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Inheritance of Mitochondria in *Pelargonium* Section *Ciconium* (Sweet) Interspecific Crosses

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Abstract: We have studied the inheritance of mitochondria in *Pelargonium* section *Ciconium* using 36 interspecific crosses generated. We designed KASP markers targeting four mitochondrial loci, belonging to the mitomes of four main crossing parents, enabling tracking the transmission of each mitome in the crosses. These markers discriminate between an individual species versus the other section *Ciconium* species. We found that maternal inheritance of mitochondria is most frequent, with occasional occurrences of paternal inheritance, while biparental inheritance is rare. For a *P. multibracteatum* crossing series, we found ambiguous results. Our results confirm those of previous studies, namely, that paternal inheritance of mitochondria can occur in *P.* sect *Ciconium* but that the instance is rare and much less common than is the case for chloroplasts.

Keywords: *Pelargonium*; mitochondrion; inheritance; CNI; *Ciconium*

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1. Introduction

Species from genus *Pelargonium* (Geraniaceae) have been used regularly as a model organism for studying cyto-nuclear incompatibility (CNI), e.g., [1–7]. The species of the genus are renowned for being relatively easy to cross, at least on the intrasectional level, and CNI between interspecific hybrids is a common occurrence [5–7]. Cyto-nuclear incompatibility is the (partial) failure or breakdown in communication between nuclear and organellar genomes. It occurs when populations, derived from a single ancestor, and having become separated in space and time, undergo secondary contact. Such populations may have acquired mutations independently from each other, creating possible reproductive barriers. This is referred to as the Bateson–Dobzhansky–Muller (BDM) model of speciation [8–10] and is thought to underly and explain the occurrence of CNI. Cyto-nuclear incompatibility can be caused by a nuclear mismatch with mitochondria (mCNI), as well as with chloroplasts (pCNI). Whereas mCNI manifests itself as dwarf growth or (partial) male sterility [11], pCNI, on the other hand, presents itself as the bleaching of the leaves (chlorosis), which is a regularly occurring phenomenon in F_1 hybrids of interspecific crosses in *Pelargonium* [5–7]. Both types of CNI seem to occur in *Pelargonium* crosses, but pCNI has been studied in far greater detail [5–13] than the inheritance of mitochondria and mCNI. Mitochondria are thought to be inherited primarily though the maternal line [14]. Strict paternal inheritance of mitochondria occurs only in Cucurbits [15,16], *Sequoiqa sempervirens* (which also displays paternal inheritance of plastids [17]), and *Musa acuminata* [18]. Paternal leakage and biparental inheritance are more common than strict paternal inheritance and may represent evolutionary intermediate forms between strictly maternal or paternal inheritance. The frequency of paternal leakage and biparental inheritance depends on the species or the clade and can vary considerably [19,20].

In *Pelargonium*, tantalizing evidence of possible biparental/paternal inheritance of mitochondria has been reported [13–15]. These observations can be explained by the direct inheritance of mitochondria, but recombination of mitomes at some point has also been

invoked as an explanation for the apparent heteroplasmy of hybrid offspring [16]. In this paper. heteroplasmy is defined as the occurrence of two or more genotypes in one organism or even an individual cell, and which can, but not necessarily does, result in different CNI phenotypes.

Recombination can occur during cell division if conditions allow (e.g., in *Oenothera* [21]) (meiosis or mitosis), in which expression of the plastid ycf and accD genes, referred to as *cytoplasmic drive* loci, appear to play an important role. It could also occur during gamete fusion when the pollen and egg contents merge, as mitochondria (and plastids) were demonstrated to be physically transmitted by pollen, as are plastids [22]. Both methods could be 'common' and allow for recombination to occur each time a cell divides or gametes fuse. Recombination could also have occurred historically (on any time scale) and have been an 'on/off event', if neutral or even beneficial (under specific circumstances). Overall, this could have resulted in fixation of the variants and effectively result in, as we define it, 'stable heteroplasmy'. Another explanation for the observed heteroplasmy is intracellular gene transfer between organelles, as was demonstrated to occur in *Geranium* [23], which is part of the sister of *Pelargonium*.

If there are selective penalties or benefits to heteroplasmy, any of these scenarios could have played a role in heteroplasmy becoming stable or recurring in *Pelargonium* at some point during the evolution of a lineage or population. Here, we take advantage of the great number of hybrids generated by our previous studies [7,11] to verify the inheritance of mitochondria in *Pelargonium* section *Ciconium* interspecific hybrids.

