Journal of Heredity 2005:96(1):66–70
doi:10.1093/jhered/esi002
Advance Access publication December 23, 2004

Chloroplast DNA Inheritance in the Orchid *Anacamptis palustris* Using Single-Seed Polymerase Chain Reaction

D. Cafasso, A. Widmer, and S. Cozzolino

From the Dipartimento di Biologia Vegetale, Università degli Studi di Napoli Federico II, Via Foria, 223, I-80139 Naples, Italy (Cozzolino and Cafasso); and Geobotanisches Institut, ETH Zürich, Zollikerstr. 107, CH-8008 Zürich, Switzerland (Widmer).

Address correspondence to Salvatore Cozzolino at the address above, or e-mail: cozzolin@unina.it.

The modality of chloroplast inheritance in orchids has been investigated only in a few species due to the difculties associated with the analysis of large progeny numbers from experimental crosses. To test chloroplast DNA inheritance in the orchid *Anacamptis palustris*, we took advantage of the presence of a highly variable minisatellite repeat located in the $tRNA^{LEU}$ intron in the chloroplast genome. Seed progeny obtained from experimental crosses between parental individuals carrying different chloroplast DNA (cpDNA) minisatellite repeat numbers were analyzed using a single-seed polymerase chain reaction (PCR) protocol. All examined seeds displayed the maternal cpDNA haplotypes, indicating that cpDNA inheritance is strictly maternal in this Mediterranean orchid species. No evidence for paternal leakage was found. This nding concurs with results obtained from PCR amplications of pollen massulae that exclude the presence of chloroplast DNA in the pollen tetrads.

With more than 20,000 species, orchids are one of the largest and most diverse families of flowering plants exhibiting a highly specialized reproductive biology (Dressler 1993). Among the unique features of orchids are the small, eight-nucleate female gametophyte and the extremely reduced seed size (among the smallest in seed plants). The seeds enclose a rudimentary embryo that contains few cells and no endosperm (Arditti 1992). The reduction in cell number of the female gametophyte and embryo allows a large number of megasporogenesis events to occur in the ovary. At the same time, during microsporogenesis, orchids produce loose aggregates of pollen grains (massulae) that form large pollen packages (pollinaria) that are easily picked up and transported by animals for pollination (Arditti 1992). When pollination occurs and massulae are deposited on the stigma, large numbers of pollen grains are delivered at once. As a consequence, one pollination event ensures the production of thousands of seeds (Benzing 1987). These peculiarities in pollination biology have strongly stimulated the use of experimental interspecific hybridization for the production of new commercial varieties (Rittershausen and Rittershausen 2000).

In recent years, the development of molecular techniques has promoted the application of chloroplast genome studies to problems in orchid population genetics (Cozzolino et al. 2003a; Soliva and Widmer 1999; Squirrell et al. 2002), systematics (Cameron et al. 1999; Cozzolino et al. 1998; Soliva et al. 2001), and for the detection of interspecific gene flow in hybrid zones (Aceto et al. 1999, 2000). In all these studies, maternal inheritance of the chloroplast genome has been assumed. The evidence in favor of maternal chloroplast inheritance derives from cytological observations of pollen grains based on epifluorescence microscopy (Corriveau and Coleman 1988; Zhang et al. 2003). To date, epifluorescence microscopy has been applied to only 17 orchid taxa (Corriveau and Coleman 1988; Zhang et al. 2003) belonging to 14 tropical genera. In other plant groups, discrepancies have sometimes been observed between independently published studies, despite the fact that the same experimental approach has been employed in both studies (Corriveau and Coleman 1988; Zhang et al. 2003). Consequently, the need for independent confirmation, preferably based on molecular techniques, of the effective mode of chloroplast inheritance has been stressed (Zhang et al. 2003).

Ideally, experimental crosses between parental plants with different chloroplast haplotypes are used to investigate the mode of chloroplast inheritance (Cafasso et al. 2001a; Mogensen 1996). In orchids, this approach has only rarely been used because of the difficulty of finding cytoplasmic variation, but also because of the costs and difficulties of experimental breeding (Chang et al. 2000). In fact, even if hundreds or thousands of first-generation hybrids can be produced by a single cross, usually only a few individuals will grow to maturity because of the highly specific developmental requirements of orchid embryos. For this reason, tests of chloroplast inheritance based on molecular markers have been performed only on a few intergeneric hybrid individuals of tropical *Phalaenopsis* and *Doritis* orchids (Chang

et al. 2000). Although these studies suggest that maternal inheritance predominates, the small sample sizes used do not allow us to assess whether some paternal or biparental chloroplast inheritance occurs (Birky 1995; Milligan 1992). To date, information on chloroplast DNA inheritance is entirely missing for nontropical orchids, such as the Mediterranean members of subtribe Orchidinae, which represents the largest group of Mediterranean orchids (Pridgeon et al. 2001).

