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Genetic diversity of Zimbabwean and exotic flue-cured tobacco varieties based on phenotypic traits and simple sequence repeats

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Determination of genetic diversity among Zimbabwean and exotic flue-cured tobacco varieties is important for variety identification and for rational use of germplasm in breeding programs. This research investigated genetic diversity among Zimbabwean and exotic flue-cured tobacco varieties using phenotypic traits and simple sequence repeats markers. A total of twenty eight flue-cured cultivars (sixteen from China, six from Brazil, two from South Africa and four from Zimbabwe) were studied. Phenotypic data for tobacco descriptor traits were collected from a field experiment carried out using standard cultural practices for flue-cured tobacco in Zimbabwe. A sample of fifteen cultivars was genotyped with simple sequence repeats markers. Low levels of diversity were detected among the cultivars using analysis of either phenotypic or DNA marker data. Pair wise genetic similarities ranged from 0.80 to 0.99. Genetic distances determined were all less than 0.4. The study suggests the need for conservation of germplasm used in Zimbabwe to avoid further genetic erosion.

Key words: Fingerprinting, genetic erosion, genetic conservation, *Nicotiana tabacum.*

INTRODUCTION

Nicotiana tabacum (2n = 48) is considered to be a product of inter-specific hybridization events between *Nicotiana sylvestris* (2n = 2x = 24) and a species closely related to modern-day *Nicotiana tomentosiformis* (2n = 2x = 24) followed by chromosome doubling (Olmstead et al*.,* 2008; Kenton et al., 1993). Limited genetic diversity has been detected among lines of cultivated tobacco (Sarala and Rao, 2008; Zhang et al*.,* 2007; Lewis and Nicholson, 2007). The genetic diversity within commercial tobacco cultivars has been narrowed due to a combination of genetic drift and conservative breeding strategies used by tobacco breeders (Moon et al., 2009a; Tripp and van der Heide, 1996). Plant breeders emphasise on crossing elite by elite lines in attempt to conform to industry market standards and thus serve as a strong force to reduce genetic diversity in breeding programs (Gepts, 2006; Rauf et al., 2010). Decreases in genetic diversity can be associated with loss of potentially useful alleles available to breeding programs (Clements et al., 2011; Upadhyaya et al*.,* 2010).

Determination of genetic diversity of existing flue-cured tobacco varieties is a prerequisite for preventing further genetic erosion. This can be achieved through the use of phenotypic, biochemical, and/or DNA-based markers (Kumar et al*.,* 2009). Shah et al. (2008) indicated that if phenotypic data are accurately collected and recorded,

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they can provide diversity information which is nearly equivalent in usefulness to that obtained using DNA-based markers. Characterization of *N. tabacum* at the molecular level has been improved by the introduction of molecular maker techniques such as simple sequence repeats (SSR) markers (Ovesna et al., 2002; Zhang et al., 2006). SSR markers for tobacco have become the most commonly used because they are highly polymorphic, co-dominant, relatively cheap, and reproducible (Semagn et al*.,* 2006). Furthermore, a dense genetic map of tobacco based on SSR markers has been developed and some markers linked to traits of interest have been reported (Bindler et al., 2007). In flue-cured tobacco genetic diversity studies, most researchers have emphasized the use of DNA based markers (Lewis and Nicholson, 2007; Sarala and Rao, 2008; Moon et al*.,* 2009b). Brown (2008) however, encouraged the analysis of genetic diversity at both phenotypic and molecular levels.

Information on the genetic relatedness of Zimbabwean and exotic flue-cured tobacco germplasm is necessary for variety identification, and for rational use of germplasm in breeding programs (Clements et al*.,* 2011). Lewis (2007) for example, emphasised the need for an examination of different sources of genetic diversity affecting resistance to various strains of PVY which induce variable reactions across various tobacco genotypes. In addition, Eigenbrode and Trumble (1994) stressed the importance of diversity for breeding for plant stress resistance and stated that crop improvement can be achieved through introduction of pathogen resistance genes from *ex situ* germplasm collections. The objective of the current study was to investigate genetic diversity of Zimbabwean and exotic flue-cured tobacco varieties using phenotypic and microsatellite marker data and to compare the two data sets for their effectiveness in grouping tobacco cultivars.

MATERIALS AND METHODS

Plant materials and phenotypic evaluation

Twenty eight flue-cured tobacco cultivars from four different countries were characterized at the phenotypic level. The names and origins and attributes of the 28 varieties studied are shown in Table 1. The 28 cultivars were evaluated in a randomized complete block design with three replications at the Kutsaga Research Station, Harare, Zimbabwe, during the 2011/2012 growing season. Kutsaga Research Station is located at latitude 17° 55' S and longitude 31° 08' E and at an altitude of 1479 m above sea level. Average annual rainfall ranges from 750 to 950 mm and mean summer temperature is 32°C while the mean winter temperature is 18°C. Soils at Kutsaga Research Station are on average light textured sand loamy which are deep and permeable. The actual site at which the study was carried out had granite sands which had the following parameters: pH 5.39, electrical conductivity 26, calcium 0.80 meq/100 g, magnesium 0.33 meq/100 g, sodium 0.08 meq/100 g, potassium 0.03/100 g, iron 134 µg/g, manganese 19 µg/g, copper 1 µg/g, zinc 3 µg/g, initial mineral nitrogen 25 µg/g and phosphate 64 µg/g. Plots consisted of single rows of 32 plants with 1.20 m inter-row and 0.56 m in-row spacing, respectively. Tobacco transplant production and field plot maintenance were performed according to tobacco production recommendations for Zimbabwe (Mazarura and Asher, 2011). Curing was done following the recommendations by CORESTA (1994a) and CORESTA (1994b).

