

PATERNAL, MATERNAL, AND BIPARENTAL INHERITANCE OF THE CHLOROPLAST GENOME IN *PASSIFLORA* (PASSIFLORACEAE): IMPLICATIONS FOR PHYLOGENETIC STUDIES¹

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Patterns of inheritance of the chloroplast genome in *Passiflora* were analyzed by examining the progeny from both interspecific and intraspecific crosses. Artificial crosses of field-collected material were performed in greenhouses at The University of Texas at Austin. DNA from fresh leaf material was analyzed by Southern blot techniques to identify the donor of the chloroplast genome. Initially, single progeny were analyzed for 11 crosses; two intraspecific crosses demonstrated maternal inheritance, whereas the nine interspecific crosses had paternal inheritance. Subsequently, the donor of the chloroplast genome was determined for multiple progeny in seven crosses. *Passiflora oerstedii* × *P. retipetala* showed strict paternal inheritance in all of 17 progeny. A series of five crosses and backcrosses between *P. oerstedii* and *P. menispermifolia* demonstrated strictly paternal inheritance. Finally, when 15 progeny were analyzed for the *P. costaricensis* × *P. costaricensis* cross, 12 of the 15 showed maternal inheritance, whereas the remaining three were biparental. Interestingly, all interspecific crosses had primarily paternal inheritance, whereas all intraspecific crosses had primarily maternal inheritance. The implications of heteroplasmy on phylogenetic analyses of chloroplast DNA are discussed.

Key words: chloroplast inheritance; heteroplasmy; *Passiflora*; Passifloraceae; paternal inheritance; phylogenetic incongruence; plastid inheritance.

The inheritance of chloroplast DNA has historically been thought to be exclusively from the maternal parent in angiosperms (Corriveau and Coleman, 1988; Birky, 1995). However, studies in recent years have documented a surprising amount of variation in inheritance patterns (Shore et al., 1994; Chat et al., 1999; McKinnon et al., 2001). With the ubiquitous use of organellar DNA in phylogenetic studies, it is critical to know the mode of inheritance if data is to be correctly interpreted (Harris and Ingram, 1991). During the course of a phylogenetic study of the genus *Passiflora* L. (Passifloraceae), we sought to determine the mode of inheritance of the chloroplast genome.

A landmark study by Corriveau and Coleman (1988) examined the potential for paternal plastid inheritance in 235 species of angiosperms from 80 different families. Using the DNA fluorochrome 4',6-diamidino-2-phenylindole (DAPI), they detected plastid DNA in either the generative cells or sperm cells in 26 genera, indicating the potential for the paternal chloroplast genome to be transmitted to offspring. This finding was surprising because paternal chloroplast inheritance was thought to be exceedingly rare in angiosperms, having only been documented in a few angiosperms such as *Medicago* (Smith, 1989b). Paternal chloroplast inheritance has subsequently been documented in a wide range of angiosperms including *Oenothera* (Chiu et al., 1988), *Pelargonium* (Metzlaff et al., 1981), and *Turnera* (Shore et al., 1994; Clément and

Pacini, 2001). Corriveau and Coleman (1988) included *Passiflora edulis* in their study and they found it to have chloroplast DNA in the pollen generative cells.

Many different mechanisms work together to prevent paternal chloroplast DNA from being transmitted during fertilization of the embryo. Most angiosperms with maternal inheritance seem to lack plastids in the generative cell, which eventually divides to form the two sperm cells (Mogensen, 1996). Exclusion of plastids from the generative cell is accomplished through polarization of the plastids prior to microspore division or degeneration of the plastid DNA. However, the presence of plastids in the generative cell does not necessarily mean they are transmitted to the embryo. Mechanisms to prevent paternal plastid DNA transmission have been documented in nearly all subsequent stages from exclusion just prior to fertilization to differential replication of maternal and paternal chloroplasts in the embryo (Birky, 1995; Mogensen, 1996; Clément and Pacini, 2001).

