

A one-step organelle capture: gynogenetic kiwifruits with paternal chloroplasts

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Androgenesis, the development of a haploid embryo from a male nucleus, has been shown to result in the instantaneous uncoupling of the transmission of the organelle and nuclear genomes (with the nuclear genome originating from the male parent only and the organelle genomes from the female parent). We report, for the first time, uncoupling resulting from gynogenesis, in *Actinidia deliciosa* (kiwifruit), a plant species known for its paternal mode of chloroplast inheritance. After pollen irradiation, transmission of nuclear genes from the pollen parent to the progeny was inhibited, but transmission of the chloroplast genome was not. This demonstrates that plastids can be discharged from the pollen tube into the egg with little or no concomitant transmission of paternal nuclear genes. Such events of opposite inheritance of the organelle and nuclear genomes must be very rare in nature and are unlikely to endanger the long-term stability of the association between the different genomes of the cell. However, they could lead to incongruences between organelle gene trees and species trees and may constitute an alternative to the hybridization/introgression scenario commonly invoked to account for such incongruences.

Keywords: gynogenesis; paternal chloroplast inheritance; phylogeny; incongruence; species tree; genomic conflicts

1. INTRODUCTION

Two of the three genomes found in eukaryotic plant cells, the chloroplast and mitochondrial genomes, are ancient free-living eubacteria now forming obligatory dependent relationships with the nucleus (Margulis 1970; Gray 1999). The long-term stability of such an association requires that the interests of the symbionts align with those of the host (Herre *et al.* 1999). In the course of evolution, the mitochondrial and chloroplast genomes have undergone massive losses of functional genes, to the point that they can no longer resume an independent existence (Maynard-Smith & Szathmary 1997; Gray 1999). Nevertheless, situations likely to generate conflicts of interest between the organelle and nuclear genomes still exist because of their contrasting mode of transmission (Eberhard 1980; Cosmides & Tooby 1981; Moran & Wernegreen 2000). The complete uncoupling of the transmission of organelle genomes from that of the nuclear genome (whereby an organelle genome is transmitted from one parent and the nuclear genome from the other parent) should, in principle, generate even more drastic genomic conflicts, potentially endangering the stability of the association and hence the very existence of the eukaryotic cell. Despite their potential interest, cases of transmission of organelle and nuclear genomes through opposite sexes have not attracted the interest of evolutionists, probably because reports of their occurrence remain extremely rare. In fact, the only well-known cases of transmission of different genomes through opposite sexes do not involve cytonuclear transmission but involve, instead, chloroplast and mitochondria, when one organelle is

paternally inherited and the other maternally inherited as found, in particular, in some conifers (see Wagner *et al.* 1987; Neale & Sederoff 1989). However, the prospects for the uncoupling of organelle transmission from that of the nucleus do exist. First, under artificial conditions, intact organelles can be cloned into donor cells of other individuals from the same species in the absence of nuclear transfer in plants (see Verhoeven & Blass 1988; Eigel & Koop 1992) as well as in animals (Pinkert *et al.* 1997), including humans (see Cohen *et al.* 1997). Second, there are reproductive processes that mimic sexual reproduction but omit syngamy (Kimber & Riley 1963; Nogler 1984), leading to the uniparental transmission of the nuclear genome, another prerequisite for such uncoupling.

In apomictic plants that reproduce asexually through seeds, embryos inheriting their diploid nucleus from one parent and their organelle genomes from the other parent have not yet been reported. In such species, found in some 40 angiosperm families (Vielle Calzada *et al.* 1996) not only meiosis but also fertilization is by-passed, giving birth to individuals that are the exact genetic copy of the seed parent (Nogler 1984; Asker & Jerling 1992) or, as recently discovered in a rare *Cupressus* species, of the pollen parent (Pichot *et al.* 2001). In maternal apomixis, even if the development of the seed is sometimes dependent on the fertilization of the polar nuclei for endosperm development, the male parent does not contribute at all to the genetic constitution of the embryo. Similarly, and quite strikingly, in *Cupressus*, the only case of paternal apomixis reported so far, the cytoplasm of the embryo is expected to be male in origin, like the nuclear genome, because both chloroplast and mitochondrial genomes are paternally inherited in *Cupressaceae* (Chesnoy 1987; Neale *et al.* 1991).