Data on potato virus Y (PVY) incidence and severity was collected 74 days after transplanting and was indexed using the scoring system by the Cooperation Center for Scientific Research Relative to Tobacco (CORESTA)'s phytopathology group (2008). This was followed by the collection of the lengths and widths of both the largest and penultimate leaves on ten plants per plot for all replications, 94 days after transplanting. The middle leaf angles from the stems and plant heights (cm) were measured and recorded at 120 and 127 days post-transplanting, respectively. Data on root-knot nematode infestation was scored on ten plants per plot for all replications at 165 days post-transplanting using the scoring system of Daulton and Nusbaum (1961), with a rating of 0 for non-infested and a rating of 8 for severely infected. After harvesting and curing plot yields were determined and quality was assessed using the Tobacco Industry and Marketing Board's official tobacco classification system for Zimbabwe (Tobacco Industry and Marketing Board, 2012). Percent nicotine and percent reducing sugars were determined for the cured leaves in middle stock position (fifth and sixth reaping) on plot basis using analytic methods recommended by CORESTA (1994a) and CORESTA (1994b), respectively. In brief, the percent nicotine determination was based on spectrophotometric measurement at three wavelengths of 236, 259 and 282 nm of a steam distillate of ground cured leaf tobacco using a Perkin Elmer Lamda 25 UV/ Vis Spectrophotometer with the use of the measurements at 236 and 282 nm for interference correction. The percent reducing sugars were determined by water extraction prior to the removal of interfering phenolic substances with saturated barium hydroxide and zinc sulphate (10%) precipitation. The tobacco extract was then reacted with a known amount of Somogyi solution. The copper II ions in the Somogyi solution are reduced to copper I with reducing sugars. After filtration the excess unreacted copper ions were read on a Varian Spectra 100 atomic absorption spectrophotometer at a wavelength of 324.8 nm.

Simple sequence repeats marker genotyping

A subset of fifteen cultivars was selected for microsatellite marker analysis. The subset was selected in such a way that all local and both South African varieties were included (these were few) and that Brazil and each of the two Chinese provinces were represented by two closely related and one distant flue-cured tobacco variety according to the phenotypic clustering. The sweet potato cultivar 'Brondal' was used a taxonomically divergent check. The cultivars included in the subset for molecular analyses are indicated by asterisks in Table 1.

Sixteen previously published microsatellite marker primer pairs (Table 2) were selected based upon previously published polymorphic information content (PIC) and genomic location (Bindler et al., 2011; Davalieva et al*.,* 2010) and were used to genotype the subset of fifteen cultivars. Primer pairs, Taq DNA polymerase (Super-Therm 250U), 25 mM $MgCl_2$, and buffer (10 X) were obtained from Inqaba Biotech in South Africa. All other reagents were supplied by Sigma Aldrich (St. Louis, MO, USA).

DNA was isolated from leaves of plants of the selected cultivars using the CTAB procedure according to Doyle and Doyle (1987). Using a GeneAmp PCR system 9700 thermal cycler, PCR reactions were performed in 50 µl final volumes containing 10 x PCR buffer, 0.2 mM dNTPs, and 10 pmol of each forward and reverse primer, 1 U of Taq polymerase (5 µ/µl), 1.5 mM $MgCl_2$, 50 ng of template DNA, and 32.7 µl of ultra pure water. PCR conditions consisted of

Table 1. List of varieties which were characterized at Kutsaga Research Station.

Variety	Country of origin	Variety	Country of origin
LH 48/34*	South Africa	Red flower and big dollar	China (Sichuan)
LH 43/34*	South Africa	Cuibi No. 1	China (Sichuan)
DM 466*	Brazil	Nanjiang-3	China (Sichuan)
DVH 301	Brazil	Jiucaiping-2	China (Guizhou)
DVH 2101*	Brazil	Guiyan-201	China (Guizhou)
AOV 405*	Brazil	Zhongyan-90*	China (Guizhou)
AOV 708	Brazil	Guiyan No.11*	China (Guizhou)
AOV 911	Brazil	H80A*	China (Guizhou)
Yunyan 85	China (Sichuan)	98	China (Guizhou)
Yunyan 87	China (Sichuan)	Guanghuang-35	China (Guizhou)
Yunyan 97*	China (Sichuan)	K RK26*	Zimbabwe
Zhongyan 103*	China (Sichuan)	T66*	Zimbabwe
Zhongyan 100	China (Sichuan)	KE1*	Zimbabwe
Nanjiang 3 Hao(ex–Liangshan)*	China (Sichuan)	KM10*	Zimbabwe

Cultivars with *'s were those contained in the subset that was genotyped. Sichuan and Guizhou are Chinese provinces where the cultivars were obtained.