To determine whether paternal plastids were actually inherited in *Passiflora*, we used restriction fragment length polymorphisms of chloroplast DNA to determine the donor parent in 78 progeny from 15 artificial intraspecific and interspecific crosses. This study was completed in two phases. In the first phase, we examined single progeny from 11 different crosses. When variation in the donor of the chloroplast genome was detected in these crosses, a second and expanded study was undertaken. For the expanded study, we tested multiple progeny from six crosses (including reciprocal and backcrosses) to determine whether multiple modes of inheritance resulted from a single cross. Our results and their implications for phylogenetic studies are discussed.

MATERIALS AND METHODS

All parental plants used in this study were collected from natural populations and grown for several years in the greenhouses of the Gilbert Lab at The

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TABLE 1. *Passiflora* parental accessions and collection localities. Greenhouse accession numbers (AN), collection localities, and voucher numbers are listed. All vouchers are held at TEX.

Species	AN	Locality	Voucher no.
<i>P. alata</i> Curtis	8021	USDA accession	Kh248
<i>P. coccinea</i> Aubl.	8030	Brazil	Kh176
<i>P. costaricensis</i> Killip	7006	Vera Cruz, Mexico	Kh228
<i>P. costaricensis</i> Killip	8016	Corcovado, Costa Rica	Kh151
<i>P. retipetala</i> Mast.	7007	Arima Pass, Trinidad	Gilbert s.n.
<i>P. garckeii</i> Mast.	9104	French Guiana	Kh177
<i>P. menispermifolia</i> Kunth	8039	Corcovado, Costa Rica	Gilbert s.n.
<i>P. oerstedii</i> Mast.	7005	Puerto Viejo, Costa Rica	Gilbert s.n.
<i>P. "pseudo-oerstedii"</i>	8053	Corcovado, Costa Rica	Gilbert s.n.
<i>P. quadrangularis</i> L.	8054	Corcovado, Costa Rica	Kh183
<i>P. racemosa</i> Brot.	8055	Brazil	Kh157
<i>P. serratifolia</i> L.	8058	Belize	Kh159
<i>P. vitifolia</i> Kunth	9137	Brazil	Kh174

University of Texas at Austin. Parents, their greenhouse accession numbers, and collection localities are listed in Table 1. Stamens of the female parent were removed before anthesis, and pollen was then transferred from the donor plant to receptive stigmas. Seeds were germinated in pots and grown in the greenhouses for variable periods of time. A total of 78 progeny from two intraspecific and 15 interspecific crosses were analyzed (Table 2). The number of progeny analyzed for each cross did not reflect the amount of viable seed produced.

Total DNA was extracted from 3–5 g of leaf tissue using the modified CTAB protocol of Doyle and Doyle (1987). This procedure was followed by ultracentrifugation in ethidium bromide gradients (Sambrook et al., 1989) to purify the DNA. Restriction site polymorphisms that uniquely identified each parent were found by digesting the DNAs with eight different restriction endonucleases (*Hind*III, *Cl*aI, *B*glII, *B*glI, *N*ciI, *B*anII, *E*coRI, and *E*coRV). The digested DNA fragments were separated on 1–1.2% TAE (Tris-acetate-EDTA) agarose gels until the dye front reached 12–15 cm. Bidirectional Southern blots (Southern, 1975) as described by Maniatis et al. (1982) were used to transfer DNA fragments to nylon membranes (Zetabind, AMF Cuno, Meriden, Connecticut).

Single probes from the *Nicotiana tabacum* L. chloroplast DNA clone bank (Olmstead and Palmer, 1992) were radiolabeled with P³² by nick-translation and hybridized to the nylon filters using the methods described by Palmer (1986). Hybridizations and subsequent washes were performed at 62°C. Filters were air dried and exposed to x-ray films at –70°C using intensifying screens.

Once unique polymorphisms were detected for the parental accessions, the procedure was repeated with parents and progeny on the same gel. After exposure to film, the filters were stripped and rehybridized with a different tobacco probe to confirm the results. In cases where a single band differed between the parents, films were overexposed to confirm presence or absence of the band in the progeny.