2. Materials and Methods

2.1. Plant Material, DNA Extraction, and Sequencing

The list of plant material, DNA extraction protocol, and standard Illumina HiSeq sequencing protocol are the same as those reported in [11]. Additional plant material was collected from herbaria and from living collections (Table 1, copied with permission from [24]), and these were subjected to the same treatment with respect to DNA extraction and sequencing as in [11]. For the sake of convenience, throughout the text, four-letter acronyms for each accession will be used (see Table 1). A total of 36 different F_1 crosses consisting of 179 accessions were evaluated ('mito-typed'), 12 of which were wild-type plants (i.e., parental material), thus, resulting in a total of 163 F_1 accessions for evaluation.

Table 1. Plant materials used in this study, along with herbarium voucher information.

Species	Herbarium Voucher Accession	Institute [1]	Acronym Used in Text
P. acetosum	1243	STEU	ACET
P. acraeum	1975	STEU	ACRA
P. alchemilloides	1885	STEU	ALCH2x
P. alchemilloides	1882	STEU	ALCH4x
P. articulatum	1972055	WAG	ARTI
P. barklyi	1972061	WAG	BARK
P. frutetorum	0754	STEU	FRUT
P. inquinans	0682	STEU	INQU
P. multibracteatum	2902	STEU	MULT
P. peltatum	1890	STEU	PELT
P. quinquelobatum	1972049	WAG	QUIN
P. ranuncolophyllum	A3651	MSUN *	RANU
P. tongaense	3074	STEU	TONG
P. zonale	1896	STEU	ZONA
P. elongatum	0854	STEU	ELON
P. aridum	1847	STEU	ARID
P. insularis	19990489	RBGE	INSU
P. yemenense*sp. *nov	1972037	WAG	YEME
P. omanense*sp. *nov	2184	RBGE	OMAN
P. somalense	V-067490	V	SOMA

[1] STEU = Stellenbosch University, RSA; AL = Albers/MSUN = Münster and * [25]. WAG = National Herbarium of the Netherlands. V = Uppsala herbarium.

2.2. Mitome Assembly

From the Illumina sequence read libraries, we assembled mitome fragments. We used getOrganelle v1.6.2e [26] and started assembly with previously Sanger-sequenced mitochondrial fragments available for *Pelargonium* [27,28]. After a first round of assembly, we evaluated the resulting contigs, which we then took and used, as a more specific reference to start our next fragment assembly on. We accepted an assembly with a base coverage >10 reads/base position as 'good'. However, for practical purposes, we sometimes accepted an assembly with a base coverage of >5 and <10 reads. We only focused on exons as it was not possible to achieve homologous noncoding sequences for all accessions.

2.3. Organellar Genotyping Using PCR and KASP Markers

We employed a competitive allele-specific PCR (currently named 'Kompetitive Allele-Specific PCR', or KASP^{TM}) from Kbioscience or LGC Genomics (http://www.lgcgenomics.com, accessed on 01 April 2020) for mitotyping. KASP was proven to be a reliable and fast technique for genotyping material and is now considered a benchmark for SNP calling [29–32]. KASP is a PCR-based assay designed to detect SNP variants by using two forward primers (containing the SNP) and one reverse primer. As a result of our crossings, our F_1 and F_2 progeny had two parents that differed by SNPs at various positions in their mitomes. The mother of each crossing series was considered the 'target', whereas the paternal parent could be any other species from section *Ciconium* and is, therefore, collectively referred to as 'Ciconium' in our assays. KASP primers were designed on (partial) assemblies of mitome exons as outlined above. Using introns for marker development is standard, as these are generally more variable, yet close to conserved exon sequences. However, in the case of *Pelargonium*, mitome introns have been found to be absent from *nad1* [28,33–35], and, in addition, exon's silent nucleotide substitution rates were found to be dramatically increased [27,33,35,36] and may, therefore, be good candidates for SNP marker development. The following exons were used as a source: *cox2*, *cox3*, *cytb*, *NAD1-exon 1*, *NAD5*, and *atp1*. We selected SNPs unique to a target, if possible, to be able to distinguish it from the other *Ciconium* species. We performed this by comparing the sequences, obtained from the mitome contigs, in separate alignments and selecting SNPs by eye as targets for the KASP primers. Our KASP thermo profile was: 5 min denaturation at 94 °C, 10 cycles of 94 °C (20 s), and 61–55 °C annealing and extension (60 s; dropping 0.6 °C in annealing temperature each cycle), followed by 30 cycles of 94 °C and 55 °C extension for final amplification. We added 10 µM of the first F-primer + 10 µM the second F-primer and 20 µM R primer and mixed them with a KASP 2x master mix (LGC Genomics. (2013) and mQ water. Template DNA working concentrations must be in the range of 0.2–10 ng/µL and these need to be added accordingly to the mix for each reaction. For KASP, we used two positive control samples which contained only the maternal and/or paternal genotype, and non-template controls ('NTC') to be able to discriminate between fluorescence signal caused by primer interactions and those with the templates.