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In androgenetic plants, however, haploid embryos are produced that originate from the development of a male gamete within the embryo sac of the female parent, resulting in uniparental paternal inheritance of the nuclear genome, whereas the organelle genomes are still contributed by the female parent. This results, therefore, in the complete uncoupling of the transmission of organelle and nuclear genomes. Androgenetic plants, although considerably rarer than apomictic plants and, to a lesser extent, than gynogenetic plants (i.e. haploid plants deriving from the development of the female gamete), have been reported several times since the early twentieth century (e.g. Clausen & Lammerts 1929; Kehr 1951; Burk 1962; Singh & Cornu 1976). They are known to occur spontaneously, at a low frequency, in various angiosperms belonging to both mono- and dicotyledons, for example, *Capsicum* (Campos & Morgan 1958), *Nicotiana* (Burk 1962), *Petunia* (Singh & Cornu 1976) and *Zea* (Chase 1963). In the 1960s, Goodsell (1961) and Chase (1963) demonstrated that maize androgenetic haploids had the organelle genomes of their mother, together with the haploid nucleus of their father. In the 1980s, the same conclusion was reached for *Nicotiana* and *Petunia* by using molecular markers (Pelletier *et al.* 1987; Raquin *et al.* 1989; Horlow *et al.* 1993).

By contrast, no case of gynogenesis leading to the uncoupling of the transmission of nuclear and organelle genomes has yet been reported. In principle, it is conceivable that a male plant could contribute its chloroplasts or mitochondria to a gynogenetic offspring, provided that paternal or biparental inheritance of the organelle genomes can occur in that species. Paternal inheritance of the chloroplast genome has been demonstrated in the genus *Actinidia* (Cipriani *et al.* 1995; Chat *et al.* 1999). Furthermore, there have been successful attempts to produce *Actinidia* haploids by using irradiated pollen (Pandey *et al.* 1990; Chalak & Legave 1997), a physical treatment well known to trigger haploidization processes (Lacadena 1974). In particular, four trihaploids were recovered from an experiment conducted in the cultivated hexaploid species *A. deliciosa* (Chalak & Legave 1997). In the present study, we examined whether the chloroplast genome of the pollen parent had been transmitted and checked if only the female parent had contributed nuclear genes to this haploid progeny using several codominant nuclear markers (Weising *et al.* 1996; Huang *et al.* 1998). In conjunction with previous reports of androgenesis, our findings raise the question of whether cases of complete uncoupling of the transmission of organelle and nuclear genomes occur in nature, for instance following interspecific hybridization. Such whole-genome horizontal transfers could explain some of the incongruences between organelle gene trees and species trees in plants and should improve our understanding of the evolutionary relationships between organelle and nuclear genomes.

2. MATERIAL AND METHODS

(a) Plant material

Kiwifruit is a dioecious species; the two hexaploid parents used in this study were 'D uno', a male plant, and 'Hayward', a female cultivar. In 1994, Hayward was pollinated with irradiated pollen from D uno to induce gynogenesis (Chalak & Legave

