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The decoupled evolution of the organellar genomes of *Silene nutans* leads to distinct roles in the speciation process

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Summary

- There is growing evidence that cytonuclear incompatibilities (i.e. disruption of cytonuclear coadaptation) might contribute to the speciation process. In a former study, we described the possible involvement of plastid–nuclear incompatibilities in the reproductive isolation between four lineages of *Silene nutans* (Caryophyllaceae). Because organellar genomes are usually cotransmitted, we assessed whether the mitochondrial genome could also be involved in the speciation process, knowing that the gynodioecious breeding system of *S. nutans* is expected to impact the evolutionary dynamics of this genome.
- Using hybrid capture and high-throughput DNA sequencing, we analyzed diversity patterns in the genic content of the organellar genomes in the four *S. nutans* lineages.
- Contrary to the plastid genome, which exhibited a large number of fixed substitutions between lineages, extensive sharing of polymorphisms between lineages was found in the mitochondrial genome. In addition, numerous recombination-like events were detected in the mitochondrial genome, loosening the linkage disequilibrium between the organellar genomes and leading to decoupled evolution.
- These results suggest that gynodioecy shaped mitochondrial diversity through balancing selection, maintaining ancestral polymorphism and, thus, limiting the involvement of the mitochondrial genome in evolution of hybrid inviability between *S. nutans* lineages.

Introduction

Plants cells are composed of three distinct genomic compartments: the nucleus, the mitochondrion, (mt) and the plastid (pt). These three compartments exist in a tight relationship, as both cytoplasmic organelles depend upon proper import of nuclear-encoded proteins for organellar protein complex function (Rand *et al.*, 2004; Greiner & Bock, 2013; Sloan *et al.*, 2018). Nuclear and organellar genomes differ in characteristics such as mutation rates and inheritance patterns, with the nucleus being inherited from both parents while the organelles are generally inherited from the mother (Rand *et al.*, 2004; Greiner *et al.*, 2014; Smith, 2015; Ramsey & Mandel, 2019). Tight coordination and coadaptation are required between organellar genomes and the nuclear genes whose gene products are targeted to the organelles, with mutation accumulation in one compartment generating selection for coevolutionary changes in the other (Osada & Akashi, 2012; Havird *et al.*, 2015). This coevolved relationship can be disrupted when crossing individuals of distant lineages, leading to cytonuclear incompatibilities and dysfunctional hybrid individuals (Yao & Cohen, 2000; Bogdanova *et al.*, 2015; Barnard-Kubow *et al.*, 2016; Zupok *et al.*, 2021). As such, cytonuclear incompatibilities are often considered as postzygotic barriers that contribute to the first steps of speciation (Burton & Barreto, 2012; Postel & Touzet, 2020).

While both constrained to coevolve with the nucleus but also to each other, the organelle genomes exhibit different features that can potentially submit them to different selective forces. In particular, contrarily to the pt genome whose structure is conserved among most angiosperms, the plant mt genome is variable in size and gene order even at the species level due to recombination through repeated sequences (reviewed in Chevigny *et al.*, 2020). Recombination can ultimately lead to the emergence of new genes, often chimeric, that will be selected for as soon as they favor their own maternal transmission by by-passing the production of viable pollen (reviewed in Chase, 2007; Delph *et al.*, 2007). This phenomenon, called cytoplasmic male sterility (CMS), involves an arms race between the mt genome that produces male sterilizing factors and the nucleus that counteracts by restoring male fertility. This can trigger the evolution from hermaphroditism to gynodioecy, that is, a polymorphic sexual system where females and hermaphrodites co-occur in populations.

Gynodioecy can be maintained either through balancing selection (Gouyon *et al.*, 1991) or follow epidemic-like dynamics, with the recurrent invasion of new sterilizing mt genomes (Frank, 1989). These two scenarios will result in different outcomes in terms of mt diversity. Indeed, balancing section is expected to favor the maintenance of old mt genomes and high mt polymorphism in gynodioecious species (Stadler & Delph, 2002; Touzet & Delph, 2009; Adhikari *et al.*, 2019), while epidemics the depletion of mt diversity though selective sweep (Ingvarsson & Taylor, 2002).

The indirect impact of these dynamics on the pt genome would depend on the strength of the linkage disequilibrium (LD) with the mt one. Because of their typical mode of maternal inheritance, pt and mt genomes are expected to be in strong linkage disequilibrium (LD) (Olson & Mccauley, 2000). However, this assumption is not always met. In some angiosperm species, evidence for mito-plastid incongruence and decay of LD between organellar genomes was identified, potentially due to paternal leakage of the organellar genomes and/or subsequent mt recombination (Houliston & Olson, 2006; Lahiani *et al.*, 2013; Govindarajulu *et al.*, 2015; Adhikari *et al.*, 2019; Ramsey *et al.*, 2019). Nevertheless, previous analysis of pt and mt genomes across *Silene* species showed correlated increases in evolutionary rates, suggesting that common evolutionary forces could shape organellar genome evolution (Sloan *et al.*, 2012).

Silene nutans L. (Caryophyllaceae) is a gynodioecious plant species with at least four genetic lineages, including an eastern lineage (E1) widespread in the north of Europe (e.g. England, Belgium, and North of France) and a western groups composed of three sublineages: W1 distributed in England, France and Belgium, W2 restricted to Spain and southwestern France, and W3 in the Alps and Italy (Martin, 2016; Martin *et al.*, 2016; Van Rossum *et al.*, 2018). A diallel cross reported a high percentage of interlineage hybrid mortality and chlorotic seedlings, suggesting strong reproductive isolation among these lineages (Martin *et al.*, 2017; Postel *et al.*, 2022). The level of reproductive isolation depended on the direction of the cross and which lineage was used as the maternal parent, suggesting that cytonuclear incompatibilities may be involved. We previously scanned pt and nuclear genetic variation in *S. nutans* to search for candidate gene pairs involved in a history of cytonuclear co-adaptation (Postel *et al.*, 2022). We focused on nuclear genes encoding gene products targeted to the pt (hereafter referred to as N-pt genes). We found that pt genes in *S. nutans* accumulated a large number of nonsynonymous mutations (i.e. mutations leading to a change of the encoded amino acid) that were differentially fixed between lineages and that there was a mirrored accumulation of substitutions in N-pt genes encoding subunits within the corresponding protein complexes. Mutation accumulation in pt genes was inferred to be mainly driven by relaxed selection, although signatures of positive selection were also identified for some of the pt genes (Postel *et al.*, 2022).