In the present study we used a recently discovered and well-characterized minisatellite repeat locus (Cafasso et al. 2001b; Cozzolino et al. 2003b) located in the chloroplast $tRNA^{LEU}$ intron of the strictly outbreeding orchid *Anacamptis* (formerly *Orchis*) *palustris* (Orchidinae) as a molecular marker and developed a single-seed polymerase chain reaction (PCR) amplification protocol for a large progeny screening. This chloroplast marker is highly variable both within and among *A. palustris* populations (Cozzolino et al. 2003a) and thus allowed us to perform experimental crosses between individuals of the same population carrying different chloroplast DNA (cpDNA) haplotypes (Cozzolino et al. 2003c).

In plants, seeds are typically composed of maternal tissues (the seed coats), endosperm (a triploid seed reserve tissue derived from the union of two female gametophyte nuclei with a male vegetative nucleus), and embryonic tissue (derived from the zygote divisions). As a consequence of the presence of maternal tissue in seeds, DNA extracted from whole seeds cannot be directly used to detect cpDNA inheritance. In contrast to the majority of seed plants, however, orchid seeds have no endosperm, enclose only a small embryo, and the seed coat is composed of only a thin layer of dried cell walls (Figure 1). Consequently, embryo cells are the only vital tissue in orchid seeds and DNA extracted from orchid seeds thus originates predominantly from the embryo. Because orchid seeds are exceptionally small, they are 0.1–0.2 mm wide and 0.2–1 mm long in Mediterranean orchids (Arditti 1992), and because they lack endosperm tissue, which often contains inhibiting compounds (e.g., polysaccharides or polyphenols) in other plant groups, whole seeds can be used directly in PCR amplifications to screen large sample sizes.

Materials and Methods

Molecular Analysis

Total DNA from parental plants was extracted according to Doyle and Doyle (1987) starting from 100 mg of fresh leaf material. PCR amplifications of the $tRNA^{LEU}$ intron region containing the minisatellite locus were performed using specific primers, APALF and APALR, as described in Cozzolino et al. (2003b). These primers are located 192 bp upstream and 30 bp downstream of the minisatellite region. PCR conditions were as described in Aceto et al. (1999), with 5 ng of total DNA used as the PCR template. Amplification products were visualized on a 2% Methaphore gel (FMS, Rockland, ME), using a 50 bp ladder as standard (Amersham Biosciences, Freiburg, Germany). PCR products and ladder were stained with ethidium bromide and photographed using a digital camera. To verify the results obtained from the fragment length analysis on the agarose gel, we also sequenced all parental individuals used for crosses (Table 1). For this purpose, PCR products were purified and sequenced in both directions using a modification of the Sanger dideoxy method as implemented in a double-stranded DNA cycle sequencing system with fluorescent dyes. Sequence reactions were then run on a 310 Automated DNA Sequencer (Applied Biosystems, Foster City, CA). Sequence Navigator software (Applied Biosystems, Foster City, CA) was used to check electropherograms.

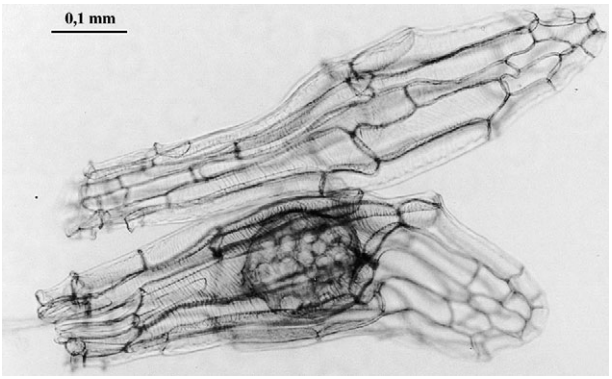

Figure 1. Viable (bottom) and nonviable (top) orchid seeds stained with 50% lactic acid.

