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Geometric morphometrics of leaves of *Anacardium microcarpum* Ducke and *A. occidentale* L. (Anacardiaceae) from the coastal region of Piauí, Brazil

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Abstract *Anacardium microcarpum* (cajuí) differs from *A. occidentale* (cashew) by its smaller drupe and hypocarp and more restricted range in *cerrados* and coastal plateaux of Brazil's Pará, Maranhão, Piauí, Ceará and Rio Grande do Norte states. Taxonomists treat *A. microcarpum* as conspecific with *A. occidentale*, but many agronomists treat it as distinct. This study used geometric morphometrics to investigate leaf shape differences between the two taxa in ten populations from coastal Piauí state, Brazil with samples of 300–380 leaves. Configurations of two landmarks and 14 semilandmarks were digitized along the leaf outline from standardized images and subjected to multivariate analysis after Procrustes alignment. Principal component analysis produced four significant principal component shape variables accounting for 79.5 % total variance. These were visualized using thin-plate spline analysis. Discriminant analysis showed a significant difference between the taxon means ($P = 0.003$). Jackknife cross-validation correctly classified only 61.3 % of *A. occidentale* individuals and 56.7 % of *A. microcarpum*. Non-Parametric MANOVA of the ten populations showed significant population differences ($P = 0.0001$). The most isolated population (Cocal da Estação)—the only one from the interior of the state—differed most. A Mantel test found no significant correlation between morphological dissimilarity and geographical distance. Although leaf shape differences both between taxa and populations were statistically significant, overlap made leaf shape difference alone ineffective for separating *A. microcarpum* from *A. occidentale*.

Keywords Cajuí · Cashew · Geometric morphometrics · Leaf shape · Northeast Brazil · Semilandmarks

Introduction

The genus *Anacardium* L. is well known because of the cashew nut, a delicious and nutritious edible nut produced by the cashew tree, *A. occidentale* L., which is marketed world-wide. Current taxonomy recognizes eleven species overall (Mitchell and Mori 1987, Mitchell 1992), of which eight are recognized in Brazil (Silva-Luz and Pirani 2014). The most recent revision of the genus (Mitchell and Mori 1987, p. 39, Map 2) states that due to its long history of use by indigenous people and European settlers, the natural distribution of *A. occidentale* remains poorly defined due to a complex history of human selection and transfer within the Neotropics. These authors hypothesize, nevertheless, that the central area for its natural distribution is very likely located in the heart of the Cerrado biome of central Brazil.

In northeast Brazil, *A. occidentale* is important socio-economically within low-income rural communities (Guanziroli et al. 2008), because the fruit crop tides people over the dry season when there is little alternative production in family-based small-holdings. The wood is used for construction and the leaves for medicinal purposes, but

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the edible seed is the most important product and has a high market value. Resin extracted from the hard fruit wall is used medicinally and in the plastics industry for its phenolic content. The pulp and juice extracted from the fleshy fruit pedicel (hypocarp) are widely sold throughout Brazil in the form of fruit juice, cakes, jams, ice cream and as a liqueur (Schultes and Raffauf 1990). The hypocarps are also used in animal food (Dantas 2004).

The wild status of populations of *A. occidentale* is difficult to establish, because the species is actively exploited and cultivated by local people (Rufino et al. 2007). However, a rather similar tree known as “cajuí” occurs as a wild plant in cerrados and coastal plateaux of the Brazilian states of Ceará, Maranhão, Pará, Piauí and Rio Grande do Norte (Barros et al. 1999). Cajuí is recognized locally as different from *A. occidentale* because of its smaller fruit and hypocarp which render it less valuable commercially, but this taxon is nevertheless important for low-income rural communities where it occurs (Rufino et al. 2007). Botanically, it corresponds to *Anacardium microcarpum* Ducke (1922), originally described from the state of Pará. According to Ducke’s description, *A. microcarpum* resembles small individuals of *A. occidentale* but differs in its more coriaceous leaves, more flattened petiole, thicker midrib, less dense inflorescence, smaller fruit and strongly acid red or yellow peduncle [= hypocarp] no longer than the fruit. Ducke (1922) did not cite a vernacular name for his plant, but his binomial has been applied by various authors studying cajuí plants in northeast Brazil, e.g. Rufino et al. (2007) who reported that trees of *A. microcarpum* can reach 25–30 m in height, as against a maximum of 8–15 m in *A. occidentale*, contradicting Ducke’s account. Mitchell and Mori (1987, p. 39), however, in their revision of the genus *Anacardium*, treated *A. microcarpum* as a synonym of *A. occidentale*, while at the same time pointing out that there was no satisfactory study of the infraspecific classification of the latter species. More recently, agronomic research studies have shown the importance of *A. microcarpum* for production of hybrids with *A. occidentale* with improved commercial characteristics of the hypocarp (e.g. Crisóstomo et al. 2002).

Rufino et al. (2007) note that the term “cajuí” is used in a vernacular sense for dwarf fruits of various species of *Anacardium*, but this applies only to fruits rather than plant populations. Using evidence from molecular studies carried out by Cunha (2002) and Barros (1991), they suggested that cajuí is probably no more than an ecotype or variety of *A. occidentale*, but they nevertheless used the species binomial *A. microcarpum* for this taxon, no doubt for practical reasons. All of these authors thus treat the cajuí of the northern part of the northeast region of Brazil as a taxon distinct—at least to some degree—from *A. occidentale*, but the appropriate taxonomic rank remains undecided. Furthermore, detailed taxonomic studies of morphological and molecular data from populations of *A. microcarpum* are lacking, in particular comparison with plants from the type locality in Pará. The absence of such studies has meant that hitherto, the taxonomic differences between *A. microcarpum* and *A. occidentale* rest on the size of drupe and hypocarp and little else.

The present study starts out from the prior assumption that these two named morphotypes represent distinct taxa, at least in the northern part of northeast Brazil. This premise is based on the existence of the fruit differences referred to previously. We refer to the two morphotypes using their existing species-category binomials, but it should be borne in mind that this is primarily for pragmatic reasons, since it is to the further investigation of their taxonomic distinctness that this study is primarily directed.

Our hypothesis is that, given their distinctness in fruit characters, there is also a quantitative difference in leaf shape between *A. microcarpum* and *A. occidentale*. More specifically, we sought to show that leaf shape differences would aggregate populations of each taxon together. The study is limited to comparisons between populations from the coastal area of Piauí state, a region which is situated more-or-less centrally within the range of *A. microcarpum*. The study is a contribution towards building a more detailed taxonomic picture of morphological and taxonomic variation in these plants.

Investigating possible distinctions in leaf shape is an obvious first step in probing new differences between the two taxa, given the importance of this character field in plant taxonomy, particularly for diagnosing distinctions between species and infraspecific taxa in the absence of flowers and fruits (Ellis et al. 2009, Gonçalves and Lorenzi 2011). Leaf shape varies between and within species of *Anacardium* (Mitchell and Mori 1987, pp. 7–8).

