Plant Ecology and Evolution 143 (2): 239–242, 2010
doi:10.5091/plecevo.2010.425

SHORT COMMUNICATION

Inheritance of the chloroplast genome in *Rhinanthus angustifolius* (Orobanchaceae)

Jérôme Vrancken* **& Renate A. Wesselingh**

Biodiversity Research Centre, Université catholique de Louvain, Croix du Sud 4-5, BE-1348 Louvain-la-Neuve, Belgium
*Author for correspondence: jerome.vrancken@uclouvain.be

Background and aims – The mode of inheritance of the chloroplast genome is an important factor in studies dealing with population and evolutionary plant biology. In this paper, we aim to determine the chloroplast DNA (cpDNA) inheritance in *Rhinanthus angustifolius*.
Methods – We studied the cpDNA inheritance using PCR-RFLP. One hundred sixty six offspring obtained from controlled crosses between individuals with two distinct chlorotypes were analysed.
Key results – All the progeny exhibited the maternal chloroplast genome. The power analysis of the binomial distribution showed that the frequency of paternal transmission of the chloroplast, if any, should not exceed 1.81% (with 95% confidence). This work indicates that maternal inheritance of the chloroplast genome in *Rhinanthus angustifolius* is a reasonable assumption.

Key words – Chloroplast DNA, inheritance, PCR-RFLP, *Rhinanthus angustifolius*.

INTRODUCTION

Formerly belonging to the family Scrophulariaceae, the genus *Rhinanthus* L. has recently been placed into the family Orobanchaceae (Olmstead et al. 2001). The taxonomy of the genus is complicated because of hybridization between species (Chabert 1899) and the high morphological intraspecific plasticity. Nonetheless, based on morphology, 25 species are recognised in Europe (von Soó & Webb 1972) and some of these species are also found in North America (van Hulst et al. 1986) and in Asia (ter Borg 2005). All *Rhinanthus* species are hemi-parasitic, summer-annuals growing in a wide range of grassland habitats on soils of low to moderate fertility (Westbury 2004). They may be found from sea level up to more than 2000 meters altitude in many different climatic conditions.

Different aspects of this genus have been studied; numerous researches have focused on the biology (e.g. Bullock et al. 2005, Lindborg et al. 2005) and ecophysiology (e.g. Cameron et al. 2005, Phoenix & Press 2005) of different species of the genus. Since a few years, our laboratory focuses on the evolution of *Rhinanthus*. Hybridization between *Rhinanthus minor* and *Rhinanthus angustifolius* (Durcarme & Wesselingh 2005) as well as the phylogeography of the genus in Europe (Vrancken et al. 2009) are under investigation.

In our studies, chloroplast DNA (cpDNA) markers have shown to be valuable. Indeed, due to the development of molecular techniques (Taberlet et al. 1991) and to some of its specific characteristics such as no recombination and a low rate of evolution (Wolf et al. 1987), cpDNA presents phylogenetically informative variation useful for plant molecular phylogeny or evolutionary studies. Another important feature of the chloroplast is that cpDNA is haploid; the effective population size for cpDNA is then reduced compared to the nuclear genome (Petit et al. 2005). Finally, as strict maternal inheritance of the chloroplast is presumed in most plant species (Birky 1995), the dispersion of this organelle genome is attributed to seed movement only. Therefore, with all these characteristics put together, the chloroplast genome is expected to present high levels of divergence through genetic drift and is likely to retain historical patterns (Comes & Kadereit 1998).

If maternal transmission inheritance is assumed to be the rule in angiosperms, numerous exceptions to this rule have been discovered where at least partial paternal transmission is suspected (Sewel et al. 1993, Rajora & Mahon 1995, Shore & Triassi 1998, Chat et al. 1999, Yang et al. 2000, Hansen et al 2007, McCauley et al. 2007, Ellis et al. 2008). Actually, bi-parental transmission of the chloroplast genome was found in 27% of 398 species investigated (Harris & Ingram 1991). The possibility of paternal or bi-parental transmission of the choloroplast implies that movement of the cpDNA genome is no longer associated with seed movements only (Petit & Vendramin 2008). The potential transfer of cpDNA trough pollen has for consequence to increase the gene flow which may strongly invalidate conclusions in studies dealing with seed dispersal, hybridization or phylogeography. Therefore, determining the mode of transmission of the cpDNA genome

is an important issue when one wants to investigate plant evolution (Raspé 2001).