Artificial Crosses

In the spring of 2003, we bagged flowers on five *A. palustris* individuals, while flowers were still in bud phase. These plants served as parentals for our experimental crosses and originated from the natural population Castelvolturno, where cpDNA haplotype variation was previously found to be high (Cozzolino et al. 2003a). Experimental pollinations were carried out by removing pollinaria as soon as the flowers opened by tipping the anther cap with a plastic toothpick and transferring pollen to the stigma of another plant. We took care to exclude the first and last flowers of the inflorescence because they sometimes fail to develop properly. A total of eight flowers were used for intraspecific crosses (Table 1). After 30 days, mature fruit capsules were collected and preserved in Petri dishes containing silica gel.

Pollen PCR Analysis

Pollinaria, from the same plants used for crosses, were collected and preserved under identical storage conditions. Pollen was then amplified directly, without prior isolation of DNA, by adding massulae directly to the PCR mixture. Duplex PCR was performed using the chloroplast-specific primers APALF and APALR and the nuclear ribosomal internal transcribed spacer I (ITS I) primers as employed in Widmer et al. (2000). DNA extracted from leaf tissue was used as positive controls.

Table 1. Experimental crosses and seed analysis in *A. palustris*

Crosses	Pollen donor	Ovule donor	Capsules	Viable seeds (%)	Examined seeds	Positive PCR amplification	cpDNA inheritance
A	36 (3 repeat)	39 (1 repeat)	5	98,9	484	406	100% maternal
B	35 (3 repeat)	38 (1 repeat)	1	87,5	96	91	100% maternal
C	38 (1 repeat)	36 (3 repeat)	1	93,4	95	74	100% maternal
D	42 (12 repeat)	36 (3 repeat)	1	91,4	96	96	100% maternal

Single-Seed PCR Amplification

Each fruit capsule was broken in a sterile Petri dish. The percentage of viable seeds was estimated by counting under a binocular microscope, and single viable seeds were collected and transferred to 0.2 ml PCR tubes with the aid of a thin plastic tip previously electrically charged by rubbing on a wool panel. PCRs were performed with specific primers APALF and APALR under the conditions described above for a total of 40 amplification cycles. Approximately 100 viable seeds from each capsule were investigated individually (Table 1). Viable and nonviable seeds can easily be distinguished using a binocular microscope, because the latter lack an embryo, whereas viable seeds contain an embryo (Figure 1). Nonviable seeds were used as negative controls for cpDNA amplification in our PCR experiments.

Results and Discussion

The five parental individuals used in the experimental crosses carried chloroplast haplotypes that differed in the number of minisatellite repeats. Two individuals carried a 1-repeat-containing haplotype (specimens 38 and 39), two individuals carried a 3-repeat-containing haplotype (specimens 35 and 36), and one individual possessed a 12-repeat-containing haplotype (specimen 42). Crossing experiments were performed between plants carrying different chloroplast haplotypes (i.e., different numbers of repeats) (Table 1).

All pollinated flowers produced fruit capsules. The eight investigated ripe capsules contained a high percentage of viable seeds, ranging from 88% to 99% (Table 1).

Single-seed PCR amplification gave a visible amplification product in 87% of examined viable seeds (Table 1). PCR amplifications gave unambiguous results. All viable seeds from which a PCR product was obtained possessed the maternal chloroplast haplotype (Table 1 and Figure 2A). No evidence for paternal or biparental inheritance was found.

Because orchid seeds typically lack endosperm, the other potential source for maternal DNA contamination may come from the orchid seed coat. However, in orchids, like in many other plants, seed coat tissues, which have a strictly maternal origin, typically do no contain plastids and are reduced to a thin layer of dead lignified cell walls (Arditti 1992). As a consequence, these cells should contain nuclear DNA (or nuclear DNA traces when the cells are dead in mature seeds), but should lack plastid DNA. However, to clearly show that contamination by the maternal tissue (i.e., the seed coat) cannot be the basis of our findings of maternal cpDNA inheritance, we also performed duplex PCRs with nonviable seeds (i.e., seeds without an embryo). We expected the internal transcribed spacer (ITS) primers to amplify nuclear DNA from the seed coat. Therefore ITS amplification could be used as a positive control for the PCR. In contrast, we expected no amplification product from the chloroplast primers, because plastid DNA should be absent from seed coat tissue. As expected, these duplex PCRs with nonviable seeds did not amplify the plastid product, but produced an amplification product with the ITS1 primers, thus confirming that cells from seed coat may still contain nuclear DNA but lack plastid DNA (Figure 2B). In combination, the successful amplification of the ITS1 product confirmed that the PCR worked and that the negative result obtained with the plastid primers is a consequence of the absence of plastid DNA from the seed coat, and not due to PCR failure.

