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Phenotypic and Agronomic Variation Within Naturalized *Medicago polymorpha* L. (Burr Medic) in Subtropical Queensland, Australia, and Relationships with Climate and Soil Characteristics

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Abstract: To characterize the naturalized population of burr medic (*Medicago polymorpha* L.), a valuable pasture legume, in subtropical Queensland, Australia, a collection of 1747 lines from 107 sites in 11 regions was grown, and 26 phenotypic and agronomic attributes were recorded. This data matrix was analyzed by cluster, principal co-ordinates, discriminant and correlation analyses to examine line relationships based on plant attributes and their association with site characteristics of climate and soil. Among the wide polymorphism of attributes across the collection zone, there were a number of notable phenotypic associations. One of these, with large green leaves, minimally dentate leaf margins, and light purple petioles, was widely distributed. Three others, one with a distinctive magenta leaf mark, dark purple petioles, and an upright habit; one with those same attributes but with a prostrate habit; and one with grey-green leaves, high frost resistance, and the ability to stay green and to produce high pod yields, were associated with climatic and soil characteristics in the north, east, and south of the collection zone, respectively. Days to flowering were longer in lines from saline soils at lower altitude, and plant vigor was greatest in lines from more fertile soils with higher rainfall. A wide variation in time to flower of lines at all collection sites contributes to the adaptation of *M. polymorpha* in subtropical Queensland and potentially to its persistence with future climate change.

Keywords: burr medic; naturalized; phenotypic; agronomic; variation; polymorphic; climate; soil; pattern analysis

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1. Introduction

Burr medic (*Medicago polymorpha* L.) has naturalized widely across subtropical southern and central Queensland, Australia, where it occurs across more than two million hectares of pastoral, cropping, and recreational land. It is a self-regenerating annual, cool

season forage legume that provides valuable, high-quality forage to cattle and sheep enterprises, particularly in the grain and pastoral zone of the state. The spread of the species has been facilitated by the movement of spiny pods in the wool of sheep and the hair of cattle. It is accepted that burr medic is adapted to soils with a slightly acid to alkaline pH, with textures ranging from silty to clay, and persists as a consequence of setting a high proportion of hard seed [1]. In the subtropical environment to which the species is naturalized, annual rainfall decreases gradationally from east to west, and the winter growing season rainfall increases from north to south, with totals subject to strong intra- and inter-seasonal variability [2].

This study was initiated through a National Program, the National Annual Medic Improvement Program (NAMIP) funded by the Grains Research and Development Corporation (GRDC), and involved coordinated research across the agricultural zones of southern, western, and subtropical Australia. In southern and central Queensland, and in New South Wales, it was deemed important (P.S. Cocks, pers. comm.) to characterize and further understand the adaptation of the naturalized annual burr medic population, a study not conducted before in a subtropical environment.

As further background to the importance of this study, annual medics have been widely used in pastoral and ley/break farming systems in the crop/pasture zones of Australia [3–5]. Annual *Medicago* spp. are not native to Australia, occurring naturally around the Mediterranean Basin and central Asia [6]. Burr medic is a native of 34 of those countries [6]. Lines from more than 20 annual *Medicago* species [7] have been introduced into Australia, initially accidentally (and without provenance) through the emigration of European settlers in the 19th Century [6], and subsequently in processes initiated largely by the Commonwealth Plant Introduction Service (CPI). This was first established in 1912 but with an increased focus since 1937 [8], and enhanced by collections by State Departments of Agriculture, particularly in New South Wales and South Australia [6,7,9].

Ten of the introduced species have naturalized throughout Australia [8,10,11]. The most widely naturalized variety of burr medic is *M. polymorpha* L. var. *polymorpha* with spiny pods while a non-spined variety, *M. polymorpha* var. *brevispina* (Benth.) Heyn [12], is less widespread. In both Queensland and New South Wales, the spiny expression of burr medic occurs the most frequently [13]. *M. polymorpha* has also naturalized in other countries, including in the Mediterranean climate of Chile [14,15].

The evaluation and commercialization of annual *Medicago* spp. cultivars in Australia up to the 1990s was a parochial process that led to the release of niche cultivars. Beginning in the early 1990s, NAMIP was established to understand adapted species better, and develop broadly adapted, superior cultivars with a better appeal to commercializing industries and primary producers.

Our project aimed to firstly understand the polymorphic makeup of the naturalized population, and factors contributing to its adaptation to the climates and soils of subtropical Queensland. Ultimately, the objective was to involve elite germplasm in the commercial release of a cultivar adapted to the subtropics, comparable to a project conducted in Sardinia [16]. It began with the collection of naturalized germplasm in subtropical Queensland, followed by a single year of growth to necessarily record and understand the importance of the phenotypic and agronomic attributes of the species. This enabled the identification of elite lines for subsequent evaluation. This paper details the findings of the initial growth of 1747 lines of *M. polymorpha* collected from 107 locations in subtropical Queensland, by describing relationships among the phenotypic and agronomic attributes of the lines and their occurrence with respect to climate and soil characteristics measured at the collection sites. This enables a better understanding of the polymorphic and adaptive attributes as an essential precursor to the improvement of the species in subtropical Queensland.

2. Materials and Methods

2.1. Collection of Medicago polymorpha Germplasm

Pods of naturalized *M. polymorpha* were collected in bulk (rather than from individual plants) during the spring/summer of 1993/94 from 111 sites in 11 regions of Queensland, Australia (Figure 1), in which climates vary gradationally in east–west and north–south transects. The regions, defined by local government boundaries, were located within an area of 620,000 km^2. Pods were collected from above the soil surface of farming and grazing lands where possible, and from parkland. Pods collected were from plants that had grown in the spring of 1993 and were the sole source of germplasm used in this study.

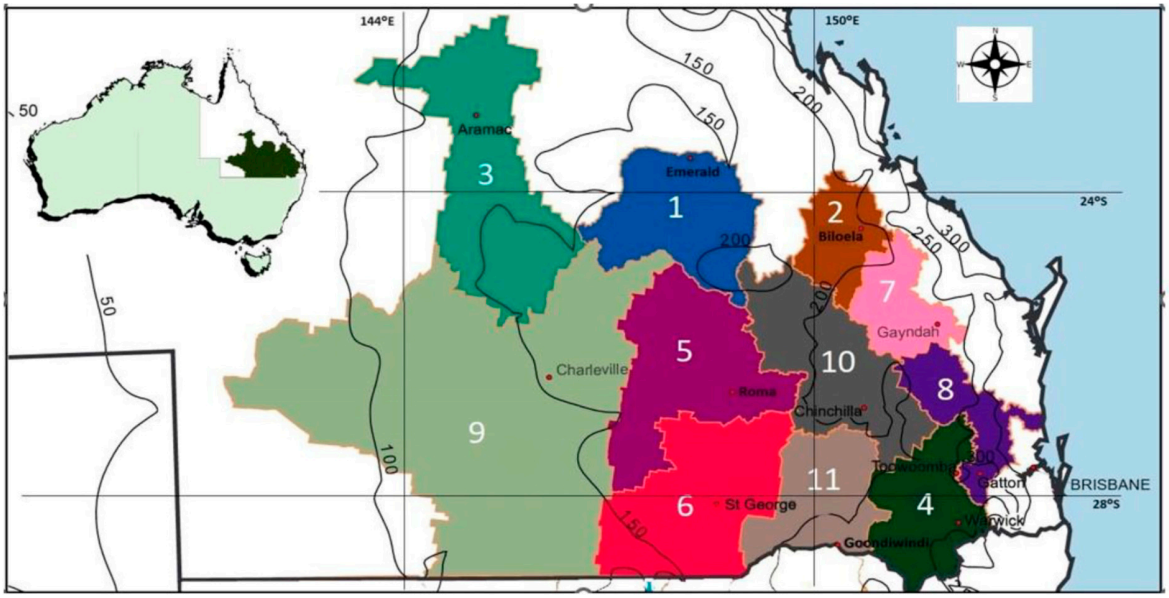

Figure 1. *Medicago polymorpha* collection regions (defined by local government boundaries) in subtropical southern and central Queensland, Australia. Numbered regions are named with their acronym and number of lines collected in parentheses: 1. Central Highlands (CH, 71); 2. Central Queensland (CQ, 171); 3. Central West (CW, 55); 4. Darling Downs (DD, 260); 5. Maranoa North (MN, 119); 6. Maranoa South (MS, 168); 7. North Central Burnett (NCB, 128); 8. South Burnett Moreton (SBM, 121); 9. South West Queensland (SWQ, 94); 10. Western Downs North (WDN, 195); 11. Western Downs South (WDS,365). Isohyets represent average growing season rainfall (April to September).

Detailed provenance data were recorded from collection sites. Surface soil (0–10 cm) samples were taken from 104 of the 111 collection sites for chemical and physical analyses as follows: pH (1:5 in $CaCl_2$ solution), electrical conductivity (mS cm^{-1}), organic carbon (C, %), total nitrogen (N, %), available phosphorus (P, Colwell bicarbonate extraction, µg g^{-1}), exchangeable cations calcium (Ca), magnesium (Mg), sodium (Na), potassium (K), and cation exchange capacity (CEC) (all as meq 100 g^{-1}), and clay content (%).

Climatic information for each collection site was obtained from the nearest recording location [17]. Data included the mean total annual rainfall (mm), mean growing season (April–September) rainfall (mm), and mean growing season maximum and minimum daily temperatures (°C). In this paper, this growing season is referred to as 'winter'.

