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Microgametophytic plastid nucleoid content and reproductive and life history traits of tribe *Trifolieae (Fabaceae)*

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Abstract: Microgametophytic plastid nucleoids were quantified for 18 species representing the four core genera of the tribe *Trifolieae* (*Fabaceae*), *Medicago, Melilotus, Trigonella,* and *Trifolium.* Generative cells of all taxa contained nucleoids, establishing that biparental plastid inheritance is common in the *Trifolieae.* Nucleoid number and volumes of pollen grains and generative cell nuclei differed among taxa. Nucleoid number was positively correlated with pollen grain and generative cell nuclear volumes, flower size and style length. These relationships disappeared after adjusting nucleoid number for pollen grain and generative cell nuclear volumes. Adjusted nucleoid numbers provided no evidence to support hypotheses that plastid content is associated with ploidy level, mating system, perenniality or size of the reproductive apparatus.

Advances in cytology and molecular biology have stimulated interest in describing the mode of organelle inheritance in plants. Data from a number of studies have shown that the assumption of strict maternal inheritance of plastids in angiosperms is not justified (review in Harris & Ingram 1991). Using the DNA fluorochrome DAPI (4′-6-diamidino-2-phenylindole), Corriveau & Coleman (1988) analyzed generative or sperm cells from 235 angiosperm species for the presence of plastid nucleoids (DNA aggregates) and found cytological evidence for potential paternal inheritance of plastids in 43 species from 26 genera. Such cytological data generally correlate closely with available genetic data on plastid DNA inheritance (Corriveau & al. 1990, Harris & Ingram 1991).

Plastid inheritance has been studied extensively in the widely cultivated taxon *Medicago sativa* L. (*Fabaceae*), which exhibits biparental inheritance of plastids with a strong paternal bias (Smith & al. 1986). Genotypic differences have been observed in the extent of plastid transmission as both a maternal and paternal parent (Smith 1989), and cytological studies using serial ultrathin sections or DAPI-stained whole cells have also demonstrated the existence of plastids and plastid nucleoids within generative (Zhu & al. 1990, Shi & al. 1991) and sperm cells (Zhu & al. 1993). These studies have also shown that the number of plastid nucleoids

in these cells is proportional to, but less than the number of plastids as determined from electron microscopic observation of serial ultrathin sections.

Very little research has addressed possible functions of plastids either with or without DNA within the microgametophyte. The observation that many conifers exhibit strongly paternal plastid inheritance (SUTTON & al. 1991, NEALE & al. 1991) as well as temporal separation between pollination and fertilization (WILLSON & BURLEY 1983) has led to the suggestion that plastids within the male gametophyte may play a functional role in reproductive success perhaps by influencing microgametophyte survival. Similarly, organelle DNA has been visualized within the cytoplasm of vegetative cells of pollen and microgametophytes from three genera in the *Orchidaceae* in which pollen is very long-lived (ALBERT & al. 1989). These observations indicate that plastids could provide an additional source of energy and/or essential metabolites to pollen, microgametophyte, or embryo (CRUZAN 1990). If this is the case, quantitative differences in plastid content might be expected to be related to microgametophyte or embryo performance, but the amount of DNA in the plastids would be unimportant. Alternatively, these organelles may produce RNA or protein important in the development of these organs (WILLSON & BURLEY 1983, CORRIVEAU & COLEMAN 1991), so that a certain minimum amount of functional DNA within plastids would then be required. Ontogenetic factors may also determine the presence of DNA-containing organelles in microgametophytes. CORRIVEAU & COLEMAN (1991) showed that nucleic acids were present in both mitochondria and plastids of generative and vegetative cells of six angiosperm taxa until pollen maturation. After that, nucleic acids were observed only in plastids in the generative cells and only in species in which plastids are inherited biparentally.