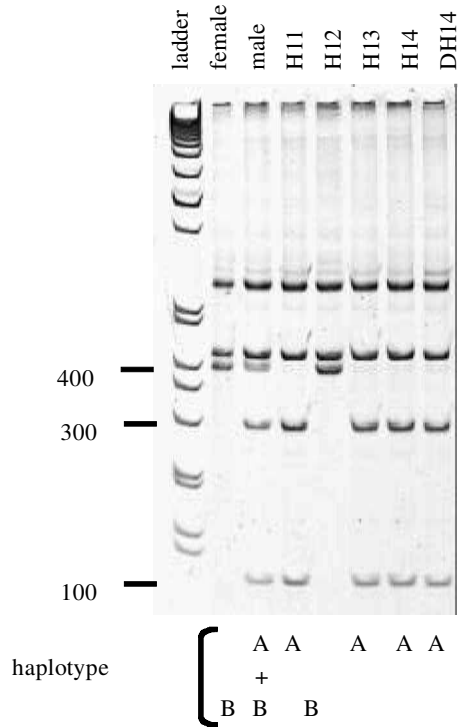


Figure 1. Inheritance of cpDNA in the four trihaploids (H11, H12, H13 and H14) and in the doubled trihaploid (DH14) resulting from H14. The *psbC-trnS* cpDNA fragment amplified by PCR (1600 bp) is digested by the *MspI* restriction enzyme. The fragment sizes (in bp) for the type A and type B specific bands are indicated on the left. Ladder: 1 kilobase molecular weight marker.

1997). Several seedlings resulting from that experiment were assessed for ploidy. Four of them were trihaploids, as determined by flow cytometry (Chalak & Legave 1997). These were designated H11, H12, H13 and H14. A shoot resulting from the spontaneous chromosome doubling of H14 during *in vitro* adventitious regeneration was also propagated and designated DH14. Forty full sibs originating from the controlled cross Hayward \times D uno performed with non-irradiated pollen were also included in the study. The two parents, the four trihaploids, the doubled trihaploid and the 40 full sibs were analysed using molecular markers. Total genomic DNA was isolated from leaf tissue according to a procedure described in Chat *et al.* (1999). All the DNA extracts were diluted to a final concentration of $5 \text{ ng } \mu\text{l}^{-1}$ to be used as a template for polymerase chain reaction (PCR) amplification.

(b) Polymorphism of the chloroplast genome

The chloroplast DNA (cpDNA) polymorphic region analysed is located between the *psbC* and *trnS* genes, coding for a *psII* protein and the tRNA-Ser(UGA), respectively. Universal primers (Demesure *et al.* 1995) were used to amplify this particular cpDNA region. Two different cpDNA haplotypes, called haplotypes A and B, are present within the species *A. deliciosa* (Chat *et al.* 1999). Haplotype B differs from haplotype A by an extra *MspI* recognition site within the *psbC-trnS* region. The pollen parent D uno possesses both haplotypes (heteroplasmic state) whereas the female parent Hayward possesses only haplotype B (homoplasmic). As demonstrated in a previous study, the heteroplasmic state of D uno (responsible for the restriction pattern observed: two strong bands of 300 and 100 base pairs (bp), specific to haplotype A, together with the faint 400 bp band specific

to haplotype B; see figure 1) is actually due to its periclinal plastid chimeric state. The second histogenic layer (LII) of D uno contains exclusively plastids of type A, and as a consequence only this type is transmitted to the next generation during sexual reproduction (Chat *et al.* 2002). This polymorphism was used to infer the plastid origin of the four trihaploids and the doubled trihaploid. Conditions for DNA amplification and electrophoresis have been described previously (Chat *et al.* 1999).

(c) Polymorphism of the nuclear genes

Nuclear composition of the trihaploids was investigated by using five microsatellite (simple sequence repeat (SSR)) primer pairs, UDK96-001, UDK96-030 (Huang *et al.* 1998) and 721, 722, 735 (Weising *et al.* 1996). As the primer pairs all revealed differences between the two parents, they were subsequently used to determine the genotype of the four trihaploids and that of the doubled trihaploid. Conditions for SSR amplification and electrophoresis have been detailed previously (Chat *et al.* 2002).

