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

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Article

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 interspecific crosses. We generated 36 different interspecific crosses. We designed KAPS markers targeting four individual mitochondrial markers, belonging to the four main crossing parents, that discriminate between an individual species and the other section *Ciconium* species. We found that maternal inheritance of mitochondria is most frequent, but we find occasional occurrences of paternal inheritance, while biparental inheritance is rare. For the *P. multibracteatum* crossing series we found ambiguous results. Our results confirm what was found before, 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*

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 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 bleaching of the leaves (chlorosis), which is a regularly occurring phenomenon in F_1 hybrids of interspecific crosses in *Pelargonium* [Geraniaceae; 1,5,6]. Both types of CNI seem to occur in *Pelargonium* crosses, but pCNI has been studied in far greater detail [1–7,12,13] than the inheritance of mitochondria and mCNI. However, tantalizing evidence of possible biparental/paternal inheritance of mitochondria in *Pelargonium* has been reported [13–15]. These observations can be explained by 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 defined as the occurrence of two or more genotypes in one organism or even individual cell and which can, but not necessarily do, result in different CNI phenotypes.

Recombination can occur during cell division if conditions allow see [17] (meiosis or mitosis). It could also occur during gamete fusion when pollen and embryo content merge as mitochondria (and plastids) were demonstrated to be physically transmitted by pollen, as are plastids [14]. Both methods

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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 a 'on off event' if neutral or even beneficial (under specific circumstances). This could have resulted in fixation of the variants and effectively result in as we define "stable heteroplasmy"). Another explanation for observed heteroplasmy is intracellular gene transfer between organelles, as was demonstrated to occur in *Geranium* [18], which is a sister genus of *Pelargonium*. If there are selective penalties or benefits to heteroplasmy any of these scenarios could have been 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 [6,7] 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 [7]. Additional plant material was collected from herbaria and from living collections (Table 1, copied with permission from [19] and these were subjected to the same treatment with respect to DNA extraction and sequencing. For the sake of convenience, throughout the text four letter acronyms for each accession will be used. The corresponding species names and their acronyms can be found in Table 1. A total of 36 different F_1 crosses consisting of 179 accessions were evaluated and of these 12 were wildtype plants (i.e., parental material). 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

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[1]STEU = Stellenbosch University, RSA; AL = Albers/MSUN=Münster & *Bakker et al. 2004. 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 [20] and started assembly with known mitochondrial fragments available for *Pelargonium* [21,22]. After a first round of assembly, we evaluated the results, which we then took and used, as a more specific target to start our next fragment assembly on. We accepted an assembly with a base coverage >10 reads/base position as 'good'. Although for practical purposes we sometimes accepted an assembly with a base coverage of >5<10. We then only focused on the 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™) from Kbioscience or LGC Genomics (http://www.lgcgenomics.com) for mitotyping. KASP was proven to be a reliable and fast technique for genotyping material and is now considered a benchmark technique for SNP calling [23–26]. KASP is a PCR-based assay designed to detect SNP variants by using two forward primers (containing the SNP) and one reverse primer. Our F_1 and F_2 progeny had two parents that differed by SNPs at various positions. The mother of each crossing series was considered the 'target' and the paternal parent can be any other species from section *Ciconium* and is therefore 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. But, in the case of *Pelargonium,* mitome introns have been found to be absent from *nad1* [22,27–29] but instead that exon silent nucleotide substitution rates were found to be significantly increased [21,27,29,30] and therefore may be good candidates for SNP marker development. The following exons were used as a source: *cox2*, *cox3*, *cytb*, *NAD1-exon 1*, *nad7*, *NAD5* and *atp1*. We selected SNPs unique to a target, if possible, to be able to distinguish it from the other *Ciconium* species. We did this by comparing the sequences, obtained from the mitome assembly procedure described above, in separate alignments and using the SNP's as targets for the KASP primers. Our KASP thermo profile was as follows: 5 minutes denaturation at 94°C, 10 cycles of 94°C 20s, 61-55°C annealing and extension for 60s (dropping 0.6°C for the annealing temperature each cycle), followed by 30 cycles of 94°C and 55°C extension for final amplification. We added 10µM F1 primer + 10µM F2 primer and 20µM R primer.. These were mixed with the KASP 2x master mix (LGC Genomics. (2013) and mQ water. Template DNA concentrations must be in the range of 0.2-10 ng/µL and these need to be added to the mix per each reaction. For KASP we used two positive control samples which contained only the maternal and paternal genotype, and non-template controls (NTC') to be able to discriminate fluorescence signal caused by primer interactions from 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 if there are differences in concentrations between alleles, we mixed DNA extracts of one parent with a 'Ciconium' allele (set to 0,2 ng/µL) with the extracts of a sample with the target (e.g., 'HORT') allele to obtain series of known concentration ratios (10:90, 25:75, 50:50, 75:25 & 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 targets, hybrids based on two targeted parents could be reciprocally evaluated and 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 done 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 themselves or if the mitomes are recombining, effectively resulting in a heteroplasmic offspring. The KASP primers can be found in OSM.

