Theor Appl Genet (1990) 79:550-555

THEORETICAL AND APPLIED GENETICS

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Cybrids of *Nicotiana tabacum* and *Petunia hybrida* have an intergeneric mixture of chloroplasts from *P. hybrida* and mitochondria identical or similar to *N. tabacum*

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Received May 22, 1989; Accepted December 12, 1989
Communicated by P. Maliga

Summary. The mitochondrial genomes of cybrids of *Nicotiana tabacum* containing chloroplasts of *Petunia hybrida* were characterized by restriction endonuclease digestion and agarose gel electrophoresis. Cybrids that displayed normal growth and development contained mitochondrial DNA indistinguishable from *N. tabacum* mitochondrial DNA. Cybrids that displayed abnormal growth and development contained mitochondrial DNA that differed from *N. tabacum* either by possessing a few additional fragments, by lacking a few fragments, or both. In spite of these differences, the mitochondrial DNA of cybrids showing abnormal growth and development was much more similar to *N. tabacum* than to *P. hybrida* mitochondrial DNA. In those cybrids that contained *P. hybrida* chloroplasts and *N. tabacum* mitochondria, cotransfer of cytoplasmic organelles did not occur. Although *P. hybrida* chloroplasts can interact compatibly with the *N. tabacum* nucleus, no cybrids were found in which *P. hybrida* mitochondria coexisted with the *N. tabacum* nucleus.

Key words: Cybrid – Mitochondrial DNA – Organelle – *Petunia* – *Nicotiana*

Introduction

Plant organelles are not completely autonomous, since they depend on the information in nuclear DNA for many of their gene products. In spite of this dependence, compatible interaction can take place between the organelles of one species and the nucleus from another, as is illustrated by alloplasmic lines, produced by sexual crosses, in which the cytoplasmic organelles in one plant are replaced with the organelles from a related species. Even intergeneric combinations of organelles and nucleus have been obtained, such as the alloplasmic line of a *Brassica* species with the cytoplasm of *Raphanus sativus* (Bannerot et al. 1974). However, this combination was accompanied by defects, such as chlorophyll deficiency and male sterility, as a consequence of incompatibility between the nucleus and the cytoplasmic organelles.

For species or genera that are not sexually compatible, a combination of nucleus and cytoplasmic organelles can only be obtained via protoplast fusion. Alloplasmic lines have been produced by protoplast fusion between different species (for a review, see Pelletier 1986) and between different genera (Pelletier et al. 1983; Glimelius and Bonnett 1986). The many cybrids produced by Glimelius and Bonnett between *Nicotiana* and *Petunia* demonstrate that an intergeneric combination of nucleus and chloroplasts can be compatible, resulting in normal plants. Recently, Thanh et al. (1988) reported that an intertribal combination of chloroplasts from *Salpiglossis* with the nucleus of *N. tabacum* could be obtained, but these plants displayed different types of abnormalities. Besides the intertribal combination, Thanh et al. (1988) attempted unsuccessfully to produce an inter-subfamilial combination of chloroplasts from *Solanum nigrum* and the *N. tabacum* nucleus.

A similar result was obtained from our investigation where abnormal plants were found after fusion of irradiated *Salpiglossis* protoplasts with albino *N. tabacum* (Glimelius et al. 1986). According to our investigations, a transfer of both organelles and nuclear DNA from *Salpiglossis* to *N. tabacum* was obtained. When species from *Datura, Solanum,* and *Capsicum* were used as donor protoplasts, no green colonies were obtained, indicating that an incompatibility existed between the chloroplasts from the donor plants and the nucleus from *N. tabacum.*

Somatic hybridization provides a fusion product in which all the genomes from the two fusion partners are combined into one cell. Somatic hybridization can result in novel combinations of chloroplasts and mitochondria, and usually results in alterations of the mitochondrial genome. Thus, the presence of chloroplasts from *Petunia* in the *N. tabacum* cybrid plants does not predict whether these cybrids contain mitochondria of *N. tabacum*, *Petunia*, mixtures of both, or some type of rearranged mitochondrial genome. Accordingly, this study was performed to determine the type of mitochondria present in the cybrids by an analysis of their mitochondrial DNA.