Cytological analysis of pollen has already been used to show maternal inheritance of the chloroplast in different species of Scrophulariaceae (Corriveau & Coleman 1988) but to our knowledge, this study is the first to collect genetic evidence for the mode of chloroplast inheritance in Orobanchaceae. In order to define the mode of chloroplast transmission, we used PCR-RFLP (Demesure et al. 1995). This PCR-based method allows analysis of a large number of individuals necessary to detect a possible biparental transmission (Milligan 1992) but is known to miss low levels of heteroplasmy (Frey et al. 2005, Welch et al. 2006). Therefore, taken into account this technical limitation, the aim of the present research is not to test for a strict maternal inheritance hypothesis but rather to evaluate the maximum probability of paternal transmission of the chloroplast in *R. angustifolius*.

MATERIALS AND METHODS

Plant material and controlled pollinations

Seeds of *Rinanthus angustifolius* C.C.Gmel. were collected in a natural Belgian population (in the nature reserve of Doode Bemde, Oud-Heverlee, 50°49'N 04°38'E) and grown in a greenhouse. We isolated thirty plants to serve as parent plants in our experiment. Before flowering, two leaves of each of the thirty plants were collected in order to determine their chlorotype (see DNA extraction, PRC and RFLP analysis). Two groups of plants were separated based on their chlorotypes and intraspecific crosses were carried out between plants of these two groups. To perform these crosses, anthers from plants of a specific group were cut and directly applied on stigmas from plants of the other group. *R. angustifolius* is predominantly outcrossing but selfing is not infrequent (Campion-Bourget 1980, Ducarme 2008), therefore, self-fertilisation was avoided by removing the anthers as soon as the flowers opened. We observed that emasculated flowers that had not been cross-pollinated did not produce any seeds, confirming that early emasculation was effective to avoid self-fertilization.

DNA extraction, PCR-RFLP and data analysis

The DNA extraction protocol was different for parents and offspring. Total DNA of the parents was isolated from the leaves following a CTAB protocol modified from Doyle & Doyle (1990) (Ducarme & Wesselingh 2005) whereas offspring DNA was directly isolated from seeds using the Invisorb Spin Plant Genomic DNA Purification Kits. Prior to extraction, seed coats were removed.

The PCR-RFLP method implemented is based on a previous study of Véronique Ducarme (2008). We used digestion with an enzyme (Eco47I) that permits the distinction between two chlorotypes (fig. 1). Chlorotype 1 (CT1) presents two bands resulting from the digestion of the chloroplast trnL (UAA) fragment, while chlorotype 2 (CT2) presents only one band. The enzyme, Eco471 (AvaII), recognises and cuts the nucleotide sequence GGTCC present in the trnL fragment of *R. angustifolius*. But some *R. angustifolius* present a different

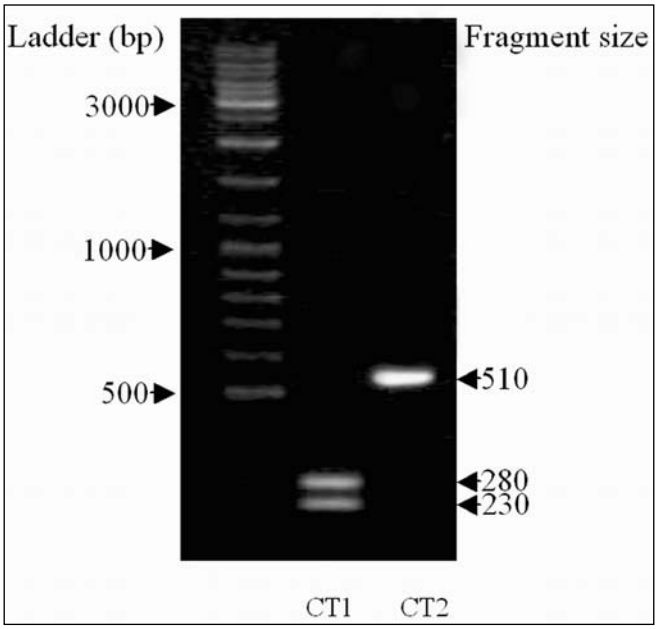

Figure 1 – Agarose gel showing trnL (UAA) fragment digested with Eco47I. Lane 1: ladder; lane 2: chlorotype 1 (CT1); lane 3: chlorotype 2 (CT2).

sequence, GGCCC, which the enzyme is unable to recognise. CT2 results from the non-digestion of the trnL (UAA) chloroplast fragment (GenBank accession number: HM222969 and HM222970).