Attempts to describe the possible evolutionary significance of the mode of plastid inheritance, and the use of such information in systematic research, have been hampered by lack of studies that have systematically sampled taxa. This study utilized a systematic approach to study plastid inheritance in the tribe *Trifolieae* (*Fabaceae*), that includes four closely related genera, *Medicago, Trigonella, Melilotus,* and *Trifolium. Parochetus* and *Ononis* are sometimes included in the tribe, but doubtfully so (SMALL 1987, 1988). The genus *Medicago* presents an uncommon opportunity to address questions related to plastid inheritance and reproductive and life history traits. The genus contains about 85 species (SMALL & JOMPHE 1989) of annual or perennial herbs or shrubs that exhibit an irreversible explosive pollination mechanism (SMALL & al. 1987). The majority of perennial *Medicago* taxa are polyploid, outcrossing, and entomophilous, while most annual species are diploid and strongly autogamous (HEYN 1963). A substantial difference in time between pollen dehiscence and fertilization would be expected between autogamous and outcrossing, entomophilous species. Although considerably less than the time lag between pollination and fertilization in conifers, the time lag and environmental stress involved in entomophily should permit testing of hypotheses regarding plastid contribution toward pollen survival in angiosperm genera.

The objectives of this research were to 1) described plastid content (as DAPI-staining plastid nucleoids) in generative cells from 18 species representing ten sections of *Medicago* and, less intensively, the three other core genera in the *Trifolieae*; and 2) determine whether plastid nucleoid content is associated with

reproductive biology and life history traits in these taxa. In an attempt to elucidate the role of plastids in reproductive biology within this tribe, we tested the specific hypotheses that microgametophytic plastid nucleoid content 1) does not differ between outcrossing and autogamous species, 2) does not differ among plants with different ploidy levels, 3) does not differ between annual and perennial species, and 4) is unrelated to size of the reproductive apparatus.

Material and methods

Plant material. Accessions of 14 species of *Medicago* from 10 sections as well as one species of *Trigonella*, one of *Melilotus*, and two of *Trifolium* were utilized (Table 1). Flowers for pollen studies were collected in Tucson, Arizona, from greenhouse-grown plants of each accession except for *M. arborea*, which was collected from field-grown plants.

Pollen collection, germination and cytology. Pollen tubes were grown using a modification of method (iv) described by Corriveau & Coleman (1988). Pollen from five to eight flowers was placed in a depression well to which 100 µl of germination medium (15% sucrose, 0.16 mM H_3BO_3, 0.20 mM KNO_3, and 0.13 mM $Ca(NO_3)_2 \cdot 4H_2O$) were added. Germination was allowed to proceed for 2.5–3 h at 22–27 °C in a petri plate con-

Table 1. Description of plants used in analysis of plastid nucleoid content within generative cells

Taxon (ploidy, chromosome number)	Section[a]	Accession[b]	Habit[c]	Mating system
Medicago arborea L. (4x = 32)	*Dendrotelis*	PI 330671	P-shrub	Cross-pollinated
M. sativa L. (4x = 32)	*Medicago*	cv. Malone	P-herb	Cross-pollinated
M. sativa L. (2x = 16)[d]	"	(M) 225467	P-herb	Cross-pollinated
M. cancellata M. Bieb. (6x = 48)	"	PI 440491	P-herb	Cross-pollinated
M. carstiensis Wulf. (2 × = 16)	*Carstiensae*	(M) 47	P-herb	Self-pollinated
M. truncatula Gaertn. (2x = 16)	*Spirocarpos*	PI 292434	A-herb	Self-pollinated
M. scutellata (L.) Miller (2n = 30)	"	PI 197821	A-herb	Self-pollinated
M. polymorpha L. (2x = 16)	"	(A) LONG	A-herb	Self-pollinated
M. lupulina L. (2x = 16)	*Lupularia*	(C) M-1231	A-herb	Self-pollinated
M. heyniana Greuter (2x = 16)	*Heynianae*	(C) M-839	A-herb	Self-pollinated
M. radiata L. (2x = 16)	*Hymenocarpos*	(C) M-225	A-herb	Self-pollinated
M. ruthenica (L.) Ledebour (2x = 16)	*Platycarpae*	(M) 2139-4x2082-8	P-herb	Cross-pollinated
M. edgeworthii Sirjaev (2x = 16)	"	(C) M-957	P-herb	Cross-pollinated
M. biflora (Griseb.) E. Small (2x = 16)	*Lunatae*	(C) 157	A-herb	Self-pollinated
M. monantha (C. A. Meyer) Trautv. (2x = 16)	*Buceras*	(C) T-133	A-herb	Self-pollinated
Trigonella foenum-graecum L. (2n = 16)	-----	PI 138685	A-herb	Self-pollinated
Melilotus officinalis (L.) Pallas (2n = 16)	-----	(A) MNLE	P-herb	Cross-pollinated
Trifolium repens L. (2n = 4x = 32)	-----	(A) DLAW	P-herb	Cross-pollinated
T. pratense L. (2n = 2x =16)	-----	cv. Kenstar	P-herb	Cross-pollinated