2.2. Characterization of Phenotypic and Agronomic Variation

The phenotypic and agronomic attributes of collected lines were recorded during growth in the field at the Hermitage Research Facility, Warwick, Queensland (28.22° S, 152.02° E), on a fertile Black Vertosol [18], sown in May 1994. In preparation, pods of *Medicago laciniata* and *M. minima* that occurred concurrently but infrequently at a number of collection sites were removed. Pods of *M. polymorpha* were then threshed and the seed

abraded to soften the hard seed component. The seeds from each collection site were sown in three rows, each 4 m long and 1 m apart, in 4 m × 3 m plots in a rectangular array. The site was irrigated for establishment and twice subsequently (the last in September), to enable plants to develop to their potential. No fertilizer was applied to the site. The winter was cold with about 80 terrestrial frosts recorded during the growing season. Daily maximum and minimum temperatures and rainfall recorded during the growing season at the site are listed in Table S1 [17].

Plants that had successfully established were thinned manually to form a spaced population of 1747 plants, at least 40 cm apart to enable individual plant identification. Phenotypic variation in seedlings, following abbreviated International Board for Plant Genetic Resources (IBPGR) conventions [19], was recorded during the manual thinning process to ensure the phenotypic variation expressed by plants from each collection site was maintained. The phenotypic attributes of individual spaced plants, herein referred to as lines, were recorded in accord with the plant attributes described in Table 1.

Table 1. List of attributes used to determine the phenotypic and agronomic similarities of 1747 lines of *Medicago polymorpha.* Attributes measured at two times (*), the first was during the seedling stage, 28 days after establishment, and the second as mature plants when each plant had produced more than three flowers.

Code	Descriptor	States
QUANTITATIVE ATTRIBUTES		
DTF	Period from sowing to flowering (emergence of three flowers)	days
DTH	Period from sowing to pod harvest	days
PODYLD	Pod yield	g 0.25 m^{-2}
ORDINAL ATTRIBUTES		
FRST	Frost susceptibility	0 most resistant; 1 slightly susceptible; 2 moderately susceptible; 3 most susceptible
HABIT; HABIT1	Habit at two times *	1 prostrate, compact; 2 prostrate, open; 3 upright compact; 4 upright open
KIKON	Stay-green trait	1 hayed off most quickly after first pod formation; 2 hayed off moderately quickly after first pod formation; 3 strong tendency to continue vegetative growth after first pod formation; 4 strongest tendency to continue vegetative growth after first pod formation
PODSP	Pod type spininess	0 spineless; 1 short spines; 2 intermediate length spines; 3 long spines
VIG; VIG1	Plant vigor at two times *	1 to 9 (low to high) visual rating of biomass production
BINARY ATTRIBUTES		
LFMN	Leaf margin	0 minimally dentate; 1 strongly dentate
LFMK; LFMK1	Leaf mark at two times *	0 no leaf mark; 1 leaf mark present
LFCOL	Leaf color	0 grey-green; 1 green
LFSZE	Leaf size	0 small; 1 large
PETCOL; PETCOL1	Petiole color at two times *	0 light purple; 1 dark purple
PLT COL	Plant color	0 grey-green; 1 green

Table 1. *Cont.*

Code	Descriptor	States
	QUALITATIVE ATTRIBUTES	
LFMKC; LFMKC1	Leaf mark color at two times *	1 narrow purple margin pertaining to inverted Y shape; 2 broad purple margin pertaining to inverted Y shape; 3 dark purple blotching; 4 light purple blotching; 5 broad magenta margin pertaining to inverted Y shape; 6 white; 7 purple and white; 8 dark or light purple blotching with cinnamon dot; 9 cinnamon; 10 light purple with magenta midrib; N.C. no comparison if LFMK or LFMK1 is 0, respectively
LFMKP; LFMKP1	Leaf mark position at two times *	1 proximal, on upper side only; 2 proximal, lower side only; 3 both upper and lower sides, not restricted proximally; N.C. no comparison if LFMK or LFMK1 is 0, respectively
LFMKS; LFMKS1	Leaf mark shape at two times *	1 midrib line only; 2 inverted Y shaped; 3 heart shaped; 4 lightly purpled inverted Y with flecking; 5 flecking; 6 midrib line, cinnamon-colored with flecking; 7 inverted Y with dark purple margin and flecking; 8 inverted Y with magenta margin and flecking; 9 inverted Y with cinnamon margin and flecking; 10 inverted Y, cinnamon dot, with flecking; N.C. no comparison if LFMK or is 0, respectively

2.3. Numerical Analysis

A plant data matrix was formed consisting of 1747 individual lines each with 24 attributes, 3 of which were quantitative, 7 ordinal, 8 binary, and 6 qualitative (Table 1). No comparison (blank entry) was made between leaf-mark-dependent attributes of color, position, or shape where no mark was present on the leaves. The complete data matrix is given in Table S2, the legend to attributes in Table S2 is given in Table 1, and the legend to geographic regions and collection sites in Table S2 is given in Table S3.

Pattern analysis [20] was conducted on the data using multivariate analysis programs for hierarchical clustering and ordination in Genstat (VSN International 2023) [21]. A similarity matrix between the 1747 medic lines was produced using the Euclidean metric for the quantitative and ordinal attributes and a simple matching coefficient for the binary and qualitative attributes. From the similarity matrix, a hierarchical cluster analysis was produced using complete linkage as the clustering strategy with results displayed as a dendrogram. From the same similarity matrix, a principal co-ordinates analysis (PCoA) [22] was also produced.

Clusters of lines were delimited in the dendrogram at a similarity index of 0.65 and the mean values of their attributes were tabulated as well as the mean values of their collection site characteristics. A diagnosis of the top furcations of the dendrogram was conducted according to the method used in the program GROUPER [23]. The disproportionate representation of certain clusters in certain collection regions was tested in a two-way contingency table for interaction by chi square (χ^2), and the CHIPERMTEST in Genstat [21] was used to account for cells containing <5 lines by the method of Rolf and Bentzen [24].

The first ten PCoA axes were diagnosed in terms of attributes by correlation analysis as described by Lance et al. [23]. Similarly, the correlation coefficients between the first five PCoA axes and the extrinsic collection site characteristics were determined. The climate characteristics of the collection sites used for these purposes were as follows: latitude

(°S), longitude (°E), altitude (m), annual rainfall (mm), winter rainfall (mm from April to September), average daily maximum winter temperature (°C), and average daily minimum winter temperature (°C). The soil characteristics of the collection sites used were as outlined in Section 2.1.

The correlation coefficients between seven agronomic attributes and collection site characteristics were determined. These agronomic attributes were the two vigor ratings (VIG and VIG1), days to flowering (DTF), days to harvest (DTH), frost susceptibility (FRST), stay green (KIKON), and pod yield (PODYLD).

The average values of the attributes of the *M. polymorpha* lines for each of the regions were determined. A linear discriminant analysis of the *M. polymorpha* lines based on plant attributes and grouping by region was conducted. For this purpose, secondary leaf mark attributes that were scored N.C. were entered as zeros in the data matrix. Correlation analyses of the discriminant scores for regions with the plant attributes and site characteristics of climate and soil were conducted.

3. Results

3.1. Descriptions, and Climate and Soil Characteristics of the Collection Sites

The provenance data from collection sites are given in Table S4. The climate and soil characteristics determined for the individual collection sites are presented in Table S5 and mean values for regions in Table S6. The range, with median in parentheses, for the climate characteristics of the collection sites were as follows: latitude 22.97–28.77° S (27.33° S); longitude 144.27–153.13° E (150.25° E); altitude 3–675 m (283 m); annual rainfall 335–1621 mm (612 mm); growing season rainfall 118–607 mm (200 mm); mean growing season maximum daily temperature 17.9–26.3 °C (22.8 °C); and mean growing season minimum daily temperature 4.3–12.2 °C (7.8 °C).

The majority of soils at collection sites were neutral to alkaline silty clays or clays of at least moderate fertility. Their characteristics were as follows (range with median values in parentheses): clay content 15–65% (38%); pH 5.78–8.76 (7.05); electrical conductivity 0.05–0.49 mS cm^{-1} (0.12 mS cm^{-1}); organic C 0.46–3.16% (1.47%); total N 0.04–0.39% (0.14%); available P 17–260 μg g^{-1} (38.2 μg g^{-1}); exchangeable Ca 3.2–41 meq 100 g^{-1} (19 meq 100 g^{-1}); exchangeable Mg 0.30–26 meq 100 g^{-1} (6.3 meq 100 g^{-1}); exchangeable Na 0.01–5.0 meq 100 g^{-1} (0.84 meq 100 g^{-1}); exchangeable K 0.36–2.6 meq 100 g^{-1} (0.9 meq 100 g^{-1}); CEC 5–68 meq 100 g^{-1} (29 meq 100 g^{-1}).

3.2. Clustering of M. polymorpha Lines

3.2.1. Hierarchical Cluster Analysis Based on Plant Attributes

The full hierarchical cluster analysis is displayed as a dendrogram in Figure 2a and as a truncated dendrogram at a similarity level of 0.65 in Figure 2b. The 15 clusters of lines delimited at the similarity level of 0.65 are designated from A to O, and the major furcations in the dendrogram are numbered from 1 to 14. The *M. polymorpha* lines composing these clusters are given in Table S7. A diagnostic table giving the average values of the 15 clusters for the plant attributes and states of qualitative plant attributes is given in Table S8. A second diagnostic table giving the average values of the 15 clusters for the collection site climate and soil characteristics is given in Table S9. A diagnostic table of plant attributes for the 14 major furcations in the dendrogram that separate 15 clusters of lines is given in Table S10, and for site climate and soil characteristics in Table S11.

