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Genetic Diversity in Tanzanian Accessions of *Brassica carinata* A. Braun

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ABSTRACT

We analyzed the extent and structure of genetic variation in *Brassica carinata*, an important sub-Saharan African leafy vegetable using RAPD markers. Sixty one accessions from 49 sites were collected in Tanzania over an area of almost 0.8 M km^2. Most variation, 88%, was among accessions, 4% was among regions and 8% within accessions. This pattern was reflected in the AMOVA, PCA and cluster analysis which failed to segregate accessions into regional or ecogeographic groups. We did find a pattern of spatial genetic structure at shorter distances with a significant relationship between genetic and geographic distance among accessions as revealed by a Mantel test combined with a significant autocorrelation effect at three geographic distances, 1, 5 and 7 km. This result corresponds well to the known exchange and sale of *B. carinata* seeds among neighbors, friends and family members in Tanzania. The recommended collection strategy based on results of this study is concentrating on collecting a larger number of accessions in a few, easily to collect areas and fewer accessions in less accessible areas.

Keywords: Ethiopian kale, genetic resources, germplasm conservation, leafy vegetable

INTRODUCTION

African indigenous vegetables, especially leafy vegetables, play an important role in food security and nutritional balance of both urban and rural populations in many sub-Saharan African countries (Abe and Imbamba 1977; Gomez 1981; Chweya 1985; Schippers 1997; Maundu *et al*. 1999). Leafy vegetables are often the main source of many vitamins, minerals and other nutrients in the diet of people, especially subsistence farmers, living in these areas (Schmidt 1971; Imbamba 1973; Okigbo 1983; Ruberto 1984; Chweya 1985; Aletor and Adeogun 1995; Raji *et al.* 1995; Barminas *et al.* 1998; Maundu *et al*. 1999; Mendlinger *et al*. 2006). They are usually grown by small-scale subsistence farmers for their personal consumption, with any extra yield sold in local and urban markets (Hawkes 1983; Brush 1989; Edmonds and Chweya 1997; Mendlinger *et al*. 2006). As they are neither a major cash crop nor an export crop, at the national level in most countries their importance is usually overlooked and underappreciated. Subsequently many of these species have not had the degree of genetic, breeding and agromanagement research that their local importance requires and too few systematic collections of landraces and primitive varieties have been conducted or genetically analyzed. This is important not only for selection and breeding programs but also for formulating efficient *in situ* and *ex situ* conservation strategies.

Brassica carinata A. Braun is an important and popular leafy vegetable in many Eastern and Southern African countries (Gildemacher 1997; Gruppen and Denton 2004; Mendlinger *et al*. 2006). It is believed to have been cultivated as far back as the 4th to 5th millennia BCE (Simmonds 1987). Young immature shoots and leaves are usually harvested every week or so until the plant begins to flower and are eaten as a relish or cooked vegetable. Yields of up to 55 t/ha have been reported (Gruppen and Denton 2004). It is a good source of xanthophylls, carotene, lysine and other essential amino acids (Stephens *et al.* 1970; FAO 1998; Punia *et al*. 2001; Singh *et al*. 2002; Gruppen and Denton 2004). In Ethiopia its seeds are an important source of edible cooking oil (Alemayehu and Becker 2001, 2002; Velasco *et al.* 2004; Nabloussi *et al*. 2006). Its oil has been examined for its potential as a source of biodiesel in developing countries (Cardone *et al.* 2002). In addition to its importance as a food source, it can be crossed with and genes from it can be introgressed into related crop species including *B. rapa*, *B. napus* and *B. juncea* (Burton *et al.* 2004; Yuan *et al.* 2004; Teklewold and Becker 2005).