The geometric morphometric (GM) approach is the most suitable method for quantitative comparison of two-dimensional biological shapes. GM using landmarks and semilandmarks has been used by various authors in recent years as a quantitative approach to studying systematic and developmental problems in plants, for example, the studies of Jensen et al. (2002) on species and their hybrid in *Acer*, Shipunov and Bateman (2005) on labellum shape in *Dactylorhiza*, Gómez et al. (2006) on zygomorphy in *Erysimum* flowers, Butterworth et al. (2009) on cotton fibre shape, Viscosi and Cardini (2011) on leaf shape in *Quercus* and Silva et al. (2012) on inter-population leaf shape variation in *Montrichardia*. Other kinds of morphometric data, reflecting ecological traits, are used with advanced methods of unsupervised multivariate analysis in ecological studies, particularly those concerning pollution bioindicators (e.g. Kosiba 2009).

Materials and methods

Study area and populations sampled

Piauí state has a coastline only 66-km long, extending from the Rio Ubatuba—the border with Ceará state—to the Rio Parnaíba on the west, which borders Maranhão state. The climate in the coastal region is hot and humid with a summer rainy season; the total annual rainfall is ca. 1,200 mm and the mean annual temperature ca. 26 °C. The delta region of the Rio Parnaíba is a major geographical feature (Silva 2004; Lustosa 2005), and the vegetation of the coastal region includes pioneer to well-established dune successional communities, tall mangrove forest, *cerrado*-like vegetation of the coastal plateaux and secondary *cerradão* (Cepro 1996).

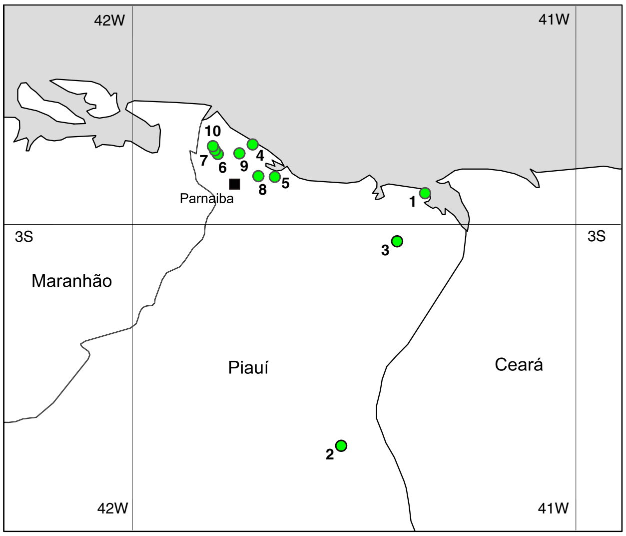


Fig. 1 Map of the 10 study populations of *Anacardium occidentale* (populations *1–5*) and *A. microcarpum* (populations *6–10*). *1* Cajueiro da Praia, *2* Cocal da Estação, *3* Morada Nova, *4* Pedra do Sal, *5* Rosápolis, *6* Baixão, *7* Cal, *8* Floriópolis, *9* Labino, *10* Tatus. Produced with SimpleMappr (Shorthouse 2010)

The five sampled populations of *A. microcarpum* were all located in the northwestern part of the coastal region of Piauí (Fig. 1; Table 1) where this taxon is most abundant—in the municipalities of Ilha Grande (Cal, Baixão, Labino, Tatus) and Parnaíba (Floriópolis). These localities border the Cajuí Extractive Reserve, a proposed area of environmental protection for the sustainable use of *A. microcarpum* trees. The five sampled populations of *A. occidentale* were more spread out in order to better represent the regional variation within this taxon. Populations in three widely separated municipalities were sampled: Parnaíba in the northwest (Pedra do Sal, Rosápolis), Cajueiro da Praia (Cajueiro da Praia, Morada Nova) in the extreme east and Cocal da Estação, which is 94 km south of Parnaíba (Fig. 1; Table 1). The latter population, the only one from the interior of the state, was chosen because of known phenotypic differences from the coastal populations of *A. occidentale* (Pedro Neves, pers, comm.).

Sampling procedure

The taxonomic determination of the sampled leaves was made as follows. First, the identification of populations as either cultivated "caju" or as wild "cajui" was made with the help of local rural farmers. Ripe drupes and hypocarps were then sampled from each of these populations and measured (Vieira et al., in prep.). The results confirmed that the populations identified locally as *A. occidentale* (caju) had significantly larger drupes and hypocarps than those of the *A. microcarpum* (cajuí) populations, although

Table 1 Populations and samples of *Anacardium occidentale* and *A. microcarpum*

Taxon	Population	Municipality	Number of trees	Number of leaves (no. used in CVA)	Voucher specimen	Latitude (decimal degrees S)	Longitude (decimal degrees W)
occidentale	Cajueiro da Praia	Cajueiro da Praia	15	30 (38)	Sousa, M.V. 260	−2.9323	−41.3429
occidentale	Cocal da Estação	Cocal da Estação	15	30 (38)	Sousa, M.V. 223	−3.49879	−41.5300
occidentale	Morada Nova	Cajueiro da Praia	15	30 (38)	Sousa, M.V. 296	−3.03960	−41.4049
occidentale	Pedra do Sal	Parnaíba	15	30 (38)	Sousa, M.V. 52	−2.8242	−41.7331
occidentale	Rosápolis	Parnaíba	15	30 (38)	Sousa, M.V. 44	−2.8961	−41.6822
microcarpum	Baixão	Ilha Grande	15	30 (38)	Sousa, M.V. 114	−2.8457	−41.8116
microcarpum	Cal	Ilha Grande	15	30 (38)	Sousa, M.V. 155	−2.8376	−41.8187
microcarpum	Floriópolis	Parnaíba	15	30 (38)	Sousa, M.V. 186	−2.8949	−41.7197
microcarpum	Labino	Ilha Grande	15	30 (38)	Sousa, M.V. 1	−2.8441	−41.7629
microcarpum	Tatus	Ilha Grande	15	30 (38)	Sousa, M.V. 31	−2.8296	−41.8243

Voucher specimens are deposited at the HDELTA herbarium (Universidade Federal do Piauí, Parnaíba Campus, Parnaíba, Piauí, Brazil) there was some overlap in the measurement ranges. Based on this procedure, leaves were identified accordingly as *A. occidentale* or *A. microcarpum*.

In each population, 30 trees were selected at random, and from each tree, two leaves were selected from a single branch in the region of the shoot where the leaves are largest. For most of the analyses, leaves from 15 trees were selected, making a total sample of 300 leaves. For the canonical variates analyses (CVA), however, four more trees were added to each population (8 leaves), increasing the sample 38 leaves per population; in CVA, it is statistically desirable (Hammer and Harper 2006) for the number of objects per defined group to exceed the number of variables—in this case 32 coordinates from 16 two-dimensional landmarks.

Data gathering

The leaves were attached by drawing pins onto a pale base with their abaxial surfaces exposed and photographed against a millimetre scale with a vertically positioned Ricoh CX6 digital camera. The abaxial surface was preferable because of the more prominent secondary veins.