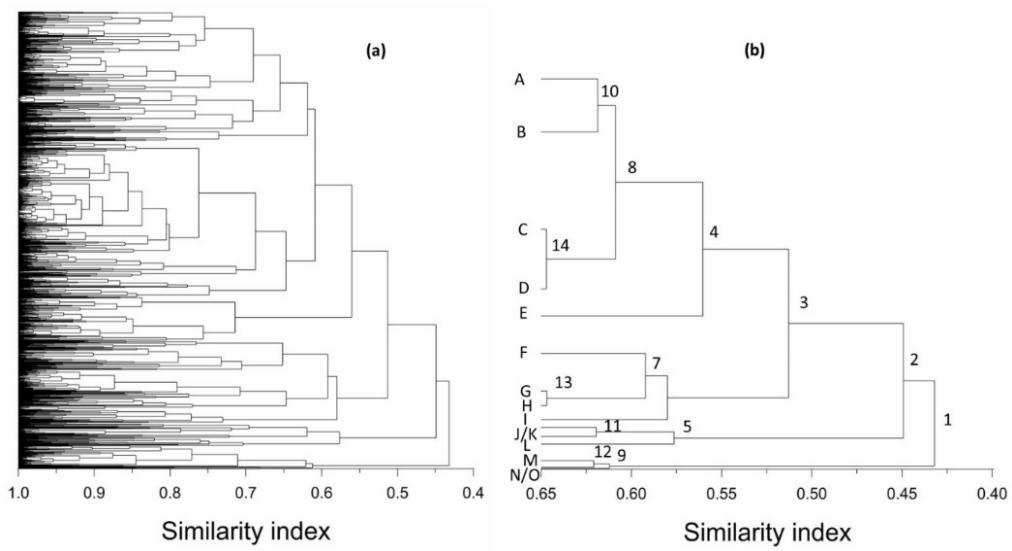

Figure 2. (**a**) Dendrogram of 1747 *Medicago polymorpha* lines depicting a cluster analysis based on 24 attributes. (**b**) The same dendrogram truncated at a similarity level of 0.65 delineating 15 clusters labelled A to O containing the following numbers of *M. polymorpha* lines given in parentheses: A (454); B (41); C (517); D (79); E (165); F (113); G (107); H (47); I (48); J (46); K (11); L (30); M (81); N (7) and O (1). Major furcations of the dendrogram are numbered 1 to 14.

3.2.2. Associations Among Phenotypic and Agronomic Attributes of *M. polymorpha* Lines Composing the Clusters and Their Occurrence Within Regions

The mean values for attributes and attribute states of the 15 clusters of *M. polymorpha* lines delimited in the hierarchical cluster analysis are presented in Table S8. Most defining attributes occurred widely across clusters (Table S8). That is, many attributes were common between clusters, defined by a similarity index of 0.65, with no single cluster separating from the other clusters at the first furcation (similarity index 0.43), the point at which clusters A–I separated from J–O. That broad separation occurred well before the first cluster, namely cluster E, separated from the other nine (similarity index 0.57).

Despite the similarities, the initial multi-group separation was based on a small number of consistent associations of mainly phenotypic attributes (Table S9). The results of the chi-square analysis of clusters within regions are given in Table S12, and with over-representation or under-representation of the 15 major clusters in the 11 regions shown in Table 2. The clusters were delineated by a high proportion of lines having attributes and geographic distributions as follows:

- Clusters A, B, C, D, and E representing 72% of the population had a combination of large green leaves with minimally dentate leaf margins, light purple petioles, and intermediate and long pod spines. The majority of these lines expressed a leafmark comprising an inverted Y with a light purple margin. These lines were widely distributed across the collection zone but most frequently in the Maranoa South, Darling Downs, North Central Burnett, South Burnett Moreton, Western Downs South, Central Highlands, and Central West regions.
- Cluster F (6.5% of the population) was characterized by lines with small green leaves with widely varying leaf marks including blotching and flecking that produced short-intermediate pod spines, and low pod yields. Lines in this cluster occurred most frequently in the Central Queensland and Maranoa North regions.
- Cluster G (6.1% of the population) was characterized by leaves of both green (77%) and grey-green (23%) color, widely varying leaf marks, long pod spines and low pod yields. This cluster was well represented in the South Burnett Moreton and Western Downs South regions.

- Cluster H (2.7% of the population) lines produced pods with short spines including a number with spineless pods, occurring with greatest frequency in Western Downs North and Western Downs South regions.
- Cluster I (2.7% of the population) expressed the greatest proportion of small grey-green leaves with a variety of leaf marks. Small grey-green leaves were expressed with the greatest frequency by lines from the Western Downs North and South West Queensland regions. Grey-green leaves were nevertheless expressed more widely.
- Clusters J, K, and L (5% of the population) had flecked and blotched leaves, with Cluster L differentiating through the expression of large grey-green leaves. These were sporadically distributed at low frequency across the collection zone, but particularly in the North Central Burnett, South Burnett Moreton, Western Downs South, Maranoa North, Maranoa South, and Central Highlands regions.
- Clusters M, N, and O (5% of the population) expressed a band of magenta coloring around a proximal inverted Y leaf mark, dark purple petioles, a strong tendency to be upright rather than prostrate, and mainly intermediate length pod spines. These attributes were not exclusive to any one cluster, but occurred with greatest frequency in the Central Queensland, Central Highlands and Central West regions. While these clusters expressed a strong level of similarity, cluster N was differentiated by the occurrence of a proportion of grey-green leaves. The differentiation of the single line cluster O from clusters M and N was associated with the rare association in O of an early time to flower and a short stay green period after flowering, with a shorter period until harvest, frost resistance, and a high pod yield.

Table 2. Two-way contingency table of the numbers of *Medicago polymorpha* lines in 15 clusters from the hierarchical cluster analysis and their 11 regions of origin. Substantial contributions of cells to the chi-square statistic * ($\chi^2 > 9$) through over-representation or under-representation of a cluster in a region are highlighted in yellow or blue, respectively. The names of regions according to their acronym, and number of lines collected in parentheses: 1. Central Highlands (CH, 71); 2. Central Queensland (CQ, 171); 3. Central West (CW, 55); 4. Darling Downs (DD, 260); 5. Maranoa North (MN, 119); 6. Maranoa South (MS, 168); 7. North Central Burnett (NCB, 128); 8. South Burnett Moreton (SBM, 121); 9. South West Queensland (SWQ, 94); 10. Western Downs North (WDN, 195); 11. Western Downs South (WDS, 365).

Regions	**Clusters**															**Total**
	A	**B**	**C**	**D**	**E**	**F**	**G**	**H**	**I**	**J**	**K**	**L**	**M**	**N**	**O**	
CH	24	0	13	10	0	4	0	0	0	8	0	2	8	2	0	71
CQ	3	1	56	14	8	34	5	0	0	2	0	0	48	0	0	171
CW	12	0	17	12	0	5	1	0	0	1	0	3	3	0	1	55
DD	71	23	96	4	17	16	6	2	8	0	1	4	11	1	0	260
MN	43	6	17	4	3	22	13	0	1	1	5	0	4	0	0	119
MS	71	1	32	7	24	12	8	1	2	0	5	5	0	0	0	168
NCB	41	0	71	0	1	0	7	0	0	0	0	8	0	0	0	128
SBM	14	3	70	0	1	1	16	0	1	15	0	0	0	0	0	121
SWQ	32	0	20	7	3	0	15	1	14	0	0	0	2	0	0	94
WDN	48	1	59	5	15	9	15	18	17	0	0	0	5	3	0	195
WDS	95	6	66	16	93	10	21	25	5	19	0	8	0	1	0	365
Total	454	41	517	79	165	113	107	47	48	46	11	30	81	7	1	1747

* Chi-square (Pearson) = 1279.59 with 140 *d.f.*, $p < 0.001$; range of values from 4999 permutations = 86.43–211.38, $p < 0.001$.

3.2.3. Association of Plant Clusters with Extrinsic Climate Characteristics

The average cluster values for latitude and longitude co-ordinates of line collection sites (Table S9), fell mainly within a limited range within the collection zone, emphasizing the extensive area from which lines within each of the 15 clusters were collected. Mid-points of 13 of the clusters based on these average coordinates occurred in a band across southern inland Queensland with one (B) in Darling Downs, five in both Western Downs North (C, F, G, L, and N) and Western Downs South (A, E, H, I and J), and one in each of Maranoa North (D) and Maranoa South (H). Two cluster mid-points were located further to the north, one in Central Queensland (M), and one, represented by a single line, in the Central West (O).

Thus, phenotypic and agronomic attributes of lines and climate and soil characteristics at their collection sites occurred broadly across the collection zone. They were not exclusive to one or more clusters and occurred without gradational change. Many lines in clusters J and B were collected from the mesic South Burnett Moreton and Darling Downs regions that receive the most winter rainfall. Lines in clusters O and K occurred in more arid locations that receive the least growing season rainfall. Lines in cluster J occurred in locations skewed to the south-east that experience the coolest growing season temperatures.