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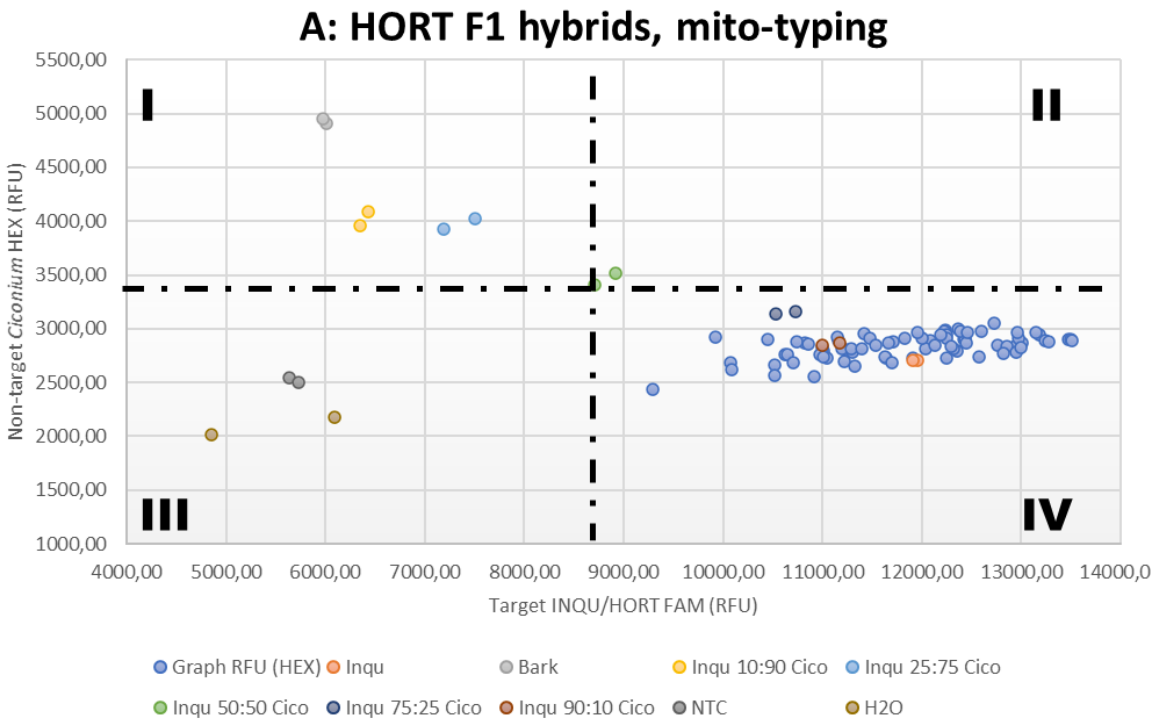