(d) Statistical analysis of the SSR data

Owing to the difficulty of distinguishing between one-dose versus multiple-dose allele conditions within the segregating populations, SSR analysis was performed based on the presence [+] versus the absence [-] of the allele. χ^2 -tests were used to determine goodness-of-fit of the segregation ratios among the 40 sexual hybrids. The inheritance of nuclear SSR markers is of polysomic type (see § 3), a finding that is in agreement with the conclusion of Testolin & Ferguson (1997) that *A. deliciosa* is an autohexaploid. As a consequence, for markers that are only present in the male parent, the probability of allele presence [+] in the male gamete corresponds to random sampling without replacement of three alleles among the six paternal ones. In the male gamete population, ratios [+] : [-] of 1 : 1, 4 : 1, 19 : 1 are expected for alleles in simplex, duplex, triplex conditions in the male parent, whereas alleles present in four copies or more in the male parent should be present in at least one copy in all male gametes produced. Considering all loci, we then calculated the probability: (i) for a zygote ($2n = 6x$) to inherit at least one paternal-specific allele; and (ii) for a haploid ($n = 3x$) to receive none of the paternal-specific allele, assuming random elimination of three alleles among the six initially present in the zygote.

3. RESULTS

(a) Chloroplast composition of the trihaploids

Three out of the four trihaploids had haplotype A only, indicating a paternal origin for their cpDNA genome (figure 1). By contrast, H12 exhibited haplotype B only. The presence of haplotype B (identical to the chloroplast genome of the mother) in one of the four trihaploids may be explained by occasional maternal inheritance of the cpDNA, possibly due to paternal cpDNA damage caused by irradiation, or by reorganization of the histogenic layer of the chimeric pollen parent D uno, leading to the presence of haplotype B in the LII layers of the father, followed by normal paternal inheritance of cpDNA (Chat *et al.* 2002).

(b) Nuclear composition of the trihaploids

The five SSR primer pairs selected produced 13 alleles specific to the male parent D uno. None of these male-specific alleles were transmitted to the trihaploids; instead,

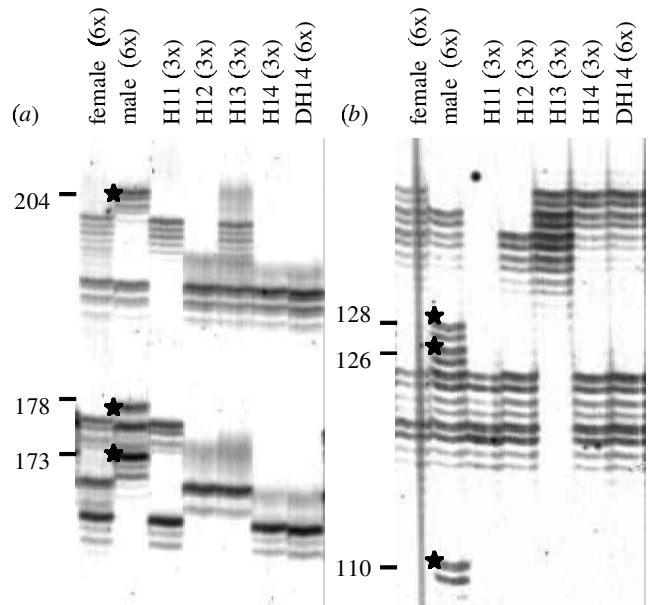


Figure 2. Segregation of nuclear SSR alleles among the four trihaploids (H11, H12, H13 and H14) and the doubled trihaploid (DH14) resulting from H14: PCR amplification of the SSR loci (a) 721 and (b) 722. Only the paternal-specific bands are indicated on the left margin by their fragment sizes (in base pairs) and on the picture by a star.

all the alleles of the trihaploids were of maternal origin (figure 2). As expected, the doubled trihaploid DH14 cannot be distinguished genetically from the trihaploid H14. Genotypes of both the parents and the trihaploids are summarized and compared with those of the full sibs in table 1. As previously reported in *Actinidia* (Huang *et al.* 1998), some of the SSR primer pairs produce more bands than expected given the ploidy level of the plant, suggesting that the locus has been duplicated at least once in the course of evolution. This was the case for locus 735 that was subsequently discarded from further statistical analyses. Among the 10 remaining paternal specific-alleles, simplex condition was the most common (table 1).