[a] For *Medicago* species only; following Small & Jomphe 1989

[b] Collection designations: PIs – USDA Plant Introduction Collection, Pullman, WA; (M) – T. J. McCoy, Dep. of Plant and Soil Sciences, Montana State University, Bozeman, MT; (A) – S. E. Smith, Dep. of Plant Sciences, University of Arizona, Tucson, AZ; (C) – E. Small, Centre for Land Biological Resources Research, Central Experimental Farm, Ottawa, ON

[c] A = annual, P = perennial. Based on Small & Jomphe (1989) Heyn (1963), and T. J. McCoy (pers. comm.) for *Medicago*, Meikle (1977) for *Trigonella*, Smith & Gorz for *Melilotus*, and Gillett (1985) for *Trifolium*

[d] Diploid plants derived from maternal haploids of 4x *M. sativa* (Bingham & McCoy 1979)

taining moistened filter paper. 100 µl of 3 : 1 (v/v) 95% ethanol : glacial acetic acid was added to the well and the contents transferred to a microcentrifuge tube. This process was repeated with an additional 100 µl of ethanol : glacial acetic acid (3 : 1 v/v). After 12–18 h storage at 4 °C, the tubes were centrifuged at 100 rpm for 5 min and the supernatant was replaced with 230 µl of 70% ethanol. This suspension was stored at 4 °C. For staining, 50 µl of the fixed pollen was spread on an acid-cleaned microscope slide and allowed to dry. Two drops of 0.05 µg/ml DAPI in Aqua-Poly/Mount (Polysciences, Inc., Warrington, PA) were placed on each slide, and covered with an acid-cleaned coverslip.

Plastid nucleoids in the generative cell were visualized at 1000 × using a Zeiss Axiophot epifluorescence microscope and the Zeiss 487902 combination of excitation and emission filters. The number of nucleoids in each generative cell was recorded from 9 to 37 intact generative cells from developing microgametophytes. Counts were taken only from cells in which nucleoids were clearly visible and there was no indication of mitotic activity. Photographs of 5–10 DAPI-stained generative cells per accession were taken using 400 ASA Tri-X Pan film. Dimensions of the nuclei of these cells were estimated by projecting the negative image at a fixed magnification, tracing their outlines on white paper, and measuring their maximum lengths and widths. Nuclear volumes were estimated by assuming that the nuclei were ellipsoids with circular cross-sections, and using the equation for the volume of an ellipsoid (volume = $4/3\ \pi b^2 a$, where b = 1/2 width and a = 1/2 length). Using phase contrast microscopy at 1000 ×, diameters along the widest and narrowest axes were measured on ungerminated, but hydrated, pollen grains. An average diameter was calculated for each grain using these values. Volume of pollen grains was estimated as $4/3\ \pi r^3$, where r = 1/2 diameter. Diameters of 10 pollen tubes for each accession were measured at a point 100 µm from the grain wall. In *M. sativa*, the cytoplasm is non-vacuolated and the number of plastids per generative cell is proportional to the cytoplasmic and nuclear volumes of the cell (ZHU & al. 1991). Therefore, to adjust for differences in cytoplasmic volume, the mean number of nucleoids per generative cell was represented as a fraction of the mean volume of generative nuclei for each taxon. Similarly, to correct for differences in the volume of pollen grains, the mean number of nucleoids per generative cell for each taxon was also presented as a proportion of the mean volume of pollen grains, under the assumption that nucleoid content of the microgametophyte is the same as in the mature pollen grain.