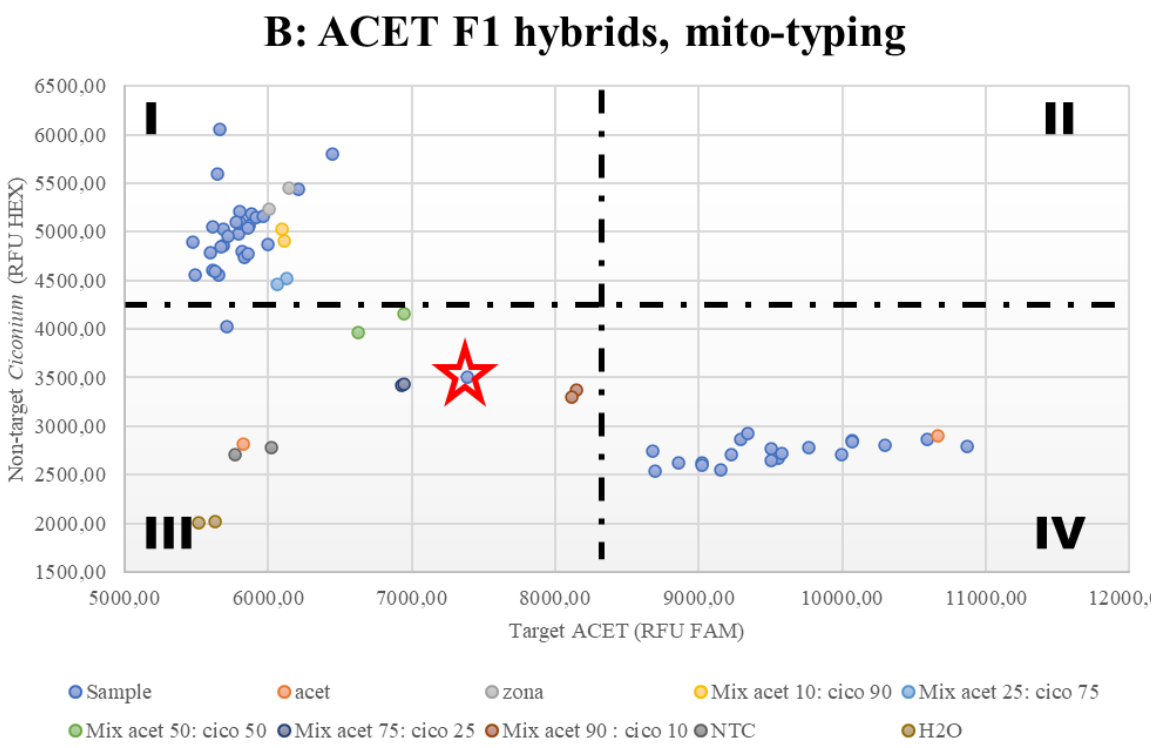

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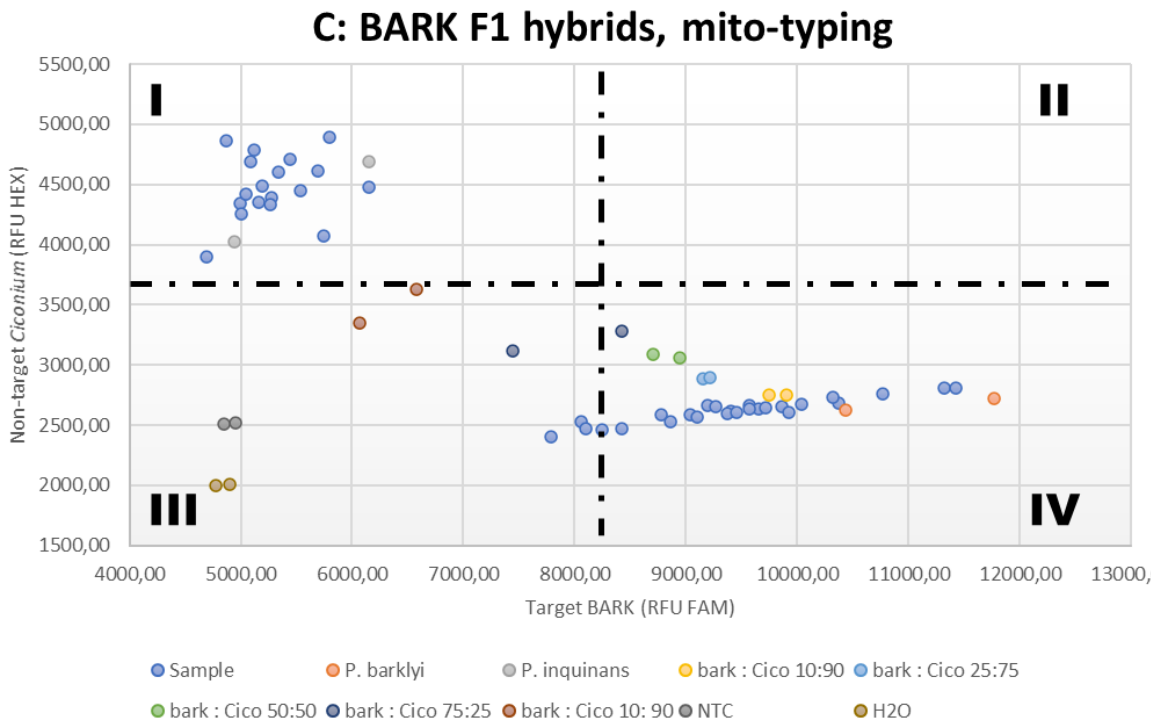