(c) Statistical analyses of the SSR loci

χ^2 -tests indicated no segregation distortion among the 40 full sibs for the 10 alleles considered. This suggests that the allelic composition of both parents had been correctly inferred and that no bias had occurred during sexual transmission. All the SSR nuclear markers polymorphic enough to examine chromosomal pairing (UDK96-030, 721, 722) displayed a polysomic inheritance. Moreover, three of the SSR loci used in this study, i.e. UDK96-001, UDK96-030 and 721, are known to segregate independently (Testolin *et al.* 2001). It is likely that all four loci examined belong to different linkage groups owing to the high basic number of chromosomes reported in *Actinidia* ($x = 29$). Consequently, the probability to detect only genotypes [-] can be calculated as the product of the probability to be [-] at each of the four loci. The probability that none of the four trihaploids inherit any of the 10 paternal-specific nuclear SSR alleles is extremely low when assuming random elimination of three sets of chromosomes after syngamy (2.9×10^{-7} ; details given in electronic Appendix A, available on The Royal Society's Publications Web site).

Table 1. Comparison of the nuclear allelic composition of the 40 full sibs population Hayward × D uno with that of the four trihaploids obtained from the same cross using irradiated pollen.

(Deviation from Mendelian segregation in the F₁ tested using χ^2 -values; n.s., non-significant at $p = 0.05$. nd, not determined.)

locus	paternal-specific SSR alleles		segregation ratio among					
	allele size (bp)	allele dosage	40 hexaploid F1			4 trihaploids		
			genetic model ([+] : [-])	observed [+]	observed [-]	χ^2	observed [+]	observed [-]
UDK96-001	238	1	1 : 1	24	16	1.60 n.s.	0	4
UDK96-030	155	1	1 : 1	18	22	0.40 n.s.	0	4
UDK96-030	123	1	1 : 1	23	17	0.90 n.s.	0	4
UDK96-030	107	1	1 : 1	21	19	0.10 n.s.	0	4
721	204	1	1 : 1	17	23	0.90 n.s.	0	4
721	178	1	1 : 1	25	15	2.50 n.s.	0	4
721	173	2	4 : 1	28	12	2.50 n.s.	0	4
722	128	1	1 : 1	20	20	0.00 n.s.	0	4
722	126	1	1 : 1	19	21	0.10 n.s.	0	4
722	110	1	1 : 1	22	18	0.40 n.s.	0	4
735	186	nd	nd	14	26	nd	0	4
735	180	nd	nd	37	3	nd	0	4
735	94	nd	nd	36	4	nd	0	4

4. DISCUSSION

The results obtained in kiwifruit demonstrate that, despite pollen irradiation, the plastids from the male plant can still be transmitted to the egg cell of the female. Indeed, the fact that the cpDNA was of paternal origin in three trihaploids indicates that the release of sperm cells, or at least of some paternal cytoplasm, into the embryo sac had occurred for these individuals, whereas no nuclear gene transmission from the father was detected. The presence of maternal-specific nuclear alleles in the trihaploids led us to exclude androgenesis, whereas the absence of paternal-specific nuclear alleles in each of the four trihaploids allowed us to reject the hypothesis of random chromosome elimination after syngamy. Thus, the hypothesis that best fits with our experimental data is gynogenesis, caused by the absence of fertilization, or by syngamy followed by preferential elimination of the male chromosomes. Empirical arguments already exist that support either hypothesis. Since the 1970s, it has been established that haploids originating after congeneric (Subrahmanyam & Kasha 1973) and intergeneric hybridization (Barclay 1975) result from the fertilization of the egg cell, followed by the selective and gradual elimination of the pollen parent chromosomes during the first mitotic divisions of the embryo. Consequently, a strong bias in favour of the elimination of the paternal chromosomes is plausible here, particularly considering that the paternal chromosomes must have been damaged by irradiation. However, the finding of diploid (instead of triploid) endosperm after pollination with irradiated pollen in kiwifruit (Musial & Przywara 1999) could indicate that double fertilization has not occurred. Figure 3 summarizes the sexual reproduction in *Actinidia* and the two processes possibly involved in haploid formation. Irrespective of whether syngamy had occurred or not, our experimental data indicate that complete chloroplast

replacement can be achieved within one generation in *Actinidia* in the absence of significant nuclear exchanges, following pollen irradiation.