Floral characteristics. Ten mature unpollinated flowers of each *Medicago* species were fixed in ethanol : glacial acetic acid (3 : 1). These were softened and cleared in 8 M NaOH for 18 h, then transferred to 0.1 M KPO_4. Under visible light microscopy, the distance from the midpoint of the stigma to the nearest and furthest ovules were measured on excised pistils, and a mean distance was calculated using these two values. The number of ovules per ovary was also recorded. Flower size was described by measurement of the length of the standard petal based on representative herbarium specimens of each taxon grown under natural conditions (SMALL 1988).

Statistical analyses. Data were analyzed using NPAR1WAY, a nonparametric one-way analysis of variance using ranked data (SAS Institute, Inc., 1985). Taxa were grouped according to taxonomy, ploidy and mating system, and the size of flowers, pollen grains, and generative nuclei. Significance of differences between these groups was determined using the Wilcoxon rank-sum test and a *t*-test approximation. Spearman rank correlations were determined for all pairs of variables. Statistical significance was assigned at $\alpha = 0.05$ throughout.

Results

Relationships among plastid nucleoid number, and microgametophytic and floral traits. Plastid nucleoids were observed within generative cells within pol-

Table 2. Pollen morphometric variables and generative cell (GC) nucleoid numbers (Standard deviation in brackets) for 14 *Medicago* species and species from the three related core genera of the *Trifolieae*

Taxon	Nucleoids per GC	Pollen grain volume ($\mu m^3 \times 10^{-3}$)	Nucleoids per GC ÷ pollen grain volume	GC nuclear volume (μm^3)	Nucleoids per GC ÷ GC nuclear volume
M. arborea	65.7 (10.9)	24.3 (2.7)	2.71	18.6 (3.6)	3.52
M. sativa (2x)	37.4 (1.7)	10.2 (0.3)	3.68	17.5 (1.4)	2.14
M. sativa (4x)	69.1 (3.1)	15.5(1.4)	4.45	31.5 (3.8)	2.19
M. cancellata	92.1 (4.7)	39.5 (5.7)	2.33	54.6 (11.6)	1.69
M. carstiensis	42.8 (1.3)	16.5(3.1)	2.60	40.0 (9.7)	1.07
M. truncatula	31.0 (1.7)	19.1 (1.2)	1.62	18.5 (2.6)	1.67
M. scutellata	43.1 (1.5)	32.2 (2.9)	1.34	28.6 (2.9)	1.51
M. polymorpha	22.4 (1.9)	7.1 (0.4)	3.16	13.0 (2.4)	1.71
M. lupulina	22.5 (2.0)	4.6 (0.1)	4.90	9.5 (1.0)	2.37
M. heyniana	24.9 (1.2)	14.3 (1.2)	1.75	18.1 (2.7)	1.38
M. radiata	27.3 (1.9)	9.2 (0.4)	2.98	14.0 (1.6)	1.95
M. ruthenica	25.9 (1.9)	8.9 (0.7)	2.90	16.5 (2.9)	1.57
M. edgeworthii	29.0 (3.3)	9.2 (0.4)	3.16	17.6 (2.3)	1.65
M. biflora	74.6 (3.9)	40.0 (2.0)	1.87	26.0 (2.7)	2.87
M. monantha	41.1 (3.0)	20.1 (0.9)	1.98	24.3 (1.9)	1.69
Trigonella foenum-graecum	21.2 (2.4)	12.5 (0.6)	1.70	30.0 (3.4)	0.71
Melilotus officinalis	30.7(2.5)	9.1 (0.4)	3.37	16.5 (1.8)	1.86
Trifolium repens	10.4 (0.5)	9.1(0.7)	1.13	16.1 (1.7)	0.64
T. pratense	14.2 (0.9)	22.8 (1.7)	0.65	11.7 (0.8)	1.22

