, flowers of *Serapias* act as shelter for their visitors (Godfery 1931; Dafni *et al*. 1981). Their sepals, petals, and the basal part of the labellum form a tube that restricts access to the flowers. Pollinators found so far are solitary bees of the genera *Eucera*, *Andrena*, *Osmia*, and *Tetralonia* (Dafni *et al*. 1981). Our finding of pollinaria on *Xylocopa iris* is the first evidence of this genus as a pollinator of *Serapias* and was quite unexpected, because *X. iris* is a relatively large insect compared to the other known pollinators.

Flowers of the genus *Ophrys* are pollinated by sexual deception. Flowers imitate female hymenoptera in colour, morphology and scent and are pollinated by sexually excited males. Pollinaria are either attached to the head (most *Ophrys* species) or abdomen (*O. fusca-lutea* complex) of the pollinator. The floral scent is highly specific (Borg-Karlson *et al*. 1993; Schiestl *et al*. 1999) and may provide a strong prezygotic barrier to gene flow.

As expected, all insects carrying *Ophrys* pollinaria were male solitary bees. A male *Andrena flavipes* collected in a population of *O. garganica* carried pollinaria on the abdomen, which indicated that they could not be from *O. garganica* because pollinaria of this species would be attached to the head of pollinators. ITS1 and ITS2 sequences obtained from these pollinaria were identical to those typically found in Italian members of the *O. fusca-lutea* species complex (M. Soliva *et al*., unpublished results). The absence of plants of the *O. fusca-lutea* complex in the vicinity of the collection site indicates that pollinators may fly considerable distances after the removal of pollinaria which may lead to long distance gene-flow among populations.

Our results further confirmed that *O. archipelagi* is pollinated by *Colletes cunicularius* and *O. sipontensis* is pollinated by *X. iris* (Paulus & Gack 1990). We observed a large number of *X. iris* in an *Opuntia* field where a number of orchid species, including *O. sipontensis*, coflowered (Table 1). ITS1 and ITS2 sequences obtained

from pollinaria collected from a *X. iris* male were identical to *O. sipontensis* ITS sequences, strongly suggesting that they were indeed removed from *O. sipontensis*. However, ITS sequences are identical among most members of the *O. sphegodes* group (M. Soliva *et al*. unpublished results), of which *O. sipontensis* is a member. To further verify the origin of the pollinaria found on the *X. iris* male, more variable markers, such as microsatellites, could be used. Preliminary results suggest that microsatellites can be amplified successfully from DNA obtained from pollinaria and pollinaria remains, which may ultimately allow us to identify the origin of pollinaria even among very closely related taxa or to assess from which and how many plants pollinaria found on a particular pollinator originate.

The method described in this paper can be expanded to other plant lineages with aggregated pollen, such as, e.g. the Asclepiadaceae, and in combination with more variable markers may provide a powerful tool for analysing the origin and composition of pollen-loads found on insects. This may be particularly interesting in plant hybrid-zones (e.g. Broyles *et al*. 1996) or in studies of gene-flow between genetically modified crop-plants and native plant species.

A cautionary note, however, must be made concerning molecular analyses of pollen found on insects. Insects carrying pollen of a given plant are not necessarily pollinators of that plant species; they could simply be occasional flower visitors. This problem may be particularly prevalent in plants with unspecialized flowers that receive visits by a large number of insects, whereas it may be rarer in orchids and Asclepiadaceae, where a mechanical fit between the plant and insect is necessary for pollinaria to be removed.

Acknowledgements

We thank A. von Foerster, A. P. Møller, Ch. Salzmann, and J. A. Shykoff for valuable help in the field, S. Graf for help in the laboratory, A. Müller and M. Török for identifying pollinators, and B. A. Roy and two anonymous referees for valuable comments. This study was supported by Swiss Federal Institute of Technology (ETH) internal grants (no. 0–20–477–98 and no. 0–20600–99) and by the PRIN program of the Italian Ministry of the University and Scientific Research.