RESULTS

Chloroplast DNA polymorphisms were detected in the progeny of 15 of the 17 crosses using restriction enzymes *Hind*III and *Cl*aI and probing with tobacco clone1. These results were confirmed by probing the filters with tobacco clones 2 or 9 (Table 2).

Of the 11 crosses for which we examined one individual, two were intraspecific and nine were interspecific. *Passiflora* "pseudo-oerstedii" is an unnamed variety of *P. oerstedii* Mast., and these taxa are considered conspecific here. Both intraspecific crosses (*P. "pseudo-oerstedii"* × *P. oerstedii* and *P. costaricensis* Killip × *P. costaricensis*) revealed maternal inheritance when single progeny were analyzed. The remaining nine interspecific crosses, in which only a single individual was

TABLE 2. Crosses made, the number of progeny analyzed, and the mode of chloroplast inheritance in *Passiflora*. The maternal parent is listed first. AN = greenhouse accession no. for the cross, NP = no. of progeny, MI = mode of inheritance (M = maternal, P = paternal, B = biparental, ND = differences not determined among the parental chloroplast types). The number after each species name is greenhouse accession number.

Cross	AN	NP	MI
<i>P. "pseudo-oerstedii"</i> 8053 × <i>P. oerstedii</i> 7005	8015	1	M
<i>P. racemosa</i> 8055 × <i>P. quadrangularis</i> 8054	8088	1	P
<i>P. racemosa</i> 8055 × <i>P. retipetala</i> 7007	9212	1	P
<i>P. oerstedii</i> 7005 × <i>P. retipetala</i> 7007	9187	1	P
<i>P. coccinea</i> 8030 × <i>P. oerstedii</i> 7005	8013	1	P
<i>P. vitifolia</i> 9137 × <i>P. coccinea</i> 8038	9223	1	P
<i>P. vitifolia</i> 9137 × <i>P. serratifolia</i> 8058	9231	1	P
<i>P. alata</i> 8021 × <i>P. garckeii</i> 9104	9214	1	P
<i>P. oerstedii</i> 7005 × <i>P. alata</i> 8021	8012	1	P
<i>P. costaricensis</i> 7006 × <i>P. costaricensis</i> 8016	9194	1	M
<i>P. costaricensis</i> 7006 × <i>P. costaricensis</i> 8016	9194	15	12M/3B
<i>P. oerstedii</i> 7005 × <i>P. retipetala</i> 7007	9187	17	17 P
<i>P. oerstedii</i> 7005 × <i>P. menispermifolia</i> 8039	8014	1	P
<i>P. menispermifolia</i> 8039 × F ₁ 8014	8098	9	ND
<i>P. menispermifolia</i> 8039 × <i>P. oerstedii</i> 7005	8101	9	P
F ₁ 8014 × <i>P. oerstedii</i> 7005	8097	11	P
F ₁ 8014 × <i>P. menispermifolia</i> 8039	8099	4	ND

analyzed, showed paternal inheritance of the chloroplast genome.

When multiple progeny were analyzed for the *P. costaricensis* × *P. costaricensis* cross, 12 of the 15 individuals showed maternal inheritance, whereas the remaining three showed biparental inheritance (Fig. 1). The interspecific cross of *P. oerstedii* × *P. retipetala* Mast. had strict paternal inheritance in all 17 progeny examined.

The series of *P. menispermifolia* Kunth × *P. oerstedii* crosses and backcrosses (Table 2) all showed paternal inheritance when restriction fragment differences were detected between the parents. Neither the *P. oerstedii* 7005 chloroplast genome nor the *P. menispermifolia* 8039 chloroplast genome appeared to be favored. We were not able to find unique patterns for the reciprocal backcrosses of the *P. oerstedii* × *P. menispermifolia* F₁ progeny or the *P. menispermifolia* parent (cross-accession nos. 8099 and 8098).