Despite its widespread range in sub-Saharan Africa, the major systematic collections and evaluations of landraces and/or primitive varieties have been from Ethiopia although collections have been made in other countries. Alemayehu and Becker (2002) examined 13 morphological traits in 36 accessions from Ethiopia and reported: (1) variation in all traits; and (2) no major geographic clustering of genes. Ethiopian accessions have been examined for molecular genetic variation using AFLP, RFLP and RAPD markers and genetic variation was found among accessions (Song *et al*. 1996; Lionneton *et al*. 2002; Burton *et al*. 2004; Genet *et al*. 2005; Teklewold and Becker 2006a, 206b; Warwick *et al.* 2006). Alemayehu and Becker (2001), Velasco *et al.* (2004) and Nabloussi *et al*. (2006) reported genetic variation in oil quality.

Unfortunately, as in many other indigenous African vegetable species, genetic erosion is most likely occurring in this species due to an array of causes including increased human migration, loss of isolation, seed exchange, habitat destruction, land scarcity due to increasing human population and crop replacement (Hawkes 1983; Brush 1989; Ingram 1990; Guarino 1995; Rouamba 1996). Due to this we conducted a systematic collection program in Tanzania in several important leafy vegetable species (Mendlinger *et al*. 2006). This paper presents the results of the extent and structure of genetic diversity in 61 accessions of *B. cari-*

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nata collected in Tanzania using presumably selectively neutral random amplified polymorphic DNA (RAPD) markers.

MATERIALS AND METHODS

Study species

Brassica carinata (A. Braun), often called Ethiopian kale or Ethiopian mustard, is an amphidiploid with one genome from *B. nigra* (L) Koch and the other from *B. oleracea* (L). It is an annual to semi-perennial, erect, branched plant, propagated via seed and includes both cultivated and weedy types. It is usually about 70-120 cm tall, but phenotypes exist that can grow to 200 cm. It has pale yellow flowers with a distinctly flat silique. While most *Brassica* species are cross-pollinating, *B. carinata* is predominantly self-pollinated as it will set seeds without any pollinator; however, when bees or other insects are present cross pollination can occur.

Sixty one accessions from 49 sites of *B. carinata* were collected in Tanzania (**Table 1**). In 38 sites collections were made from only 1 farm; in 10 sites collections were made from 2 farms 0.5-3 km apart with each farm's collection given its own accession number; and in 1 site from 3 farms several km apart. The accessions were collected from farms located over most of the country except the Southeast and Lake Tanganyika regions, an area of almost 0.8 M km^2. Seeds were taken directly from 4-15 plants growing in a farmer's field (the number was a function of how many plants were present). In several sites the plants were immature and we asked the farmers for some of their stored seeds; we were never refused. All seeds from a farm were bulked as an accession. Each farm was classified into its respective region. Additional information on each accession can be found in Mendlinger *et al*. (2006).

Table 1 Important geographic and climatic data on the 49 *B. carinata* collecting sites in Tanzania.