These observations raise questions about whether mt genes are also involved in reproductive isolation and cytonuclear incompatibilities, while being under the effect of gynodioecy dynamics.

In this study, we compared the evolutionary patterns of the mt and pt genomes of *S. nutans* individuals from each of the four major lineages. We first looked at nucleotide genetic diversity in mt and pt genes to test whether mutation accumulation followed the same trend between the two organellar genomes. Then, we conducted tests of selection to assess whether selective forces were equivalent in both organellar genomes. We also tested for a history of recombination/reassortment within and between the organellar genomes. The outcome of the tests shows that the organellar genomes exhibit striking differences, pointing to distinct evolutionary paths with implications for the speciation process of *Silene nutans*.

Materials and Methods

Genomic data acquisition and assemblies

We acquired genomic data for both the mt and the pt genes through gene capture (Supporting Information Fig. S1; see sampling details in Postel *et al.*, 2022). Briefly, 47 individuals from 24 populations (1–2 individuals per population) from UK, France, Belgium, Luxembourg, Germany, and Finland were sampled from the DNA collection of the unit Evo-Eco-Paleo (UMR 8198 – CNRS University of Lille; see Martin *et al.*, 2016 for DNA extraction procedure). These populations covered four genetic lineages of *S. nutans* based on pt SNP markers (Martin *et al.*, 2016), with 16 individuals belonging to E1, 15 individuals to W1, and eight individuals each to W2 and W3. Genomic sequences for each individual were obtained through gene capture with a myBaits® target capture kit (Daicel Arbor Biosciences, Ann Arbor, MI, USA; https://arborbiosci.com/). DNA probes were defined from the published sequence of the organellar genomes of *Silene latifolia* (NCBI accessions NC_016730.1 and NC_014487.1) and previous Illumina data from one mt genome of *S. nutans* (Genoscope PRJEB54044). Enriched libraries were pooled and sequenced on an Illumina MiSeq (2 × 150 with dual indexing) at the LIGAN platform (UMR 8199 LIGAN-PM Genomics platform – Lille, France).

Because of differences in read depth between mt (mean of 50×) and pt (mean of 600×) regions, we applied different strategies to obtain gene sequences from the two genomes. For the pt genes, we assembled the reads with SPAdes v.3.0.0 (Bankevich *et al.*, 2012) and then blasted *S. latifolia* reference sequences against the assembly as described previously (Postel *et al.*, 2022). Mt sequence coverage was more fragmented due to the lower read depth, so we used HybPiper (Johnson *et al.*, 2016) to extract the genes sequences, after read cleaning with Trimmomatic using the following parameters: 'LEADING:10 TRAILING:10 – SLIDINGWINDOW:4:20 – MINLEN:36'. We first ran the HybPiper pipeline only on the reads from W1 individuals, which represented the least fragmented dataset, using the *S. latifolia* mt genome as the reference. Then, we ran BamBam (samtools package) (Page *et al.*, 2014) on these read alignments to create a new reference. Finally, we ran HybPiper on the reads of all individuals of *S. nutans* using this new W1 reference.

Mt data quality was assessed using Igv (Thorvaldsdóttir *et al.*, 2013), MultiQC (Fig. S2), and FastQC (Ewels *et al.*, 2016; Andrews *et al.*, 2018). To assess how complete our

mt dataset was, we also counted the number of mt genes annotated in *Silene nutans* compared to those annotated in *Silene latifolia* (Sloan *et al.*, 2010).

Variant detection for both mt and pt data

To detect pt and mt variants, we ran the Genome Analysis Toolkit (GATK) HaplotypeCaller and performed joint genotyping using GenotypeGVCFs (v.3.8) (Mckenna *et al.*, 2010) with *S. latifolia* reference genomes and a min-cover of 10. Detected variants were filtered using VCFTOOLS v.0.1.16 with the following options: (1) only keep SNPs (*--remove-indel*), (2) remove variant sites with a minor allele frequency (*maf*) lower than 0.001 (*--maf 0.001*), (3) exclude variant sites with a mean read depth value of < 10 across all included individuals (*--min-meanDP 10*), (4) exclude genotype calls from an individual if its read depth for the site is < 5 (*--minDP 5*), (5) another round of filtering with *--maf 0.001*, and (6) remove variants in resulting call set with a quality score of < 100 (*--minQ 100*).

We then reported the number of polymorphic sites, per gene: (1) within individuals (i.e. apparent cases of heteroplasmy); (2) within lineages; (3) shared between lineages; and (4) fixed between lineages, using an in-house BioPython script to parse the final vcf files.

Alignment construction and analysis

For the pt genes, 68 previously constructed gene alignments for *S. nutans* individuals were already available (Postel *et al.*, 2022). These alignments also contained the reference sequences of both *S. paradoxa* and *S. latifolia*. As mt reference sequences for *S. paradoxa* were not available, we only worked with *S. latifolia* as an outgroup and removed *S. paradoxa* from the pt gene alignment using SeqKit (Shen *et al.*, 2016). We then realigned the sequences using Muscle (Edgar *et al.*, 2004) and checked the alignment for correct open reading frames (Fig. S1).

For the mt genes, we took the reference sequence build using all W1 reads and for each individual, using the variant calling results, we put the corresponding nucleotide at variable positions, using an in-house BioPython script (Fig. S1). Because we detected positions that were variable within individuals for both mt and pt DNA coding sequences, the ratio of alternative alleles varied among polymorphic sites within an individual. Haplotype reconstruction within these apparently heteroplasmic individuals was not feasible with our short-read data. Thus, we constructed three different alignment datasets to deal with this issue, regarding the file format required for each analysis (Fig. S1). Details for each of the dataset are provided below and the different version of the BioPython script used to construct these sequences are available on GitHub (https://github.com/ZoePos/Alignement_construction).