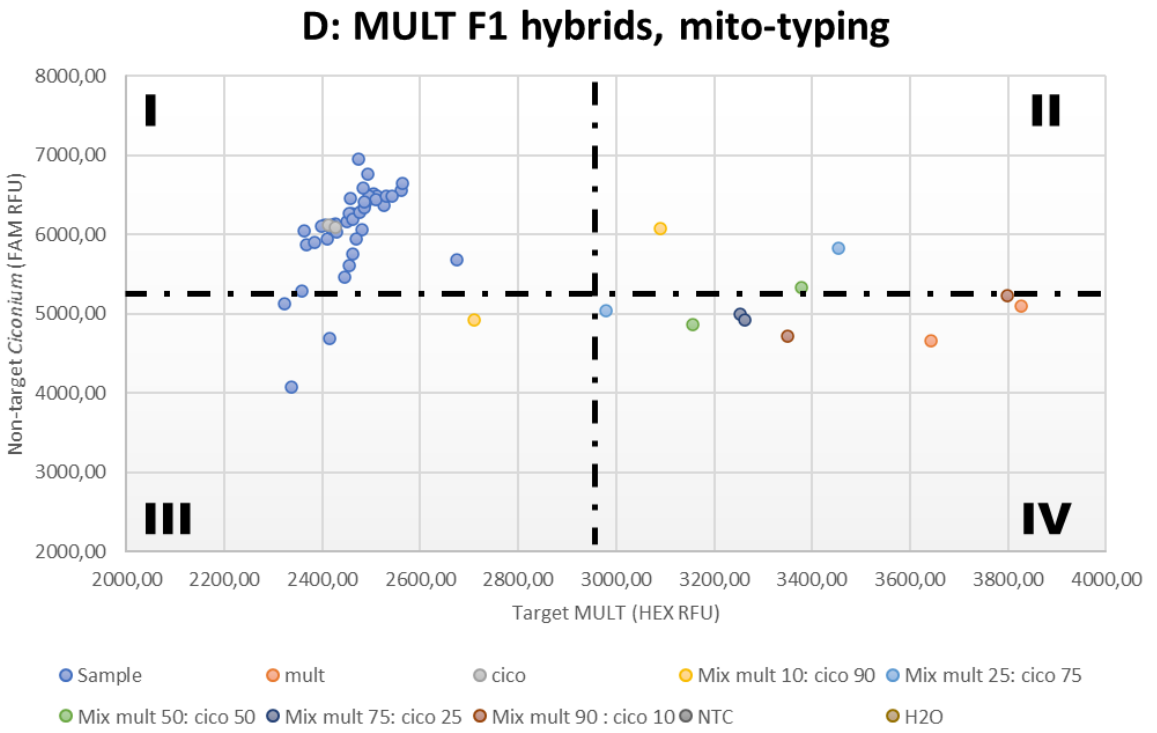

Figure 1. KASP allelic discrimination plots for F_1 hybrid accessions from crosses involving *P. x hortorum* (A) *P. acetosum* (B), *P. barklyi* (C) and *P. multibracteatum* (D)F1 hybrid samples. Each data point represents the fluorescence signal of an individual DNA sample, with the x-axis indicating 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

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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 x ACET progeny. 'sample'= , 'mult/hort//bark/acet'- , 'cico' = , 'mix mult 10: cico 90' = ,a mix of the multbreacteatum target and the non-target Ciconium in ratios of 10/90 (or other) 'NTC' = non target control (parent), 'H2O' is the water control.

3. Results

3.1. Confirmed F_1 Hybrids

The resulting hybrids are described in our previous studies [6,7,19], but a short summary is given here for convenience. These studies established a total of 30 different F_1 interspecific hybrids and hundreds of plants. The majority of these came from two (nearly) comprehensive attempts of crossing series with *P. x hortorum* ('Pinto White' diploid cultivar) X *Ciconium spp.* and *P. multibracteatum* X *Ciconium spp*. Other successful F_1 interspecific hybrids series were from *P. barklyi* X *Ciconium spp*. and *P. acetosum* X *Ciconium spp*. Thus, a wide range of crosses were established covering four clades from the section, as estimated based on plastome comparison by [31].

3.2. Mitome Assemblies

We were able to assemble mitome fragments with a read depth >10 for most accessions (see OMS1). For some accessions however, markers were assembled with a base coverage of 5-10 and these are considered moderately reliable. These moderately reliable sequences were incorporated for practical and comparative purposes, but not used for primer design. Our final dataset, only counting the concatenated exons, was 4,353 bp long and the assembly lengths for each fragment were: *COX2*: 687 bp; *COX3*: 1028 bp; *cytb*: 487 bp; *nad1 exon 1*: 311 bp; *nad5*: 502 bp; *nad7*: 582 bp and *atp1*: 756 bp. Length differences were observed in *nad1 exon 1* and *nad5* (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 Figure 1. Excluding the MULT X *Ciconium* series (see below) and the wildtype plants (the F_0 parental populations), there were 117 F_1 plants in total. 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* Table 2), and 0.8% (one plant) displayed evidence of biparental inheritance (F_1 TONG X ACET). This last example was confirmed by a second reaction with another KASP marker (shown in Table 2) confirming the result. 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, eight others displayed an apparent paternal mitotype. We tested the wildtype plants with all markers available and these displayed conflicting results (biparental/heterozygous).