Accession No.-Site	Latitude	Longitude	Elevation (ft)	Agro-climate[a]	Rainfall (mm)	Region
BG3-Hadiha	3°18.361′S	37°07.757′E	3306	4	800-1000	Kilimanjaro
BG4-Amanda Koola	3°11.387′S	37°03. 222′E	4235	2	1000-1200	Kilimanjaro
BG5-Mush (2 accessions)	3°11.875′S	37°13.633′E	5171	2	1200-1400	Kilimanjaro
BG6-Engerasia	3°17.464′S	37°10.305′E	3385	2	1200-1400	Kilimanjaro
BG8-Himo-Pofo	3°22.429′S	37°32.172′E	3100	4	700-800	Kilimanjaro
BG9-Himo-Pofo	3°22.444′S	37°32.109′E	3109	4	700-800	Kilimanjaro
BG10-Mwanga (2)	3°39.952′S	37°36.214′E	2825	4	600-700	Kilimanjaro
BG13-Bombani	5°07.948′S	38°42.189′E	634	2	1200-1400	Tanga
BG14-Pongwa	5°07.650′S	38°42.280′E	789	2	1200-1400	Tanga
BG15-Judith John	5°07.538′S	38°42.235′E	689	2	1200-1400	Tanga
BG18-Uluguru	7°03.506′S	37°34.506′E	3703	2	1200-1400	Tanga
BG20-Bigwa	6°49.015′S	37°49.015′E	1712	3	1000-1200	Morogoro
BG21-Mkata	5°47.064′S	38°17.814′E	1403	4	800-1000	Morogoro
BG22-Kibena (2)	8°10.580′S	35°23.295′E	5682	2	800-1000	Iringa
BG23-Nulolo (2)	8°30.236′S	35°04.909′E	5985	2	1000-1200	Iringa
BG25-Inyala	8°51.281′S	33°38.668′E	5028	2	800-1000	Mbeya
BG27-Nsalaga	8°53.653′S	33°33.147′E	5913	2	1000-1200	Mbeya
BG29-Mpejele	8°53.751′S	33°18.839′E	4482	3	1400-1600	Mbeya
BG30-Tukuyu	9°15.684′S	33°38.565′E	4934	3	1600-2000	Mbeya
BG31-Ushirika	9°19.017′S	33°39.441′E	4213	3	1400-1600	Mbeya
BG35-Itokela	9°05.584′S	33°33.547′E	5859	2	1200-1400	Mbeya
BG38-Mbimba	9°05.147′S	32°57.479′E	5188	3	1400-1600	Mbeya
BG39-Hanseketwa	9°08.606′S	32°53.119′E	4908	3	1000-1200	Mbeya
BG40-Kiumba	8°54.935′S	34°50.204′E	5653	2	1200-1400	Iringa
BG41-Kiumba	8°56.349′S	34°49.172′E	5752	2	800-1000	Iringa
BG42-Lialamu	9°01.287′S	34°49.596′E	5996	2	800-1000	Iringa
BG43-Njumbe	9°19.648′S	34°45.071′E	6275	2	1200-1400	Iringa
BG44-Wikichi	9°20.259′S	34°42.413′E	6717	2	1000-1200	Iringa
BG45-Igagala	9°20.909′S	34°39.111′E	6909	2	800-1000	Iringa
BG46-Nundu	9°25.426′S	34°50.463′E	6550	2	1000-1200	Iringa
BG47-Kinyangezi	9°27.100′S	34°49.068′E	6767	2	1000-1200	Iringa
BG48-Uwemba	9°28.530′S	34°47.167′E	7082	2	1000-1200	Iringa
BG49-Yakobi	9°26.641′S	34°55.361′E	5960	2	1200-1400	Iringa
BG50-Lowangu	9°28.741′S	34°59.868′E	5777	2	1400-1600	Iringa
BG51-Hagafilo	9°23.663′S	34°49.023′E	6392	2	1200-1400	Iringa
BG52-Ndiwili	7°54.643′S	35°47.685′E	5531	3	500-600	Iringa
BG53-Igula (2)	7°51.840′S	35°46.966′E	5535	2	600-700	Iringa
BG54-Kitayawa (2)	7°50.079′S	35°44.699′E	5231	2	600-700	Iringa
BG55-Kitayawa	7°50.235′S	35°44.469′E	5233	2	600-700	Iringa
BG57-Marangu	3°16.250′S	37°30.933′E	5198	2	1200-1400	Kilimanjaro
BG58-Lyasongoro	3°14.703′S	37°31.039′E	6092	2	1400-1600	Kilimanjaro
BG61-Utegi	1°19.028′S	34°12.832′E	4057	3	800-1000	Mara
BG64-Nyabangu (2)	1°31.773′S	33°51.584′E	3757	3	800-1000	Mara
BG67-Mabula-1	1°45.765′S	33°51.432′E	4158	4	1000-1200	Mara
BG68-Mabula farm	1°45.810′S	33°51.490′E	4172	4	1000-1200	Mara
BG109-Itawa	1°21.771′S	31°46.583′E	4267	3	2000-2400	Kagera
BG114-Kishera	1°27.807′S	31°44.004′E	4514	3	1400-1600	Kagera
BG115-Ibwera	1°29.558′S	31°39.260′E	3808	2	1400-1600	Kagera
BG117-Katoro	1°24.115′S	31°30.566′E	3815	2	1200-1400	Kagera