As stated above, KASP is a PCR-based technique that can determine the relative concentration of each genotype as well. In order to test for differences in concentrations between alleles, we mixed DNA extracts of one parent containing a 'Ciconium' allele (set to 0.2 ng/µL) with the extracts of an accession with the target (e.g., 'HORT') allele, to obtain a series of known concentration ratios (10:90, 25:75, 50:50, 75:25, and 90:10.) This allowed us to determine if there were different ratios of mitochondria of either parent present in the hybrid accessions, and thus enable quantification after 30 cycles. Because markers were designed for four different mitome exon targets, hybrids based on two targeted parents could be reciprocally evaluated as such. We have done so for offspring that was the result of 'MULT, ACET, BARK, and HORT' crosses. The full results are visualized in Figure 1 as allelic discrimination plots. Mitome type-calling was carried out as follows: a plant can have a maternally (M), paternally (P), biparental (B), or conflicting mitome type (B/H/R). The last category can occur if the parental plants happen to be heteroplasmic (H) themselves

or if the mitomes are recombining (R), effectively resulting in a heteroplasmic offspring. The KASP primers' sequence and info can be found in OSM.

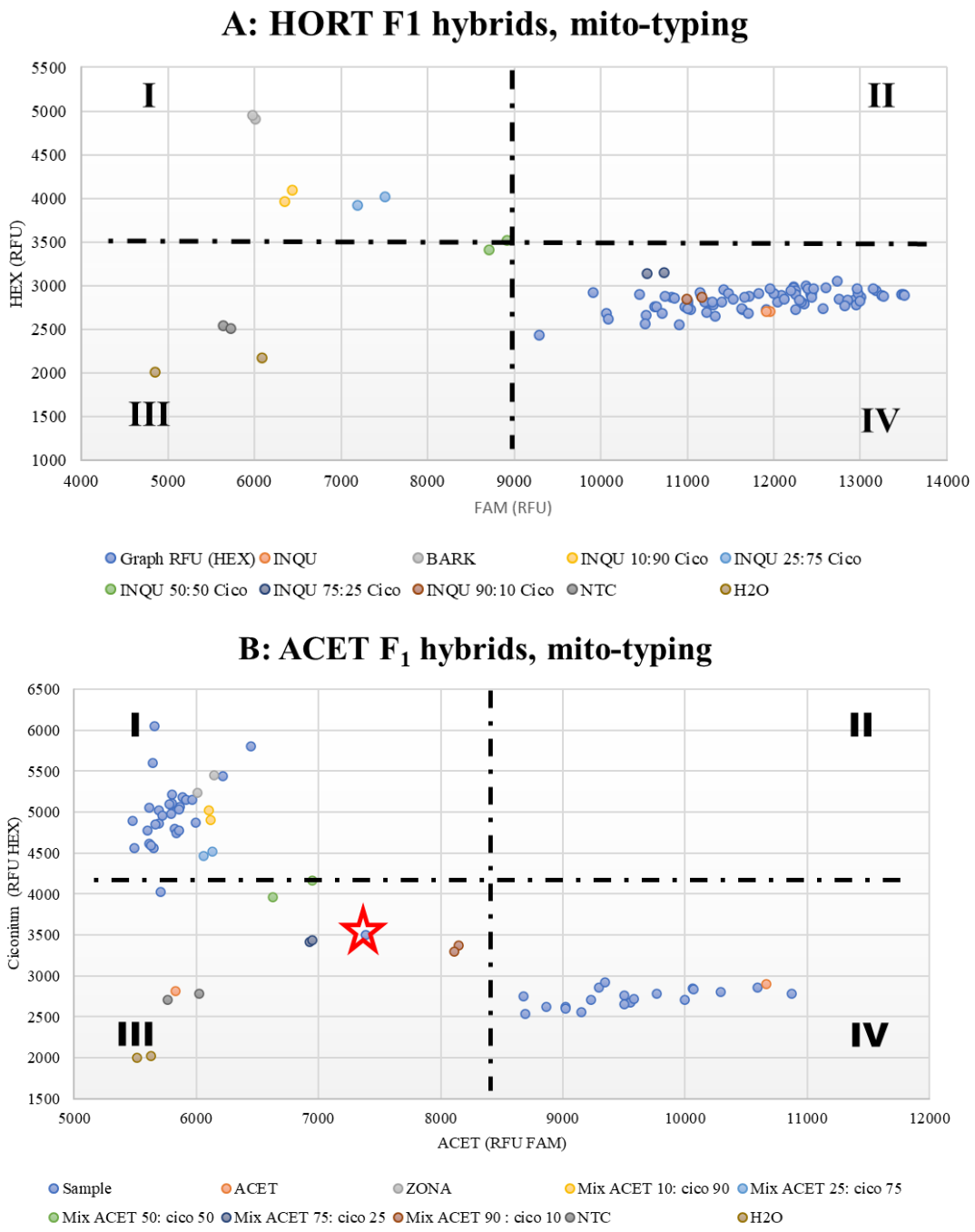

Figure 1. *Cont.*

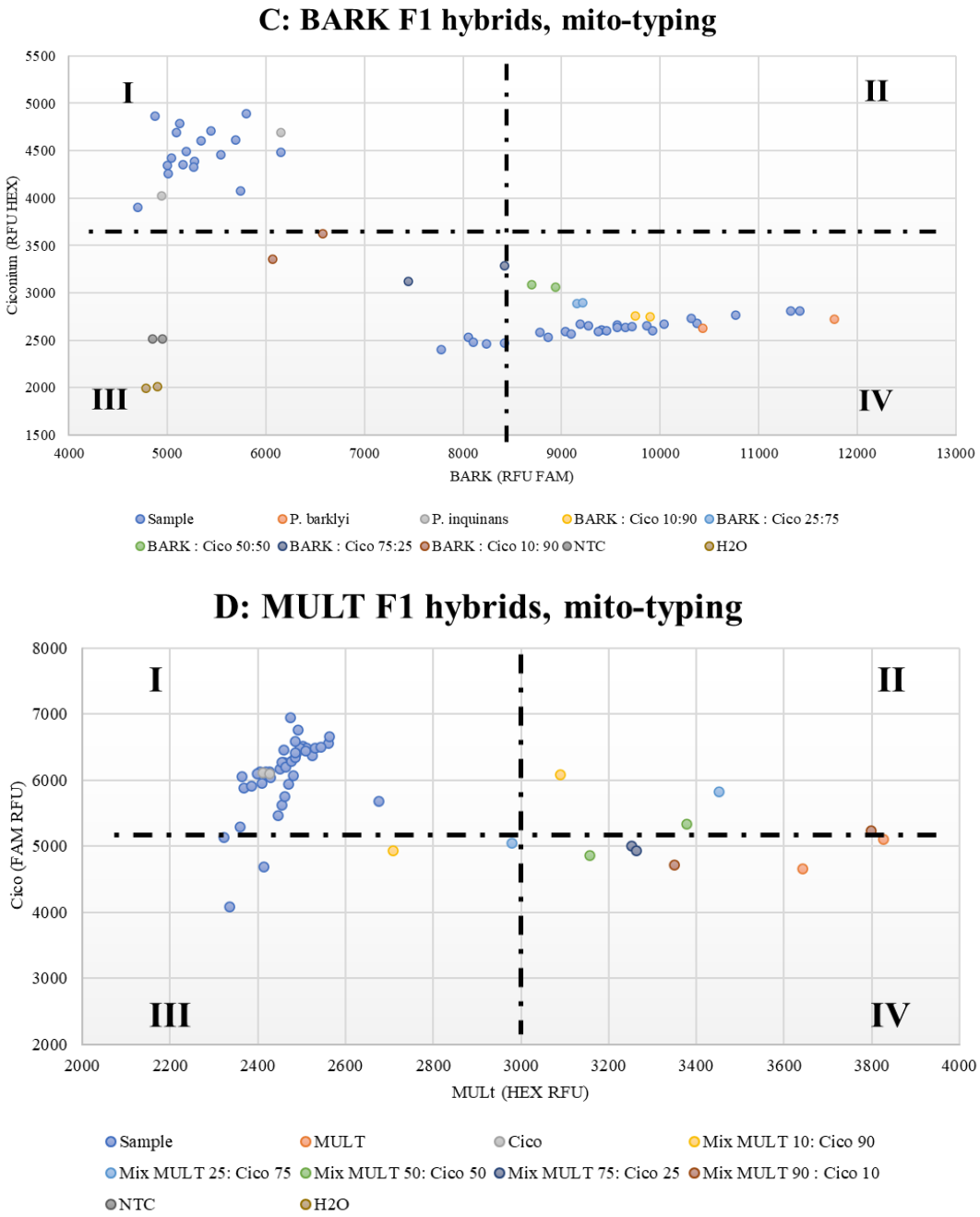

Figure 1. KASP allelic discrimination plots for F_1 hybrid accessions from crosses involving *P. x hortorum* (**A**) *P. acetosum* (**B**), *P. barklyi* (**C**), and *P. multibracteatum* (**D**) F1 hybrid accessions. Each data point represents the fluorescence signal of an individual DNA sample, with the x-axis indicating the amount of FAM fluorescence and the y-axis the HEX fluorescence for each