These results show convincingly that the chloroplast amplification products obtained from viable seeds originate from the embryo, and not from the maternal seed coat tissue. Thus the potential presence of paternal chloroplasts in the embryo would not be masked by the presence of maternal chloroplasts in the seed coat.

Similarly, duplex PCR amplifications carried out on pollen aggregates (Figure 2B) produced the expected ITS1 amplification product (~370 bp long), but did not amplify the chloroplast locus. This suggests that chloroplast DNA is absent from *A. palustris* pollen tetrads, and also rules out the hypothesis that the exclusion of chloroplasts may occur during a later fertilization stage. These results, based on

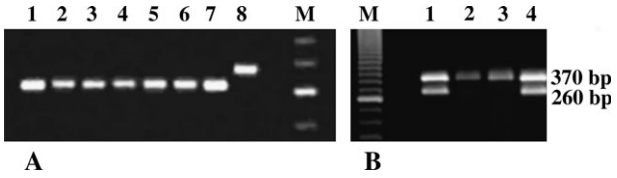

Figure 2. Maternal inheritance of cpDNA in *A. palustris*. (A) Seed PCR amplifications of chloroplast minisatellite locus in progenies (lanes 2–7) from a cross between a one-repeat-containing plant (female plant as ovule donor, lane 1) and a three-repeat-containing plant (male plant as pollen donor, lane 8); 50 bp DNA molecular ladder (M). (B) ITS1 (370 bp) and chloroplast minisatellite locus (260 bp) amplifications of a viable seed (lane 1), a nonviable seed (lane 2), pollen massulae (lane 3), and plant leaf DNA (lane 4); 50 bp DNA molecular ladder (M).

molecular markers, confirm previous epifluorescence microscopy observations that revealed the absence of cytoplasmic DNA in orchid sperm cells (Zhang et al. 2003).

The main difficulties associated with the study of chloroplast inheritance in orchids are the long generation times, the rarity of chloroplast variation, the extremely reduced seed size, and the complexity of the orchid seed germination process, which depends on the establishment of highly specific mycorrhizal interactions during the early stages of embryo development (Arditti 1992). All these complications strongly reduce the chance of efficiently analyzing large progeny arrays from experimental crosses in orchids (Chang et al. 2000). On the other hand, pollination experiments in orchids can be carried out easily and a single pollination event produces thousands of zygotes for investigation. Our single-seed PCR protocol in combination with the use of a suitable molecular marker, such as the minisatellite locus used here, allows examination of large progeny arrays from a single crossing experiment by simultaneously reducing experimental time and costs.

Organelle inheritance is a complex phenomenon and, in performing a correct analysis of the mode of organelle inheritance, it is crucial to distinguish between a strictly maternal inheritance and a rare paternal or biparental inheritance, in which at least some progeny contain plastids derived from the male parent. The validity of the analysis is clearly sensitive to the degree of biparental transmission and on the statistical power of the available observations to detect leakage at a given level.

Using the equations of Milligan (1992), we estimated that, thanks to our large sample size (667 positive amplifications), we would have been able to detect paternal leakage, which would result in paternal or biparental plastid transmission, with a probability of 95% if it had occurred at a rate as low as 0.45%. The lack of any evidence for paternal plastid transmission in our experiments thus shows that maternal plastid transmission is the rule in *A. palustris*. Thus this single-seed PCR protocol may allow the modality of organelle transmission to be tested experimentally and statistically, if suitable molecular markers are available, in other members of this fascinating plant group.

Acknowledgments

We would like to thank V. Avolio, S. Giorgio, and R. Salatiello of Naples Botanical Garden for help with plant collection and cultivation. Charlotte Salzmann kindly provided Figure 1. Tina Coppola was most helpful with crossing experiments and data collection. A. Musacchio, M. Baltisberger, and J. Mant provided helpful discussions. This study was supported by the PRIN program of the Italian Ministry of University and Scientific and Technological Research.

References

Aceto S, Caputo P, Gaudio L, Nazzaro R, and Cozzolino S, 2000. Molecular approach to the identification and characterization of natural hybrids between *Orchis pauciflora* and *Orchis quadripunctata* (Orchidaceae). Bot Helv 110:31–39.

Aceto S, Cozzolino S, Gaudio L, Nazzaro R, and De Luca P, 1999. Pollination flow between *Orchis morio* and *O. papilionacea* in hybrid formation in different habitats. Int J Plant Sci 160:1153–1156.

Arditti J, 1992. Fundamentals of orchid biology. New York: John Wiley & Sons.