Materials and methods

Plant material

Cybrid plants were produced between *Nicotiana tabacum* cv "Turkish Samsun" and *Petunia hybrida* cv "Comanche" using the donor-recipient method (Glimelius and Bonnett 1986). As donor of cytoplasmic organelles, protoplasts derived from *Petunia* were γ-irradiated to eliminate the nuclear genome before fusion. As recipient material, protoplasts from an albino mutant of *N. tabacum* were used. These cybrids are referred to as *Nicotiana-(Petunia)* cybrids. A sample of *Nicotiana-(Petunia)* cybrids was selected for analysis of mitochondrial DNA (mtDNA). Based on morphology and reproductive behavior, the cybrids were placed into two groups. Group I comprised plants with normal vegetative and reproductive development; Group II comprised plants with disturbances in development (Table 1).

MtDNA analysis

Cybrids were evaluated for mtDNA by restriction enzyme analysis. Three different sources of material were used for isolation of mtDNA. (1) Young plants were grown from seeds of cybrids that were self-fertile or that could be cross-pollinated with "Turkish Samsun" pollen. The mtDNA was isolated from 30–100 siblings, 12–19 weeks old. (2) Leaf callus material was obtained from cybrids from which viable seed could not be obtained. Cell suspensions were established and 150–200 g of cells were used for the isolation of mtDNA. (3) Plants were regenerated from protoplasts from two of the fertile cybrids. MtDNA was isolated from leaves of these plants.

The isolation of DNA, digestion with restriction enzymes, and electrophoresis in agarose gels were performed according to the procedures reported by Håkansson et al. (1988).

Chromosome determination

Chromosome counts were made according to the methods described in Glimelius and Bonnett (1981).

Results

MtDNA of Nicotiana and Petunia

Clear differences were found in mtDNA between the two parental species, *N. tabacum* and *P. hybrida*, for the restriction enzymes BamH1, Pst1, and Xho1. Of the 35–40 clearly visible fragments obtained after restriction of the mtDNA with each enzyme, only 7–9 fragments were shared or were identical for the two species in each instance (Figs. 1, 4, and 6).

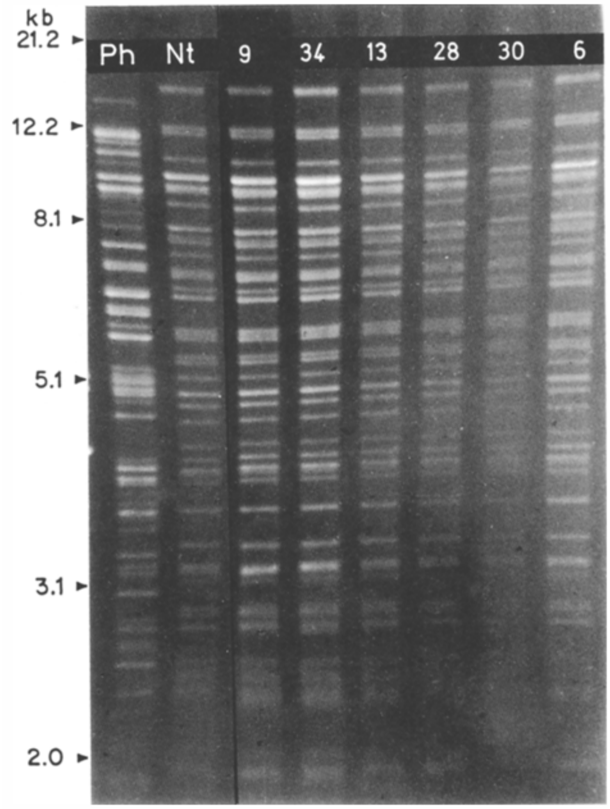

Fig. 1. Restriction pattern of mtDNA from *N. tabacum, P. hybrida,* and six cybrids belonging to Group I, restricted with BamH1. All cybrids display a pattern of restriction fragments indistinguishable from *N. tabacum* mtDNA. In contrast, *N. tabacum* and *P. hybrida* mtDNA show little similarity

Table 1. Description of cybrids by group, chromosome number, fertility, and material from which mtDNA was isolated