a – 2 = high potential area
3 = moderate potential area
4 = semi-arid area

DNA extraction and PCR amplification

RAPD analysis was performed using DNA from 5 plants per accession. DNA was purified from freshly harvested young leaves of two-three week old plants (150 µg) using the CTAB protocol (Scott *et al.* 1985). RAPD reaction was run with Operon primers (series OPA, OPB, OPC, OPF and OPV) and UBC primers (University of British Columbia). Of these, we found six primers that produced a total of 31 scorable and repeatable bands (**Table 2**).

Each amplification was performed in 25 µl total volume containing one tenth volume 10X Taq buffer (Fisher), 1 unit of *Taq* DNA polymerase (Fisher), 2.5 mM $MgCl_2$, 200 µM dNTPs, 1 µM of the primer, and 50-70 ng of template DNA. The main criterion for selection was production of clear amplified polymorphic bands that were replicable in two test reactions.

The RAPD reactions were carried out in an Eppendorf Mastercycler gradient thermocycler under the following conditions (Bhaskar *et al.* 2002): 94°C for 1 min, 37°C for 37 s, 72°C for 1 min, followed by 40 cycles of 94°C for 5 s, 37°C for 15 s, 72°C for 1 min. A final 7-min extension was carried out at 72°C after cycles were completed. The amplification products were separated by electrophoresis in 1.5% TBE agarose gel (Hispanagar, Spain), stained with ethidium bromide (0.5 µg/ml) and photographed under UV light. All reactions were repeated at least twice and only reproducible bands were scored for statistical analysis.

Data analysis

Bands were scored as present (1) or absent (2) by at least two authors using the SYNGENE gel imaging system. Bands of identical size amplified with the same primer were considered to be the same locus consisting of two alleles.

A matrix of genetic distances among accessions was computed from the accession allele frequencies using Nei's (Nei 1972) index of genetic distance. This matrix was used in a Principal Coordinates Analysis (PCoA) and cluster analysis with UPGMA (unweighted pair-group method, arithmetic average) clustering algorithm. The relationship between geographic distance and genetic similarity between accessions was analyzed by a Mantel test and spatial autocorrelation analysis which measures the genetic similarity between pairs of individuals whose geographic separation fell within specified distance classes (from 1 to 1000 km). Statistical significance of the autocorrelation coefficient was estimated using 1000 random permutations.

Analysis of molecular variance (AMOVA) (Excoffier *et al.* 1992) was carried out to estimate variance components due to differences within and among accessions/within regions and among regions. Five regions with more than one accession were used.

All the above analyses were done with GeneAlEx (Peakall and Smouse 2006) except cluster analysis which was performed using NTSYSpc version 2.0 (Rohlf 1998).

RESULTS

Accessions were found and collected in almost all eco-geographic regions of Tanzania traversed except in the more extreme semi-arid and arid areas (no sites found at under 500 mm annual rainfall and only 5 sites at under 700 mm annual rainfall) and at altitudes over 2121 m. The primers scored produced a total of 31 bands with a mean of 5.17 bands/primer (**Table 2**). This was sufficient to distinguish all accessions from one another except for two geographically close accessions (neighboring farms) from the Mara region. On average 5.5% of loci were polymorphic within accessions, but 15 out of 61 accessions were monomorphic for all loci tested. Expected heterozygosity, H_e, in most accessions was low, ranging from 0–0.079 with an average of 0.023 ± 0.002. There were no significant differences among regions in H_e (**Table 3**).

Table 2 Primers used in the RAPD analysis, their sequence, number of bands and size range. "OP" and "UBC" series are primers from Operon Technologies Inc. and University of British Columbia, respectively.