Benzing DH, 1987. Major patterns and processes in orchid evolution: a critical synthesis. In: Orchid biology: reviews and perspectives, vol IV (Arditti J, eds). Ithaca, NY: Cornell University Press; 33–78.

Birky CW, 1995. Uniparental inheritance of mitochondrial and chloroplast genes: mechanisms and evolution. Proc Natl Acad Sci USA 92:11331–11338.

Cafasso D, Cozzolino S, Caputo P, and De Luca P, 2001a. Maternal inheritance of plastids in *Encephalartos* Lehm. (Zamiaceae, Cycadales). Genome 44:239–241.

Cafasso D, Pellegrino G, Musacchio A, Widmer A, and Cozzolino S, 2001b. Characterization of a minisatellite repeat locus in the chloroplast genome of *Orchis palustris* (Orchidaceae). Curr Genet 39:394–398.

Cameron KM, Chase MW, Whitten WM, Kores PJ, Jarrell DC, Albert VA, Yukawa T, Hills HG, and Goldman DH, 1999. A phylogenetic analysis of the Orchidaceae: evidence from rbcL nucleotide sequences. Am J Bot 86:208–224.

Chang SB, Chen WH, Chen HH, Fu YM, and Lin YS, 2000. RFLP and inheritance patterns of chloroplast DNA in intergeneric hybrids of *Phalaenopsis* and *Doritis*. Bot Bull Acad Sin 41:219–223.

Corriveau JL and Coleman AW, 1988. Rapid screening method to detect potential biparental inheritance of plastid DNA and results for over 200 angiosperm species. Am J Bot 75:1443–1458.

Cozzolino S, Aceto S, Caputo P, Gaudio L, and Nazzaro R, 1998. Phylogenetic relationships in *Orchis* and some related genera: an approach using chloroplast DNA. Nord Bot 18:79–87.

Cozzolino S, Cafasso D, Pellegrino G, Musacchio A, and Widmer A, 2003a. Fine-scale phylogeographic analysis of Mediterranean *Anacamptis palustris* (Orchidaceae) populations based on chloroplast minisatellite and microsatellite variation. Mol Ecol 12:2783–2792.

Cozzolino S, Cafasso D, Pellegrino G, Musacchio A, and Widmer A, 2003b. Molecular evolution of a plastid tandem repeat locus in an orchid lineage. J Mol Evol 57:1–9.

Cozzolino S, Noce ME, Musacchio A, and Widmer A, 2003c. Variation at a chloroplast minisatellite locus reveals the signature of habitat fragmentation and genetic bottlenecks in the rare marsh orchid, *Anacamptis palustris* (Orchidaceae). Am J Bot 90:1681–1687.

Doyle JJ and Doyle JL, 1987. A rapid DNA isolation procedure for small amounts of fresh leaf tissue. Phytochem Bull 19:11–15.

Dressler RL, 1993. Phylogeny and classification of the orchid family. Portland, OR: Dioscorides Press.

Milligan BG, 1992. Is organelle DNA strictly maternally inherited? Power analysis of a binomial distribution. Am J Bot 79:1325–1328.

Mogensen HL, 1996. The hows and whys of cytoplasmic inheritance in seed plants. Am J Bot 83:383–404.

Pridgeon AM, Cribb PJ, Chase MC, and Rasmussen FN, 2001. Genera Orchidacearum. Vol. 2: Orchidiodeae (part 1). Oxford: Oxford University Press.

Rittershausen B and Rittershausen W, 2000. The practical encyclopedia of orchids: the complete guide to orchids and their cultivation. London: Anness.

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

Soliva M and Widmer A, 1999. Genetic and floral divergence among sympatric populations of *Gymnadenia conopsea* s.l. (Orchidaceae) with different flowering phenology. Int J Plant Sci 160:897–905.

Squirrell J, Hollingsworth PM, Bateman RM, Tebbit MC, and Hollingsworth ML, 2002. Taxonomic complexity and breeding system transitions: conservation genetics of the *Epipactis leptochila* complex (Orchidaceae). Mol Ecol 11:1957–1964.

Widmer A, Cozzolino S, Pellegrino G, Soliva M, and Dafni A, 2000. Molecular analysis of orchid pollinaria and pollinaria-remains found on insects. Mol Ecol 9:1911–1914.

Zhang Q, Liu Y, and Sodmergen 2003. Examination of the cytoplasmic DNA in male reproductive cells to determine the potential for cytoplasmic inheritance in 295 angiosperm species. Plant Cell Physiol 44:941–951.

Received February 11, 2004
Accepted June 21, 2004

Corresponding Editor: John Burke