Mito-typing results are visualised as KASP allelic discriminant plots in Figure 1. Plants with predominantly maternal mitotypes with a non-target *Ciconium* mother and a target *Ciconium* father (e.g., FRUT x BARK) nearly always ended up in quadrant I (Figure 1 A-C) as expected and those with the target as mother and non-target Ciconium as father in quadrant IV (Figure 1 A-C). One population (F_1 HORT x QUIN) displayed discrete bi-parental inheritance with plants displaying either the QUIN (paternal) or HORT (maternal) genotype. One accession, (F_1 of TONG x ACET), has a KASP result close to the non-target 'Ciconium': target 'acetosum' ratio 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 the exception to the rule 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 x QUIN, F_1 ACET x ZONA and F_1 BARK x INQU. Two F_2 plants of HORT x ACET also displayed a paternal (ACET) genotype

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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 wildtype marker. * are F1 plants for which (one of the) parents may have been heteroplasmic. ° plant treated as wildtype, but the result of a hybridisation process. **WT** plants are excluded from the final calculation of total and percentages. The fertility phenotypes are given as F which is fully fertile; P: partial fertile (pollen observed); MS: male sterile / no pollen observed, '- -' 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_x_frut	2	1	P	M
acet_x_inqu	1	2	P	M
acet_x_zona	12	1	--	M (11) P (1)
alch(4x)_x_bark	1	1	--	M
alch(4x)_x_yeme	2	1	P	P
bark_x_frut	3	1	MS	M
bark_x_inqu	1	2	--	P
bark_x_mult	3	2	MS	M
bark_x_quin	2	1	--	M(2) P(1)
frut_x_acet	1	1	P	M
frut_x_bark	3	1	MS	M
hort(4x)_x_arti(4x)	8	1	MS	M
(hort_x_zona)_x_arid	5	1	MS	M
hort_x_acet	3	2	P	M
hort_x_acra	1	1	P	M
hort_x_alch	1	1	MS	M
hort_x_arid	6	2	MS	M
hort_x_bark	2	1	--	M
hort_x_frut	1	1	F	M
hort_x_mult	1	1	MS	M
hort_x_quin	15	1	MS	M(8) P(7)
hort_x_tong	8	1	P	M
hort_x_tong(4x)	1	1	P	M
hort_x_zona	26	1	P	M
tong_x_acet	7	1-2	P	B(1)/P
yeme_x_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	

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P. aridum	1	3	F	**WT**
P. quinquelobatum	1	3	F	**WT**
P. alchemilloides	1	3	F	**WT**
P. tongaense	1	1	F	**WT**
P. articulatum	1	1	F	**WT**
P. multibreacteatum	1	2	F	**WT/H**
mult_x_acet	8	2-3	--	B/H*
mult_x_alch	14	2	P	P/H*
mult_x_arid	3	1	MS	P/H*
mult_x_bark	5	2	MS	B/H*
mult_x_inqu	3	1	--	P/H*
mult_x_pelt	3	1	MS	P/H*
mult_x_quin	6	2	P	P/H*
mult_x_ranu	9	1-2	P	P/H*
mult_x_tong	2	1	MS	P/H*
mult_x_zona	2	2	MS	P/H*

Figure 1. KASP allelic discrimination plots.

4. Discussion

Our results support the long-held notion that mitochondria inherit both paternally and maternally in *Pelargonium* section *Ciconium* [7,13–15]. 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]6,7]. Our results do point to maternal inheritance as the prevailing mode of inheritance. This phenomenon has intrigued people since long. And it has been considered the result of some imperfect 'sorting mechanism' in which paternal leakage is occurring at low frequency [13,32] preferentially selecting a certain type. For a review of proposed mechanisms see [33], or for an example [17]. We hypothesize that the high incidence of maternal inheritance of mitomes means that, even if these mechanisms are in place in *P.* sect *Ciconium*, they do not function perfectly and the 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]. Another explanation for the stronger maternal effect could be that mCNI is much stronger than chloroplast effects, especially because our controlled growth environment is optimized for the survival of every chlorotic plant, but not for embryos. Even though we did employ embryo rescue, this does not salvage any CNI 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. So, 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 [6]. 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 [14,34]) are sorted out and removed early in development as well [13,32]. Nevertheless, they can be present in all tissue early on [15] before most of them are removed eventually.

Finally, recombination has also been invoked as a, partial, explanation for observed patterns of heteroplasmy and cannot be excluded to play a role as well [16,35]. Basically, recombination is thought to affect occurrence of varying mitome types in a cell or plant and occasionally male sterile phenotypes and result in a more random distribution of mitome variants. We think that this

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explanation is less likely as this would have been a more rare occurrence. The relatively high occurrence of both paternal and maternal (and even bi-parental) modes inheritance across the section as well as persistence throughout generations (see [6] for an example from plastids), evidenced by the recovery of our SNP markers in offspring, are easier explained by simple inheritance.