len tubes of all taxa examined with mean number per cell varying by more than 4 × among *Medicago* species (Table 2, Fig. 1). Significant variation was also observed among taxa in the volume of pollen grains and generative cell nuclei. Among *Medicago* taxa, plastid nucleoid number represented as a fraction of the volume of pollen grains and generative nuclei varied by more than 3 ×.

The number of plastid nucleoids per generative cell was positively correlated with the volume of pollen grains and generative cell nuclei as well as two measures of floral size (length of the standard petal and the style) (Table 3). However, the number of plastid nucleoids per generative cell adjusted for the size of the pollen grain (nucleoid density) was not related to any of the microgametophytic or floral characteristics measured. When represented relative to the size of the generative cell nucleus, plastid nucleoid number was negatively correlated only with pollen grain volume. Diameter of the pollen tube and the number of ovules per ovary were not significantly correlated with any of the basic reproductive characteristics measured (data not shown).

Reflecting the basic relationship between the number of plastid nucleoids per generative cell and generative cell nucleus and pollen grain size, significantly more nucleoids per generative cell were observed in *Medicago* taxa that were polyploid or have large flowers, large pollen grains or large generative cell nuclei (Table 4). Significantly more plastid nucleoids per generative cell were also observed in all *Medicago* taxa than in representatives from *Trigonella, Melilotus,* and *Trifolium.* Nevertheless, when plastid nucleoids per generative cell were cor-