Because interspecific pollen (like irradiated pollen) can stimulate the production of haploids, the occurrence of spontaneous haploids is conceivable in kiwifruit. In such cases, gynogenesis associated with paternal transmission of the chloroplast could lead to the instantaneous chloroplast transfer across species barriers and, hence, to the uncoupling of the transmission of the organelle genomes from that of the nuclear genome. This will result in new cytonuclear associations. For the organelles, such new cytonuclear associations could act as a bridge between two distinct host species provided two conditions are met: (i) the haploids produced must be fertile; and (ii) the haploids produced must be males (otherwise their plastids will not be transmitted further). In the genus *Actinidia*, there have already been reports of haploids following manual interspecific hybridization (Chat *et al.* 1996), in line with the observation that polyploid species are particularly prone to producing haploids (Kimber & Riley 1963). In addition, the production of unreduced gametes (Chat *et al.* 1996; Yan *et al.* 1997) or the spontaneous somatic doubling of the chromosomes (Chalak & Legave 1997) observed in *Actinidia* could restore the fertility of the haploids. However, it seems unlikely that a female plant would give rise to a male plant through gynogenesis because of the probable presence of sexual chromosomes, the male being the heterogametic sex in kiwifruit (Harvey *et al.* 1997b; Gill *et al.* 1998). Residing in a female plant, the captured chloroplast genome cannot normally invade the new taxon, because females do not transmit their chloroplast genome (Cipriani *et al.* 1995; Chat *et al.* 1999). However, rare cases of biparental chloroplast inheritance seem to occur in *Actinidia* (Chat *et al.* 2002). Furthermore, although considered to be ancestral in *Actinidia*

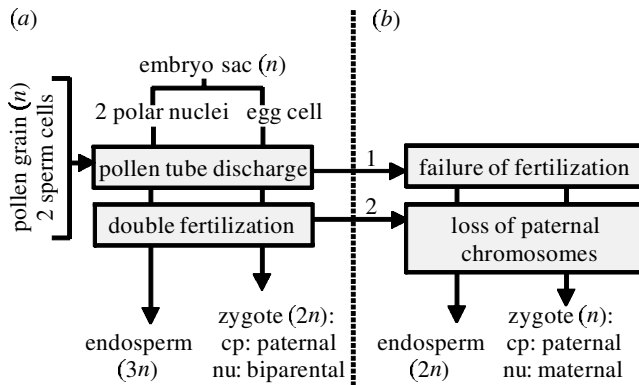


Figure 3. Possible mechanisms involved in haploid formation in kiwifruit, compared with typical sexual reproduction.

(a) Sexual reproduction. After pollen deposition and germination on the stigma, the pollen tube grows down the style until it reaches the embryo sac (Hopping & Jerram 1979). It then releases two sperm nuclei: one fuses with the egg nucleus whereas the other fuses with the two polar nuclei to produce the $3n$ endosperm (Harvey & Fraser 1988). The $3n$ ploidy of the endosperm was deduced from cytological observations (Harvey & Fraser 1988) and further confirmed by chromosome counting (Machno & Przywara 1997). The embryo inherits the chloroplast genome from its father (Cipriani *et al.* 1995; Testolin & Cipriani 1997; Chat *et al.* 1999). (b) Haploid formation after pollen irradiation. Haploids result from gynogenesis caused by the absence of fertilization (pathway 1) or by elimination of paternal chromosomes following fertilization (pathway 2). Most, if not all, of the haploids inherit the chloroplast genome from their father. The endosperm ($2n$) develops autonomously (Musial & Przywara 1999). cp, chloroplast genome; nu, nuclear genome.