Group	Cybrid	Chromosome no.	Reproductive behavior	Material for isolation of mtDNA*
I	3	–	self-fertile	a
	6	48	self-fertile	a
	9	48	self-fertile	a
	13	48	self-fertile	a
	17	48	self-fertile	a
	26	48	self-fertile	a
	28	48	self-fertile	a
	30	48	self-fertile	a
	34	48	self-fertile	a
II	1	48	self-fertile	a, b, c
	10	48	self-sterile	a
	14	–	sterile	b
	15	96	self-sterile	a
	16	48	self-fertile	a
	20	48	self-sterile	a
	40	48	self-fertile	a, b, c
	43	48	sterile	b
	49	48	sterile	b

* a – Young plants from seeds; b – Cell suspensions; c – Plants grown from protoplasts isolated from cybrid leaves

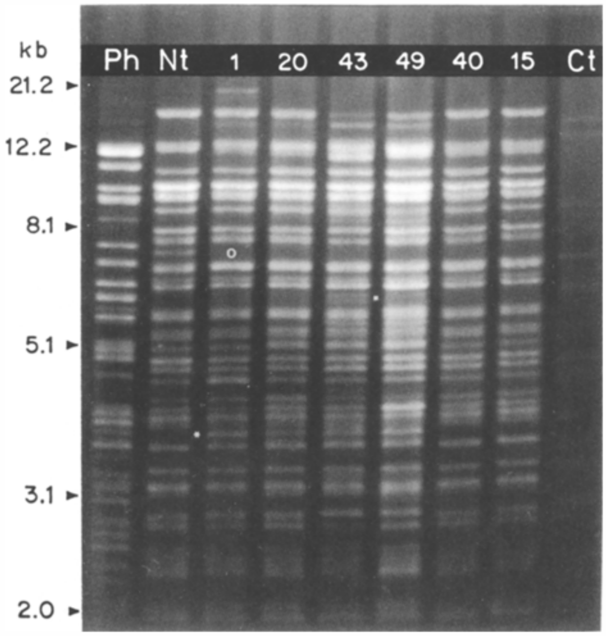

Fig. 2. Restriction pattern of mtDNA from *N. tabacum, P. hybrida,* six cybrids belonging to Group II, and *P. hybrida* chloroplast DNA, all restricted with BamH1. In the lane for Cybrid 1, the "o" indicates a 7.4-kb *N. tabacum* fragment that is absent from all the cybrids. The "*" indicates the location of a fragment present in several cybrids but absent in *N. tabacum*. In the lane of cybrid 49, the "■" indicates a pair of fragments that appear to be novel but that co-electrophorese with a pair of chloroplast fragments and, therefore, are assumed to represent chloroplast DNA contamination of the mtDNA preparation

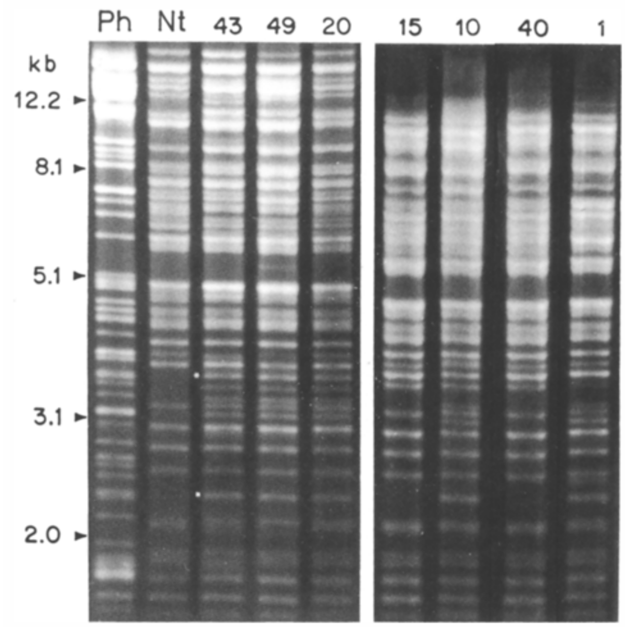

Fig. 4. Restriction pattern of mtDNA for *N. tabacum, P. hybrida,* and seven cybrids belonging to Group II, restricted with Xho1. The "*", at the left of the lane for cybrid 43, indicates the location of a fragment of 3.5 kb present in all the cybrids but absent in *N. tabacum*. The "■" indicates another fragment of 2.3 kb that is present in six of the eight cybrids examined. Both of these fragments appear at locations similar to fragments of *P. hybrida* mtDNA. Four of the cybrids were run on a separate gel and, therefore, do not align well with the kb-ladder shown in this figure