McDonald Kreitman tests

We applied the McDonald Kreitman test (MKT) (McDonald & Kreitman, 1991) to detect signatures of positive selection by comparing the number of synonymous/non-synonymous polymorphic sites within *S. nutans* (P_s and P_n) and synonymous/nonsynonymous divergent sites between *S. nutans* and *S. latifolia* (D_s and D_n). Alignments constructions for this test is described in Fig. S1. We used these data to estimate the proportion of substitutions fixed by natural selection (α) and calculate the Neutrality Index (NI), which quantifies the direction and degree of departure from neutrality (i.e. equality of the two ratios) (Rand & Kann, 1996). NI values > 1 indicate negative (purifying) selection, whereas values < 1 indicate positive selection. The mt gene *rps13* appears to have been pseudogenized in *S. latifolia* (Sloan *et al.*, 2010), so this gene was excluded from this analysis. We used PopGenome R package (Pfeifer *et al.*, 2014) to run the MKT using the options *include.unknown = TRUE* when loading the data to allow for missing data. The MKT was run on each pt and mt gene separately and for concatenations of genes encoding subunits of the same pt or mt enzyme complex. Because *S. nutans* lineages exhibit strong reproductive isolation and could be considered incipient species, we also ran the MKT on each *S. nutans* lineage separately, with *S. latifolia* as an outgroup.

Recombination

To assess the number of reassortment events within and between organellar genomes, we conducted four gamete tests (FGTs) on the mt and pt data. Considering that homoplasy (i.e. repeated mutations at the same position) is expected to be rare (infinite-sites assumption), the presence of four different allelic combinations between pairs of segregating sites most likely results from recombination. Alignments constructions for this test is described in Fig. S1.

We implemented FGTs with a BioPython script (https://github.com/ZoePos/Four-gamete-test) on the concatenated gene sets of all organellar genes. Genotype calls with evidence of intraindividual polymorphism (heteroplasmy) were treated as missing data.

Divergence (K_S) with *S. latifolia*

To assess whether the high level of polymorphism observed in the mt data was the result of recombination or elevated mutations rates, we calculated synonymous divergence (K_s) between *S. nutans* and *S. latifolia* (Table S1). Synonymous mutations can be viewed as 'silent' mutations in the sense that they do not lead to a change of the amino acid sequences. As such, they are expected to experience weaker selective pressures and evolve relatively neutrally even though they are known to have some effects on fitness (Chamary *et al.*, 2006). Although selection on linked sites will alter effective population size and polymorphism levels, the long-term substitution rate (e.g. interspecific divergence between *S. nutans* and *S. latifolia*) at neutral sites is expected to be equal to the mutation rate and independent of population size (Kimura, 1983). Thus, the synonymous divergence between *S. nutans* and *S. latifolia* should reflect the mutation rate. Mean K_s was estimated for each mt and pt gene, using DNAsp 6.12.03

(Rozas *et al.*, 2017), first with all *S. nutans* individuals and then separately for each lineage. To test for significant differences between pt and mt genes, we conducted Mann–Whitney *U*-tests in R v.4.1.2 (package STATS4).

Checking for numts

Numts (nuclear mt DNA) are mt DNA sequences transferred to the nucleus (Parakatselaki & Ladoukakis, 2021). Such transfers are ongoing processes and the insertions can be very large (Fields *et al.*, 2022). Once integrated into the nuclear genome, they are generally nonfunctional and subject to degradation (Hazkanicovo *et al.*, 2010). Misidentifying numts as true mt DNA is a common cause of erroneous conclusions about phylogenetic relationships, biparental inheritance of mt genomes, and *de novo* mutations (Bensasson *et al.*, 2001; Wu *et al.*, 2020; Lutzbonengel *et al.*, 2021). Therefore, we assessed whether our data were contaminated with numt sequences via additional analyses.

First, we checked the mt DNA data and excluded mt genes that exhibited any variant that created an early stop codon, as these can indicate sequence degradation and nonfunctionality of the mt genes. Second, we assessed whether any variants were found exclusively in heteroplasmic individuals, as these would be candidates for derived changes in a numt sequences that, therefore, must always co-occur with the true mt allele. Finally, we randomly selected 100 nuclear loci in the transcriptome that was available for several individuals of the four lineages to build a nuclear phylogeny and compare it with the mt phylogeny (Muyle *et al.*, 2021). We randomly sampled 100 nuclear loci excluded the one that might be interacting with mt and/or pt genes, to be sure that whatever dynamics is occurring in mt/pt genomes, we would be truly looking at nuclear genes experiencing evolutionary dynamics of the nuclear genome only. We constructed alignments for these 100 nuclear loci. We also blasted these alignments on *S. latifolia* transcriptome (PRJEB39526) to use it as an outgroup and add *S. latifolia* sequences to the *S. nutans* alignments. Finally, we checked the alignments to be sure that no early stop codons were presents. For nuclear alignments, we followed methods used by Postel *et al.* (2022). With these alignments, we concatenated mt genes and nuclear loci separately and constructed phylogenies, using RAxML with a GTR gamma model of nucleotide substitutions (Stamatakis, 2014) and bootstrap analysis for node support.

Results

Data acquisition

Our sequencing approach recovered most of the pt genes annotated in *S. latifolia* and *S. paradoxa* pt reference genomes (Postel *et al.*, 2022). The read depth was lower for the mt genome (*c.* 50×) than the pt genome (*c.* 600×) (Postel *et al.*, 2022), which likely reflects the higher number of pt genome copies per cell in plant leaf tissue. Most mt genes were found using *Silene latifolia* mt genome as reference, resulting in the analysis of 27 mt protein-coding genes (Table 1).

Table 1 Number of genes annotated and analyzed in *Silene nutans* individuals compared to *Silene latifolia*.

Complexes	*S. latifolia*	*S. nutans*	Analyzed
OXPHOS complex I	9	9	9
OXPHOS complex II	1	2	0 (2 pseudogenes[1])
OXPHOS complex III	1	1	1
OXPHOS complex IV	3	3	3
OXPHOS complex V	5	5	4 (numts suspicion[2])
Cytochrome *c* biogenesis	4	4	4
mttB	1	1	1
matR	1	1	0 (numts suspicion[2])
Large ribosomal subunit	1	1	1
Small ribosomal subunit	4 (1 pseudogene[1])	4	2 (numts suspicion[2])
rRNA genes	3	2	0
tRNA genes	7	8	0
Total	40	41	24

The number of genes in *Silene latifolia* were taken from Sloan *et al*. (2010).
[1]Mitochondrial pseudogenes in *S. latifolia* and *S. nutans* (*rps13*, *sdh3*, *sdh4*).
[2]Mitochondrial genes excluded because of numts suspicion (*atp6*, *matR*, *rps4*, *rps14*).