sample. Negative control samples are expected to be placed around the origin of the plot. Samples of the same mitotype will have generated similar levels of fluorescence and will therefore cluster together on the plot. Each graph is divided into four quadrants (I–IV). Here, the y-axis displays the values assigned to the non-target *Ciconium* genotypes. Hence, quadrant I holds the non-target *Ciconium* genotypes. Quadrant IV holds the target genotypes with target fluorescence values displayed on the x-axis. Quadrant III either contains the control samples or samples that displayed neither genotype. The star in 5B indicates biparental, F_1 TONG × ACET progeny. 'sample' = 'MULT/HORT/BARK/ACET', 'Cico' represents all other Ciconium species. 'mix MULT 10: Cico 90' = a mix of the multibracteatum target and the non-target Ciconium in ratios of 10/90 (or other), 'NTC' = non-target control (parent), and 'H2O' = the water control.

3. Results

3.1. Confirmed F_1 Hybrids

The resulting hybrids are described in our previous studies [7,11,24], but a short summary is given here for convenience. These studies established a total of 30 different F_1 interspecific hybrids comprising hundreds of plants. The majority of these came from two (nearly) comprehensive attempts of crossing series with *P. x hortorum* ('Pinto White' diploid cultivar) × *Ciconium* spp. and *P. multibracteatum* × *Ciconium* spp. Other successful F_1 interspecific hybrids series were from *P. barklyi* × *Ciconium* spp. and *P. acetosum* × *Ciconium* spp. Thus, a wide range of crosses were established covering four clades, and, hence, plastome-types, from the section, as estimated based on a plastome comparison by [37].

3.2. Mitome Assemblies