Sixteen landmarks were selected based on the relative positions of the primary and secondary veins of the leaf to the margins (Table 2; Fig. 2). Landmarks 1 and 2 qualify best as full homologies (Gunz and Mitteroecker 2013), corresponding respectively to the junction of petiole and primary vein, and most distal point of the primary vein. Landmarks 3–5, 7–9, 10–12 and 14–16 are semilandmarks (Zelditch et al. 2012), i.e. points determined by the relative positions of neighbouring homologies. The secondary veins have a brochidodromous pattern (Ellis et al. 2009) forming a looping inframarginal vein by apical bifurcation well within the leaf margin. The fourteen semilandmarks were defined by continuing to the margin the course maintained by the secondary vein prior to its distal bifurcation. Secondary vein number varies between leaves and is often different on each side of the leaf blade, making it difficult to assign homology to veins when comparing different leaves and the two sides of a single leaf. Following a similar procedure adopted by Klingenberg et al. (2012), we therefore opted to select only the apical and basal three veins on each side of the leaf. Semilandmarks 6 and 13 served to capture a central point along each margin, as defined by the order of the secondary veins. In these two cases, if the number of veins on a side was odd, the semilandmark was placed on the margin at the point of intersection of the projection of the central secondary vein; with an even number of veins, the semilandmark was placed on the margin at the midpoint between the two central secondary veins.

Table 2 Description of landmarks in leaves of *Anacardium microcarpum* and *A. occidentale*

Landmark	Description
1	Petiole-leaf blade midrib junction
2	Apex of leaf blade midrib
3	Intersection of left-hand leaf margin and the projection of the 1st secondary vein on the left from the leaf apex
4	Intersection of left-hand leaf margin and the projection of the 2nd secondary vein on the left from the leaf apex
5	Intersection of left-hand leaf margin and the projection of the 3rd secondary vein on the left from the leaf apex
6	Intersection of left-hand leaf margin and the projection of the central secondary vein on the left, if there is an odd number of veins on that side. If the number of veins is even, then the landmark is the point along the leaf margin intermediate between the projections of the two central secondary veins onto the leaf margin
7	Intersection of left-hand leaf margin and the projection of the 3rd secondary vein on the left from the leaf base
8	Intersection of left-hand leaf margin and the projection of the 2nd secondary vein on the left from the leaf base
9	Intersection of left-hand leaf margin and the projection of the 1st secondary vein on the left from the leaf base
10	Intersection of right-hand leaf margin and the projection of the 1st secondary vein on the right from the leaf base
11	Intersection of right-hand leaf margin and the projection of the 2nd secondary vein on the right from the leaf base
12	Intersection of right-hand leaf margin and the projection of the 3rd secondary vein on the right from the leaf base.
13	Intersection of right-hand leaf margin and the projection of the central secondary vein on the right, if there is an odd number of veins on that side. If the number of veins is even, then the landmark is the point along the leaf margin intermediate between the projections of the two central secondary veins onto the leaf margin
14	Intersection of right-hand leaf margin and the projection of the 3rd secondary vein on the right from the leaf apex
15	Intersection of right-hand leaf margin and the projection of the 2nd secondary vein on the right from the leaf apex
16	Intersection of right-hand leaf margin and the projection of the 1st secondary vein on the right from the leaf apex

Left–right definitions are made viewing the leaf from the abaxial side. See also Fig. 2

The images were imported into the TpsDig program (Rohlf 2010) which was used to digitize the landmarks, producing a configuration of 32 coordinates in 16 pairs (x, y) for each leaf. The data for all 300 leaves were stored in the ".TPS" file format.

Fig. 2 Positions of the two landmarks (*1–2*) and fourteen semilandmarks (*3–16*) on the leaf blade margin of *Anacardium microcarpum* and *A. occidentale*. Digitized with the tpsDig software (Rohlf 2010)

Data analysis

Statistical comparisons of leaf shape were made using the landmark techniques of geometric morphometrics, further details of which can be obtained from Monteiro and Reis (1999), Zelditch et al. (2012) and Viscosi and Cardini (2011).

The TPS file of digitized configurations of landmark coordinates was imported into the software package PAST version 2.17c (Hammer et al. 2001), which rearranges the data of each configuration automatically into a single row of alternating x and y values, producing a matrix of 300 rows (380 for CVA analyses) and 32 columns; PAST was used for all subsequent analyses. Procrustes analysis (Zelditch et al. 2012) was carried out on the raw coordinate matrix, and this normalized matrix was used for further analysis.

Multivariate statistical analyses of various kinds were then carried out. Useful references for further details of the techniques mentioned below can be found in the following sources. Manly (1994) and Hammer and Harper (2006) give concise and more easily understandable accounts. Reyment (1991) and Davis (1986) are more detailed and very useful, while Legendre and Legendre (2012) give a comprehensive and up-to-date account based on ecological applications. Green and Carroll (1978) is an excellent account of the basic linear algebra needed for a deeper understanding of multivariate methods. Conventions for statistical symbols, e.g. the probability symbol P, follow Sokal and Rohlf (1995).

Principal component analysis (PCA) of the whole matrix was used to study the global variation of the landmark configurations and identify the most important trends in leaf shape—PCA treats all configurations as a single population. This method was used as an objective means to summarize variation when a priori knowledge of the populations to which individuals belonged was not considered (Everitt 1978). The shape trends, corresponding to the principal component axes (PCs), were visualized using thin-plate spline reconstructions of the shapes corresponding to the minimum, mean and maximum values of the first few PCs, i.e. those capable of meaningful interpretation beyond the effects of random variation (Legendre and Legendre 2012, p. 449). Their number was estimated using the scree plot and broken-stick model implemented in PAST (ver. 2.17c, Hammer 2012).

Discriminant analysis (DA) was used to verify whether the leaf shapes of the two sampled taxa, *A. occidentale* and *A. microcarpum*, were significantly different and diagnosable using a cross-validation classification test. In PAST, the separation of the two groups, shown in contrasting colours, is visualized by a frequency bar chart. Hotelling's T^2 test was used to estimate the significance of the difference between the means of the two taxa.

Canonical variate analysis (CVA) was used to compare the two taxa, because the implementation in PAST allows the visualization of the mean shapes of each taxon using a thin-plate spline reconstruction. CVA was also used to compare leaf shape variation in the ten populations, visualizing as thin-plate splines the minimum, mean and maximum shapes on each of the first two canonical variate axes. CVA is used to ordinate population means considering variance and covariance among characters within and among populations (Everitt 1978).

MANOVA and non-parametric MANOVA (NPMANOVA) were used to test the significance of the differences between the taxa and between the populations, estimated as Mahalanobis distance. PAST also provides two cross-validation tests. In the first, each individual is assigned to the nearest group, after computing the Mahalanobis distances between it and every group centroid. In the second—the jackknife test—the CVA is calculated using a matrix from which the individual concerned has been deleted, followed by computation of its Mahalanobis distances from the group centroids—in this way, the individual plays no part in the computation of the discriminating axes (Hammer 2012). This "leave-one-out" procedure is repeated for all individuals.