DISCUSSION

In recent years, much progress has been made toward understanding the mechanisms behind chloroplast inheritance, and the mode of inheritance has been examined for many different genera of flowering plants and gymnosperms (Stine et al., 1989; Shore et al., 1994; McKinnon et al., 2001). Although gymnosperms inherit their chloroplast genome primarily from the paternal parent (Stine et al., 1989), paternal inheritance is rarely the dominant mode in angiosperms (Birky, 1995). Both angiosperms and gymnosperms seem to have primarily uniparental inheritance (Birky, 1995; Mogensson, 1996; Clément and Pacini, 2001); however, occasional biparental inheritance has been detected at a low frequency in several genera such as *Iris* (Cruzan et al., 1993), *Medicago* (Johnson and Palmer, 1989), and *Turnera* (Shore et al., 1994). These findings are congruent with the data presented here; we only

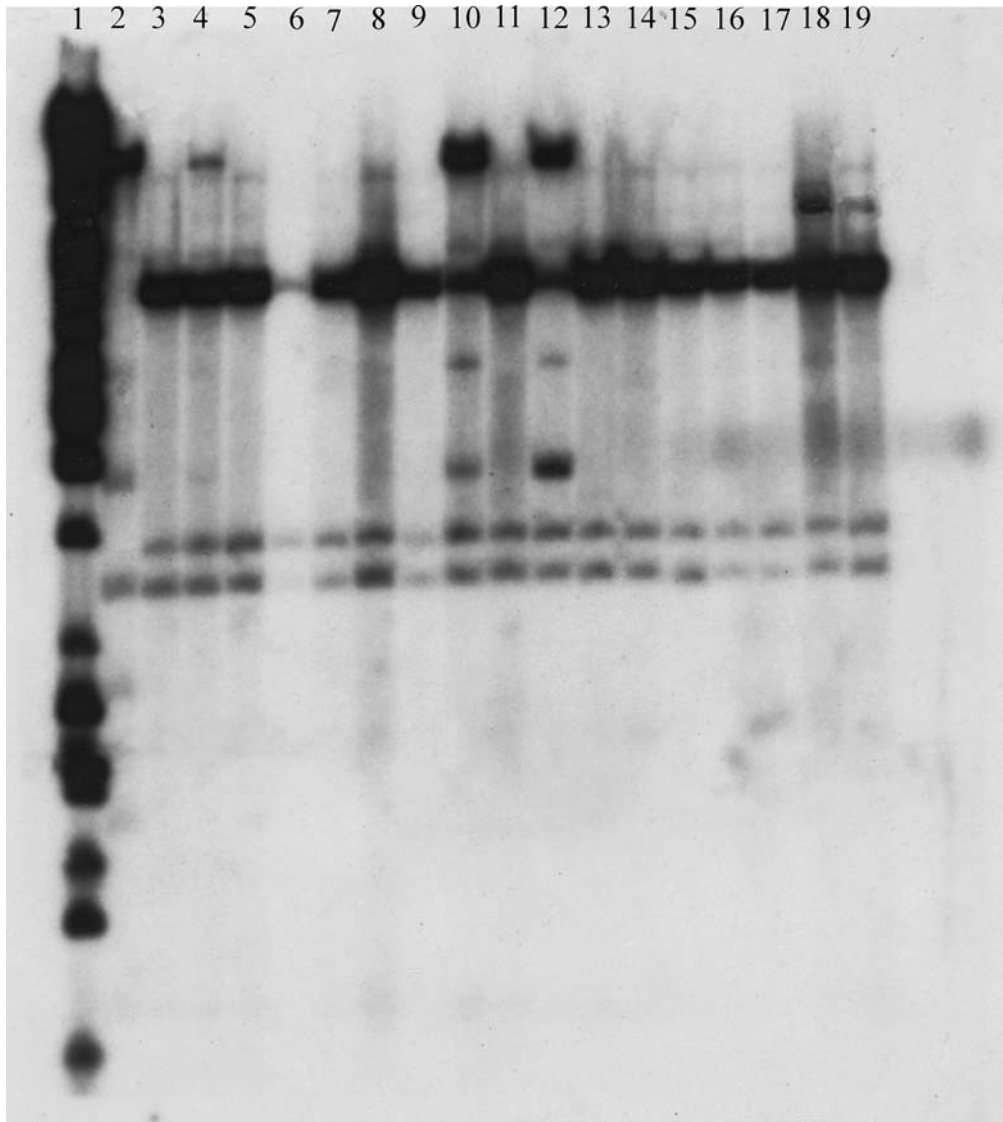


Fig. 1. Scanned image of the autoradiograph used to detect chloroplast inheritance patterns in *Passiflora*. This intraspecific cross, *P. costaricensis* × *P. costaricensis*, demonstrated both maternal and biparental inheritance of the chloroplast genome in *Passiflora*. Total DNAs were digested with restriction enzyme *Ban*II and hybridized to probe 1 of the chloroplast tobacco genome. Lanes 1, lambda molecular size marker; 2, paternal parent, *P. costaricensis* (8016); 3, maternal parent, *P. costaricensis* (7006); 4, 10, and 12, biparental inheritance; 5–9, 11, 13–19, maternal inheritance.

detected biparental inheritance in 4.7% of the *Passiflora* progeny analyzed.

A wide variety of documented mechanisms work to some extent in all plant groups to prevent biparental transfer of chloroplast and mitochondrial DNA (reviewed in Birky, 1995, 2001; Mogenson, 1996). This, along with the relative rarity of documented cases of biparental inheritance, implies a selective advantage to uniparental inheritance (Mogenson, 1996). However, exactly what that benefit might be is not well understood. Most commonly, the prevention of recombination, which might interfere with the expression of the coadapted photosynthetic genes within the chloroplast, is suggested to act as a strong selectional pressure to prevent biparental inheritance (Birky, 1995; Mogenson, 1996). Although recombination of chloroplasts has only been documented in the unicellular algae *Chlamydomonas* (Harris, 1989) and in the gymnosperms