We were able to assemble mitome fragments with a read depth >10 for most accessions (see OMS1). For some, however, markers were assembled with a base coverage of 5–10, and these are considered moderately reliable, and were incorporated for practical and comparative purposes, but not used for primer design. Our final dataset, only counting the concatenated exons, was 4353 bp long and the assembly lengths (in bp) for each fragment were as follows: *COX2*: 687; *COX3*: 1028; *cytb*: 487; *nad1 exon 1*: 311; *nad5*: 502; *nad7*: 582; and *atp1*: 756. Length differences were observed in *nad1 exon 1* and *nad5* (see OSM with alignments). For *P. yemenense*, the *cytb* sequence remained incomplete despite repeated attempts at assembly.

3.3. Mitotyping

We have successfully determined the mitome type of 179 plants. All results are summarized in Table 2, and visualized in allelic discriminant plots in Figure 1. Excluding the MULT × *Ciconium* series (see below) and the wild-type plants (the F_0 parental populations), there were 117 F_1 plants in total to base our analysis and conclusions on. Of these, 89% displayed the maternal mitotype, 8.5% the paternal type, 0.8% (one plant) displayed a conflicting signal, but this involved a 'bridge cross' in which an F_1 plant was crossed with a third species (an F_1 HORT x ZONA plant was crossed with *P. aridum;* see Table 2), and 0.8% (one plant) displayed evidence of biparental inheritance (F_1 TONG × ACET). This last example was confirmed by another KASP marker (shown in Table 2). Finally, four F_1 plants failed to yield any result.

For the *P. multibracteatum* series, 10 F_1 crosses (55 plants) were evaluated. Of these, two crosses displayed apparent biparental genotypes, while eight others displayed an apparent paternal mitotype. We tested the wild-type plants with all markers available and these displayed conflicting results (biparental/heterozygous).