The chloroplast intron trnL (UAA) was amplified by PCR using about 20 ng total DNA, 2.5 µl AmpliTaq buffer 10 ×, 2.5 µl $MgCl_2$ 25 mM, 2µl BSA 2.5 mg mL^{-1}, 2 µM of each dNTP, 0.25 µl of each primer 20 µM (c and d in Taberlet et al. 1991), and 0.125 µl Taq 5U μL^{-1} in a 25 µl volume. The amplification programme started with a denaturation step of 5 min at 94°C, followed by 30 cycles of 1 min at 94°C, 1 min at 55°C and 2 min at 72°C. A final elongation step of 7 min at 72°C terminated the reaction. Aliquots (10 µl) of the PCR product were digested with 0.125 µl Eco47I 5U μL^{-1} during 4 hours at 36°C. Digestion products were finally separated on 1.5% agarose gels with ethidium bromide and revealed under UV light, a 10000bp ladder (GeneRuler, Fermentas) was used as size marker.

The maximum likely rate of paternal transmission of the chloroplast was assessed with the power analysis test of a binomial model (Milligan 1992). When all progeny in a given sample size (N) possess the maternal cpDNA, the equation $P = 1-(1-\beta)^{1/N}$ give the probability of a paternal transmission of the chloroplast (P) with β as the power of the test.

RESULTS AND DISCUSSION

The screening of the thirty parent plants revealed that twelve individuals carried CT1 and eighteen individuals had CT2. Two hundred and fourteen crosses were performed between flowers of plants showing different chlorotypes, 99 CT1 × CT2 and 115 CT2 × CT1. From these 214 crosses, 206 capsules containing up to ten seeds were collected and DNA was extracted from 100 seeds of each cross combination (one seed

Table 1 – Description of experimental crosses and seed analysis to determine chloroplast inheritance in *Rhinanthus angustifolius*.

Cross type	Maternal × paternal	n of cross pollination	Collected capsules	Examined seeds	Positive PCR amplification	Chlorotype of the seeds
1	CT1 × CT2	99	93	100	99	CT1
2	CT2 × CT1	115	113	100	67	CT2

from 100 different capsules in CT2 × CT1 crosses and one or two seeds from 93 different capsules in CT1 × CT2 crosses). In 34 seeds produced by various maternal plants from both chlorotype groups, the cpDNA fragment failed to amplify, so that chlorotypes of 166 progeny could be determined. None of these showed the paternal chlorotype, or a combination of both chlorotypes (table 1). All possessed the same chloroplast marker as the female parent, irrespective of the direction of the cross. With a sample size of 166 progeny and a 95% confidence level ($\beta = 0.95$), the power analysis test revealed that the paternal transmission probability is less than 1.81% ($P = 0.0181$). If the confidence level is set to 99% ($\beta = 0.99$) the probability of a paternal transmission of the chloroplast is then less than 2.7% ($P = 0.027$). This probability of paternal transmission may undoubtedly be reduced by studying a larger number of progeny (Milligan 1992), but as shown by Birky et al. (1989) the low probability of paternal inheritance obtained with our data only slightly influences results and conclusions in studies of population genetics.

Since seeds are typically composed of embryonic tissue (derived from zygotic divisions), endosperm (a triploid seed reserve tissue derived from the union of two female nuclei with a male vegetative nucleus) and maternal tissues (the seed coat), the presence of maternal chloroplasts is always expected in seed tissues. In this study, the seed coat was removed prior to DNA extraction and a recent study using microsatellites showed that this operation allows extraction of the offspring's DNA only (Ducarme 2008).