3.2.4. Association of Plant Clusters with Extrinsic Soil Characteristics

Clusters containing lines that had associations with various extrinsic soil characteristics (Table S9) were as follows:

- Cluster H (mid-point in Western Downs South) was associated with soils with the highest electrical conductivity and exchangeable Mg and Na, and thus soils with a higher level of salinity than for those in cluster K (mid-point in Maranoa South), in which the levels of those cations were the lowest;
- Cluster C (mid-point in Western Downs North) was associated with soils with the highest organic C and total N, and thus higher soil fertility than for soils associated with cluster N (mid-point in Western Downs North) and O (in Central West), in which those levels were the lowest;
- Cluster B (mid-point in Darling Downs close to the border with Western Downs North) was associated with soils with the lowest pH, P and Ca.

The average clay content of soils at collection sites for 14 of the clusters of *M. polymorpha* ranged from 37.1% (Cluster N) to 46.1% (Cluster I). An exception was the collection site of the single line cluster O with only 18.0% clay. The fertility of the soil at that site was low; the values for electrical conductivity, total N, organic C, and Ca were the lowest of all clusters, but the site had the highest levels of Mg, K, and CEC. This combination suggests an unusual soil on which that line had naturalized.

3.3. Principal Co-Ordinates Analysis

3.3.1. Principal Co-Ordinates Analysis (PCoA) of *M. polymorpha* Lines Based on Phenotypic and Agronomic Attributes

The latent roots from the PCoA are given in Table S13. The percentage variation in the population accounted for by the latent roots is shown as a scree plot in Figure 3. The first, second, and third PCoA axes accounted for 17.3%, 11.2%, and 8.2%, respectively, of the variation, for a cumulative total of 36.7%, while the first five and first ten latent roots of the PCoA accounted for 51% and 76.5%, respectively, of the total variation in the plant attributes of the population.

The latent vectors (co-ordinates) for the 1747 *M. polymorpha* lines on the first ten PCoAs are given in Table S14. The positions of those lines are shown on the plane of PCoA 1 and 2 in Figure 4a and on the plane of PCoA 1 and 3 in Figure 4b.

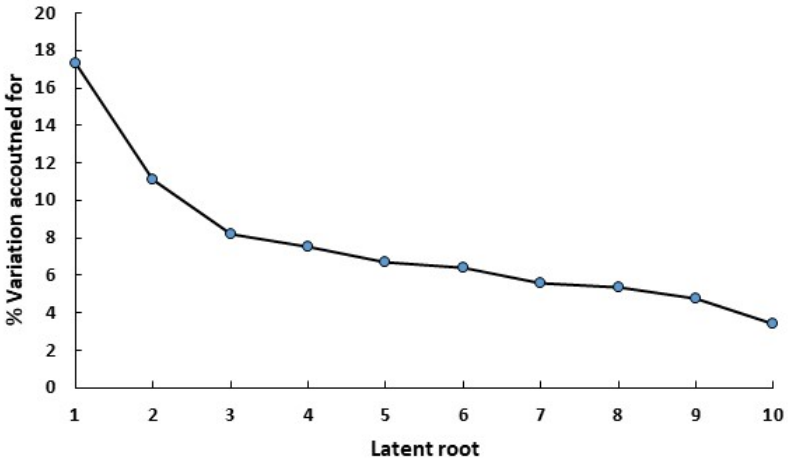

Figure 3. Scree plot of the percentage variation accounted for by latent roots from the PCoA of 1747 lines of *Medicago polymorpha*.

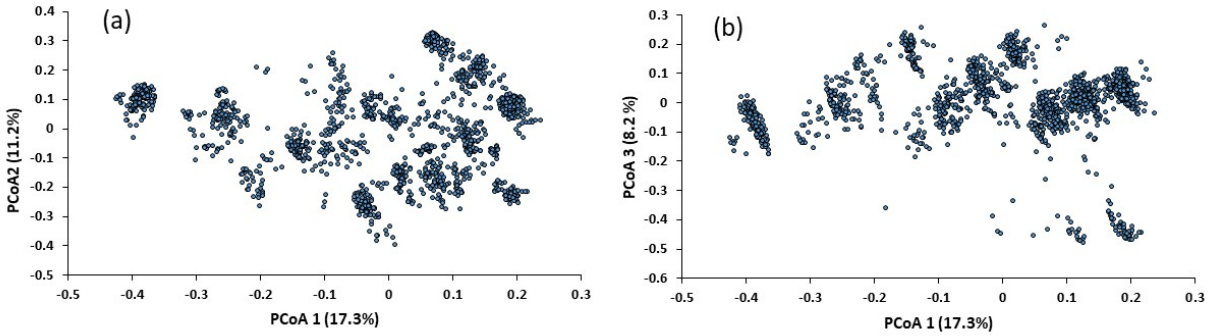

Figure 4. Projections of the 1747 lines of *Medicago polymorpha* as points on the planes of (**a**) axes 1 and 2, and (**b**) axes 1 and 3 from the principal co-ordinates analysis (PCoA). Values in parentheses are the percentage of the total variation explained by each PCoA axis out of the 24 attribute axes.

3.3.2. Diagnosis of the Principal Co-Ordinates Analysis by Intrinsic Plant Attributes

Correlations of the plant attributes with the PCoA vectors are given in Table S15. Those attributes that were significantly ($p < 0.001$) correlated ($r > 0.1$) with the first three principal co-ordinates are given in Table 3.

Plant attributes significantly correlated with PCoA 1 (Table 3, Figure 4a) enable the following deductions. Leaf markings positively correlated with this main axis were an inverted Y in a proximal location on the upper laminae, with cinnamon markings and light purple blotching on juvenile plants. Lines with the distinctive inverted Y with magenta margins and intensely purple petioles, were also strongly associated with PCoA1. Leaves and plants were mainly green rather than grey-green. Negative correlations, mainly with less frequently expressed leaf marks, included white flecking, dark purple blotches, and cinnamon-colored dots, thus implying the absence of these marks.

Plant attributes correlated with PCoA 2 (Table 3, Figure 4a) indicate that leaves of lines along this axis generally had no lower laminae markings, were cinnamon-colored on the upper laminae, were blotched light purple as juveniles only, with significant numbers having a midrib line only, and some having no leaf mark. Some expressed an inverted Y mark with purple margins. Lines were more vigorous as adults, with large green leaves, light green petioles, produced pods with longer spines, and were later flowering. They were mainly prostrate and frost-resistant, with little or no expression of the magenta margin leaf mark color.

Table 3. Attributes and attribute states (indicated by S following the attribute name) having significant ($p < 0.001$) correlation coefficients ($r > 0.1$) with the first three vectors from the principal co-ordinates analysis (PCoA) based on 1747 lines of *Medicago polymorpha*. (Table 1 provides a full description of each attribute and attribute states where appropriate).

Attribute	PCoA_1	Attribute	PCoA_2	Attribute	PC0A_3
LFMKP_1_S1	0.7385	LFMKC_1_S9	0.728	LFMKP_S2	0.4739
LFMK	0.7192	LFMKC_S4	0.6899	LFMKC_S4	0.4127
LFMK_1	0.7188	LFMKS_S1	0.6068	LFMKC_1_S9	0.2842
LFMKP_S1	0.6876	LFMKP_S1	0.4696	LFMKC_1_S4	0.2079
LFMKC_1_S9	0.5249	LFMKS_1_S1	0.377	LFMKP_1_S1	0.1428
LFMKS_1_S2	0.5029	PODSP	0.3045	LFMKC_1_S8	0.1426
LFMKC_S4	0.3711	LFCOL	0.2535	LFMK	0.1341
LFMKS_S2	0.3188	PLTCOL	0.1771	LFMKS_1_S2	0.1331
LFCOL	0.2819	LFSZE	0.1587	LFMKS_S2	0.1014
PLTCOL	0.229	VIG_1	0.1583	LFMKC_S1	0.0997
LFMKC_1_S5	0.162	DTF	0.131	VIG_1	−0.0968
PETCOL	0.1606	LFMK_1	−0.0814	PODSP	−0.1034
PETCOL1	0.1556	LFMKC_1_S1	−0.1402	LFMKS_1_S7	−0.1073
DTH	0.1265	HABIT_1	−0.1971	LFMKS_1_S8	−0.1229
LFMKC_S5	0.1195	FRST	−0.2034	PLTCOL	−0.1273
HABIT_1	0.0937	LFMK	−0.2198	LFCOL	−0.1327
LFMKS_1_S4	−0.0985	LFMKC_S2	−0.2507	KIKON	−0.1368
LFMKS_1_S6	−0.1119	LFMKC_S5	−0.2736	LFMKP_1_S3	−0.1428
LFMKC_1_S8	−0.1217	LFMKC_1_S5	−0.278	LFMKC_S3	−0.1436
LFMN	−0.1229	PETCOL1	−0.2833	LFSZE	−0.1541
LFMKS_1_S9	−0.1292	PETCOL	−0.288	LFMKC_1_S3	−0.1562
LFMKS_S7	−0.1356	LFMKS_1_S2	−0.3442	PODYLD	−0.1623
LFMKC_S3	−0.1473	LFMKC_1_S4	−0.3533	DTF	−0.232
LFMKC_S6	−0.1562	LFMKC_1_S8	−0.4267	DTH	−0.2374
LFMKC_1_S4	−0.1574	LFMKC_S1	−0.5102	LFMKP_S1	−0.3922
LFMKC_S2	−0.1702	LFMKP_S2	−0.543	HABIT_1	−0.4429
LFMKC_1_S6	−0.1887	LFMKS_S2	−0.5622	LFMKC_1_S5	−0.8045
LFMKS_S5	−0.2054			LFMKC_S5	−0.8058
LFMKS_S6	−0.2264			PETCOL1	−0.8092
LFMKS_1_S5	−0.242			PETCOL	−0.8226
LFMKC_S1	−0.2737				
LFMKC_S7	−0.3076				
LFMKS_1_S10	−0.3796				
LFMKP_S2	−0.3923				
LFMKC_1_S3	−0.4231				
LFMKC_1_S7	−0.444				
LFMKS_1_S7	−0.5028				
LFMKS_S4	−0.5651				
LFMKP_S3	−0.6589				
LFMKP_1_S3	−0.7385				

Plant attributes correlated with PCoA 3 (Table 3, Figure 4b) enable the following deductions to be made. Lines had predominantly smaller grey-green leaves and light purple petioles giving the plants a grey-green appearance, were of low vigor, mainly prostrate, produced shorter pod spines, flowered and were harvested earlier, did not stay green for long after flowering, and produced lower pod yields. Leaf marks of most lines were on the proximal lower leaf laminae as juvenile plants but on the upper sides as adults, and expressed mainly as a proximal inverted Y with light purple margins. The leaves of some lines showed purple blotching with a cinnamon dot, while many were tinged cinnamon.