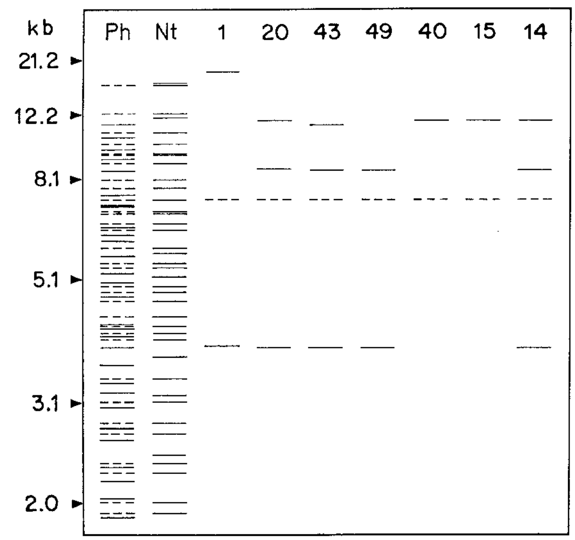

Fig. 3. Diagram representing the results presented in Fig. 2. All *N. tabacum* mtDNA fragments are shown by *solid lines*. The mtDNA fragments of all other lanes are referenced to *N. tabacum*. If an *N. tabacum* fragment is missing in one of the other lanes, a *dotted line* appears. If one of the other mtDNA sources contains a fragment that is not shown by *N. tabacum*, then a *solid line* appears

MtDNA of the progeny of the cybrids from Group I

A total of nine different self-fertile cybrids, obtained from four independent fusion experiments, were selected for analysis. MtDNA from siblings of the cybrids showed no restriction fragment length polymorphisms (RFLPs) in comparison to *N. tabacum* with all three enzymes. The results using BamH1 are shown in Fig. 1.

MtDNA of the cybrids of Group II

Analysis of mtDNA isolated from progeny of cybrid plants, from cell suspensions derived from cybrid plants, or from plants regenerated from protoplasts of Group II cybrids revealed differences from both *N. tabacum* and *Petunia* mtDNA. Each of the nine cybrids studied from three different fusion experiments had a pattern largely similar to *N. tabacum*, but with some RFLPs. After restriction with BamH1, all the restriction fragments of *N. tabacum* were found except one at 7.4 kb, which was missing in all the cybrids (Figs. 2 and 3). Several extra fragments were found that co-migrated with fragments of *Petunia* mtDNA, and a few fragments were found that differed from both parents.

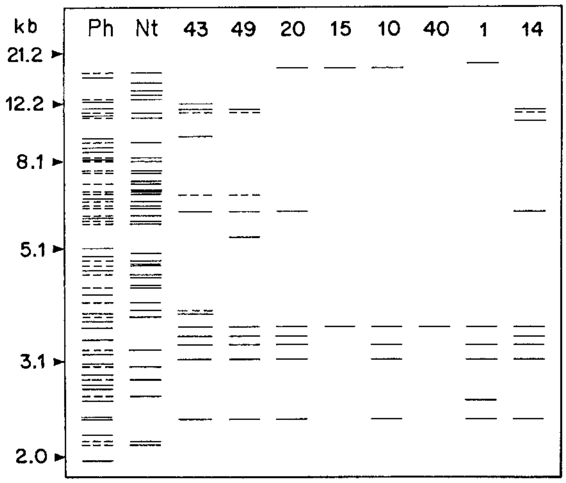

Fig. 5. Diagram representing restriction pattern for mtDNA from *N. tabacum, P. hybrida,* and eight of the cybrids, restricted with Xho1. A group of additional fragments around 3.1 – 3.6 kb in size appears as a feature common to most of the cybrids. Representation of fragments is as described in the caption to Fig. 3