Plants with predominantly maternal mitotypes with a non-target *Ciconium* mother and a target *Ciconium* father (e.g., FRUT × BARK) nearly always ended up in discriminant plot quadrant I (Figure 1A–C) as expected. Those with the target as the mother and non-target *Ciconium* as the father were grouped in quadrant IV. One population (F_1 HORT × QUIN) displayed discrete biparental inheritance with plants displaying either the QUIN (paternal) or HORT (maternal) genotype. One accession, F_1 of TONG × ACET, has a KASP result close to a ratio of non-target '*Ciconium*': target 'acetosum' of 25:75 (indicated by a star in Figure 1B). In this single case, there is more of the paternal type (~75%), but the maternal type is also present. This plant appears to be exceptional and may be one of those rare individuals which show heteroplasmy at significant levels for mitochondrial types. Three F_1 samples show a strictly paternal genotype: these are F_1 BARK × QUIN, F_1 ACET × ZONA, and F_1 BARK × INQU. Two F_2 plants of HORT × ACET also displayed a paternal (ACET) genotype.

Table 2. Mitome type determined per F_1 cross of *P.* sect *Ciconium* species. 'M' and 'P' denote maternally and paternally inherited mitome marker, respectively. 'B' denotes biparental inheritance of the mitochondrial marker(s), 'B/H/R' denotes plants which showed biparental inheritance, but one of the parents could also have been heteroplasmic or there could even have been recombination. '**WT**' refers to parental stocks displaying the wild-type marker. * are F1 plants for which (one of the) parents may have been heteroplasmic. ° plant are treated as wild-type, but the result of a hybridisation process. **WT** plants are excluded from the final calculation of total and percentages of mitome occurrences. The fertility phenotypes are given as 'F' which is fully fertile; 'P' partially fertile (pollen observed); 'MS' male sterile/no pollen observed; and '--', which could not be evaluated because the plants did not flower.

F1 Types	# Plants/Cross	# Marker Pairs/Cross	Fertility Phenotype	(M), (P), (B), (H), (R), (WT)
ACET_×_ FRUT	2	1	P	M
ACET_×_ INQU	1	2	P	M
ACET_×_ ZONA	12	1	--	M (11) P (1)
ALCH(4X)_×_ BARK	1	1	--	M
ALCH(4X)_×_ YEME	2	1	P	P
BARK_×_ FRUT	3	1	MS	M
BARK_×_ INQU	1	2	--	P
BARK_×_ MULT	3	2	MS	M
BARK_×_ QUIN	2	1	--	M(2) P(1)
FRUT_×_ ACET	1	1	P	M
FRUT_×_ BARK	3	1	MS	M
HORT(4X)_×_ ARTI(4X)	8	1	MS	M
(HORT_×_ ZONA)_×_ ARID	5	1	MS	M
HORT_×_ ACET	3	2	P	M
HORT_×_ ACRA	1	1	P	M
HORT_×_ ALCH	1	1	MS	M
HORT_×_ ARID	6	2	MS	M
HORT_×_ BARK	2	1	--	M
HORT_×_ FRUT	1	1	F	M
HORT_×_ MULT	1	1	MS	M
HORT_×_ QUIN	15	1	MS	M(8) P(7)
HORT_×_ TONG	8	1	P	M
HORT_×_ TONG(4X)	1	1	P	M
HORT_×_ ZONA	26	1	P	M
TONG_×_ ACET	7	1–2	P	B(1)/P
YEME_×_ ALCH(4X)	1	1	P	P
P. inquinans	1	1	F	**WT**
P. peltatum	1	1	F	**WT**
P. salmoneum	1	2	F	**WT**
P. x hortorum_4x	1	1	P	**WT** *
P. quinquelobatum	1	3	F	**WT**
P. yemenense	1	3	F	**WT**
P. barklyi	1	3	F	
P. aridum	1	3	F	**WT**
P. quinquelobatum	1	3	F	**WT**
P. alchemilloides	1	3	F	**WT**
P. tongaense	1	1	F	**WT**

Table 2. *Cont.*

F1 Types	# Plants/Cross	# Marker Pairs/Cross	Fertility Phenotype	(M), (P), (B), (H), (R), (WT)
P. articulatum	1	1	F	**WT**
P. multibracteatum	1	2	F	**WT/H**
mult_×_ acet	8	2-3	--	B/H *
mult_×_ alch	14	2	P	P/H *
mult_×_ arid	3	1	MS	P/H *
mult_×_ bark	5	2	MS	B/H *
mult_×_ inqu	3	1	--	P/H *
mult_×_ pelt	3	1	MS	P/H *
mult_×_ quin	6	2	P	P/H *
mult_×_ ranu	9	1–2	P	P/H *
mult_×_ tong	2	1	MS	P/H *
mult_×_ zona	2	2	MS	P/H *