Polymorphism

Overall, more polymorphic sites were identified in mt genes than in pt genes, and this was true for all four *S. nutans* lineages analyzed individually. At the lineage level, the proportion of polymorphic sites was approximately 10^{-3} for the mt genes vs 10^{-4} for the pt genes (Table 2). Polymorphism was also detected within individuals, which suggests heteroplasmy. Depending on the *S. nutans* lineage, there were 6–20 sites in the pt genome and 170–365 sites in the mt genome that exhibited apparent heteroplasmy in at least one individual (Table 2).

The two organellar genomes exhibited striking differences in the amount of shared polymorphism and fixed substitutions between lineages. In the pt genes, only a few polymorphic sites were shared between lineages. In other words, most of the polymorphic sites with *S. nutans* were fixed differences between the four lineages. By contrast, we did not detect any fixed substitutions between lineages in the mt genes; instead, there were numerous shared polymorphic sites even among E1 and the western lineages (Tables 2, 3).

Selection

We used the MKT to search for signatures of selection in pt and mt genes at *Silene nutans* level (i.e. combining all lineages). Here too, patterns differed between the two organellar genomes. Significant signatures of negative selection were detected on several pt proteins complexes (e.g. photosystem II, ATP synthase, NDH),

Table 2 Number and proportions of polymorphic sites (including indels and SNPs) in the mitochondrial and plastid gene concatenations: (1) within individuals (at the read level); (2) within lineage; (3) shared between all lineages; (4) differently fixed between lineages.

		Mitochondrial genes				Plastid genes			
	Lineage	Intra-individual	Private (one lineage)	Shared between lineage	Fixed substitutions	Intra-individual	One lineage	Shared between lineage	Fixed substitutions
Numbers of polymorphic sites for mitochondrial and plastid gene concatenations	E1	45	74	477	0	7	3	7	139
	W1	48	24			14	1		
	W2	4	17			3	10		
	W3	15	26			9	2		
Proportions of polymorphic sites for mitochondrial and plastid gene concatenations	E1	1.3e-3	2.1e-3	1.3e-2	0	4.6e-5	2.0e-5	4.6e-5	9.1e-4
	W1	1.3e-3	6.7e-4			9.2e-4	6.6e-6		
	W2	1.1e-4	4.7e-4			2.0e-5	6.6e-5		
	W3	4.2e-4	7.3e-4			5.9e-4	1.3e-5		

Length of the concatenation of the mitochondrial genes: 35 849 bp; Length of the concatenation of the plastid genes: 151 736 bp.

Table 3 Details for the shared polymorphism between lineages.

Genome	Lineages	Homoplasmic variants	Heteroplasmic variants	Homoplasmic + Heteroplasmic variants	Total number of shared variants
Mitochondrial	All lineages	16	8	156	180
	E1 + W1	3	13	14	30
	E1 + W2	5	1	9	15
	E1 + W3	1	3	8	12
	E1 + W1 + W2	2	8	27	37
	E1 + W1 + W3	19	11	28	58
	E1 + W2 + W3	0	1	1	2
	Western lineages	6	11	23	40
	W1 + W2	6	31	15	52
	W1 + W3	9	14	22	45
	W2 + W3	3	1	2	6
	Total	70	102	305	477
Plastid	All lineages	0	1	0	1
	E1 + W1	0	2	0	2
	E1 + W3	0	1	0	1
	W1 + W3	0	2	1	3
	Total	0	6	1	7

Homoplasmic variants: The two alternative alleles are only found in homoplasmic individuals; no heteroplasmic individuals identified (e.g. individuals have either an A or a T but not both); Heteroplasmic variants: One of the alleles is exclusively found in 'heteroplasmic' individuals (e.g. individuals can be homoplasmic for A or heteroplasmic for A/T, but no individuals are homoplasmic for T); Homoplasmic + heteroplasmic variants: Both alleles are found in some homoplasmic and some heteroplasmic individuals (e.g. individuals with heteroplasmic A/T, homoplasmic A, and homoplasmic T are all present).

whereas no positive selection was detected (Table 4). On the mt side, we only detected positive selection on the cytochrome *c* oxidase protein complex (Table 4) and more specifically on *cox3*, as well as for the concatenation of all mt genes (Table S2).

Because *S. nutans* lineages can be considered as four subspecies given the high level of reproductive isolation between them, we also conducted MKTs independently for each lineage. On the pt side, we did not detect any lineage-specific selection patterns, except for signatures of positive selection in W1 for the concatenation of all pt genes (Table S3). For the mt data, we detected positive selection on the concatenation of all mt genes and on the cytochrome *c* oxidase gene set in each of the four lineages, as we found when considering all four *S. nutans* lineages combined (Tables S2, S3).

Recombination

With the four-gamete test (FGT), we detected possible recombination/reassortment events within and between genes in both pt and mt genomes (Fig. 1). The numbers of between-gene positive results of the FGT were much higher for the mt genes compared to the pt ones (i.e. 542 between pt genes vs 10 401 for the mt ones). The same pattern was observed for within-gene positive FGTs: 22 within pt genes vs 521 within mt genes. Recombination/reassortment both within and between genes was detected for all mt genes except for *rpl5* (Fig. 1a), which is less variable compared with the other genes. For the pt data, almost all of the 46 pt genes with any variable sites exhibited reassortment events, either within or between genes, except for *ndhB* and *ndhK*

Table 4 Results of McDonald Kreitman tests for the mitochondrial and plastid gene concatenations per protein complex.