The Case of P. multibracteatum

The *P. multibracteatum* crossing series showed ambiguous results. The reciprocal comparison of the results for the other markers with the MULT marker were contradictory (see Table 2 & OSM1). The subsequent reciprocal comparison between the other markers (ACET, BARK and HORT) clearly showed that these three confirmed each other, leaving 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 (not shown). 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 fluorophore measurements can be found in the supporting materials.

Evolutionary Effects of mCNI

Our results lend support for both the idea that bi-parental inheritance of organelles could provide an escape from CNI [36] and the hypothesis that organellar changes, resulting in CNI, have a profound influence of speciation ([37,38]. Further support for these two hypotheses comes the fact that second generation of plants segregate for chlorosis [6,7] with only one plastid type present, showing that selection for organelle management and expression genes acts immediately after the first generation of hybridization [6,37]. Different organelle types induce different CNI in crosses with equal nuclear genomic backgrounds (as can be seen for the chloroplast in *P.* x *hortorum* crosses published in [7]). 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 a section [39] was suspected to be an ancient relict of a now extinct group (section) of species because of the unique floral morphology and its 'single branch' status in current phylogenetic trees (e.g., [31]) usually grouping as a sister to clade A2, based on the clade systematics established by [40]. Other cases can be seen in *P.* sect. *Hoarea* where the occurrence of non-monophyletic species has been attributed to 'chloroplast capture' [41]. 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 done by using more markers from the nuclear genomes. For instance, the repeatome appears promising as a source of phylogenetic markers [42,43]) 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 [44,45]. 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 an 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 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 [16] 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 plants size (an indication of possible mitochondrial CNI effects), flower shape, 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 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 website of this paper posted on Preprints.org, OSM1: Mitome fragment assembly results. Plant lists with mitotypes per plant and KASP primers. OSM2: Fasta alignments of mitome fragments.

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References

1. Baur E. Das Wesen und die Earblichkeitsverhältnisse der 'varietates albomarginatae hort' von *Pelargonium zonale*. Z. Indukt. Abstammungs-Vererbungsl. **1909**, 1, 330–351. Doi: 10.1007/BF01990603.
2. Tilney-Bassett, R. A. E. The control of plastid inheritance in *Pelargonium* II. Heredity **1973**, 30, 1–13. doi: 10.1038/hdy.1973.1
3. Tilney-Bassett, R. A. E. The control of plastid inheritance in *Pelargonium* III. Heredity **1974**, 33, 353–360. doi: 10.1038/hdy.1974.102
4. Tilney-Bassett, R. A. E. "Genetics of variegated plants," in Genetics and biogenesis of mitochondria and chloroplasts. Eds. C. W.Birky P. S Perlman, and T. J Byers (Columbus: Ohio State University Press), **1975**, 268–308.
5. Horn W. Interspecific crossability and inheritance in *Pelargonium*. Plant Breeding **1994**, 113, 3–17. Doi: 10.1111/j.1439-0523.1994.tb00696.x.
6. Breman F.C.; Snijder R.C.; Korver J.W.; Pelzer S.; Sancho-Such M.; Schranz M.E.; Bakker F.T. Interspecific Hybrids Between *Pelargonium* × *hortorum* and species From *P.* section *Ciconium* reveal biparental plastid inheritance and multi-locus cyto-nuclear incompatibility. Front. Plant Sci. **2020**, 11:614871. doi: 10.3389/fpls.2020.614871
7. Breman F.C. Exploring patterns of cytonuclear incompatibility in *Pelargonium* section *Ciconium*. PhD thesis, Wageningen UR, the Netherlands; **2021**. Doi: https://doi.org/10.18174/551565
8. Bateson W. Heredity and variation in modern lights. In Darwin and modern science (ed. A. C. Seward), pp. 85– 101. Cambridge, UK: **1909**. Cambridge University Press.
9. Dobzhansky T. Studies on hybrid sterility. II. Localization of sterility factors in *Drosophila pseudoobscura* hybrids. Genetics **1936**, 21, 113–135.
10. Müller, HJ. Isolating mechanisms, evolution, and temperature. Biol. Symp. **1942**, 6, 71–125