To plot the distances between the centroids of the populations in two dimensions, we used non-metric multidimensional scaling (NMDS) which provides an optimal fitting in two dimensions of the objects in a distance matrix. Two plots were carried out, one with squared Mahalanobis inter-population distance derived from the MANOVA/CVA and one using the matrix of inter-population values of the F statistic computed by NPMANOVA.

The Mantel test is a non-parametric technique for estimating the correlation between two matrices derived from the same objects but each with different data (Mantel 1967), in this case geographic distance and morphological dissimilarity between the ten populations. The pairwise morphological dissimilarities were represented by a Euclidean distance matrix computed from a data matrix in which the rows consisted of the mean Procrustes-aligned landmark coordinates for each population. The geographic distance between the populations was calculated as Euclidean distance using a matrix of latitude and longitude in decimal degrees.

Results

Principal component analysis (PCA)

Figure 3 shows the distribution of individuals in the space of the first two principal components (PCs) with *A. occidentale* and *A. microcarpum* coloured differently; no group differentiation is discernible. The main purpose of the PCA in this study was to reveal the major trends in leaf shape variation within the whole dataset of 300 leaves. Each orthogonal PC represents a distinct shape variable and hence an independent trend.

The eigenvectors (Table 3) show that up to eleven PCs are needed to express 95 % of the total variation; however, only the first four are meaningful according to the broken-stick model. Figure 4 shows the shape trends represented by the first four PCs, representing together 79.5 % of the total variance. PCs 1 and 2 are symmetric shape variables, while PCs 3 and 4 are asymmetric in relation to the principal axis of the leaf (Fig. 2). In PC1, low score values are accompanied by an expansion of the leaf base and a longitudinal contraction in the upper half of the leaf. High values are accompanied by a squeezing of the base and longitudinal expansion in upper half. In PC2, the variation focusses on alternating expansion and contraction at the leaf base and apex. PC3 involves expansion and contraction of each side of the leaf accompanied by a shear (Slice et al. 2014), while PC4 involves a warping of the leaf to the right (low values) or left (high values).

Discriminant analysis (DA) and canonical variate analysis (CVA)

Two Taxa, A. occidentale and A. microcarpum

The results of DA showed a significant difference between the means of the two taxa (Fig. 5, Hotelling's $T^2 = 67.99$, $F = 1.9037$, $P = 0.0034$), but since only 68.33 % of the individuals were correctly classified, it was not possible to identify with confidence a given leaf as one of the two taxa.

The multivariate analysis of variance (MANOVA) showed a significant difference between the two taxon means (Wilk's lambda = 0.8201, $df1 = 32$, $df2 = 347$, $F = 2.379$, $P = 7.1E{-}05$; Hotelling's P [Bonferroni corrected] = 7.1E−05). Non-parametric ANOVA (NPMANOVA), as

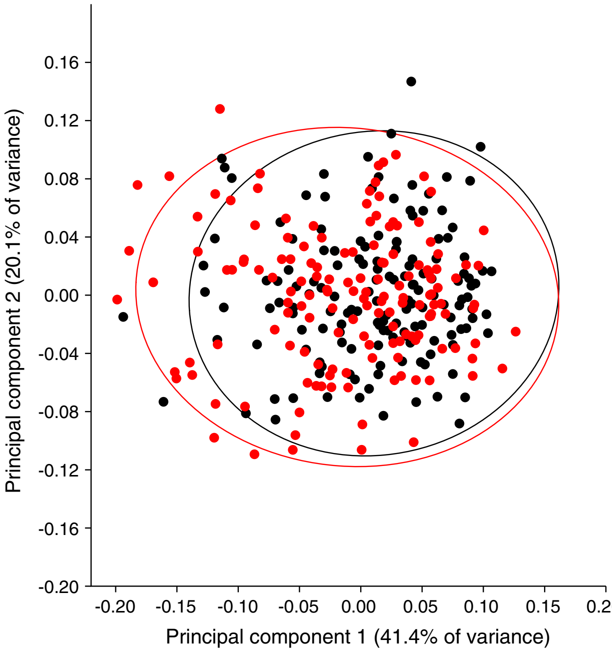


Fig. 3 Principal component analysis (PCA) of the leaf shape of 10 populations of *Anacardium occidentale* (five populations) and *A. microcarpum* (five populations). Plot of principal components 1 and 2 (PC1, PC2), using a matrix of Procrustes-aligned landmark coordinates, showing 95 % confidence ellipses. *Black dots A. occidentale*, *pale* (*red*) *points A. microcarpum*. Computed in PAST ver. 2.17c (Hammer et al. 2001). (Color figure online)

Table 3 The first twelve eigenvalues of a principal components analysis (PCA) of leaf shape in 10 populations of *Anacardium occidentale* (five populations) and *A. microcarpum* (five populations)

PC	Eigenvalue	% Variance	Cumulative % variance
1	0.004362	41.378	41.378
2	0.002115	20.066	61.444
3	0.00104	9.8673	71.3113
4	0.000859	8.1467	79.458
5	0.000532	5.0446	84.5026
6	0.000372	3.532	88.0346
7	0.000222	2.1094	90.144
8	0.000183	1.7349	91.8789
9	0.000167	1.5876	93.4665
10	0.000141	1.3334	94.7999
11	9.22E−05	0.87424	95.67414
12	7.43E−05	0.70452	96.37866

Only the first four are significant according to the broken-stick model. Eigenvalues derived from a matrix of 300 Procrustes-aligned configurations of 16 landmarks. Computed with PAST ver. 2.17c (Hammer et al. 2001)

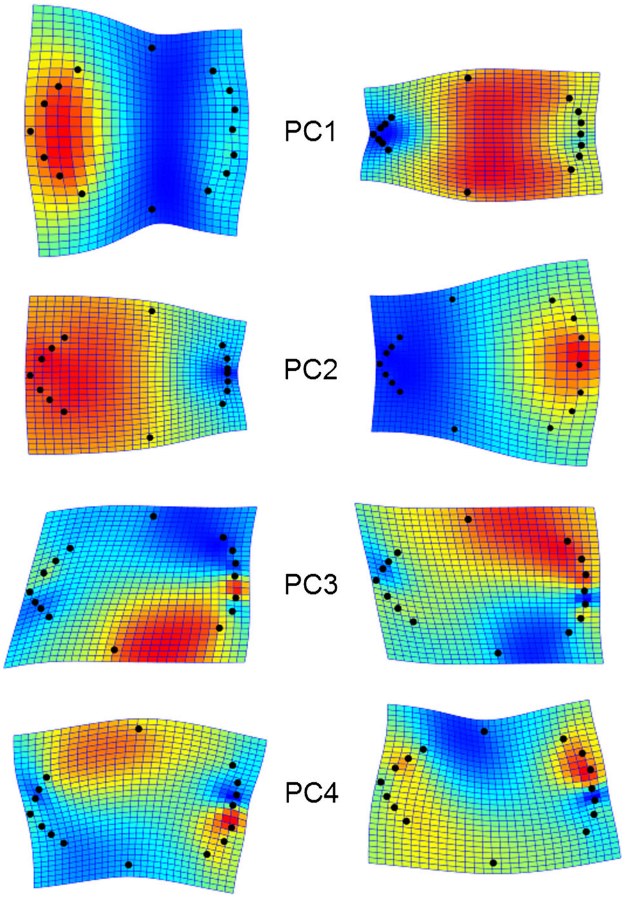


Fig. 4 Principal component analysis (PCA) of the leaf shape of 10 populations of *Anacardium occidentale* (five populations) and *A. microcarpum* (five populations). Leaf deformations represented by the first four principal components. The apex of the leaf is to the *right*. The *left-hand figures* show the leaf shape corresponding to minimum values of the PC, and the *right-hand* ones the maximum values. In *colour*, a *green background* indicates lack of distortion from the mean shape, *blue* indicates compression and *red* indicates expansion. Computed in PAST ver. 2.17c (Hammer et al. 2001). (Color figure online)