References

Aceto S, Caputo P, Cozzolino S, Gaudio L, Moretti A (1999) Phylogeny and evolution of Orchis and allied genera based on ITS DNA variation: morphological gaps and molecular continuity. *Molecular Phylogenetics and Evolution*, **13**, 67–76.

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

Broyles SB, Vail C, Sherman-Broyles SL (1996) Pollination genetics of hybridization in sympatric populations of *Asclepias exaltata* and *A. syriaca* (Asclepiadaceae). *American Journal of Botany*, **83**, 1580–1584.

van der Cingel NA (1995) *An atlas of orchid pollination*. A.A. Balkema, Rotterdam.

Dafni A, Ivri Y (1979) Pollination ecology of, and hybridisation between, *Orchis coriophora* L. and *O. collina* Sol. ex. Russ. (Orchidaceae) in Israel. *New Phytologist*, **83**, 181–187.

Dafni A, Ivri Y, Brantjes NBM (1981) Pollination of *Serapias vomeracea* Briq. (Orchidaceae) by imitation of holes for sleeping solitary male bees (Hymenoptera). *Acta Botanica Neerlandica*, **30**, 69–73.

Doyle JJ, Doyle JL (1987) A rapid DNA isolation procedure for small amounts of fresh leaf tissue. *Phytochemical Bulletin*, **19**, 11–15.

Eberle G (1974) Nektarausscheidung im Sporn des Wanzenknabenkrautes (*Orchis coriophora* L.)? *Die Orchidee*, **25**, 222–225.

Godfery MJ (1931) The pollination of *Coeloglossum*, *Nigritella*, *Serpias* etc. *Journal of Botany*, **69**, 129–130.

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

Neiland MRM, Wilcock CC (1995) Maximisation of reproductive success by European Orchidaceae under conditions of infrequent pollination. *Protoplasma*, **187**, 39–48.

Paulus HF, Gack C (1990) Pollinators as prepollinating isolation factors: evolution and speciation in *Ophrys* (Orchidaceae). *Israel Journal of Botany*, **39**, 43–79.

Peisl P, Forster J (1975) Zur Bestäubungsbiologie des Knabenkrautes *Orchis coriophora* L. ssp. *fragrans*. *Die Orchidee*, **26**, 172–173.

Pridgeon AM, Bateman RM, Cox AV, Hapeman JR, Chase MW (1997) Phylogenetics of subtribe Orchidinae (Orchidoideae, Orchidaceae) based on nuclear ITS sequences. 1. Intergeneric relationships and polyphyly of *Orchis Sensu Lato*. *Lindleyana*, **12**, 89–109.

Schiestl FP, Ayasse M, Paulus HFL *et al.* (1999) Orchid pollination by sexual swindle. *Nature*, **399**, 421–422.

Vöth W (1975) *Trielis villosa* var. *rubra*, Bestäuber von *Orchis coriophora*. *Die Orchidee*, **26**, 170–172.

White TJ, Bruns T, Lee S, Taylor J (1990) Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: *PCR Protocols: a guide to methods and applications* (eds Innis M, Gelfand D, Sninsky J, White T), pp. 315–322. Academic Press, San Diego.

Widmer A, Baltisberger M (1999) Molecular evidence for allopolyploid speciation and a single origin of the narrow endemic *Draba ladina* (Brassicaceae). *American Journal of Botany*, **86**, 1282–1289.

This study is part of an ongoing collaboration between the laboratories of Salvatore Cozzolino, Alex Widmer, and Amots Dafni on the molecular ecology and evolution of orchid–pollinator relationships. Giuseppe Pellegrino and Marco Soliva are PhD students in the laboratories of S. Cozzolino and A. Widmer, respectively, and apply molecular markers to the genera *Serapias*, *Ophrys* and *Orchis*.