4. Discussion

Our results support the long-held notion that mitochondria inherit both paternally and maternally in *Pelargonium* section *Ciconium* [11,13,22,38]. If we exclude the *P. multibracteatum* population and its derived hybrids, the instance of heteroplasmy in hybrid offspring seems to be less common than in the case of plastids, for which heteroplasmy was found more frequently previously [7,11]. Our results do point to maternal inheritance as the prevailing mode of inheritance, a phenomenon that has intrigued workers sincelong. This is similar to what is observed in other plant groups [20]. On the spectrum between paternal and maternal inheritance, *P.* sect. *Ciconium* displays a biparental model of inheritance. This is a more common mode of inheritance in angiosperms (though still rare) [19], and may represent evolutionary intermediate forms between strictly maternal or strictly paternal inheritance.

Biparental inheritance has been considered the result of some imperfect 'sorting mechanism' in which paternal leakage is occurring at a low frequency [13,39], preferentially selecting a certain type. For a review of proposed mechanisms see [14], or for an excellent example involving *cytoplasmic drive* loci in *Oenothera* see [21]. We hypothesize that the high incidence of maternal inheritance of mitomes means that, even if these sorting mechanisms are in place in *P.* sect *Ciconium*, they appear not to function perfectly and the resulting amount of paternally inherited mitochondria is more than mere 'parental leakage'. These studies mainly target plastid inheritance and plastome variation, but the same or similar mechanisms may underly mitochondrial inheritance and selection as well [13]. Environmental and genetic factors also seem to influence inheritance, especially when paternal leakage or biparental inheritance is the dominant type of inheritance [40,41]. If one or more of these mechanisms also play a role in *P.* sect. *Ciconium,* this should, for instance, show up in follow-up experiments under tighter temperature control than what we employed. Our experiments were conducted during a full growth season (March–November) across multiple years (2017–2021) in northwest Europe in semi-controlled greenhouses. This means that major temperature fluctuations could have influenced the occurrence of paternal inheritance in our experiments.

Paternal inheritance is thought to have evolved to select against pollen mitochondrial genomes that are considered too large due to acquired numbers of structural mutations, resulting in deleterious mutations [19,42]. This hypothesis is supported by the fact that paternal inheritance occurs in species with very large, repeat rich mitomes [19]. There are some indications that *Pelargonium* mitomes also suffer from an increased size [27,34,43], but an annotated mitome is, as of yet, unavailable for *Pelargonium*. Our results do not counter the enlarged mitome hypothesis and this is one that should be tested in *Pelargonium* by sequencing a full mitome.

Another explanation for the stronger maternal effect could be that mCNI is much stronger than chloroplast effects, especially because our controlled growth environment used is optimized for the survival of every chlorotic plant, but not for that of embryos. Even though we did employ embryo rescue [7,11] this does not salvage any mCNI effects that would occur directly upon fertilization. Plastids are undeveloped during and directly after fertilization and seed development, whereas the mitochondria are active during these phases. Therefore, any mCNI effect would be stronger than pCNI at these crucial early developmental stages. This would explain the high number of aborted embryos and empty seeds found on all our F_1 plants [7]. Given that the mother plant is 'responsible' for supplying energy to the development of the seeds, it is logical that there is a strong maternal bias. However, plastids which are introduced to the embryo via the pollen [22,44]) are sorted out and removed early in development as well [13,31]. Nevertheless, they can be present in all tissue early on [38] before most of them are removed eventually.

Finally, recombination has also been invoked as a partial explanation for the observed patterns of heteroplasmy and cannot be excluded as having played a role as well [43,45]. Basically, recombination is thought to affect the occurrence of varying mitome types in a cell or plant, and to result in a more random distribution of mitome variants. We think that this explanation is less likely as this would have been a rarer occurrence. The relatively high occurrence of both paternal and maternal (and even biparental) modes of inheritance across the section, as well as persistence throughout generations (see [7] for an example from plastids), evidenced by the recovery of our SNP markers in offspring, are more parsimoniously explained by simple inheritance.