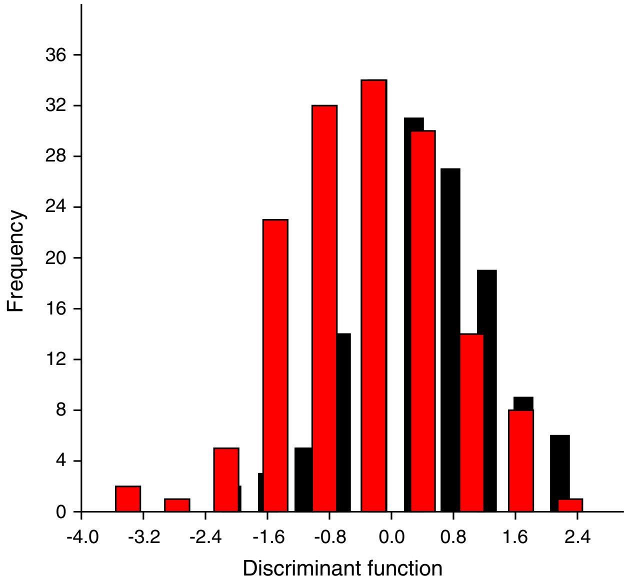


Fig. 5 Discriminant analysis (DA) of the leaf shape of 10 populations of *Anacardium occidentale* five populations) and *A. microcarpum* (five populations). *Black bars A. occidentale*; *pale* (*red*) *bars A. microcarpum*. Hotelling's $T^2 = 67.99$, $F = 1.90$, $P = 0.003$. Computed in PAST vers. 2.17c (Hammer et al. 2001). (Color figure online)

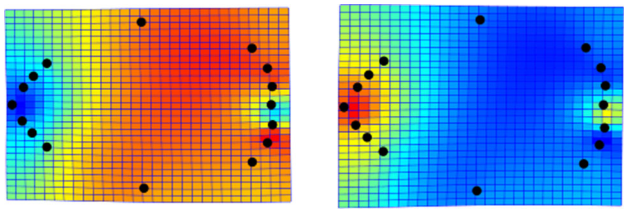


Fig. 6 Canonical variates analysis of the leaf shape of two taxa, *Anacardium occidentale* and *A. microcarpum*. Mean leaf shape of each taxon along the single canonical variate axis. *A. occidentale* (*left*), *A. microcarpum* (*right*). In *colour*, *red background* corresponds to expansion from overall mean shape and *blue* to compression. Computed in PAST ver. 2.17c (Hammer et al. 2001). (Color figure online)

implemented in PAST, also found a significant difference between the taxon means using Mahalanobis distance. With 9,999 permutations, the result was total SS = 379.1, within group SS = 376.9, $F = 2.137$, $P = 0.0002$.

Using CVA, the differences in leaf shapes of the two taxon mean values along the single canonical variate axis could be visualized (Fig. 6). These were only slight and consisted of a localized longitudinal compression at the base and an extension in the upper two thirds in *A. occidentale*, and the reverse effect in *A. microcarpum*.

In the first cross-validation test (Table 4a), 72.7 % of *A. occidentale* individuals and 68.0 % of *A. microcarpum* were correctly classified. In the second jackknife test (Table 4b), only 61.3 % of *A. occidentale* and 56.7 % of *A. microcarpum* were correctly classified.

Ten populations, five of A. occidentale and five of A. microcarpum

To carry out the CVA for 10 populations, it was necessary to increase the sample to 38 leaves per population (the smallest overall sample made per population in the original field survey) so that there were more objects than variables in each group. With this matrix, the results showed only weak inter-population differentiation using MANOVA, the only significant differences being population pairs involving the Cocal da Estação population.

NPMANOVA computed with Mahalanobis distance showed stronger inter-population differences: total SS = 379.1, within group SS = 363.1, $F = 1.807$, $P = 0.0001$,

Table 4 Cross-validation matrices from canonical variates analysis (CVA) of leaf shape in *Anacardium occidentale* and *A. microcarpum*

	A. occidentale	*A. microcarpum*	Total	% correct
A. Confusion matrix without jackknife				
A. occidentale	109	41	150	72.67
A. microcarpum	48	102	150	68.00
Total	157	143	300	
B. Confusion matrix with jackknife				
A. occidentale	92	58	150	61.33
A. microcarpum	65	85	150	56.67
Total	157	143	300	

A. Classification using Mahalanobis distance computed from the original matrix. B. Jackknife classification ("leave-one-out"). In each row (taxon), the cell values represent the number of individuals classified as the taxon whose name is in the column heading. Computed in PAST ver. 2.17c. (Hammer et al. 2001)

9,999 permutations. Table 5 shows *P* values (sequential Bonferroni significance) of comparisons between population pairs. This table shows a slight differentiation between the two taxa in that apart from Cocal da Estação, the pairs involving populations from the same taxon tend to be more similar. The most conspicuous feature of Table 5, however, is the difference between Cocal da Estação and all the other populations. This result is also clearly seen in the cross-validation tests (Table 6) in which Cocal da Estação emerges as the population with the highest percentage of correctly assigned individuals (76.3 and 63.2 % in the respective tests). In almost all other populations, a majority of the individuals are misclassified in both tests.

Figure 7 shows a non-metrical multidimensional scaling (NMDS) plot computed using the matrix of population-pairwise *F* values computed by NPMANOVA as the inter-population distances; a minimum spanning tree is superimposed to show closest neighbours. In this figure, the *A. occidentale* populations are more closely associated than those of *A. microcarpum*. An alternative plot is shown in Fig. 8, derived from the matrix of population-pairwise squared Mahalanobis distances resulting from the CVA of 10 populations. This result is similar, but the closest population pairs are not always the same.

The most important shape deformations, as represented by the extreme values along the first two canonical variate axes, were visualized (Fig. 9). Since these are the axes which separate the 10 populations most completely, the deformations represent the most contrasting leaf forms among them. On the first canonical variate axis (CV1) the leaf shape varies from obovate (leaves with low values) to broadly elliptic (leaves with high values) with an associated asymmetry, and for high values an expansion of the extreme apex. In CV2, the shape change is slighter, consisting of a basal expansion and an apical compression (leaves with low values) and the inverse in leaves with high values.