Primer	Sequence	Number of scorable bands	Size range of scorable bands
OPB-08	5'GTCCACACGG3'	5	700-1640
OPF-12	5'ACGGTACCAG3'	5	750-1500
OPV-03	5'CTCCCTGCAA3'	4	780-1800
OPV-12	5'ACCCCCCACT3'	5	300-1300
UBC-490	5'AGTCGACCTT3'	8	250-1600
UBC-534	5'CACCCCCTGC3'	4	344-2000
Total		31	

Table 3 Average accession genetic diversity calculated for 6 regions in Tanzania.

Region	n	He ± SE
Kilimanjaro	11	0.022 ± 0.006
Tanga	4	0.040 ± 0.011
Morogoro	2	0.019 ± 0.007
Iringa	23	0.024 ± 0.004
Mbeya	12	0.023 ± 0.005
Mara	5	0.026 ± 0.012
Kagera	4	0.000 ± 0.000

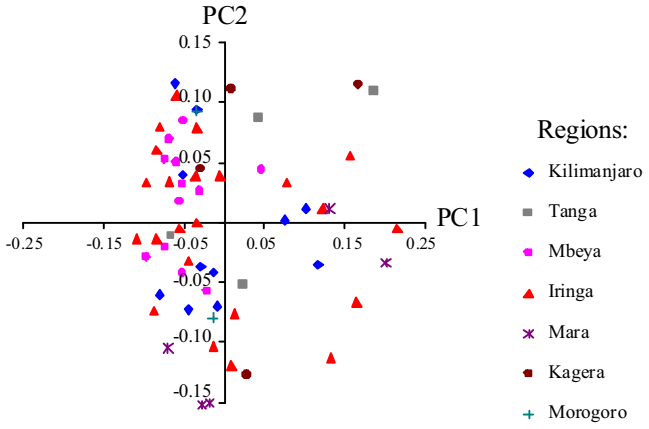

Fig. 1 A PCoA plot with the first two axes accounted for 29.6 and 20.6%, respectively, of the total variation.

The structure of genetic variation was examined by partitioning the total genetic variation into that due to among regions, among accessions in a region and within accessions. The results of the AMOVA showed that 88% of genetic variation was among accessions, 8% within accessions and 4% among regions (**Table 4**). PCoA, in which the first two axes accounted for 50.2% of variation, failed to cluster accessions according to regions (**Fig. 1**).

While we did not find any significant regional differentiation we did find a pattern of spatial genetic structure at shorter distances. A Mantel test revealed a weak but significant relationship between genetic and geographic distance among accessions (r = 0.134, p = 0.03). The spatial autocorrelation analysis detected a significant autocorrelation effect for the three shortest distance classes, 1, 5 and 7 km (r = 0.321, 0.180 and 0.143, p < 0.001, 0.01 and 0.05, respectively) (**Fig. 2**). No significant autocorrelations were found at distances beyond 7 km. This autocorrelation pat-

Table 4 Results of AMOVA. P-value is derived from 1000 permutations and denotes the probability of observing a larger component of variance by chance alone.

Source of variation	df	SSD	MSD	Variance component	Φ_{PT}	p-value
Among regions	6	229.1	38.2	0.229	Φ_{RT} 0.035	0.01
Among accessions	54	1578.1	29.2	5.774	Φ_{AR} 0.919	0.01
Within accessions	244	123.2	0.5	0.505		

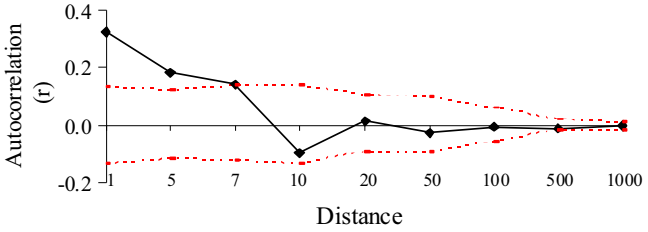

Fig. 2 The autocorrelogram with 95% confidence interval (dotted lines) for autocorrelation coefficient *r* (solid line).

tern was reflected in the cluster dendrogram produced by using UPGMA (**Fig. 3**). Excluding a clumping of 7 of the 23 Iringa accessions, 5 of the 12 Mbeya accessions and 2 accessions each from Kilimanjaro and Mara (most of the accessions within each clump were geographically close to each other), no clumping via region was found.