(Harvey *et al.* 1997a), dioecy is sometimes relaxed in this genus (McNeilage 1991). These two facts could contradict our first expectations.

The possibility of chloroplast exchanges in the absence of significant nuclear gene flow must be considered when reconstructing species phylogenies, as a growing number of incongruences between chloroplast gene trees and species trees have been reported (reviewed by Rieseberg & Soltis 1991; Doyle 1992). The most frequently invoked hypotheses involve a first event of interspecific hybridization followed by several successive backcrosses until the nuclear genome of the recipient species is completely replaced, a process called 'cytoplasmic capture' (reviewed by Rieseberg & Soltis 1991) or 'pollen swamping' (Potts & Reid 1988; Petit *et al.* 1997; Belahbib *et al.* 2001) (depending on the relative importance of pollen and seed flow and on whether species' ranges are considered to be dynamic). Another possibility involving hemigamy has been proposed for *Gossypium* (Wendel *et al.* 1991). Hemigamy is caused by an abnormal fertilization resulting in a chimeric embryo with haploid tissues of paternal and maternal origin (Turcotte & Feaster 1967). Androgenesis or gynogenesis associated with maternal or paternal inheritance of organelle genes could also be involved, as discussed above for *Actinidia*. In fact, only in these two cases could one speak of 'cytoplasmic capture' in its strictest sense, i.e., the instantaneous capture of an organelle genome of one individual by another one without concomitant transfer of nuclear genes.

Results such as those presented here for the kiwifruit are also relevant to the understanding of intergenomic conflicts and evolutionary interdependence of the organelle and nuclear genomes. Whereas the contrasting inheritance patterns between the organelle genomes, the sex chromosomes and the autosomes have already been identified as a source of genomic conflicts within eukaryotic organisms (see Eberhard 1980; Cosmides & Tooby 1981; Maynard-Smith & Szathmary 1997), the question of the obligatory transmission of organelle genomes together with nuclear chromosomes across generations has not yet been explored, possibly because of the perceived absence of exceptions to this rule. Studies with plants and, to a lesser extent, animals (androgenesis has been reported in insects; Mantovani & Scali (1992); Tinti & Scali (1996), and molluscs; Komaru *et al.* (1998), and can be induced in fishes and molluscs; Corley-Smith & Brandhorst (1999)) indicate that the barriers preventing organelle genomes from being transmitted independently of the nuclear genome are not total and deserve to be studied, both empirically and theoretically. Such independent transmissions of the organelle genomes are expected to exacerbate already-existing conflicts of interest between the organelle symbionts and the host cell, because horizontal transmission of whole organelle genomes to new hosts should favour their selfishness and their moves towards parasitism (Maynard-Smith & Szathmary 1997). Although horizontal transmissions of organelle genomes sometimes take place in nature through pseudo-sexual reproduction events, as discussed here for the kiwifruit, they seem to be kept at low levels and/or to lead to an evolutionary cul-de-sac (as when newly captured organelle genomes end up in the non-transmitting sex). This may reflect the fact that the nuclear genome should readily suppress selfish organelle genes owing to its disproportionately high number of loci, a phenomenon called 'central control' by Maynard-Smith & Szathmary (1997). In fact, endosymbiotic organelles have been considered to be 'encapsulated slaves' rather than mutualists, because the advantages of the relationship for the organelles are impossible to quantify in the absence of free-living stages (Douglas & Smith 1989). We argue that the extreme rarity of events whereby organelles are 'freely' transmitted (i.e. independently of the nucleus) reinforces this view.

The process described here is limited to transfers between taxa showing compatible nucleo-cytoplasmic interactions, that is, between closely related species (Grun 1976). Horizontal transfers of whole organelle genomes between more divergent lineages have been inferred in algae but they result from secondary endosymbioses. Such events are fundamentally different because they involve the capture of whole eukaryotic cells—instead of isolated organelles—followed by the progressive elimination of the endosymbiotic nucleus during evolution (McFadden *et al.* 1996).

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