Correlation between inter-populations morphological and geographical distance: Mantel test

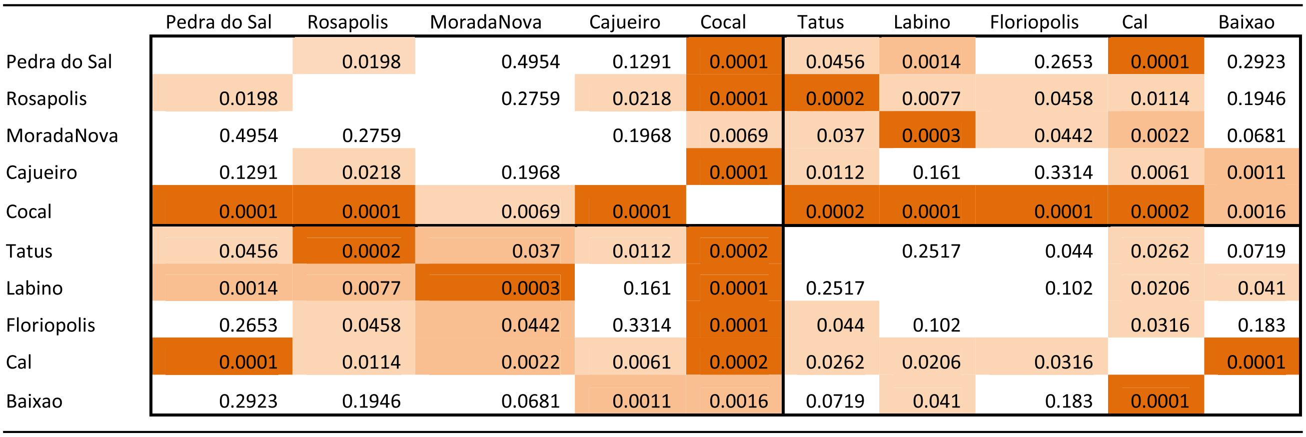
The Mantel test for 10 populations showed no significant correlation between morphological distance and the geographic distance between the populations. The value of the correlation coefficient was low ($R = 0.2886$) and not significant ($P = 0.1766$). A similar test for correlation among the five populations within each species also found no significant result (*A. occidentale*: $R = 0.5668$, $P = 0.0816$; *A. microcarpum*: $R = 0.2931$, $P = 0.1138$).

Discussion and conclusions

Furuta et al. (1995), Yamanaka et al. (2001), Iwata et al. (2002) and Yoshioka et al. (2004), among others, showed that symmetrical shape variables showed a stronger correlation with genetic factors than asymmetrical ones, using elliptic Fourier analysis of leaves and petals in cultivated varieties of *Glycine max* (L.) Merr., *Citrus* spp. and *Primula* spp. It is thus reasonable to infer that the more important symmetric principal components of our study, such as PCs 1 and 2, reflect genetically determined shape change more strongly than the asymmetric PCs 3 and 4, and that the leaf shape differences observed in this study are thus at least partly determined genetically.

The results of the DA and CVA analyses comparing the two taxa *A. occidentale* and *A. microcarpum* showed that leaf shape, at least in the way that it was captured in this study, does not differentiate them sufficiently to allow identification. Only 57–61 % of the sampled leaves were correctly assigned to their taxa when subjected to cross-validation using the Jackknife procedure. Despite this, the comparisons showed a significant difference in their mean leaf shapes. We can therefore conclude that a statistical difference in leaf shape does exist but that this is largely

Table 5 Non-parametric multivariate analysis of variance (NPMANOVA) of leaf shape in 10 populations of *Anacardium occidentale* (five populations) and *A. microcarpum* (five populations), using Mahalanobis distance



	Pedra do Sal	Rosapolis	MoradaNova	Cajueiro	Cocal	Tatus	Labino	Floriopolis	Cal	Baixao
Pedra do Sal		0.0198	0.4954	0.1291	0.0001	0.0456	0.0014	0.2653	0.0001	0.2923
Rosapolis	0.0198		0.2759	0.0218	0.0001	0.0002	0.0077	0.0458	0.0114	0.1946
MoradaNova	0.4954	0.2759		0.1968	0.0069	0.037	0.0003	0.0442	0.0022	0.0681
Cajueiro	0.1291	0.0218	0.1968		0.0001	0.0112	0.161	0.3314	0.0061	0.0011
Cocal	0.0001	0.0001	0.0069	0.0001		0.0002	0.0001	0.0001	0.0002	0.0016
Tatus	0.0456	0.0002	0.037	0.0112	0.0002		0.2517	0.044	0.0262	0.0719
Labino	0.0014	0.0077	0.0003	0.161	0.0001	0.2517		0.102	0.0206	0.041
Floriopolis	0.2653	0.0458	0.0442	0.3314	0.0001	0.044	0.102		0.0316	0.183
Cal	0.0001	0.0114	0.0022	0.0061	0.0002	0.0262	0.0206	0.0316		0.0001
Baixao	0.2923	0.1946	0.0681	0.0011	0.0016	0.0719	0.041	0.183	0.0001	

Each cell value is the probability *P* of the pair (column and row) being from the same population (sequential Bonferroni significance). The intensity of shading in each cell reflects the level of significance of the probabilities. See Table 1 for details of the populations. Computed in PAST ver. 2.17c. (Hammer et al. 2001)

Table 6 Cross-validation matrices from canonical variates analysis (CVA) for leaf shape in 10 populations, five each of *Anacardium occidentale* and *A. microcarpum*

	PedradoSal	Rosapolis	MoradaNova	Cajueiro	Cocal	Tatus	Labino	Floriopolis	Cal	Baixao	Total	% correct
A. Confusion matrix without jackknife												
PedradoSal	14	3	5	1	3	3	4	2	2	1	38	36.8
Rosapolis	2	16	3	2	2	1	4	2	2	4	38	42.1
MoradaNova	4	5	14	1	3	0	1	3	4	3	38	36.8
Cajueiro	2	3	4	17	1	0	6	2	2	1	38	44.7
Cocal	1	1	3	0	29	0	3	0	1	0	38	76.3
Tatus	2	3	3	3	0	19	3	1	2	2	38	50.0
Labino	3	2	0	5	2	4	13	2	4	3	38	34.2
Floriopolis	2	1	2	3	3	1	2	18	3	3	38	47.4
Cal	2	0	1	1	2	4	4	0	23	1	38	60.5
Baixao	0	6	2	1	6	4	3	2	2	12	38	31.6
Total	32	40	37	34	51	36	43	32	45	30	380	
B. Confusion matrix with jackknife												
PedradoSal	10	3	7	1	3	3	4	3	2	2	38	26.3
Rosapolis	5	6	7	2	3	1	4	3	3	4	38	15.8
MoradaNova	4	7	7	2	3	2	1	4	4	4	38	18.4
Cajueiro	3	3	5	10	1	2	6	4	2	2	38	26.3
Cocal	1	1	5	0	24	0	4	1	1	1	38	63.2
Tatus	2	5	4	4	0	11	3	1	3	5	38	28.9
Labino	4	2	0	8	3	5	6	2	5	3	38	15.8
Floriopolis	3	1	5	5	3	1	3	10	3	4	38	26.3
Cal	2	3	1	1	2	7	4	3	13	2	38	34.2
Baixao	1	6	3	1	7	6	3	4	2	5	38	13.2
Total	35	37	44	34	49	38	38	35	38	32	380	