Genome	Complexes	Neutrality index	alpha	Fisher *P* value	DoS
Mitochondrial	All mitochondrial genes	0.70	0.30	0.06	–
	OXPHOS complex I	1.25	−0.25	0.78	–
	OXPHOS complex IV	0.25	0.75	0.00	Positive
	OXPHOS complex V	1.55	−0.55	0.17	–
	Cytochrome *c* biogenesis	0.46	0.54	0.16	–
	Membrane protein	0.00	1.00	0.12	–
	Large ribosomal subunit	0.00	1.00	1.00	–
	Small ribosomal subunit	Inf	−Inf	0.55	–
Plastid	All plastid genes	2.95	−1.95	0.00	Negative
	Photosystem I	2.22	−1.22	0.46	–
	Photosystem II	3.65	−2.65	0.06	Negative
	ATP synthase	5.14	−4.14	0.05	Negative
	Cytochrome b6/f	4.80	−3.80	0.24	–
	Rubisco	0.00	1.00	1.00	–
	NDH	4.92	−3.92	0.00	Negative
	RNA polymerase	1.23	−0.23	0.68	–
	Large ribosomal subunit	4.92	−3.92	0.00	Negative
	Small ribosomal subunit	10.75	−9.75	8.93E-07	Negative
	Other functions	1.07	−0.07	1.00	–

DoS, Direction of Selection depending of the value of the NI index: if NI > 1 = negative selection; if NI < 1 = positive selection.

(Fig. 1b). Reassortment between pt and mt genes was also detected for the same genes that exhibited recombination within each organellar genome (Fig. 1c).

Divergence

The mean divergence between *S. nutans* and *S. latifolia* was calculated using K_s, and was significantly lower for mt genes (0.0284) than for pt genes (0.0437) (Fig. 2) (Mann–Whitney *U* tests = 1210.5, *P*-value = 4.6×10^{-5}). Consistently, when comparing K_s between mt and pt genes within each lineage of *S. nutans*, the K_s was significantly higher for each lineage in pt genes (Fig. S3).

Are mt sequences contaminated by numts?

To assess whether the studied mt sequences were derived from numts or genuine mt genes, we asked the three following questions:

(**1**) Do we find early stop codons in genes that could be the signature of numts rather than mt genes?

(**2**) Are identified variants found exclusively in heteroplasmic individuals, as would be expected if one of the 'alleles' was actually a derived change in a numt copy?

(**3**) Does the phylogeny of 100 nuclear genes sampled by chance generate a phylogeny as inconsistent with the lineage tree as the mt gene tree?

For the first question, we found only four mt genes in which early stop codons were identified in the nucleotide sequences: *atp6*, *matR*, *rsp4*, *rps14*. To be conservative, these genes were excluded from the analyses (Table 1).

For the second question, variants found in the heteroplasmic state could also be found in the homoplasmic state (i.e. both nucleotides were found in at least on homoplasmic individuals). This was the case among the shared polymorphic variants, where most of the heteroplasmic variants identified were also homoplasmic for other individuals: 0.75 were heteroplasmic for some individuals and homoplasmic for others (305 of 407), while only 0.25 were exclusively heteroplasmic (102 of 407) (Table 3). The fact that both alleles for most of the variants were found in at least some homoplasmic individuals implies that they are true mt polymorphisms. This observation does not rule out the possibility that some individual cases of apparent heteroplasmy are actually due to a numt sequence, but it does indicate that these variants arose in the mt genes and were not derived mutations that occurred in a nuclear copy. Meanwhile, the recombination data described above provide evidence that heteroplasmy is a real phenomenon in this system because it is a prerequisite for producing recombinant haplotypes.

For the final question, the nuclear gene phylogeny was highly supported (bootstrap nodes > 80%) and similar with the tree based on concatenated pt genes, with a strict clustering by lineage. Conversely, the mt phylogeny exhibited lower bootstraps values and no lineage clustering (Fig. S4). SNPs shared among lineages would be the signature of ancestral polymorphism, which does not concur with the idea of recent numts or the phylogenetic patterns recovered for known nuclear genes. Overall, even though we cannot rule out the possibility that some SNPs and apparent heteroplasmies are the result of numts, it appears that the identified variants are primarily from authentic mt sequences.

Discussion

Mt and pt genomes differ in their pattern of diversity

In the pt genes, most of the identified substitutions were differently fixed between lineages, whereas not a single mt variant