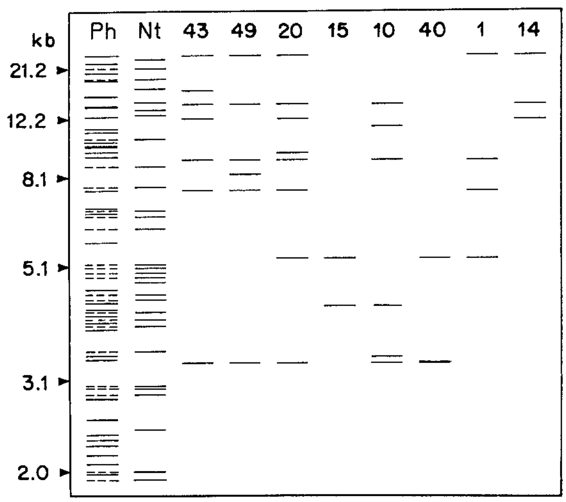

Fig. 6. Diagram representing the restriction pattern for mtDNA from *N. tabacum, P. hybrida,* and eight of the cybrids, restricted with Pst1. Representation of fragments is as described in the caption to Fig. 3

A different pattern was obtained after restriction of the mtDNA with Pst1 and Xho1. With Xho1, most fragments typical for *N. tabacum* were found in all nine cybrids (Figs. 4 and 5). Several additional fragments were present, some of which co-migrated with *Petunia* mtDNA. One extra fragment found at 3.5 kb was found in all cybrids (Figs. 4 and 5). With Pst1, all the fragments present in *N. tabacum* were found in all the cybrids (Fig. 6). Of the additional fragments, many appeared in several of the cybrids.

With one exception (cybrid 40), no differences were detected when the materials for mtDNA isolation were leaves from progeny of a cybrid, cell suspensions derived from cybrid leaf tissue, or plants regenerated from cybrid protoplasts. Cybrid 40 showed one fragment from suspension cell mtDNA, which was not present in plant tissue (data not shown).

Chromosome numbers of cybrid plants

Counts of root-tip chromosomes of the cybrid plants showed them to contain the normal chromosome number for *N. tabacum* ($2n = 4x = 48$), except for one cybrid from Group II, which was tetraploid (Table 1). The abnormal growth of Group II cybrids was not accompanied by aneuploidy.

Discussion

In the *Nicotiana-(Petunia)* cybrids, organelle segregation resulted in a combination of chloroplasts from *Petunia* and mitochondria from *N. tabacum*, either identical to (Group I) or slightly altered from *N. tabacum* mtDNA (Group II). Thus, in spite of screening for the transfer of *Petunia* chloroplasts (Glimelius and Bonnett 1986), transfer of *Petunia* mitochondria did not occur.

Our results suggest that some type of incompatibility between the nucleus of *N. tabacum* and the mitochondria from *Petunia* exists. Similar results were obtained by Aviv et al. (1984) in the interspecific combinations of cytoplasm from *N. sylvestris* and *N. rustica,* where a cytoplasm of a mixed organelle composition rather than a cytoplasm with both chloroplasts and mitochondria from *N. sylvestris* was detected. Also, in the intertribal combinations between *N. tabacum* and *Salpiglossis sinuata,* cybrids with chloroplasts from *Salpiglossis* were obtained, while no cybrids with mitochondria from *Salpiglossis* were found (Thanh et al. 1988). These results contrast with those of other investigators (Menczel et al. 1983; Medgyesy et al. 1985; Barsby et al. 1987; Aviv and Galun 1988), also using the "donor-recipient" method (Zelcer et al. 1978) for transfer of organelles, where co-transmission of chloroplasts and mitochondria was found. Furthermore, Pelletier (1986) reported that only certain specific cybrids were obtained, when combining cytoplasmic male-sterile *Brassica napus* containing the cytoplasm of *R. sativus* with fertile *B. napus* or *B. campestris,* which could be due to limited cooperation between certain chloroplast and mitochondrial types. Barsby et al. (1987) also reported that one specific combination of chloroplasts from the cytoplasmic male-sterile Polima variety of *B. napus* and mitochondria from the fertile variety with *B. campestris* cytoplasm was not recovered in their experiments. Taken together, these results indicate that there are compatibility requirements, both on the interspecific and intergeneric levels, between either certain organelles and the nuclear genome or certain mitochondria and chloroplasts.

The cybrids of Group II, which showed varying degrees of abnormal morphology and reduced fertility, contained mtDNA that differed from the parental *N. tabacum* type. Although the mtDNA pattern was very similar to *N. tabacum*, RFLPs were found representing unique fragments. Some unique fragments co-migrated with *Petunia* fragments, while others were different from both *Petunia* and *N. tabacum*. Such alterations in mtDNA are probably due to events occurring in the heteroplasmic state in the original fusion product (Nagy et al. 1983) and might be due to recombinational events (Belliard et al. 1979; Galun et al. 1982; Rothenberg et al. 1985; Vedel et al. 1986).