A. Classification using Mahalanobis distance computed from the original matrix. B. Jackknife classification ("leave-one-out"). In each row (population), the cell values represent the number of individuals classified as the population whose name is in the column heading. Computed in PAST ver. 2.17c. (Hammer et al. 2001). See Table 1 for further details of the populations

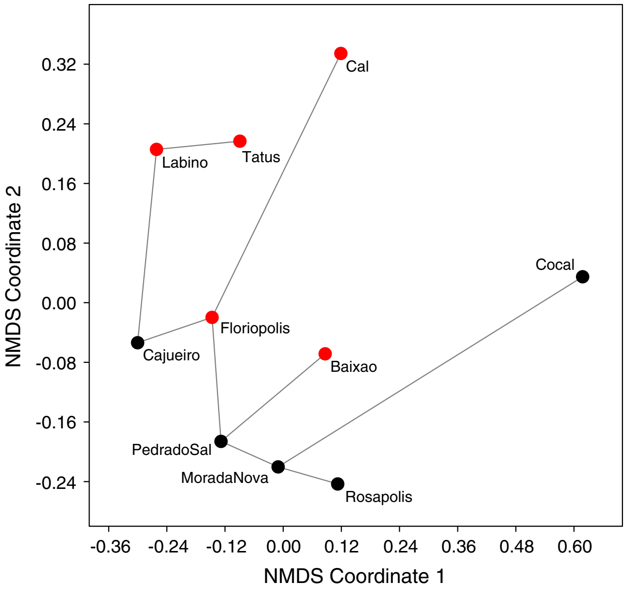


Fig. 7 Non-metric multidimensional scaling (NMDS) of the leaf shape of 10 populations of *Anacardium occidentale* (five populations) and *A. microcarpum* (five populations). The distances between populations are derived from a population-pairwise matrix of *F* values computed by non-parametric MANOVA (NPMANOVA) from a matrix of Procrustes-aligned configurations of 16 landmarks. *Black points A. occidentale*; *pale* (*red*) *points A. microcarpum*. The *lines* represent the minimum spanning tree. Computed in PAST ver. 2.17c (Hammer et al. 2001). (Color figure online)

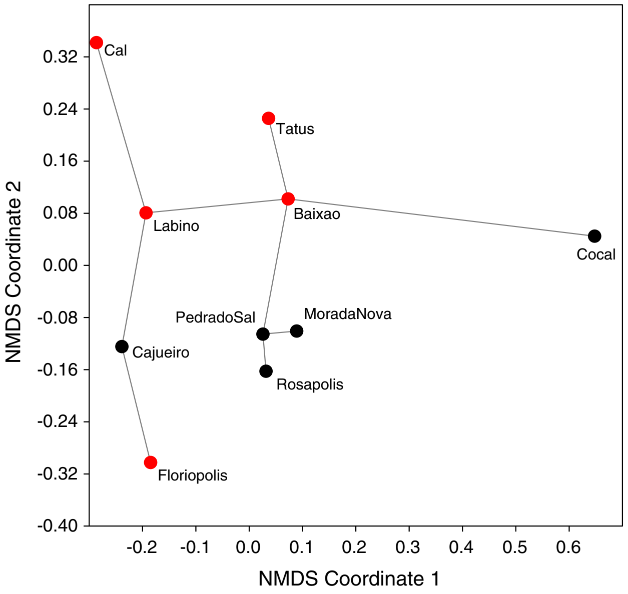


Fig. 8 Non-metric multidimensional scaling (NMDS) of the leaf shape of 10 populations of *Anacardium occidentale* (five populations) and *A. microcarpum* (five populations). The distances between populations are derived from a population-pairwise matrix of squared Mahalanobis distances computed by canonical variates analysis (CVA) from a matrix of Procrustes-aligned configurations of 16 landmarks. *Black points Anacardium occidentale*; *pale* (*red*) *points A. microcarpum*. The *lines* represent the minimum spanning tree. Computed in PAST ver. 2.17c (Hammer et al. 2001). (Color figure online)

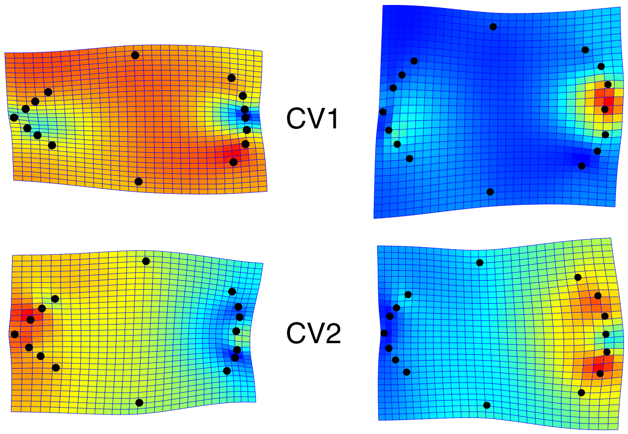


Fig. 9 Canonical variates analysis of the leaf shape of 10 populations of *Anacardium occidentale* (five populations) and *A. microcarpum* (five populations). Leaf shapes shown are deformations from the overall along the first two canonical variate axes (CV1, CV2). The leaf apex is on the *right*. The *left-hand figures* show the leaf shape corresponding to minimum values of the CV, and the *right-hand ones* the maximum values. In *colour*, a *green background* indicates lack of distortion from the mean shape, *blue* indicates compression and *red* indicates expansion. Computed in PAST ver. 2.17c (Hammer et al. 2001). (Color figure online)

obscured for all practical purposes by a large degree of overlap.

There is a weak indication of leaf shape differentiation between *A. occidentale* and *A. microcarpum* in the NPMANOVA result (Table 5), which suggests a tendency for pairwise comparisons of populations from the same species to be less distant than comparisons of populations from different species. The marked differentiation of the Cocal population of *A. occidentale* may be related to its more distant geographic position from the rest, well into the interior of the state of Piauí, to the south—all the other populations are from the coastal region (Fig. 1). This leaf shape difference could be due to ecological and environmental differences between the coastal and interior regions and strongly suggests that wider and more detailed comparisons are needed between populations from the two regions.

The distribution of population means (Figs. 7, 8) suggest that, excepting Cocal da Estação, the leaves of *A. occidentale* are somewhat more similar in shape to one another than those of *A. microcarpum*, despite the populations of the latter being geographically more concentrated (Fig. 1). However, the connections of the minimum spanning trees show that there is no consistent inter-taxon or even inter-population difference—some populations are closest to those of the other taxon, and these connections vary according to the distance metric used. This overall picture is also supported by the NPMANOVA tests in which the significance of the difference in taxa and in populations is approximately the same (respectively $P = 0.0002$, $P = 0.0001$). It may thus be stated that leaf shape differences between the two taxa are no greater than those between population pairs.