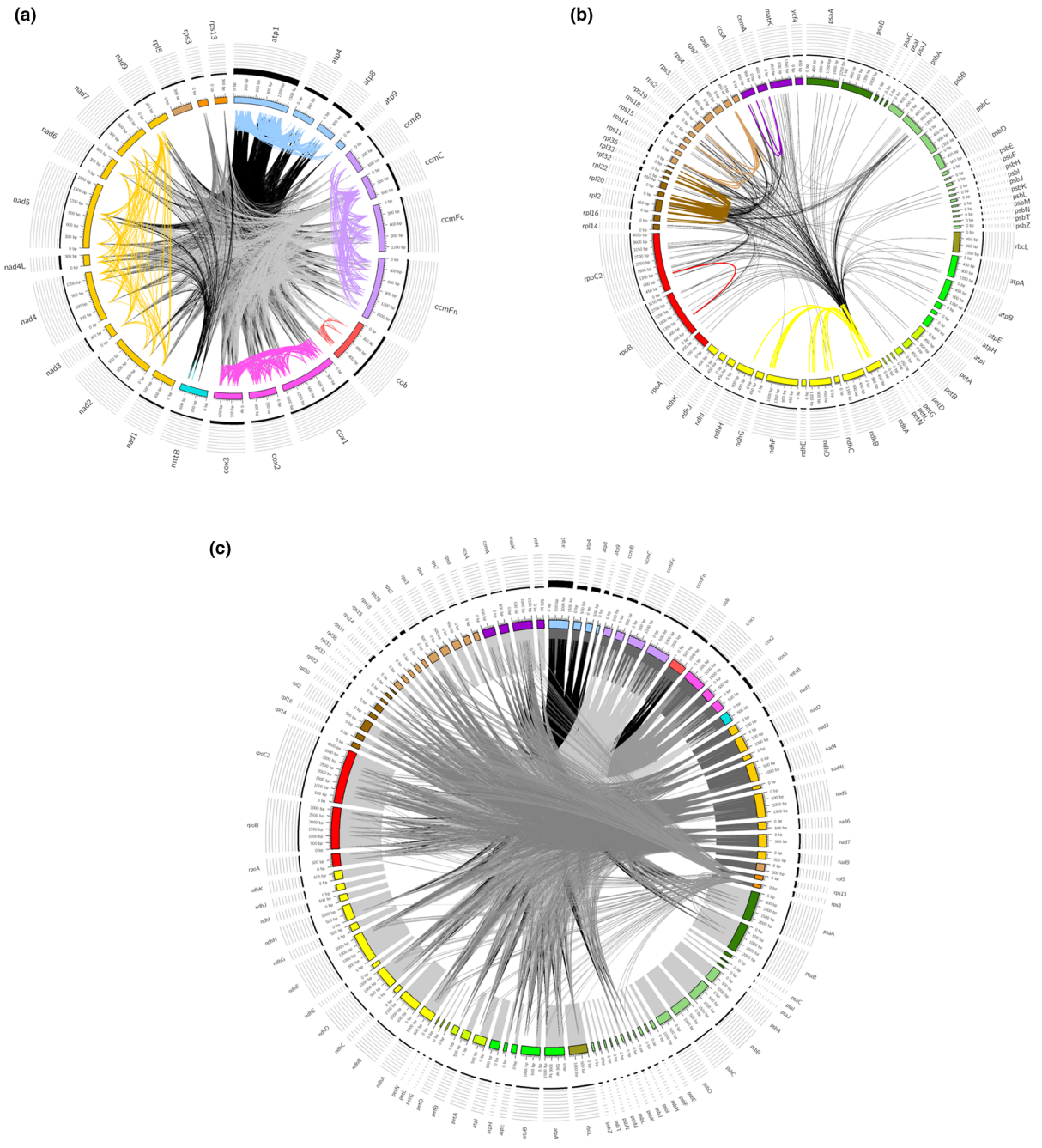

Fig. 1 CIRCOS figures to represent the number of recombination events and recombination blocks. (a) Recombination within and between mitochondrial genes. (b) Recombination within and between plastid genes. (c) Recombination between plastid and mitochondrial genes. Black rectangle heights above each gene are proportional to the levels of polymorphism.

differentiated the four lineages. Overall, there were 30-fold more shared polymorphic sites between the four lineages for mt genes than for pt genes.

Signatures of selection also differed between the organellar genomes. The MKT identified negative selection acting on most of the pt gene concatenations but not for the mt genes. These results are in accordance with previous studies comparing both genomes, where pt genes appeared to be under a stronger purifying selection than mt ones (Muse, 2000). Signatures of positive selection were identified for only one mt gene concatenation (cytochrome *c* oxydase) and only one mt gene (*cox3*).

The comparison of synonymous divergence (K_s) with *S. latifolia* between pt and mt genes suggests that the pt mutation rate is higher than the mt one, which is consistent with previous findings (Drouin *et al.*, 2008). Yet, when looking at the polymorphism level within each lineage, it was higher for mt than pt genes. This could be due partly to a lower purifying selection pressure on mt genes, consistent with the result of the MKT.

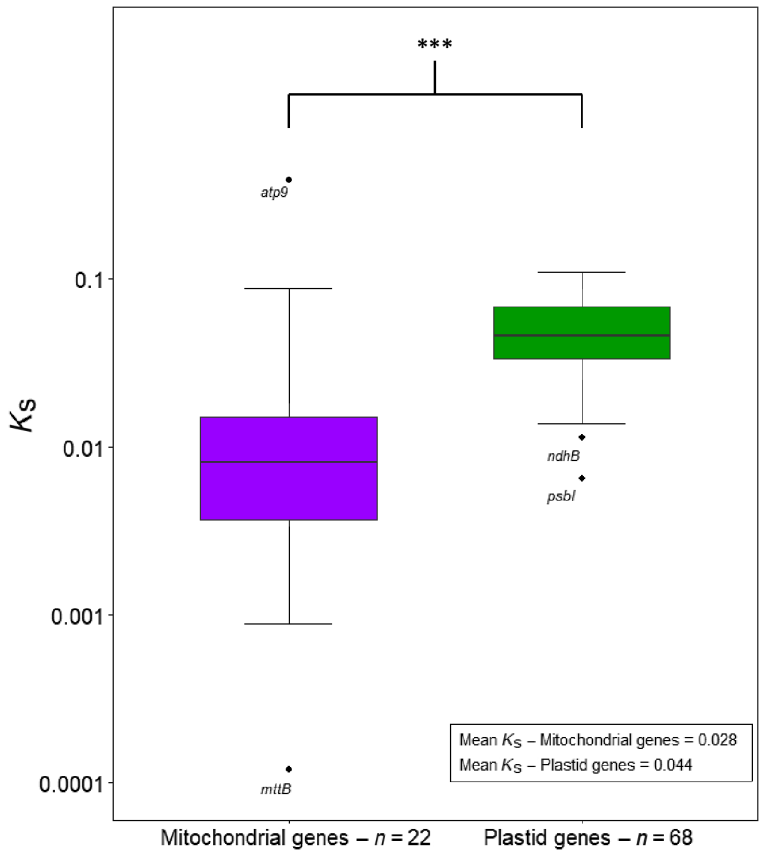

Fig. 2 K_s between *Silene nutans* and *Silene latifolia* for plastid and mitochondrial genes. Values of K_s ($\log_{10}$ transformation). Results of the Mann–Whitney test are also shown: ***, *P*-value < 0.001. Horizontal lines within the boxplot represent the median values and the outliers are displayed as black dots.

Furthermore, we detected many reassortment events in the mt genome, whereas they were scarce in the pt genome. Recombination might be one of the main factors shaping the mt genome diversity in the species. Surprisingly, we also detected some recombination events between pt genes. These rare recombination event signatures in the pt genome could also be due to homoplasy. True recombination in organelle genomes would imply the past co-occurrence of two haplotypes in a given individual, that is heteroplasmy, due to a shift from strict maternal inheritance of the organellar genomes (i.e. biparental inheritance or paternal 'leakage') (Kmiec *et al.*, 2006; Ramsey & Mandel, 2019; Gonçalves *et al.*, 2020; Parakatselaki & Ladoukakis, 2021). Interestingly, paternal leakage of both organellar genomes has been documented in the congener *S. vulgaris* (McCauley *et al.*, 2005, 2007).