Pinus (Marshall et al., 2001) and *Cycas* (Huang et al., 2001), Medgyesy et al. (1985) showed that recombination in angiosperms was possible through somatic fusion in *Nicotiana*. Birky (1995) suggested that the selective pressures likely vary among species and that no one selective regime is responsible for uniparental inheritance of plastid DNA in all organisms.

The majority of the progeny (71.9%) analyzed in this study demonstrated paternal inheritance. However, it is notable that paternal inheritance was only detected in interspecific crosses. The two intraspecific crosses, *P. "pseudo-oerstedii"* × *P. oerstedii* and *P. costaricensis* × *P. costaricensis*, had primarily maternal inheritance. The correlation of intraspecific crosses having maternal inheritance with interspecific crosses having paternal inheritance is a phenomenon that has been observed in other taxonomic groups such as *Iris* (Cruzan et al., 1993), *Daucus* (Boblenz et al., 1990), *Hordeum* × *Secale* (Soliman,

1987), *Larrea* (Yang et al., 2000), and *Medicago* (Lee, 1988). In fact, most cases of paternal inheritance in the literature involved interspecific hybrids. There are three notable exceptions. First, in kiwifruit, multiple progeny from an intraspecific cross demonstrated strict paternal inheritance (Chat et al., 1999). Second, in a family closely related to the Passifloraceae, intervarietal crosses in the *Turnera ulmifolia* complex (Turneraceae) also demonstrated paternal inheritance (Shore et al., 1994). The authors of this last study point out that the degree of reproductive isolation among these varieties is more typical of interspecific taxa; therefore, paternal inheritance may not be the norm in *Turnera*. The third exception is also found in *Turnera*. Shore and Triassi (1998) examined progeny from 43 intraspecific crosses of *T. ulmifolia* and found that 64% of the time, the chloroplast donor was the paternal parent. However, *T. ulmifolia* is a known allohexaploid (Belaousoff and Shore, 1995). To our knowledge, no cases of primarily paternal inheritance have been documented in crosses of noncrop angiosperms that do not involve interspecific hybrids. Therefore, the primarily paternal inheritance of chloroplast DNA could be a function of incompatibility between interspecific or modified genomes where the mechanisms for paternal exclusion fail. At present, we have very little understanding of the likelihood and mechanisms for primarily paternal inheritance in natural populations of angiosperms.