11. Schnable P.S.; Wise R.P. The molecular basis of cytoplasmic male sterility and fertility restoration. Trends Plant Sci **1998**, 3:175–180. Doi: 10.1016/S1360-1385(98)01235-7
12. Tilney-Bassett R.A.E.; Almouslem A.B.; Amoate H.M. Complementary genes control biparental plastid inheritance in *Pelargonium*. Theor. Appl. Genet. 1992, 85:317-324. Doi: 10.1007/BF00222876
13. Weihe A.; Apitz J.; Salinas A.; Pohlheim F.; Börner T. Biparental inheritance of plastidial and mitochondrial DNA and hybrid variegation in *Pelargonium*. Mol. Genet. Genomics **2009**, 282:587–593. Doi: 10.1007/s00438-009-0488-9
14. Kuroiwa T.; Kawazu T.; Ushida H.; Ohta T.; Kuroiwa H . Direct evidence of plastid DNA and mitochondrial DNA in sperm cells in relation to biparental inheritance of organelle DNA in *Pelargonium zonale* by fluorescence/electron microscopy. Eur. J. Cell Biol. **1993**, 62:307–313.
15. Guo F.L.; Hu S.Y. Cytological evidence of biparental inheritance of plastids and mitochondria in *Pelargonium*. Protoplasma. **1995**, 186:201-207. Doi: 10.1007/BF01281330.
16. Apitz J.; Weihe A.; Pohlheim F.; Börner T. Biparental inheritance of organelles in *Pelargonium*: evidence for intergenomic recombination of mitochondrial DNA. Planta **2013**, 237:509–515. Doi: 10.1007/s00425-012-1768-x.
17. Sobanski J.; Giavalisco P.; Fischer A.; Kreiner J.M.; Walther D.; Schöttler M.A.; Pellizzer T.; Golczyk H.; Obata T.; Bock R.; Sears B.B.; Greiner S. Chloroplast competition is controlled by lipid biosynthesis in evening primroses. PNAS **2019** Doi: www.pnas.org/cgi/doi/10.1073/pnas.1811661116
18. Park S.; Grewe F.; Zhu A.; et al. Dynamic evolution of Geranium mitochondrial genomes through multiple horizontal and intracellular gene transfers. The New Phytologist. **2015**, 208(2):570-583. DOI: 10.1111/nph.13467.
19. Breman F.C.; Korver J.W.; Snijder R.C.; Bakker F.T. Plastid-encoded RNA polymerase variation in *Pelargonium* sect. *Ciconium*. HORTIC. ADV. **2024**, 2. 1 doi: https://doi.org/10.1007/s44281-023-00015-2.
20. Jin J.J.; Yu W.B.; Yang J.B.; et al. GetOrganelle: a fast and versatile toolkit for accurate de novo assembly of organelle genomes. Genome Biol. 2020, 21, 241 doi: 10.1186/s13059-020-02154-5
21. Bakker F.T.; Breman F.C.; Merckx V. DNA sequence evolution in fast evolving mitochondrial DNA nad1 exons in Geraniaceae and Plantaginaceae. Taxon 2006, 55:887–896. Doi: 10.2307/25065683.
22. Grewe F.; Zhu A.; Mower J.P. Loss of a trans-splicing nad1 intron from Geraniaceae and transfer of the maturase gene matR to the nucleus in *Pelargonium*. Genome Biol Evol. 2016, 30;8(10):3193-3201. doi: 10.1093/gbe/evw233.
23. Hiremath P.J.; Kumar A.; Penmetsa R.V.; Farmer A.; Schlueter J.A.; et al. Large-scale development of cost-effective SNP marker assays for diversity assessment and genetic mapping in chickpea and comparative mapping in legumes. Plant Biotechnol. J. **2012**, 10:716–32. Doi: 10.1111/j.1467-7652.2012.00710.x.
24. Semagn K.; Babu R.; Hearne S. Single nucleotide polymorphism genotyping using Kompetitive Allele Specific PCR (KASP): Over view of the technology and its application in crop improvement. Mol. Breed. **2014**, 33:1–14. doi: 10.1007/s11032-013-9917-x
25. Islam M.S.; Thyssen G.N.; Jenkins J.N.; Fang D.D. Detection, validation, and application of genotyping-by-sequencing based single nucleotide polymorphisms in upland Cotton. Plant Genome **2014**, 8:1–10. Doi: 10.3835/plantgenome2014.07.0034.
26. Ka S, Quinton-Tulloch MJ, Amgai RB, et al. Accelerating public sector rice breeding with high-density KASP markers derived from whole genome sequencing of indica rice. Mol. Breed. **2018**, 38:38. Doi:10.1007/s11032-018-0777-2.
27. Bakker F.T.; Culham A.; Pankhurst C.E.; Gibby M. Mitochondrial and chloroplast DNA-based phylogeny of *Pelargonium* (Geraniaceae). Amer. J. Bot. **2000**, 87: 727– 734. Doi: 10.2307/2656859
28. Palmer J.D.; Adams K.L.; Cho Y.; Parkinson C.L.; Qiu Y.L.; Song K. Dynamic evolution of plant mitochondrial genomes: mobile genes and introns and highly variable mutation rates. Proc. Natl. Acad. Sci. U.S.A. **2000**, 97: 6960–6966. Doi: 10.1073/pnas.97.13.6960
29. Choi K.; Weng M-L.; Ruhlman T.A.; Jansen R.K. Extensive variation in nucleotide substitution rate and gene/intron loss in mitochondrial genomes of *Pelargonium*. Mol. Phyl. and Evol. **2021**, 155, (106986). Doi: 10.1016/j.ympev.2020.106986
30. Mower J.P.; Touzet P.; Gummow J.S.; Delph L.F.; Palmer J.D.; Extensive variation in synonymous substitution rates in mitochondrial genes of seed plants. BMC Evol. Biol. **2007**, 7:135. doi: 10.1186/1471-2148-7-135.
31. Kerke, van de S.J.; Shrestha B.; Ruhlman T.A.; Weng M-L.; Jansen R.K.; Jones C.S.; Schlichting C.D.; et al. Plastome based phylogenetics and younger crown node age in *Pelargonium*. Mol. Phyl. and Evol. **2019**, 137:33–43. 10.1016/j.ympev.2019.03.021.
32. Kirk J.T.O.; Tilney-Bassett R.A.E. The plastids. Freeman and Co., London **1967**, United Kingdom.
33. Greiner S.; Sobanski J.; Bock R. Why are most organelle genomes transmitted maternally? Bioessays **2015**, 37, 80–94. doi: 10.1002/bies.201400110
34. Kuroiwa H.; Kuroiwa T. Giant mitochondria in the mature egg cell of *Pelargonium zonale*. Protoplasma **1992**, 168, 184–188 Doi: 10.1007/BF01666264.