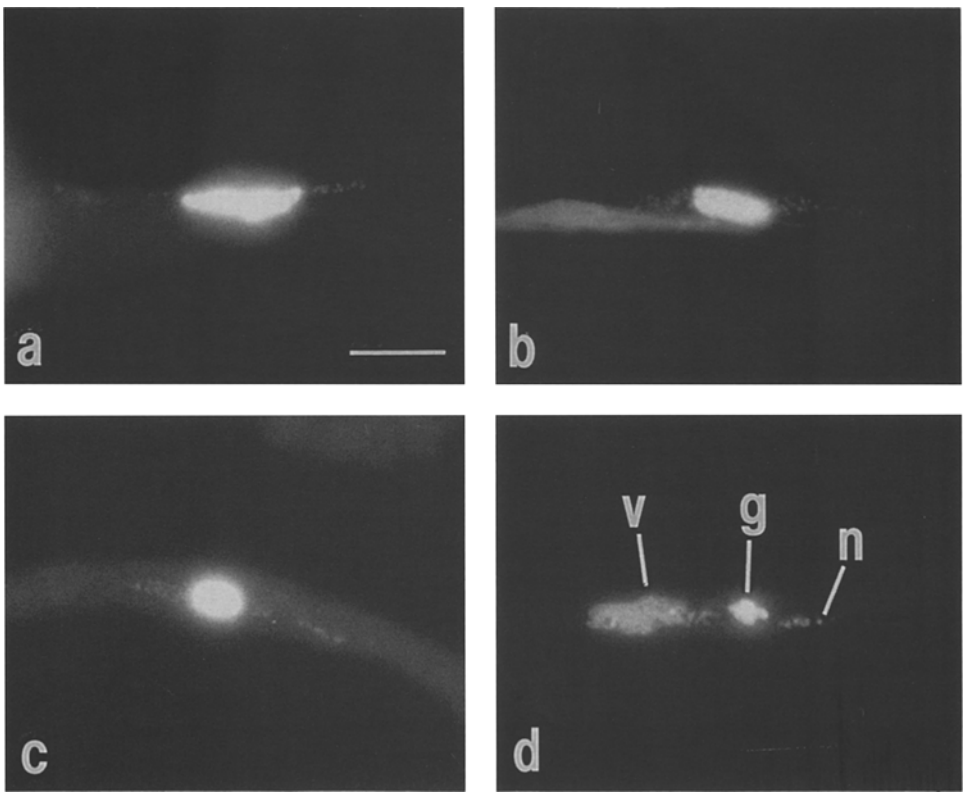

Fig. 1. Epifluorescence micrographs of DAPI-stained pollen tubes showing relative sizes of generative nuclei. *a Medicago sativa* (4x = 32), *b M. scutellata* (2n = 30), *c M. sativa* (2x = 16), *d M. lupulina* (2x = 16); *g* generative nucleus; *n* cluster of plastid nucleoids; *v* vegetative cell nucleus. The entire components of plastid nucleoids are not visible in the generative cells because of limited focal plane.– Bar: *a* – *d* 10µm

rected for the volume of the pollen grain or generative cell nucleus, significant differences were observed only for the value corrected for pollen size between *Medicago* taxa grouped by pollen size. In this case, nucleoid densities were higher in taxa with small pollen grains than in those with large pollen grains.

Discussion

Our data establish that paternal transmission of plastids is probable throughout the genus *Medicago*. Moreover, these data suggest that biparental plastid inheritance may be more common in the tribe *Trifolieae* of the *Fabaceae* than had been previously thought. Our observation of plastid nucleoids within generative cells of *Trifolium repens* and *T. pratense* disagrees with earlier reports based on observation of DAPI-stained ungerminated pollen (CORRIVEAU & COLEMAN 1988). Subsequent analysis by CORRIVEAU & COLEMAN (1991) showed that plastid nucleoids were present in pollen of *T. pratense* before maturation but were not visible after maturation. Differences between these results may reflect intraspecific variation in plastid nucleoid number within generative cells (CORRIVEAU & al. 1989; R. N.

Table 3. Spearman rank correlation coefficients and significance values (in brackets) between male gametophyte and floral characteristics for 14 *Medicago* species. *GC* generative cell

	Pollen grain volume	GC nuclear volume	Flower size[a]	Style length[b]	Nucleoids per GC ÷ GC nuclear volume	Nucleoids per GC ÷ pollen grain volume
Nucleoids per GC	0.875 (0.001)	0.879 (0.001)	0.871 (0.001)	0.815 (0.001)	0.211 (0.451)	– 0.318 (0.248)
Pollen grain volume		0.846 (0.001)	0.833 (0.001)	0.825 (0.001)	0.029 (0.920)	– 0.704 (0.003)
GC nuclear volume			0.880 (0.001)	0.771 (0.003)	– 0.200 (0.475)	– 0.496 (0.598)
Flower size				(0.921 (0.001)	0.036 0.891)	– 0.443 (0.099)
Style length					0.248 (0.437)	– 0.425 (0.169)
Nucleoids per GC/ pollen grain volume						0.468 (0.079)

[a] Length of standard petal
[b] Mean distance from stigma to most proximal ovule and distance from stigma to most distal ovule

KEYS unpubl.). However, in species where at least some plastid nucleoids have been observed in generative cells, no individual plant has yet been identified that consistently produces generative cells without plastid nucleoids.

Previous quantitative analyses of the number of plastid nucleoids in the generative cell of different genotypes of individual species (CORRIVEAU & al. 1989, SHI & al. 1991) have not considered the size of the cells of the microgametophyte when making comparisons. Differences in cellular volume among closely related taxa could be the result of a variety of factors, most notably ploidy (JULÉN 1944). Our results show that the number of plastid nucleotids is proportional to the volumes of both the pollen grain and the generative cell nucleus in the *Medicago* taxa examined. Accessions of diploid and tetraploid *M. sativa* exhibited very similar numbers of nucleoids relative to the volume of the generative cell nucleus (Table 2). This supports the contention that this correction for inherent differences in cell size provides the most accurate values for characterization of individual taxa. The volume of the generative cell nucleus has been shown to be closely correlated with the cytoplasmic volume per generative cell in *M. sativa* (ZHU & al. 1991) suggesting that plastid nucleoid number per generative cell is proportional to that cell's volume. It is noteworthy that these relationships among cell size, nuclear volume, ploidy and plastid number were also found by BUTTERFASS (1979) for mesophyll, leaf epidermal and guard cells of many species.