These correlations with PCoA vectors support the existence of limited associations between some phenotypic and agronomic attributes of lines that have been defined by cluster analysis, namely, with leaves with a proximal inverted Y on the upper laminae; leaves with a distinctive magenta margin around a proximal inverted Y, with dark purple petioles; small, grey-green leaves; shorter pod spines; early flowering lines; and lines producing lower pod yields. On the other hand, leaf blotching and cinnamon markings occurred more randomly across the population.

3.3.3. Correlations of the Principal Co-Ordinates with Extrinsic Collection Site Characteristics

Correlations between principal coordinates and climate and soil characteristics at the collection sites are given in Table S16 and significant correlations ($p < 0.05$) are presented in Table 4. Correlation between the ten PCoA vectors and site characteristics indicate that lines with attributes associated with seven of those PCoA vectors are significantly correlated with climate and soil characteristics, with three correlated with soil characteristics only.

Table 4. Site climate and soil characters with significant ($p < 0.05$, $n = 107$ for climate and 101 for soil) correlation coefficients with the site centroids from the first ten vectors from the principal co-ordinates analysis (PCoA) based on plant attributes of 1747 lines of *Medicago polymorpha*. Highlighting indicates statistical probability of the correlation coefficient with yellow $p < 0.01$ and green $p < 0.05$.

Character	PCoA	Character	PCoA
	PCoA 1		**PCoA 7**
Na	0.2405	Longitude	0.2636
Temp Max Winter	0.2276	Rainfall Winter	0.2203
Temp Min Winter	0.2025	Rainfall Annual	0.2058
Org_C	−0.1893	Org_C	0.1983
Total_N	−0.1936		**PCoA 8**
CEC	−0.2448	Latitude	0.2064
Ca	−0.2562	Mg	−0.2055
Clay_%	−0.2955	Temp Max Winter	−0.2094
Altitude	−0.3009		**PCoA 9**
	PCoA 2	Temp Min Winter	0.2301
pH	0.2132	P_Colwell	0.1957
	PCoA 4		**PCoA 10**
Ca	0.201	K	−0.227

Lines positioned along PCoA Vector 1 were correlated with soil and climate characteristics that associate with sites which experience high growing season temperatures and at low altitudes thus, by implication, across a range of warm growing season, low-altitude environments, namely in an arc across northern regions and into the south-west of the collection zone, and in soils with a lower fertility as defined by negative correlations with clay content, organic C, total N, CEC, and Ca, and those of greater sodicity. Amongst the remaining six vectors, lines associated with PCoA 7, which explained only a small percentage (5.6%) of the variation in the population, occurred in eastern parts of both the north and south of the collection zone that experience higher annual and winter rainfall. These associations have some geographic specificity but nevertheless occur widely across regions (Tables S4 and S9).

3.4. Discriminant Analysis

3.4.1. Discriminant Analysis of Regions Based on Plant Attributes

The average values of plant attributes for the eleven regions in Queensland are given in Table S17, and the data matrix used for discriminant analysis is given in Table S18. The latent roots from the discriminant analysis are given in Table S19, and the percentages of variance accounted for by the six statistically significant latent roots are presented in Figure 5. Further results from the discriminant analysis are correlations between plant attributes and discriminant functions for regions (Table S20), mean discriminant scores for the 11 regions (Table S21), and Mahalanobis D-squared distances between regions (Table S22). The distribution of the 1747 *M. polymorpha* lines by region on discriminant function 1 is shown in Figure 6. The positions of regional means on the plane defined by discriminant functions 1 and 2 are given in Figure 7. Major correlations between plant attributes and the first two discriminant functions are given in Table 5. Correlation coefficients between discriminant scores for the 11 regions based on plant attributes and mean regional climate and soil characteristics are given in Table S23, and statistically significant correlations with discriminant scores 1 and 2 are presented in Table 6.

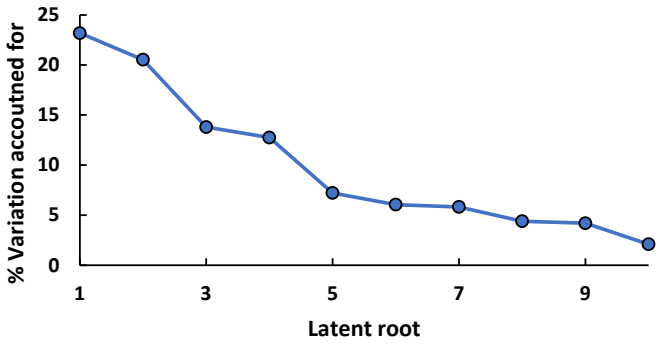

Figure 5. Scree plot of the percentage variation accounted for by latent roots from the discriminant analysis of regions based on phenotypic and agronomic attributes of 1747 lines of *Medicago polymorpha*.

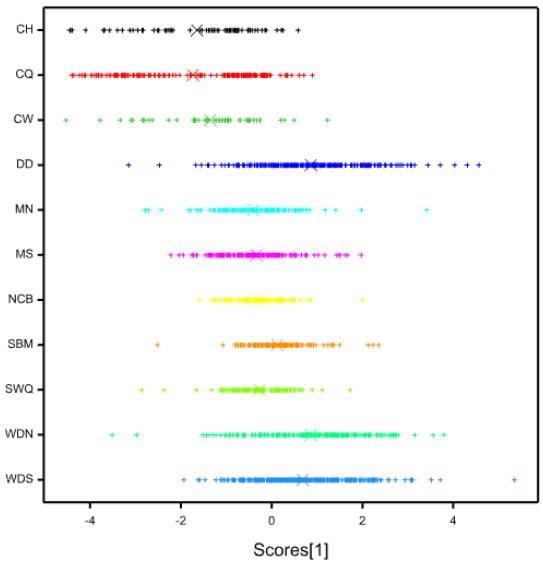

Figure 6. Distribution of the 1747 *Medicago polymorpha* lines grouped by region on the first discriminant axis from discriminant analysis based on plant attributes, which accounted for 23.5% of the variation. Region acronyms and numbers of lines are: Central Highlands (CH, 71); Central Queensland (CQ, 171); Central West (CW, 55); Darling Downs (DD, 260); Maranoa North (MN, 119); Maranoa South (MS, 168); North Central Burnett (NCB, 128); South Burnett Moreton (SBM, 121); South West Queensland (SWQ, 94); Western Downs North (WDN, 195); Western Downs South (WDS, 365).

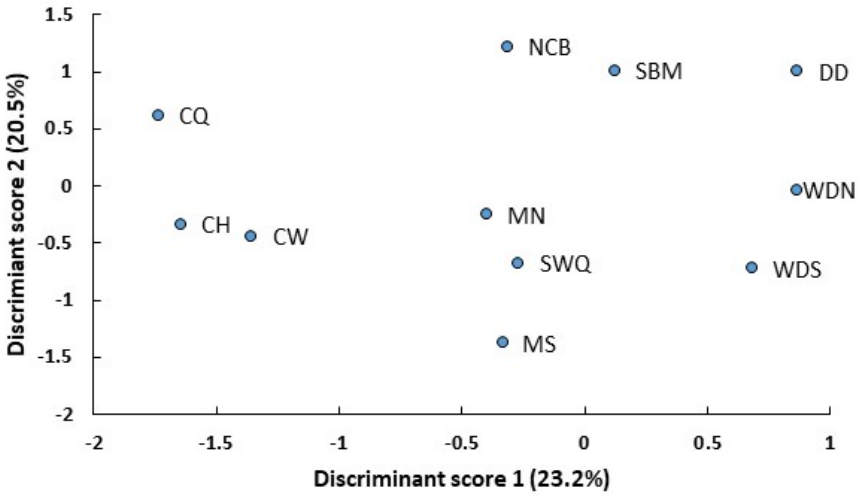

Figure 7. Distribution of the 11 regional means of discriminant scores 1 and 2 from the discriminant analysis of regions based on plant attributes of 1747 lines of *Medicago polymorpha*. Region acronyms and numbers of lines are: Central Highlands (CH, 71); Central Queensland (CQ, 171); Central West (CW, 55); Darling Downs (DD, 260); Maranoa North (MN, 119); Maranoa South (MS, 168); North Central Burnett (NCB, 128); South Burnett Moreton (SBM, 121); South West Queensland (SWQ, 94); Western Downs North (WDN, 195); Western Downs South (WDS, 365).