The existence of multiple modes of inheritance in *Passiflora* requires caution when interpreting phylogenetic data. For example, it is not possible to make inferences regarding the seed or pollen parent of hybrid taxa. Although only a single natural hybrid has been unequivocally confirmed in *Passiflora* (Lorenz-Lemke et al., 2005), there is ample morphological and chromosomal evidence to suggest that hybridization in natural systems is not uncommon (Escobar, 1988; Vanderplank, 1996; Hansen et al., 2006; Hansen, personal observation). Based on our present results, the interpretation of the origin of hybrids based on chloroplast data is limited in this genus.

Heteroplasmy, on the other hand, has a greater potential to be problematic for phylogenetic inference in *Passiflora*. Because divergent plastids within an individual are functionally paralogous (Wolfe and Randle, 2004), the chloroplast phylogeny may not accurately represent ancestor-descendant relationships. An unambiguous example of this scenario is discussed by Hansen et al. (2006). In this case, a single, heteroplasmic individual of *P. microstipula* contained two extremely divergent chloroplast types resulting in this species' placement in two different clades. Another phylogenetic study of cpDNA in *Passiflora* found, as suspected, *P. ovalis* (shown as *Tetrastylis ovalis*) as a sister group to the rest of *Passiflora* using *rps4*, whereas the *trnL-trnF* spacer region nested *P. ovalis* within the "x = 9 group," which is not consistent with morphology (Muschner et al., 2003). Although there are alternative explanations to heteroplasmy for the situation in *P. ovalis*, we would argue, based on the evidence presented here and in Hansen et al. (2006), that it is plausible. Fortunately, the phylogenetic placement of *P. microstipula* and *P. ovalis* were in such stark contrast to the available morphological and chromosomal data that red flags were immediately raised, thus prompting further exploration into *P. microstipula*. A more likely scenario, as a result of the hybridization barriers between the major groups in *Passiflora* (L. Gilbert, University of Texas, unpublished data), is that the chloroplast types in a heteroplasmic individual would be less divergent than in the

examples described, leading to the misinterpretation of relationships. Because two cases of confirmed heteroplasmy have been detected in *Passiflora* (*P. costaricensis* and *P. microstipula*), caution is warranted when interpreting chloroplast phylogenetic data in this genus.

While the perils of paralogous copies of nuclear genes in phylogenetic inference have been extensively addressed in the literature (see Alvarez and Wendel, 2003; Bailey et al., 2003), they have largely been ignored for chloroplast data (but see Harris and Ingram, 1991; Wolfe and Randle, 2004). As Wolfe and Randle (2004) point out, nearly one third of surveyed angiosperms have at least occasional biparental inheritance of cpDNA (Smith, 1989a), resulting in functionally paralogous copies within an individual. Therefore, it is probable that heteroplasmy occurs on a limited scale in most groups of angiosperms. Indeed, Birky (1995, 2001) suggested that plastid inheritance might occur on a continuum, making the classification of discrete states (maternal, paternal, biparental) artificial.

Further, the ubiquitous use of phylogenetic relatedness to justify the assumption of uniparental, maternal inheritance seems misguided, at least at the family level. In a recent survey of pollen generative cells in 295 species, Zhang et al. (2003) detected more than one mode of inheritance (either biparental or a combination of maternal and biparental) in 37% of the families for which multiple taxa were sampled. Although we do not dispute their contention that there might be a phylogenetic component to the mode of plastid transmission, it seems an unreliable predictor given these data. The advent of PCR methods for determining the donor of the chloroplast genome has made generating this information relatively inexpensive and easy. These types of studies would be a useful prelude to systematic investigations using chloroplast DNA, especially those focusing on hybridization.

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