Mitchell and Mori (1987, pp. 7–8, 46, their Fig. 20) illustrate intra-specific variation in leaf shape and size for several species of *Anacardium*, including *A. occidentale*. These authors noted that despite its great economic importance, no work had been carried out at that time on the infraspecific taxonomy of *A. occidentale* although cultivar selection was in progress (Mitchell and Mori 1987, p. 46).

Natural inter-crossing between *A. occidentale* and *A. microcarpum* is very probable given that they are often in close proximity, and this makes it possible that introgression plays a part in the breeding behaviour of these plants, although there appear to be no studies yet available from natural populations. Mitchell and Mori (1987, p. 22) mention that in the Planalto of central Brazil, three sympatric species of the genus, including *A. occidentale*, have very similar flowering ecology and that phenotypically intermediate individuals are probably the result of natural hybridization. Mitchell and Mori (1987, p. 40, 46) discuss size and shape differences between the leaf blade and petiole of ecotypes of *A. occidentale* from the *cerrado* of central Brazil and the *restinga* of northeastern Brazil, noting that the former have more undulate, thickly coriaceous leaf blades, shorter and stouter petioles and usually smaller and sometimes more acidic hypocarps—it may be noted that there are some resemblances in this character combination with those given in Ducke's original diagnosis of *A. microcarpum* (Ducke 1922), suggesting that the cajuí of coastal Piauí should be compared to *cerrado* populations of *A. occidentale* in central Brazil.

While *A. microcarpum* populations can be regarded as natural (e.g. Rufino et al. 2007), the same cannot be said for those of *Anacardium occidentale* (e.g. Mitchell and Mori 1987, pp. 39–40, 43) due to their intensive exploitation by local people—*A. occidentale* is not only cultivated but also adventive, i.e. in suitable habitats, it forms apparently natural population from originally cultivated plants without the aid of human intervention. It is therefore to be expected that the patterns of genetic and morphological characters that exist among the populations of both taxa in the region are complex and require population level studies using molecular markers.

Studies of variation in *Anacardium* using morphometric or molecular markers have often made use of germplasm collections rather than direct sampling of natural populations, especially the cashew germplasm bank (BAG: Embrapa Agroindústria Tropical) at Pacajus, Ceará state. Understandably, most studies have focussed on finding economically important variation to breed improved clones for agricultural purposes. Examples of the relatively few population level studies made to date of *A. microcarpum*, *A. occidentale* and *A. humile* are briefly mentioned here. Rufino (2004), as part of a study of the extractive use of cajuí by rural communities in Piauí, sampled 23 plants from native *A. microcarpum* stands in the municipalities of Ilha Grande and Parnaíba—i.e. from the same region as the present study. She found a marked variability within the sample in four morphological and eleven chemical attributes of the hypocarp. Lucena (2006) studied 14 economically significant, quantitative morphological and chemical variables of the fruits and hypocarps in a natural population of *A. occidentale* in Roraima, Amazonia. She used multivariate analysis to estimate genetically determined variation of these attributes and showed that the natural population contained a potentially important reservoir of genetic variation in economically important characteristics. Pessoni (2007) carried out a major multivariate genetic (ISSR markers) and morphometric study based on a sample of 45 individuals from a subspontaneous population in the state of Rio de Janeiro and 91 germplasm accessions at Embrapa's Pacajus cashew germplasm bank, determined as *A. occidentale*, *A. othonianum*, *A. humile* and *A. microcarpum* (two individuals from Ceará). Among his conclusions were the importance of studying natural populations of *A. othonianum* and *A. humile* to clarify their taxonomic status as species and for the purposes of sustainable use and conservation, considerations which apply equally to the taxon *A. microcarpum* focussed in the present study. The morphometric study of this author did not include leaf characters. One result, interesting from the standpoint of the objectives of the present study, was that *A. othonianum*, regarded as a synonym of *A. occidentale* by Mitchell and Mori (1987), was found to be morphologically distinct, with 100 % correct allocation in discriminant analysis. Pessoni also carried out a phenetic analysis of 18 qualitative descriptors, four of which were from the leaf (blade shape, leaf apex shape, angle of leaf insertion on stem, cross-sectional shape of leaf blade). Carvalho (2011) found a high level of intra-population genetic variation in 11 populations of *A. humile* from the *cerrado* of central Brazil, using RAPD molecular markers, with a total sample of 122 plants; eight morphological characters, including leaf length and maximum width, were also studied. Soares et al. (2013) sampled 58 individuals from field populations in three widely separated localities in Goiás, Minas Gerais and Bahia states, to test the transferability of microsatellite primers from *A. occidentale* on individuals from natural populations of *A. humile* in Brazil's *cerrado* biome. Their study aimed to build a foundation for future population genetic studies of *A. humile*, which is also socio-economically important in rural communities of the *cerrado* region. They found a high degree of genetic variation in their sample, sufficient to fingerprint individual plants.

These studies show that natural populations harbour considerable variation and that the current circumscription of *A. occidentale* may admit further taxonomic subdivision once a range of more detailed data on both morphological and molecular patterns becomes available (see especially Pessoni 2007). The outcrossing pollination biology of *Anacardium occidentale* highlighted by Mitchell and Mori (1987) and Freitas et al. (2002) provides one explanation for the variation so far recorded. However, a better understanding of the geographical patterns of this variation in natural populations is needed as a foundation for an infraspecific taxonomy of *A. occidentale* and thus for evaluating the status of morphotypes like *A. microcarpum*.

We conclude that there is a statistically significant difference in mean leaf shape between *A. microcarpum* and *A. occidentale*, but the overlap is considerable and negates the use of leaf shape as a diagnostic character to distinguish the two taxa. Furthermore, inter-population differences are just as great as those between the taxa and the populations did not form consistent separate clusters corresponding to the taxa, neither was there any evidence of correlation of inter-population leaf shape variation and geographical distance. Our initial hypothesis is thus only partially upheld.

While the status of *A. microcarpum* as a taxon distinct from *A. occidentale* could be said to have been slightly strengthened by these results, at the same time, the most important basic premise of their difference—fruit and hypocarp size—has proved also to be a quantitative rather than a qualitative distinction (Vieira et al. in prep.), and the taxonomic picture remains unresolved. This is not especially surprising, since the present study focussed on a single narrowly defined character field—leaf shape under the strict definition of geometric morphometrics (Zelditch et al. 2012)—without considering size or any other phenotypic attributes.

Further investigations of taxonomic differences between field populations of *A. microcarpum* and *A. occidentale* are needed using a wider array of phenotypic and genetic characters and are justifiable in view of the socio-economic importance of both taxa, the need for a detailed understanding of their biodiversity and because of current efforts in Piauí to preserve the wild populations of *A. microcarpum*, for which a strong foundation of biological knowledge is an essential support.

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