Table 5. Major correlations (r > 0.1) between intrinsic phenotypic and agronomic attributes of 1747 lines of *Medicago polymorpha* and the first two discriminant functions of regions from a discriminant analysis.

Attribute	DF1	Attribute	DF2
PODYLD	0.280	LFMKP_S1	0.475
LFMKC_1_S8	0.213	LFMKS_1_S2	0.411
PODSP	0.210	LFMK_1	0.398
KIKON	0.192	PODSP	0.359
LFMN	0.169	LFMKP_1_S1	0.347
LFMKC_S1	0.166	LFMK	0.302
VIG	0.151	DTF	0.291
LFMKP_S2	0.126	LFMKS_S2	0.265
LFCOL	−0.106	LFMKC_S3	0.230
LFMKP_S1	−0.154	DTH	0.212
PLTCOL	−0.164	VIG	0.205
FRST	−0.184	PODYLD	0.202
HABIT_1	−0.228	LFMKC_1_S3	0.193
VIG_1	−0.236	LFMKC_S5	0.181
LFMKC_S5	−0.266	LFMKC_1_S9	0.159
PETCOL	−0.312	PLTCOL	0.150
PETCOL1	−0.317	PETCOL	0.139
LFMKC_1_S1	−0.321	PETCOL1	0.137
LFMKC_1_S5	−0.347	LFMKC_1_S5	0.134
		LFMKC_S7	0.120
		KIKON	0.116
		LFCOL	0.112
		LFMKC_S4	0.104
		LFMKC_1_S4	−0.116
		LFMKS_1_S1	−0.118
		LFMKC_S2	−0.184
		FRST	−0.203
		LFMN	−0.231
		LFMKP_S2	−0.272

Table 6. Significant correlation coefficients (r) between the first two mean discriminant scores of eleven regions based on attributes of *Medicago polymorpha* lines and mean climate and soil characteristics of regions. Highlighting indicates statistical probability of the correlation coefficient (n = 11) with red $p < 0.001$, yellow $p < 0.01$, and green $p < 0.05$.

Site Characteristics	r
Discriminant Score 1	
Temperature max winter	−0.898
Temperature min winter	−0.767
Latitude	0.834
Discriminant Score 2	
Organic C	0.897
Rainfall annual	0.817
Total N	0.809
CEC	0.746
Longitude	0.703
Rainfall winter	0.675
Mg	0.666

The first, second, third and fourth roots accounted for 23.5%, 20.5%, 13.8% and 12.7%, respectively, of the variation in the data set, for a cumulative total of 70.2%, with the first five latent roots accounting for 77.5% of the total (Figure 5).

The breadth of the distribution of the scores of the *M. polymorpha* lines along discriminant axis 1 overlapped for all regions (Figure 6), thus representing the extensive, not strongly associated, polymorphism of the species within and among collection regions. However, *M. polymorpha* lines from the three most northerly regions of Central Highlands, Central Queensland, and the Central West were positioned more toward the negative end of discriminant axis 1 than lines from the other eight regions. Western Downs North, Western Downs South, and Darling Downs had similar midpoints, with the majority of lines within the same range. Maranoa North and Maranoa South had similar midpoints, but the range within Maranoa North was wider.

The position of the regions along discriminant axis 1 based on the attributes of *M. polymorpha* lines in those regions (Figure 7) further indicates a contrast between the three northern regions of Central Queensland, Central Highlands, and Central West, and the more southerly Darling Downs, Western Downs North, and Western Downs South. Discriminant score 2 further indicates an east–west separation (Figure 7). Together the two discriminant scores suggest grouping of the regions into four based on *M. polymorpha* attributes, with the Central Queensland, Central Highlands, and Central West regions in the north forming one group, the regions Maronoa North, Maronoa South, and South West Queensland in the southwest, and the regions North Central Burnett and South Burnett Moreton in the mid-east. The Darling Downs region appeared to share affinities with this grouping as well as with Western Downs North and Western Downs South.

3.4.2. Correlations of Regional Discriminant Scores with Plant Phenotypic and Agronomic Attributes

Significant correlations between phenotypic and agronomic attributes of the 1747 lines (Table 5) and the first two discriminant functions (Figure 7) enable a broad picture to be formed of associations of plant phenotypic attributes with the collection regions, as detailed below:

- Lines associated with discriminant function 1 had mainly grey-green leaves, strongly dentate leaf margins, light purple petioles, and a grey-green appearance. The lines

were vigorous as seedlings, mainly prostrate, and relatively frost-resistant. After flowering, they stayed green and produced high pod yields, with pods having long spines. Leaves were marked on the lower laminae with light and dark purple blotches as juvenile plants and displayed proximal inverted Y markings with purple margins as adults. Light and dark purple blotching occurred on the lower or underside of the leaf laminae in juveniles and on both upper and lower sides of the laminae as adults.

- Lines associated with discriminant function 2 had green, minimally dentate leaves, dark purple petioles, and a green appearance. The lines were vigorous as seedlings, frost-resistant, had long pod spines, flowered late and stayed green after flowering, leading to a longer period before pod harvest, and with high pod yields. Leaves were marked on the upper laminae with a proximal inverted Y with magenta margins, were tinged cinnamon, and had light and dark purple blotching. As juveniles, leaves were flecked purple and white.

3.4.3. Correlations of Regional Discriminant Scores with Site Characteristics

Correlations between discriminant scores and site characteristics (Table 6) showed that the regional discrimination based on plant attributes was largely associated with temperature as shown by negative correlations with maximum and minimum growing season temperatures and the positive correlation with latitude along major discriminant axis 1. The next most important discrimination of regions based on plant attributes along discriminant axis 2 was associated with the climate characteristics influencing water supply as shown by positive correlations with annual and growing season rainfall, and longitude. Soil fertility characteristics namely, organic C, total N, CEC, and exchangeable Mg, were also positively associated with discriminant axis 2.

The following inferences can be drawn from these analyses:

- Lines associated with discriminant function 1 described in Section 3.4.2, with mainly grey-green leaves with a range of leaf marks, a prostrate habit, a relatively high resistance to frosting, an ability to stay green after flowering and produce high pod yields, with pods having long spines (Table 5), were associated with sites in higher latitudes and lower maximum and minimum winter temperatures, and thus were found in the southern regions of the collection zone.
- Lines associated with discriminant function 2 with green, minimally dentate, leaves with a proximal, inverted Y leaf mark with distinctive magenta margin that was tinged cinnamon and/or blotched, dark purple petioles, that flowered late, stayed green after flowering, and produced high pod yields, were associated with fertile soils in higher rainfall and higher longitude environments, and thus were found in the eastern regions of the collection zone.

3.5. Correlation of Agronomic Attributes with Climate and Soil Characteristics of Collection Sites

Correlation coefficients of plant agronomic attributes with climate characteristics at 107 sites and soil characteristics at 101 sites are given in Table S24, and statistically significant correlations are given in Table 7.

Significant correlations between agronomic plant attributes and collection site characteristics were as follows.

- Days to flower was positively associated with the electrical conductivity, exchangeable Na, and pH levels of soils, and negatively with altitude, with later flowering lines occurring widely in lower altitudes across the collection zone.
- The vigor of mature plants was positively correlated with soils with higher organic C, with exchangeable Ca and K levels in locations of higher longitude receiving higher

rainfall, and thus was greatest in lines from sites with more fertile soils to the east of the collection zone.

- Frost damage indicating susceptibility to frosting was positively correlated with altitude and negatively correlated with soils with lower pH in areas of lower winter temperature, and thus was widely distributed across the collection zone.
- The trait enabling plants to stay green after flowering was positively correlated with latitude, and thus was greatest in lines widely distributed across the south of the collection zone.
- Pod yield was positively correlated with latitude and electrical conductivity, and negatively with annual rainfall, and thus was greatest in lines distributed in drier locations across the south of the collection zone. By association, pod yield was frequently greatest in lines with the ability to stay green after flowering.

Table 7. Significant correlation coefficients (r) between five agronomic attributes of *Medicago polymorpha* lines and climate characteristics of 107 sites and soil characteristics of 101 sites. Highlighting indicates statistical probability of the correlation coefficient with red $p < 0.001$, yellow $p < 0.01$, and green $p < 0.05$.

Site Characteristics	r	Site Characteristics	r
Days to flowering (DTF)		**Frost susceptibility (FRST)**	
EC	0.422	Altitude	0.346
Na	0.357	pH	−0.220
pH	0.283	Temperature max. winter	−0.279
Altitude	−0.287	**Stay-green (KIKON)**	
Vigor later (VIG1)		Latitude	0.313
Organic C	0.313	**Pod yield (PODYLD)**	
Longitude	0.274	Latitude	0.315
Ca	0.231	EC	0.211
Rainfall annual	0.217	Rainfall annual	−0.231
K	0.198		

3.6. Regional Variation in Flowering Time of M. polymorpha Lines

Climatically, the collection regions are distinctly different (Table S4). The impact of regional differences on adaptation was explored through the analysis of flowering time, which impacts on the ability of lines to set seed, particularly in marginal, subtropical environments. The number of days to flower of lines collected from the different regions is presented in Figure 8. The mean flowering time of lines from all regions ranged 100–107 days after sowing. However, lines collected from three of the driest regions, Central West, South West Queensland, and Maranoa South flowered, on average, slightly earlier, between 100 and 101 days after sowing than lines from other regions. The range in days to flower among these three regions was higher in Maranoa South than in the other regions. Nevertheless, there was an extremely wide variation in the flowering times of lines from within all collection regions, with a small number of lines flowering in fewer than 90 days after sowing, and one line flowering 130 days after sowing.