35. Maréchal A.; Brisson N. Recombination and the maintenance of plant organelle genome stability. New Phytol. **2010**, 186: 299–317
36. Barnard-Kubow K.B.; McCoy M.A.; Galloway L.F. Biparental chloroplast inheritance leads to rescue from cytonuclear incompatibility. New Phytol. **2017**, 213(3):1466-1476. Doi: 10.1111/nph.14222.
37. Barnard-Kubow K.B.; So N.; Galloway L.F. Cytonuclear incompatibility contributes to the early stages of speciation. Evolution **2016**, 70(12):2752-2766. Doi: 10.1111/evo.13075.
38. Greiner S.; Bock R. Tuning a ménage à trois: Co-evolution and co-adaptation of nuclear and organellar genomes in plants. Bioessays **2013**, 35: 354–365. Doi: 10.1002/bies.201200137.
39. Röschenbleck J.; Albers F.; Müller K.; Weinl S.; Kudla J. Phylogenetics, character evolution and a subgeneric revision of the genus *Pelargonium* (Geraniaceae). Phytotaxa **2014**, 159(2), 31–76. 10.11646/phytotaxa.159.2.1.
40. Bakker F.T.; Culham A.; Daugherty L.C.; Gibby M. A trnL-F based phylogeny for species of (Geraniaceae) with small chromosomes in *Pelargonium*. Plant Species Biol. **1999**, 216, 309–324.
41. Bakker F.T.; A. Culham A.; Marais E.M.; Gibby M. Nested radiation in Cape *Pelargonium*. Pp. 75–100 in Bakker FT, Chartrou LW, Gravendeel B, Pielser PB, eds. Plant species-level systematics: new perspectives on pattern and process. A. R. G. Ganter Verlag K. G., Ruggell, Liechtstein **2005**.
42. Dodsworth S.; Jang T.S.; Struebig M.; Chase M.W.; Weiss-Schneeweiss H.; et al. Genome-wide repeat dynamics reflect phylogenetic distance in closely related allotetraploid *Nicotiana* (Solanaceae). Pl. Syst. and Evol. **2017**, Doi: 10.1007/s00606-016-1356-9.
43. Breman, F.C.; Chen G.; Snijder R.C.; Schranz M.E.; F.T. Repeatome-Based Phylogenetics in *Pelargonium* Section *Ciconium* (Sweet) Harvey, Genome Biol. and Evol., **2021**, 13(12), evab269, https://doi.org/10.1093/gbe/evab269
44. Knuth R. Geraniaceae. - In Encler A., (Ed.): Das Pflanzenreich 4, 1–9. – Leipzig (1912), Germany Engelmann.
45. Van der Walt J.J.A.; Albers F.; Gibby M. Delimitation of *Pelargonium* sect. *Glaucophyllum*. PI. Syst. Evol. **1990**, 171: 15—26. Doi: 10.1007/BF00940594.