Because the relationships among plastid number, cell size and nuclear DNA content in photosynthetic cells, BUTTERFASS (1979) suggested that plastid number

Table 4. Mean number of nucleoids per generative cell and per generative cell nuclear volume for selected *Trifolieae* taxa grouped by taxonomy, ploidy, and mating system and size of flowers, pollen grains, and generative cell nuclei. *GC* generative cell

Characteristic, taxon	No. of taxa	Nucleoids per GC	Nucleoids per GC ÷ pollen grain volume	Nucleoids per GC ÷ GC nuclear volume
Diploid, *Medicago*	11	34.4	2.78	1.82
Polyploid, *Medicago*	4	67.5	2.71	2.23
		(0.030)[a]	(0.749)	(0.556)
Annual, *Medicago*	9	35.6	2.67	1.93
Perennial, *Medicago*	6	48.8	2.89	1.94
		(0.456)	0.527)	(0.604)
Small flowers, *Medicago*[b]	7	27.0	3.22	1.82
Large flowers, *Medicago*	8	57.4	2.36	2.03
		(0.008)	(0.077)	(0.865)
Small pollen grains, *Medicago*[c]	8	32.3	3.37	1.87
Large pollen grains, *Medicago*	7	55.8	2.06	2.00
		(0.026)	(0.021)	(0.865)
Small GC nuclei, *Medicago*[d]	8	27.5	3.02	1.81
Large GC nuclei, *Medicago*	7	61.2	2.47	2.08
		(0.007)	(0.244)	(0.692)
Self-pollinated, *Medicago*	10	35.9	2.53	1.79
Cross-pollinated, *Medicago*	5	58.0	3.21	2.22
		(0.181)	(0.264)	(0.316)
All *Medicago*	15	43.3	2.76	1.93
Trigonella, Melilotus, Trifolium	4	19.1	1.71	1.11
		(0.030)	(0.139)	(0.524)

[a] Significance level, t-test approximation, Wilcoxon rank-sum test
[b] Small flowers: length of standard petal < 7.0 mm
[c] Small pollen grains: volume < 17×10^3 μm^3
[d] Small GC nuclei: nuclear volume < 18.6 μm^3

should be adjusted by ploidy level for comparisons among taxa. Based on our results with plastid-containing generative cells, we would also stress the importance of representing nucleoid numbers as a proportion of cell volumes when comparing taxa whose inherent cell volumes vary significantly. This would be especially important when examining relationships between plastid number per generative cell and microgametophyte function among organisms.

While the absolute number of plastids per generative cell differed significantly among groups of taxa based on ploidy, life history, size of the reproductive apparatus, or mating system, plastid nucleoid density was not generally related to any of these strategic characteristics (Table 4). These results suggest that the presence of plastid nucleoids in the microgametophyte confers no obvious selective advan-

tage. Some members of the *Trifolieae* lack an inverted repeat found in chloroplast DNA of other legumes (LAVIN & al. 1990). The genera reported by CORRIVEAU & COLEMAN (1988) and HARRIS & INGRAM (1991) to express biparental inheritance of plastids also purportedly lack the inverted repeat. These genera display frequent rearrangement of the genome (PALMER & al. 1988), possibly mediated through intramolecular recombination (PALMER 1983). Assuming nuclear control of plastid replication (BUTTERFASS 1979), extensive plastome rearrangement might somehow disrupt the mechanisms of this control during microsporogenesis in genera displaying biparental plastid inheritance. If this is the case, the presence of plastid nucleoids in the microgametophyte would be simply an artifact. On the other hand, the methodology employed in this study centered on general morphological and life history traits, and the selective advantage of plastid nucleoids may only be detectable at other levels of measurement and under certain environmental or genetic circumstances.

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