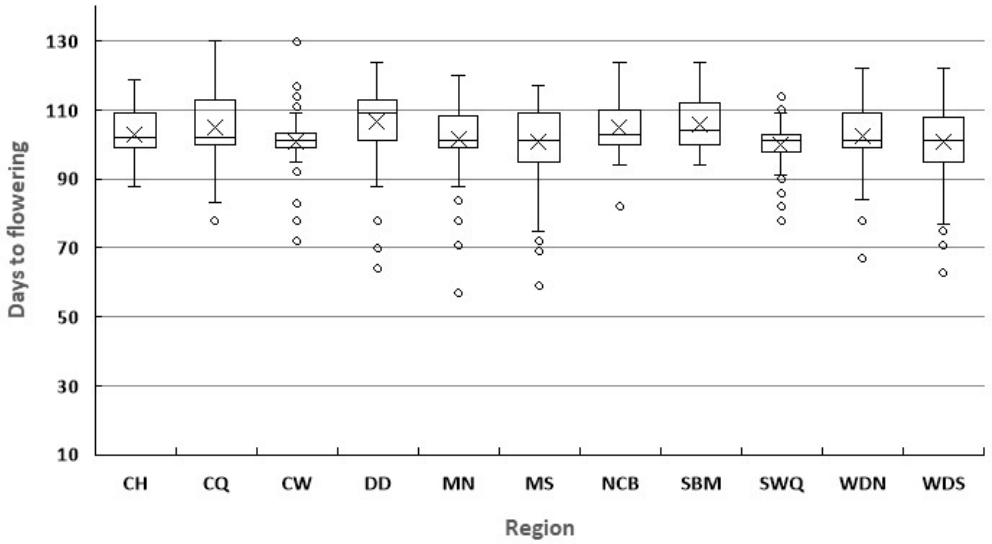

Figure 8. Box and whiskers plot of days to flowering of 1747 lines of *Medicago polymorpha* within regions. The upper and lower extremities of the box denote the upper and lower quartile values, respectively. The upper and lower extremities of the whiskers denote the maximum and minimum values, while the marked points are considered outliers. The median is shown as a horizontal line while the mean is shown as a cross. The maximum *Flsd* ($p = 0.05$) for difference between means is 3.1 days. Region acronyms and numbers of lines are: Central Highlands (CH, 71); Central Queensland (CQ, 171); Central West (CW, 55); Darling Downs (DD, 260); Maranoa North (MN, 119); Maranoa South (MS, 168); North Central Burnett (NCB, 128); South Burnett Moreton (SBM, 121); South West Queensland (SWQ, 94); Western Downs North (WDN, 195); Western Downs South (WDS, 365).

4. Discussion

This study is the first to characterize a population of *M. polymorpha* that has naturalized in the subtropics based on phenotypic and agronomic plant attributes, and to explore the relationships of those attributes with climate and soil characteristics at the collection sites. Through pattern analysis, the underlying intrinsic polymorphism of *M. polymorpha* is emphasized, with a number of plant attributes and extrinsic site characteristic relationships defined.

4.1. Associations Among Phenotypic and Agronomic Attributes of Lines in the Population

Pattern analysis comprising hierarchical cluster, principal coordinates, and discriminant analyses, has demonstrated that a wide array of associated phenotypic and agronomic attributes defines plant types that occur in almost all collection regions, thus confirming our understanding that *M. polymorpha* is a species with a widely distributed polymorphism. The single line cluster O was the most frost-resistant line in the collection. That, associated with its adaptation to an unusual soil, may have been the critical attribute in separating it from the other lines. These analyses have identified a small number of phenotypic and agronomic attribute relationships that occur together, with some associated with climate and soil characteristics. Some vary in a north–south pattern related to temperature, and others in an east–west pattern related to rainfall and soil fertility, with no one association confined to any collection region, as detailed below:

- Lines with large green leaves with a wide range of leaf marks, minimally dentate margins and light purple petioles and a variety of leaf markings that occurred frequently across all collection regions and represented a large proportion of the population, reflecting a widespread commonality within the broad polymorphism of the population.
- Lines with green leaves with a distinctive magenta margin around a proximal inverted Y leaf mark, dark purple petioles, a more upright than prostrate habit, and producing

pods with intermediate length pod spines, occurred with much lower frequency, but most frequently in regions in the north of the collection zone. Nevertheless, a small portion of the lines with this leaf mark, but including leaf blotching, sometimes tinged cinnamon, having a more prostrate habit, and with long pod spines, occurred in the east of the collection zone. The small proportion of lines expressing these attributes were distinct within the population.

- Lines with small, grey-green leaves, with short pod spines, flowering earlier and often producing lower pod yields, occurred widely but with low frequency. These lines, located predominantly in regions in the south-east, south-west, and north of the collection zone in relatively low numbers, were readily distinguishable. This leaf color was aligned with lines with grey-green leaves that were not exclusively small, that were prostrate, relatively frost-resistant, able to stay green after flowering, and produced high pod yields, with pods having long spines. These occurred in the southern regions of the collection zone.

The significance of these associations was that the leaf color attributes were identifiable within the broad polymorphism expressed, along with the fact that these attributes were reproduced in sowings of short-listed lines made in both pots and in the field in 1995 (data unpublished).

The intensive measurement of the phenotypic attributes of the lines described the polymorphism of a naturalized population of *M. polymorpha* from a subtropical environment. At the same time, the identification of three groups of associated lines in which specified leaf colors were dominant has enabled a visible recognition of these groups of lines. Despite lines with a striking magenta leaf mark per se occurring more widely, those that expressed an upright habit occurred largely in the northern regions of the collection zone, implying adaptation to that area.

4.2. Associations Among the Plant Phenotypic and Agronomic Attributes, and Climate and Soil Characteristics at the Collection Sites

Pattern analyses have suggested from the distribution of lines across the collection zone with intrinsic attributes specified in Section 4.1 that *M. polymorpha* has naturalized in different climate and/or soil environments. For example, lines associated with PCoA 1, 2 and 3 (describing 36.1% only, of the variance in the population) including, but not exclusively, lines that expressed a proximal inverted Y with striking magenta margin and purple petioles, had a significant association with soils with a low fertility in environments experiencing higher winter temperatures. These site characteristics were widely distributed, with these lines occurring mainly in low-fertility soils in a wide arc from the northern to the southwestern regions. These analyses confirmed that this readily identifiable phenotype with dark purple petioles and a striking magenta margin leaf mark, but with a more upright habit, occurred mainly in the three northern collection regions in soils with a lower fertility and clay content.

Discriminant analysis nevertheless confirmed that a proportion of lines with the same leaf mark and petiole color, but with a prostrate habit, and producing a high pod yield, occurred on fertile soils in the eastern collection regions. It also confirmed that lines with mainly grey-green leaves, a prostrate habit, a high level of frost resistance, and an ability to stay green after flowering, and produce a high pod yield, were associated with regions in the cooler south of the collection zone.

Apart from this, differences in the phenotypic and agronomic associations of lines generally were not strongly associated with the climate and soil characteristics of the collection regions.

Nevertheless, a number of unassociated plant agronomic attributes were correlated with a small number of site characteristics that occurred widely but sporadically across collection regions. These were associated with the widespread distribution of similar soil types on which the population has naturalized, across the whole collection zone. For example, lines flowering later were associated with soils with a higher level of salinity in lower altitude locations (these higher levels of salinity were well below the levels at which plant growth is compromised). Nevertheless, lines having a greater ability to stay green after flowering and produce higher pod yields were collected from higher latitudes and altitudes, an association thus limited to the more elevated south-east of the collection zone. Similarly, lines with a growth response in which the vigor of lines in their mature growth stage was greatest, occurred on fertile soils in the more mesic east of the collection zone.

This contrasts with the findings of studies carried out in Mediterranean climates. In Sardinia [25], there were clear associations in which lines of *M. polymorpha* from mountainous areas with a lower temperature had the attributes of *M. polymorpha* var. *vulgaris*, and those from warmer, coastal areas were from *M. polymorpha* var. *polymorpha*. In Chile [14], a gradational difference in the agronomic attributes of the population was strongly defined from the warmer drier north compared to the colder wetter south of the collection zone.

Studies have been carried out with native populations of *M. polymorpha* in Mediterranean environments in which the expression of pod spines has strongly featured [14,25,26]. In our study, nearly all lines produced pods with spines that varied in length from short to long, but with a small percentage producing no spines at all. These lines with spineless pods were collected from one site only, side by side with lines that produced spined pods. This unique occurrence suggests that these lines were collected from a paddock, more recently sown to the spineless cv. Santiago (based on the phenotypic attributes measured). In Sardinia [25] and in Sicily [26], no spineless lines were found. However, Del Pozo et al. [14] found spineless ecotypes in northern, semi-arid Chile, but spined lines only in colder, more mesic southern locations. Notably, the Australian cv. Santiago was derived directly from Chilean germplasm [27].