4.1. The Case of P. multibracteatum

The *P. multibracteatum* crossing series showed ambiguous results. The reciprocal comparison of the results for the other markers with those of the MULT marker were contradictory (see Table 2 and OSM1). While the reciprocal comparison between the other markers (ACET, BARK, and HORT) clearly showed that these three confirmed each other, we were left us with the MULT markers' ambiguous results. To assess performance issues of the MULT KASP marker, we re-analyzed the *P. multibracteatum* series with a second KASP marker and we included another *P. multibracteatum* accession. This again resulted in a near-universal '*Ciconium*' call, and, this time, the positive control also resulted in a '*Ciconium*' call. We, therefore, conclude that we do not have consistent results for the mito-typing of the *P. multibracteatum* series. Rather, we believe that the patterns we see is caused by innate heteroplasmy of *P. multibracteatum*. The full overview of all reactions and raw data of fluorophore measurements can be found in the Supplementary Materials.

4.2. Evolutionary Effects of mCNI

Our results lend support for both the idea that biparental inheritance of organelles could provide an escape from CNI [46], involving 'vegetative sorting' of incompatible types, and the hypothesis that organellar changes, resulting in CNI, have a profound influence on speciation [47,48]. Further support for these two hypotheses comes from the fact that the second generation of plants segregate for chlorosis [7,11] with only one plastid type present, showing that selection for organelle management and expression genes acts immediately after the first generation of hybridization [7,47]. Different organelle types induce different CNI in crosses with equal nuclear genomic backgrounds (as can be seen for the chloroplast in *Pelargonium* × *hortorum* crosses [11]). The preference for one type, as well as preferentially backcrossing with one of the parents (introgression), after a historical hybridization event could explain the problematic position of taxa in phylogenetic trees due to conflict between plastid and nuclear genomic markers. For instance, the four-petalled *P. nanum* which is currently not assigned to any section [49] was suspected to be an ancient relict of a now extinct group (section) of species because of its unique floral morphology and its 'single branch' status in current phylogenetic trees (e.g., [37]). *P. nanum* usually group as a sister to clade A2 [30,39,50]. Other cases can be seen in *P.* sect. *Hoarea*, where the occurrence

of non-monophyletic species has been attributed to 'chloroplast capture' [25]. Such taxa would have retained the organelle of one species, while displaying the morphology and nuclear genomic type of another. Further testing of such incongruencies could be carried out by using more markers from the nuclear genomes. For instance, the repeatome appears promising as a source of phylogenetic markers [51,52]) as it provides resolution at a low taxonomic level and provides a genome-wide overview represented by the most abundant parts of the non-coding DNA (repeats).

Naturally occurring hybrids in *Pelargonium* are rarely found (pers. comm. Powrie Kirstenbosch RSA), but not unheard off [53,54]. This is logical given the reduced fitness characteristic of most hybrid offspring which will result in lower chances of surviving to the reproductive life stage. However, despite this post-zygotic barrier, our study also shows that species can be highly compatible as we obtained many (~30) interspecific crosses, some of which are fully green and fertile. Therefore, we do not exclude that hybridization does play an additional, if minor, role in *Pelargonium* evolution. Two cases of possible natural hybrids from section *Ciconium* are known. The first is a herbarium specimen of a wild hybrid between *P. peltatum* and *P. alchemilloides* at RBGE (M. Gibby pers. comm.). The second case is *P.* x *salmoneum* (from our own collections). We have analyzed both the plastome and mitome of *P.* x *salmoneum* and found that it carries the *P. inquinans* plastome and a mix of *P. inquinans* and *P. acetosum* mitochondrial genotypes (OSM1), potentially due to mitome recombination [43] or historical biparental inheritance. The morphology of *P.* x *salmoneum* is intermediate between *P. inquinans* and *P. acetosum*. *Pelargonium* x *salmoneum* is a fully fertile, green plant which segregates for numerous traits such as plant size (an indication of possible mitochondrial CNI effects), flower shape, and leaf shape, indicating it is not a 'stable' species (yet), but a hybrid. We propose that *P. x salmoneum*, irrespective of whether it arose naturally or was the result of human crossing activities, is a genuine interspecific hybrid with equal fitness to either of its proposed parents and that it contains possible traces of mitochondrial recombination. We hypothesize that embryonic organelle sorting is absent or impaired in *Pelargonium* section *Ciconium* and conclude that the 'sorting out' of mitochondria is stronger than that of plastids. Heteroplasmy is rare for mitochondria, in all *P.* sect. *Ciconium species*, but seems to be innate to the *P. multibracteatum* population used in this study. This may imply that selective barriers are stronger for mitochondria than for chloroplasts.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/ijpb15030044/s1. OSM1: Mitome fragment assembly results. Plant lists with mitotypes per plant and KASP primers. OSM2: FASTA alignments of mitome fragments.

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