DISCUSSION

The accessions collected in Tanzania had levels of genetic variation similar to those found in a comparable study using RAPDs by Teklewold and Becker (2006b) which examined 29 accessions from Ethiopia and 14 breeding lines with unknown origins from Australia, Pakistan, Spain and Zambia. We had a mean number of bands/primer of 5.2 whereas they had a mean of 5.5. Genetic variation in *B. carinata* accessions and breeding lines, mostly originating in Ethiopia, was found for important agronomic traits (Alemayehu and Becker 2002), oil quality (Alemayehu and Becker 2001; Velasco *et al.* 2004; Nabloussi *et al*. 2006) and in AFLP molecular markers (Genet *et al*. 2005; Warwick *et al.* 2006). The accessions from this study are presently being tested in field trials for their agronomic potential.

What is new and relevant for both conservationists and breeders is that we found that 88% of the molecular variation was among accessions and only 4% among regions even though our collecting area was over 700,000 square kilometers which ranged from semi-tropical cool mountains to semi-arid hot plains. This was reflected in the AMOVA, PCoA and cluster analysis which gives no support to potential segregation of accessions into regional or ecogeographic groups. Similar lack of regional clumping was found in Ethiopian accessions using morphological and agronomic traits (Alemayehu and Becker 2002) and AFLP molecular markers (Genet *et al*. 2005; Warwick *et al.* 2006). However, these studies did not quantify the partitioning of the variation or reported such small amount of among region and within accession variation. For conservationists who need to design cost efficient collection and conservation strategies which are labor saving and parsimonious for *in situ* and *ex situ* seed banks, these results point to developing a strategy of concentrating on collecting a larger number of accessions from a few, easily to collect areas and fewer number of accessions from harder and more expensive areas to collect (this strategy is based on Brown's (1989) recommendation for core collections). While such a strategy may not be perfect, minimizing costs and time has become important for collectors.

As for breeders, the results of molecular genetics does not always correspond well with agronomic traits, as Tekleword and Becker (2006a) found in examining heritability