4.3. Variation in Flowering Times

Factors in adaptation are complex. Nevertheless, the time to flower of individual lines is one important factor in the adaptation of annual medics to any environment [11]. The mean times to flower of lines collected from the geographically separate regions varied significantly but not widely (100–107 days after sowing), with lines from three of the drier regions, including the two driest, flowering, on average, earlier than those from more mesic regions [28]. There was, nevertheless, wide variation in the flowering times of individual lines from every region, with a small proportion flowering in fewer than 90 days and one line after 130 days, comparable with the variation in lines collected in Tunisia [29]. The absence of a direct correlation between climatic variables and days to flower across the whole population suggests that line-by-line variation in time to flower is an important factor in the adaptation of *M. polymorpha* to this subtropical environment. Across the whole collection zone, this enhances the opportunity for one or more lines in each region to set seed in an environment which, overall, is considered marginal for the adaptation of annual medics. This marginality is attributable to rainfall during the growing season being low and sporadic, and contributing 30–40% only, to the annual total.

This is a point of difference between the traditional role of time to flower of lines in Mediterranean environments where, in more arid locations with shorter growing seasons, lines with a shorter time to flower are best adapted; in more mesic environments, lines with a longer growing season are able to capitalize on their later time to flower [11,14,30]. In the Mediterranean climate of Chile [14], and, to a lesser extent, in Sicily [26], there were strong

gradational differences in the flowering time of lines, in which those from the more arid locations flowered earlier.

Flowering in lines of annual *Medicago* spp. is controlled by different levels and extents of photoperiod, vernalization, and temperature [31–35]. One study [35] demonstrated that the flowering time of a commercial line of *M. polymorpha* (cultivar not specified) was driven by intermediate level responses to temperature and photoperiod, with flowering occurring earlier at warmer and later at cooler sites. Thus, the flowering times of the lines in this study will vary if sown in different locations from the location of this study. This plasticity, expressed as ranges in time to flower among cultivars of annual *M. polymorpha* [11], suggests that the wide variation in time to flower that was measured among lines collected at each site would continue to be represented by a wide, but not necessarily the same, range of flowering times among lines from each collection site.

With regard to the effects of soil properties on flowering time, it is noteworthy that *M. polymorpha* lines collected from more saline sites at lower altitudes that occurred widely through the study area had somewhat longer times to flower.

4.4. The Future for Naturalized M. polymorpha in Subtropical Queensland

Naturalized *M. polymorpha* has an important but sporadic role to play in the feed year budgets of livestock, mainly beef cattle but also sheep, in the Queensland subtropics [3–5]. Forage production from temperate annual legumes varies widely from year to year based on the variability in winter rainfall [2,29]. The consequences of climate change on the long-term persistence of a temperate annual legume in this subtropical region are open for speculation. In all regions in Queensland in which burr medic is naturalized, the climates are predicted to change, with all encountering warmer winters with fewer frosts, hotter summers with more days of extreme heat, and reduced rainfall, especially in winter and sometimes spring [36].

These conditions will place greater pressure on the successful establishment (often dependent on late summer–autumn rains) and subsequent growth of annual medics, resulting in a lesser, and less frequent, supply of forage. The pressure to establish, grow, and set seed in potentially drier and hotter locations may be compensated by a greater efficiency in using growing season rainfall for medic growth in warmer and drier environments [37]. It is accepted that *M. polymorpha* has naturalized in this environment, largely because of the high proportion of hard seed that is set when conditions are suitable. It is most likely that the population of *M. polymorpha* will be sustained at least in the medium term by the ongoing ability of the species to produce high proportions of hard seed, with seed remaining in the seed bank for up to 10 years. This will be enhanced by the implications of individual plants flowering across a range of time following establishment (this study), and that establishment itself may occur over a range of times as seeds soften [38]. It is also likely that attribute plasticity [39,40] and attribute drift will occur, influenced by changing environmental conditions.

5. Conclusions

Three strongly defined associations of limited numbers of mainly phenotypic attributes of 1747 lines of *M. polymorpha* collected in subtropical Queensland, Australia, within an otherwise widespread distribution of phenotypic and agronomic attributes have been identified. One large group that expressed large green leaves with minimally dentate leaf margins, light green petioles, and a variety of leaf markings occurred widely across the collection zone. Conversely, one small group that expressed a striking magenta leaf mark and a more upright habit occurred in soils with lower fertility and clay content within

three contiguous regions in the warmer north of the collection zone. These findings have provided novel information about the species in subtropical Queensland.

Relationships between the agronomic attributes of lines, and the climates and soils of the collection zone, appear limited by an absence of wide extremes in site characteristics that occur across the collection zone. There is limited variation in altitude and in climatic characteristics across the zone. This is complemented by the wide and sporadic distribution of similar soil types across the collection zone. Nevertheless, three broad groups of lines associated with climatic and soil attributes were identified: one in the southern and western regions, one in the three contiguous regions in the north, and the third in the eastern regions of the zone.

In addition, lines that flowered later were strongly associated with more saline soils in locations at lower altitudes. Lines from lower latitudes in the south had the ability to stay green after flowering. In drier locations in the south, those lines produced higher pod yields, particularly in soils with higher electrical conductivity. The stay-green ability of these lines appeared linked through discriminant analysis with vigor as mature plants when growing in soils with high fertility. Nevertheless, lines with higher vigor were widely distributed, occurring more widely across the more mesic south-east of the collection zone.

In this study in subtropical Queensland, the times to flower of individual lines from each collection region varied greatly. This attribute is likely to contribute to the adaptation and naturalization of *M. polymorpha* and its ongoing survival in the face of potential future climate change.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/agronomy15010139/s1. Table S1. Daily maximum and minimum air temperatures and daily rainfall from April to December 1994 at the Hermitage Research Facility [17]; Table S2. Individual *Medicago polymorpha* line values for 24 phenological and agronomic attributes. Attribute descriptions are provided in Table 1. Region and site details are provided in Table S4; Table S3. Legend to regions and collection sites of *Medicago polymorpha* lines; Table S4. Collection site descriptive information; Table S5. Collection site climate and soil data. Climatic information was obtained from the nearest recording locations from the Australian Government Bureau of Meteorology [17]). Winter is designated as April–September and maximum and minimum temperatures are average daily values for that period; Table S6. Mean site characteristics of the 11 regions, based on 107 sites for climate characteristics and 101 sites for soil characteristics. Climatic information was obtained from the nearest recording locations from the Australian Bureau of Meteorology [17]. Winter is classified as April–September and maximum and minimum temperatures are average daily values for that period; Table S7. Code numbers of *Medicago polymorpha* lines forming 13 clusters designated A to M at similarity level 0.65 from the hierarchical cluster analysis with number of lines in each cluster in parentheses; Table S8. Diagnostic table of 15 clusters from the hierarchical cluster analysis for assessed plant attributes (details provided in Table 1); Table S9. Diagnostic table of 15 clusters of *Medicago polymorpha* lines from the hierarchical cluster analysis in terms of collection site characteristics (107 sites with climate data, and 101 sites with soil data); Table S10. Diagnosis of the 14 major furcations of the hierarchical cluster analysis in terms of plant attributes; Table S11. Diagnosis of the 14 major furcations of the hierarchical cluster analysis of *Medicago polymorpha* lines in terms of collection site characteristics (107 sites for climate and 101 sites for soil); Table S12. Values of (observed-expected)2/expected for a contingency table of 15 clusters × 11 regions, where over-representation and under-representation are highlighted in yellow and blue, respectively; Table S13. Latent roots of the principal co-ordinates analysis of 1747 *Medicago polymorpha* line; Table S14. Co-ordinates of the 1747 *Medicago polymorpha* lines on the first ten latent vectors from the principal co-ordinates analysis; Table S15. Correlation coefficients of PCoA vectors with attributes and attribute states arranged in descending order, based on 1747 lines of *Medicago polymorpha*. Highly significant correlations ($p < 0.001$) are highlighted in yellow; Table S16. Correlation coefficients between site centroids on the first ten principal co-ordinates for lines of *Medicago polymorpha* and site characteristics of climate

(107 sites, 1797 lines) and soil (101 sites, 1694 lines). Statistically significant correlations ($p < 0.05$) are highlighted in yellow; Table S17. Average values for attributes of *Medicago polymorpha* lines from 11 regions of Queensland; Table S18. Data matrix of 1747 lines of *Medicago polymorpha* with states of secondary leaf mark attributes scored as binary (0,1) for discriminant analysis. Attribute descriptions are provided in Table 1. Region and site details are provided in Table S3; Table S19. Latent roots from the discriminant analysis of regions based on plant attributes; Table S20. Correlations between attributes of 1747 lines of *Medicago polymorpha* and discriminant functions for regions. Attribute descriptions are provided in Table 1; Table S21. Mean discriminant scores for the eleven regions based on plant attributes; Table S22. Inter-regional distances (Mahalanobis D-squared) from discriminant analysis based on plant attributes; Table S23. Correlations coefficients between discriminant scores based on plant attributes for the 11 regions and site characters of climate and soil. Highlighting indicates statistical probability of the correlation coefficient with red $p < 0.001$, yellow $p < 0.01$ and green $p < 0.05$; Table S24. Correlation coefficients between seven agronomic attributes of *Medicago polymorpha* lines (site mean values) and climate and soil characters at 107 and 101 sites, respectively. Highlighting indicates statistical probability of the correlation coefficient with red $p < 0.001$, yellow $p < 0.01$ and green $p < 0.05$.

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