To conclude, this study is, to our knowledge, the first attempt to evaluate the mode of inheritance of the cpDNA genome in Orobanchaceae. We did not detect any evidence for paternal or bi-parental inheritance of the chloroplast so that maternal inheritance of the chloroplast genome in *Rhinanthus angustifolius* is a reasonable assumption. This observation performed in *R. angustifolius* may probably be extended to the other close species of the genus *Rhinanthus*. Therefore, the chloroplast genome may be reliably used as a seed-specific marker to investigate geographic structure, evolution and hybridization of *Rhinanthus* species.

ACKNOWLEDGMENTS

We thank the Fonds Spécial de Recherche (FSR) of the Université catholique de Louvain and the Fond National de Recherche Scientifique (FNRS) for their financial support to this study. We thank Linda Dhondt and Christine Noël at the Université catholique de Louvain for their help in the laboratory. This is publication no. BRC180 of the Biodiversity Research Centre of the Université catholique de Louvain.

REFERENCES

Birky C.W., Fuerst P., Maruyama T. (1989) Organelle gene diversity under migration, mutation, and drift: equilibrium expectations, approach to equilibrium, effects of heteroplasmic cells, and comparison to nuclear genes. Genetics 121: 613–627.

Birky C.W. (1995) Uniparental inheritance of mitochondrial and chloroplast genes: mechanisms and evolution. Proceedings of the National Academy of Sciences of the USA 92: 11331–11338.

Bullock J.M., Pywell R.F. (2005) Rhinanthus: a tool for restoring diverse grassland? Folia Geobotanica 40: 273–288.

Cameron D.D., Hwangbo J.K., Keith A.M., Geniez J.-M., Kraushaar D., Rowntree J., Seel W.E. (2005) Interactions between the hemiparasitic angiosperm Rhinanthus minor and its host: from the cell to the ecosystem. Folia Geobotanica 40: 217–230.

Campion-Bourget F. (1980) L'hybridation interspécifique expérimentale chez les cinq principales espèces de Rhinanthus L. de France. Revue de Cytologie et de Biologie végétales – le Botaniste 3: 221–280.

Corriveau J.L., Coleman A.W. (1988) Rapid screening method to detect potential biparental inheritance of plastid DNA and result for over 200 angiosperm species. American Journal of Botany 75: 1443–1458.

Chabert A. (1899) Etude sur le genre Rhinanthus. Bulletin de l'Herbier Boissier 7: 512–542.

Chat J., Chalak L., Petit R.J. (1999) Strict paternal inheritance of chloroplast DNA and maternal inheritance of mitochondrial DNA in intraspecific crosses in kiwifruit. Theoretical and Applied Genetics 99: 314–322.

Comes H.P., Kadereit J.W. (1998) The effect of Quaternary climatic changes on plant distribution and evolution. Trends in Plant Sciences 3: 432–438.

Demesure B., Sodzi N., Petit R.J. (1995) A set of universal primers for amplification of polymorphic non-coding regions of mitochondrial and chloroplast DNA in plants. Molecular Ecology 4: 129–131.

Doyle J.J., Doyle J.L. (1990) Isolation of plant DNA from fresh tissue. Focus 12: 13–15.

Ducarme V., Wesselingh R.A. (2005) Detecting hybridization in mixed populations of Rhinanthus minor and Rhinanthus angustifolius. Folia Geobotanica 40: 151–162.

Ducarme V. (2008) Ecological and genetical aspect of natural hybridization in Rhinanthus L. PhD Thesis, Université catholique de Louvain, Louvain-la-Neuve, Belgium.

Ellis J.R., Bentley K.E., McCauley D.E. (2008) Detection of rare paternal chloroplast inheritance in controlled crosses of the endangered sunflower Helianthus verticillatus. Heredity 100: 574–580.

Frey J.E., Frey B., Forcioli D. (2005) Quantitative assessment of heteroplasmy levels in Senecio vulgaris chloroplast DNA. Genetica 123: 255–261.

Hansen A.K., Escobar L.K., Gilbert L.E., Jansen R.K. (2007) Paternal, maternal, and biparental inheritance of the chloroplast genome in Passiflora (Passifloraceae): implications for phylogenetic studies. American Journal of Botany 94: 42–46.

Harris S.A., Ingram R. (1991) Chloroplast DNA and biosystematics: the effects of intraspecific diversity and plastid transmission. Taxon 40: 393–412.