A striking result was that the same alterations appeared as a common theme in several of the cybrids in spite of the fact that they were independently derived. For example, the BamH1 fragment of 7.4 kb present in *N. tabacum* was absent in all Group II cybrids tested. An extra fragment of 3.5 kb was found in all Group II cybrids after restriction with Xho1. In general, most RFLPs were found in several different cybrids. These results indicate that the interaction between the mtDNA of *P. hybrida* and *N. tabacum* was not random, but was rather restricted to specific regions on the genome. Similar results have been obtained in other hybrids and cybrids where mitochondria of different types have been combined, such as in *Solanum* (Kemble et al. 1986) and *Daucus* (Kothari et al. 1986). Although intramolecular recombination of mtDNA occurs at specific regions of the DNA that contain repeats (Lonsdale et al. 1984; Palmer and Shields 1984), investigations by Vedel et al. (1986) of *B. napus* cybrids and by Rothenberg and Hanson (1987) of *Petunia* cybrids revealed that the repeat regions were not the sites of intermolecular interaction. Rothenberg and Hanson proposed that the nonrandom appearance of unique fragments could be due to the presence of specific regions of the mtDNA in the two species, which have homology to the repeated regions. These regions would permit recombination to occur after the stress exerted during the fusion and tissue culture processes.

Kemble et al. (1988) argued that tissue culture methods could affect the degree of rearrangements found in the mtDNA. However, in the production of the *Nicotiana-(Petunia)* cybrids, the same culture procedures were used for all plants, yet the mtDNA of Group I cybrids differed from Group II cybrids. Similar results were obtained by Kothari et al. (1986) for three *Daucus* cybrids, one of which contained a new type of mtDNA, while the other two were identical to the parents, and Landgren and Glimelius (1989) for *Brassica* hybrids. In their experiments, several of the hybrids contained mtDNA with rearranged and probably also recombined DNA, while others had the parental pattern, even though all 30 hybrids were produced and cultured using the same procedures. Thus, there are several independent investigations which stress that the alterations of mtDNA are more likely the result of fusion-induced events than of tissue-culture-induced variations.

A plausible explanation for the rearrangements found in Group II cybrids could be that some nuclear DNA from *Petunia* was incorporated, thus enabling some *Petunia* mtDNA to be present. The abnormality of the plants could also be explained by the presence of *Petunia* nuclear DNA. Investigations of chromosomes and isoenzymes (data not shown) did not reveal any nuclear DNA from *Petunia*. However, as stressed by Thanh et al. (1988), genetic material could still be present, which might only be detected with more sensitive methods such as RFLP analysis. The rearrangements recorded in the mtDNA could result from early events in the development of the fusion products, when the presence of nuclear DNA from *Petunia* could have sustained *Petunia* mitochondria long enough to enable recombination to occur.

A well-known phenomenon in interspecific hybridization of *Nicotiana* is the appearance of cytoplasmic male sterility, when the mitochondria of one species are partially incompatible with the *N. tabacum* nucleus. The abnormalities recorded in the plants of Group II were both vegetative and reproductive. Although plants were obtained that did not produce pollen, these plants also showed a reduction in female fertility. Several plants produced flowers with a split corolla or petaloid stamens, traits frequently shown in male-sterile lines of *Nicotiana*. These plants failed to set seed following pollination, and, thus, were reproductively sterile.

Since none of the *Nicotiana-(Petunia)* cybrids analyzed contained mitochondria identical or similar to *Petunia* mitochondria and none showed cytoplasmic male sterility, we conclude that the *P. hybrida* mitochondrial and *N. tabacum* nuclear genomes have diverged to such a degree that unaltered *P. hybrida* mitochondria and *N. tabacum* nuclei cannot coexist.

Acknowledgements. This work was supported by the Competitive Research Grants Program of the USDA and grants from the Swedish Natural Science Council. We are grateful to Wilma Hu, Department of Crop Science, North Carolina State University, for her instruction in the methodology of mtDNA preparation, and to Gunilla Håkansson and Judith Horstmann for their excellent technical assistance.

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