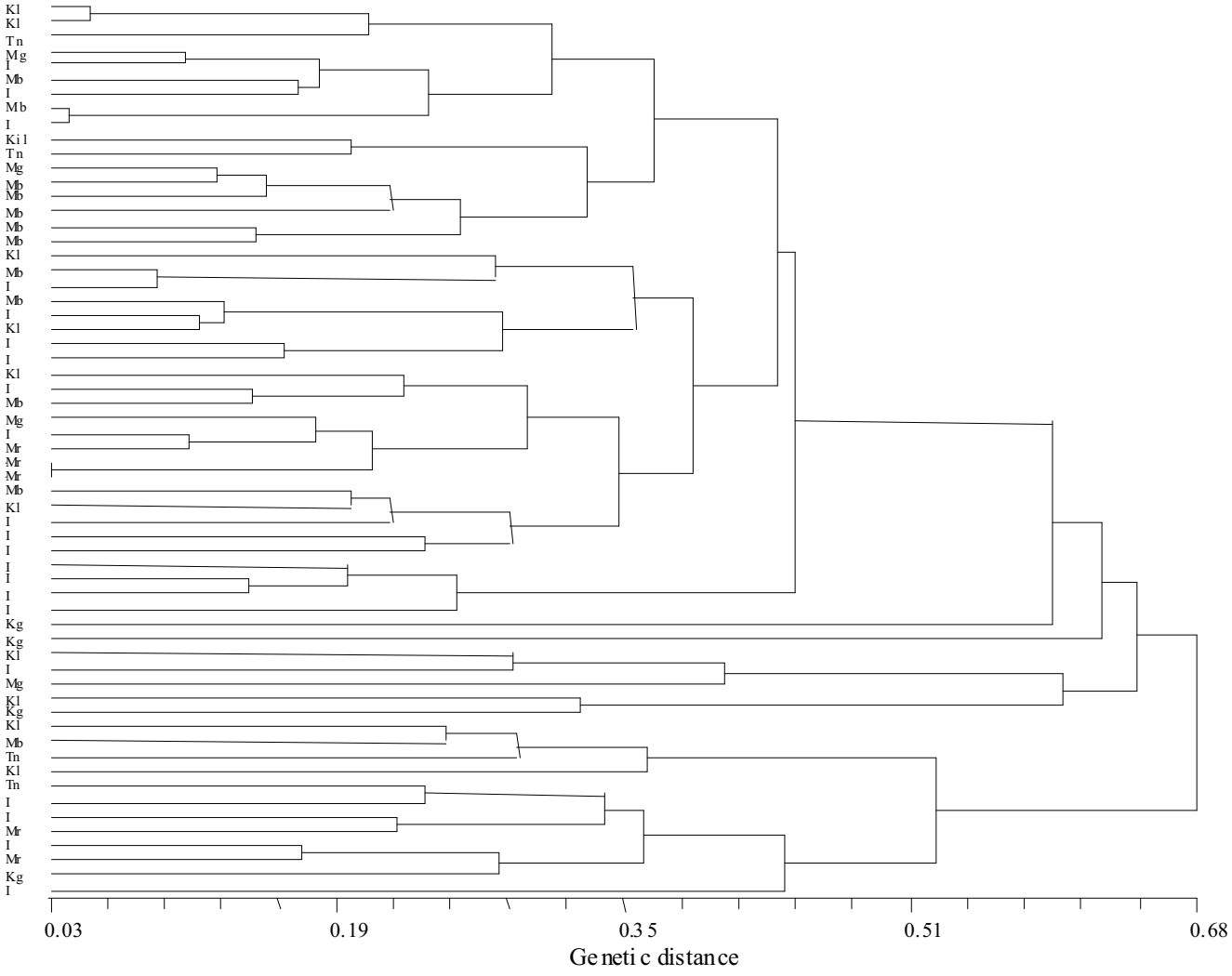

Fig. 3 UPGMA dendrogram of analyzed accessions classified by regional identity. Abbreviations: Kl – Kilimanjaro, Tn – Tanga, I – Iringa, Mg – Morogoro, Mb – Mbeya, Mr – Mara, Kg – Kagera.

estimates and combining ability in *B. carinata* accessions. This is especially so as, unlike molecular markers, many agronomic traits have been actively selected for by farmers. Therefore collection and screening programs need to reflect this difference. The collection strategy proposed here takes this into account by suggesting that while one needs to collect several accessions from many areas for area or regional specific alleles, large scale in depth collections need only be done in a few. The same strategy can be used for screening programs.

The significant autocorrelations found within a few km of a farm may represent traditional social and family norms. Mendlinger *et al*. (2006) found considerable exchange and/or sale of seed of *B. carinata*, as well as other vegetable crops, among neighbors, friends and family members in Tanzania. Usually this exchange is done with people who live close to a farmer. The significant autocorrelation detected within a distance up to but not beyond 7 km, appears to reflect this. That no regional autocorrelation was found may indicate that regional genetic differentiation or selection may not be occurring or is very weak, that some seed exchange may be occurring over large areas and/or genetic drift is occurring. Our study was not designed to differentiate among these possibilities.

Despite critique of RAPD utility for systematic and genetic diversity studies, our results contribute to advocating that RAPD can be not only an inexpensive but also an efficient method of screening molecular diversity (Geleta *et al*. 2007; Hüseyin Karataş and Sabit Ağaoğlu 2007; Volis *et al*. 2009).

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