Lindborg R., Cousins S.A.O., Eriksson O. (2005) Plant species response to land use change Campanula rotundifolia, Primula veris and Rhinanthus minor. Ecography 28: 29–36.

McCauley D.E., Sundby A.K., Bailey M.F., Welch M.E. (2007) Inheritance of chloroplast DNA is not strictly maternal in Silene vulgaris (Caryophylaceae): evidence form experimental crosses and natural populations. American Journal of Botany 94: 1333–1337.

Milligan B.G. (1992) Is organelle DNA strictly maternally inherited? Power analysis of a binomial distribution. American Journal of Botany 79: 1325–1328.

Olmstead R.G., DePamphilis C.W., Wolfe A.D., Young N.D., Elisons W.J., Reeves P.A. (2001) Disintegration of the Scrophulariaceae. American Journal of Botany 88: 348–361.

Petit R.J., Duminil J., Fineschi S., Hampe A., Salvini D., Vendramin G.G. (2005) Comparative organization of chloroplast, mitochondrial and nuclear diversity in plant populations. Molecular Ecology 14: 689–701.

Petit R.J., Vendramin G.G. (2008) Plant phylogeography based on organelle genes: an introduction. In: Weiss S., Ferrand N. (eds) Phylogeography of Southern European refugia: 23–97. Dordrecht, Springer.

Phoenix G.K., Press M.C. (2005) Effects of climate change on parasitic plants: the root hemiparasitic Orobanchaceae. Folia Geobotanica 40: 205–216.

Rajora O.P., Mahon J.D. (1995) Paternal plastid DNA can be inherited in lentil. Theoretical and Applied Genetics 90: 607–610.

Raspé O. (2001) Brief communications: Inheritance of the chloroplast genome in Sorbus aucuparia L. (Rosaceae). The Journal of Heredity 92: 507–509.

Sewell M.M., Qiu Y.-L., Parks C.R., Chase M.W. (1993) Genetic Evidence for Trace Paternal Transmission of Plastids in Liriodendron and Magnolia (Magnoliaceae). American Journal of Botany 80: 854–858.

Shore J.S., Triassi M. (1998) Paternally biased cpDNA inheritance in Turnera ulmifolia (Turneraceae). American Journal of Botany 85: 328–332.

Taberlet P., Gielly L., Pautou G., Bouvet J. (1991) Universal primers for amplification of 3 noncoding regions of chloroplast DNA. Plant Molecular Biology 17: 1105-1109.

ter Borg S. (2005) Dormancy and germination of six Rhinanthus species in relation to climate. Folia Geobotanica 40: 243–260.

van Hulst R., Thériault A., Shipley B. (1986) The systematic position of the genus Rhinanthus (Scrophulariaceae) in North America. Canadian Journal of Botany 64: 1443–1449.

von Soó R., Webb D.A. (1972) Rhinanthus L. In: Tutin T.G., Heywood V.H., Burges N.A., Moore D.M., Valentine D.H., Walters S.M., Webb D.A. (eds) Flora Europaea vol. 3 – Diapensiaceae to Myoporaceae: 276–280. Cambridge, Cambridge University Press.

Vrancken J., Brochmann C., Wesselingh R.A. (2009) How did an annual plant react to Pleistocene glaciations? Postglacial history of Rhinanthus angustifolius in Europe. Biological Journal of the Linnean Society 98: 1–13.

Welch M.E., Darnell M.Z., McCauley D.E. (2006) Variable populations within variable populations: quantifying mitochondrial heteroplasmy in natural populations of the gynodioecious plant Silene vulgaris. Genetics 174: 829–837.

Westbury D.B. (2004) Biological Flora of the British Isles: Rhinanthus minor L. Journal of Ecology 92: 906–927.

Wolfe K.H., Li W.H., Sharp P.M. (1987) Rate of nucleotide substitution vary greatly among plant mitochondrial, chloroplast and nuclear DNAs. Proceeding of the National Academy of Sciences of the USA 84: 9054–9058.

Yang T.W., Yang Y.A., Xiong Z. (2000) Paternal inheritance of chloroplast DNA in interspecific hybrids in the genus Larrea (Zygophyllaceae). American Journal of Botany 87: 1452–1458.

Manuscript received 12 Feb. 2010; accepted in revised version 2 Jun. 2010.

Communicating Editor: Myriam Heuertz.