The consequence of paternal leakage and subsequent heteroplasmy might be different for pt and mt genomes. Indeed, recombination between two pt types is expected to be rare, as fusion of the pt is uncommon (Chiu *et al.*, 1988; Birky, 2001; Sakamoto, 2003). Instead, the pt compete with each other for fixation within the cells (Sobanski *et al.*, 2019). By contrast, mt are known for frequent fusion events (Segui-Simarro *et al.*, 2008; McCauley, 2013; Greiner *et al.*, 2014; Ramsey *et al.*, 2019). Recombination between distinct mt haplotypes could then be the result of paternal leakage, increasing polymorphism within each lineage. We also detected polymorphic sites within individuals, suggesting heteroplasmy. Overall, the different outcomes for pt and mt genomes following biparental *trans*mission could explain the distinct genetic patterns observed in the organellar genomes of *S. nutans*.

Mt genetic diversity link to gynodioecy

Silene nutans is a gynodioecious species (i.e. presence of female and hermaphroditic individuals). Gynodioecious species often exhibit intergenomic conflict resulting from cytoplasmic-male sterility (CMS) factors found in the mt genome (Budar *et al.*, 2003; Delph *et al.*, 2007). According to theoretical models, paternal leakage of the mt could maintain stable mt polymorphism and limit the risk of stochastic loss of CMS factors, which would be beneficial for the maintenance of gynodioecy (Wade & McCauley, 2005). Additionally, this inheritance pattern offers opportunity for heteroplasmy and recombination among mt haplotypes, which in turn can lead to the emergence of new CMS genes (McCauley & Olson, 2008). Finally, paternal mt genomes are more likely to carry fertile cytotype than maternal ones, which would reduce the probability of local extinction due to pollen limitation (McCauley, 2013; Breton & Stewart, 2015; Ramsey & Mandel, 2019). Because of that, gynodioecy could select for paternal leakage of the mt genome (McCauley, 2013; Breton & Stewart, 2015; Ramsey & Mandel, 2019). We previously hypothesized that selection favoring sterilizing mt haplotypes might have led to hitchhiking and fixation of pt haplotypes (Postel *et al.*, 2022). However, the low observed LD between pt and mt genomes likely limits the effects of linked selection between the two genomes.

Maintenance of gynodioecy has been hypothesized to result from two different mechanisms: maintenance of CMS factor through balancing selection with negative frequency-dependence or recurrent invasion of CMS factors in population (epidemic-like dynamics) (Frank, 1989; Gouyon *et al.*, 1991). These mechanisms would result in distinct patterns of genetic diversity of the mt genome (Ingvarsson & Taylor, 2002; Stadler & Delph, 2002; Touzet & Delph, 2009). The level of mt polymorphism, and in particular shared between lineages, corresponding to trans-specific polymorphism, is in favor of the hypothesis of balancing selection to explain the maintenance of gynodioecy in *S. nutans*, in accordance with previous studies that were conducted on two mt genes (Touzet & Delph, 2009; Lahiani *et al.*, 2013).

The decoupled evolutionary pathways of the pt and mt genomes in *Silene nutans*

This study highlights that mt and pt genomes in *S. nutans* lineages do not share a common evolutionary history (Fig. 3). LD between the organellar genomes seems to be loose enough to result in two decoupled evolutionary pathways. When considering pt genetic diversity at the lineage level, a large number of nonsynonymous fixed mutations were documented, pointing to the involvement of plastid-nuclear incompatibilities in reproductive isolation between *S. nutans* lineages (Postel *et al.*, 2022). The low-recombining nature of the pt genome might favor the occurrence of speciation genes just as it is the case in any low recombining genomic regions for example chromosomal inversions (Schluter & Rieseberg, 2022). By contrast, no fixed mt variants were detected between lineages, with balancing selection most likely preventing the effect of drift observed on the pt genome. Balancing selection of the mt genome in a gynodioecious species might limit lineage-specific coevolution between the mt and the nuclear genomes and thus the accumulation of mito-nuclear incompatibilities. This could also imply that CMS mt genomes and their corresponding nuclear restorer genes might be distributed and maintained at the level of the *Silene nutans* species complex and would not contribute to hybrid

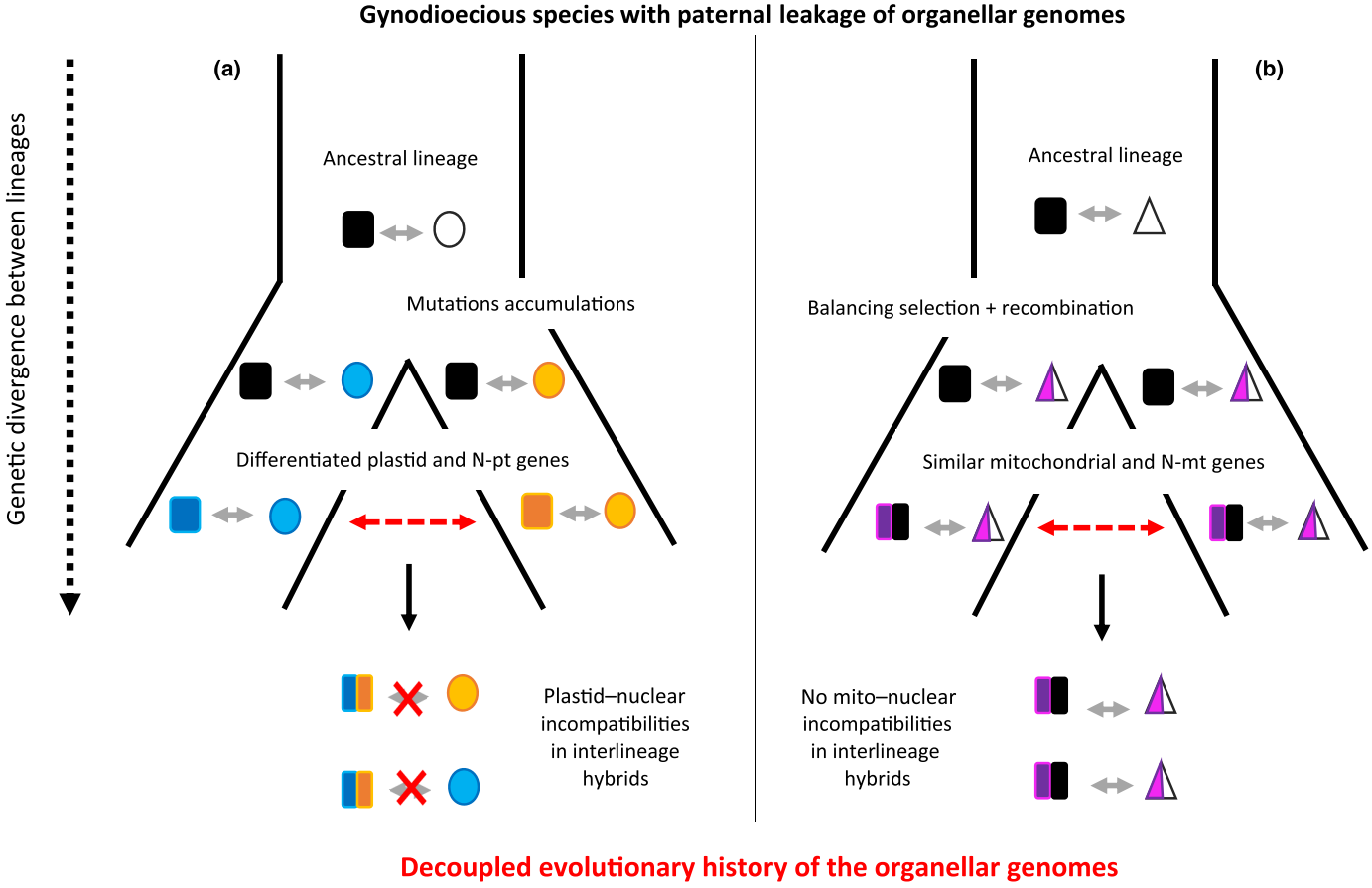

Fig. 3 Summary of the scenario for decoupled evolution of mitochondrial and plastid genome in a gynodioecious species exhibiting paternal leakage. (a) Evolutionary scenario for the plastid genome and (b) for the mitochondrial genome. The grey arrows represent interactions between nuclear (the squares) and organellar genomes (the circles for the plastid genome and the triangles for the mitochondrial one). The red dotted arrows represent reproduction between divergent lineages. The red 'X' represent mis-interactions between genomic compartment and generation of nucleo-cytoplasmic incompatibilities. N-mt, nuclear genes encoding gene products targeted to the mitochondria; N-pt, nuclear genes encoding gene products targeted to the plastid. Because the species is gynodioecious, balancing selection might act on the mitochondrial genome, maintaining ancestral polymorphism shared between diverging lineages. Additionally, the presence of paternal leakage of the organellar genomes might further mix the mitochondrial genomes of the diverging lineages through intermitochondria recombination. Contrastingly, despite this paternal leakage, plastid genomes of the diverging lineages do not recombine together, resulting in highly differentiated plastid genome between lineages and potential plastid–nuclear incompatibilities in interlineages hybrids.

incompatibility between lineages. Thus, unlike the general case of strong LD between the organellar genomes, which can make it difficult to identify which genome might be involved in a given trait, the present study and prior work (Postel & Touzet, 2020) suggest a larger role for the pt genome than the mt genome in postzygotic reproductive isolation in the *S. nutans* species complex.

To what extent alternative dynamics of CMS could lead to hybrid incompatibility between lineages? And more globally, how mitochondrial genomes could be involved in the speciation process? First, it must be noted that in most cases the emergence of a CMS does not lead to a stable gynodioecy (Charlesworth & Ganders, 1979; Dufaÿ *et al.*, 2007). Either the invasive CMS mt genome and its corresponding nuclear alleles that restore male fertility go to fixation (like in the epidemic-like model) (Frank, 1989) or the CMS emergence is under a neutral process that does not imply this arms-race dynamic (see the 'agnostic' model in Fishman & Sweigart, 2018).

In the case of a secondary contact, male sterility might be revealed in hybrids. For example, this was observed in hybrids between *Mimulus nasutus* a selfing species and the outcrosser *M. gutttatus* individuals from a given hermaphrodite population where CMS had been fixed as well as its specific nuclear restorer of fertility (Fishman & Willis, 2006; Case *et al.*, 2016). However, as pointed out by Fishman & Sweigart (2018), hybrid incompatibility might easily be bypassed through the subsequent selection of the introgressed nuclear restorer alleles. Therefore, it is not very likely that CMS leading to hybrid incompatibility might be involved in postzygotic reproductive isolation and the speciation process in the long term. In which situation then might mitochondrial genomes be involved in reproductive isolation? In the case of nongynodioecious species where the mt mutation rate has been found to be exceptionally high, the subsequent selection for compensatory mutations on the Nu-mt genes could lead to mitonuclear incompatibilities between species. Some cases of species with fast mt evolution rate have been documented (Mower *et al.*, 2007; Sloan *et al.*, 2009) and could be good candidate to test this hypothesis.

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Competing interests

None declared.

Author contributions

PT, J-SV and SG planned and designed the research. ZP, SG, CG, ES, SM, LD performed the experiments. ZP, PT and DBS analyzed the data and wrote the manuscript.

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Data availability

Sequence data are available in NCBI: PRJEB54044 for initial mt data, PRJNA745523 for mt and pt reads.

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Supporting Information

Additional Supporting Information may be found online in the Supporting Information section at the end of the article.

Fig. S1 Workflow of the analyses for the plastid and mitochondrial genes.

Fig. S2 Per sequence and per base quality scores plots, generated with MultiQC, after reads cleaning.

Fig. S3 K_s calculated per lineage for mitochondrial and plastid genes.

Fig. S4 Phylogenies constructed running RAxML – GTR Gamma model of nucleotide substitutions on the nuclear and mitochondrial genes concatenations.

Table S1 Number of individuals per gene for calculating K_s.

Table S2 Results of the MK test calculated with all individuals of *Silene nutans* for mt and pt genes per protein complex.

Table S3 Results of the MK test for mitochondrial and plastid gene concatenations per protein complex per lineage.

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