Table 2. Forward (F) and reverse (R) sequences of the SSR primer pairs used to genotype selected flue cured tobacco cultivars.

Primer name	Sequence 5´-3´	Primer name	Sequence 5´-3´
PT20172	F: ACACCTCCTTCTTCCTGC R: CCAAAATGGTTCACTGGA	PT30077	F: CTTCCTCTGCCACCTTTCAG R: GCTTCCATAGTTGGTAAAGCCA
PT30392	F: CGAGGACGATGTAAATGCTT R: GCTAAGCTTGAAATCACATTCA	PT30096	F: GAAGTTTCAAAGTAGCACCAACAA R: GCACCCTATTTGGTCTCCC
PT30021	F: CATTTGAACATGGTTGGCTG R: CTCAACTCTCGTCGCTCTTG	PT30274	F: GACAGCTAAGCTAATAACAGTAAATG R: GGACTTTGGAGTGTCAAATGC
PT30480	F: AAAGGGAAACATGGACATTG R: TAGGCGAGATTGTGGGATTC	PT50519	F: TCGAATCAAATTATGTCGATGG R: CCAACTGAATTACCCGTTACC
PT20242	F: TCCAAAGTTGGACCAGAA R: GTCCTACATGGGGCTCTT	PT54294	F: ACGTCTCTGTTGAAACCTGC R: GCCATCCCAAGAGAAACAAA
PT30378	F: TCAAATGAGGGTTGTAGCCA R: TGCAATGGCTACACAAGAAGA	PT51635	F: TGTTATCACAACTCGACATTTATGAG R: GGCCTTGTCATCATATTGGG
PT30144	F: TGATTTGTATTGACAGCGTGAAG R: TTGTTTAGTTACCCTATTTGACTTGC	PT51299	F: TAACCCGCGCAACAAACTAT R: TGACATTGTATAGCCGCTCTC
PT20445	F: CAAGAACAATGTGTCAACTGTGAA R: ATGGCAAAATGTTGCATCTC	PT30292	F: AAGACAGATTGGTGCGGAAC R: AGCACTTGGACAGGCGAATA

The primers were selected from the primer lists of Bindler et al. (2011) and Davalieva et al. (2010).

an initial denaturation step at 94°C for 10 min, 35 amplification cycles of denaturation at 94°C for 10 s, primer annealing at 55°C for 45 s, DNA extension at 72°C for 1 min and a final extension at 72°C for 20 min. PCR products were separated on 1.5% agarose gels (80 min at 110 volts and 2 amps). Amplified bands were recorded as binary data, where the presence of each reproducible polymorphic DNA band at a particular location on the gels was scored as a 1, while its absence was indicated by a 0.

Data analysis

Multivariate analysis of variance (MANOVA) for the phenotypic traits was carried out using Genstat 14 (Harding and Payne, 2011). The total number of marker fragments, the number of polymorphic loci, and the band size ranges for each marker were recorded for primer evaluation and quantification of molecular diversity. The data was used to calculate the proportion of polymorphic loci using the

formular; P = n_{pj}/n_{total}; where P is the proportion of polymorphic loci, n_{pj} is the number of polymorphic loci, and n_{total} is the total number of loci. The average number of alleles per locus was also calculated by dividing the sum of all detected alleles in all loci by the total number of loci. The genetic distances (GDs) between the closest and most distant varieties were calculated using the formula; GD = 1– 2Nij/(Ni + Nj) by Nei and Li (1979), where Nij is the number of fragments common to accessions i and j, and Ni + Nj is the total number of fragments in accessions i and j. The UPGMA (unweighted paired group method using arithmetic averages) based similarities were produced to determine relatedness among the fifteen flue-cured tobacco varieties. Hierarchical cluster analysis of the phenotypic, molecular (SSR), and combined (phenotypic and SSR marker) data were carried out to produce dendrograms using Euclidean's distance with the UPGMA algorithm (Harding and Payne, 2011).

RESULTS

Analysis based on phenotypic data

Multivariate analysis of variance (MANOVA) using the phenotypic data indicated significant differences ($P < 0.05$) among varieties for each of the traits studied. The Chinese varieties, H80A and Guanghuang 35, exhibited the highest nicotine levels of 2.66 and 2.56%, respectively. Zimbabwean varieties, KRK 26 and T66, were the least affected by root-knot nematodes with nematode severity ratings of 0.9 and 0.4, respectively. LK48/34 was the shortest cultivar (46 cm tall), while Guiyan 11 (85 cm) and H80A (90 cm) were the tallest. Generally, local cultivars exhibited the highest values for yield, quality, and other measured agro-morphological traits. The varieties tested also exhibited differential responses to PVY. All the varieties were affected by PVY, but the South African varieties had the highest percentage of unaffected plants while the Zimbabwean, KRK 26, was among the most affected varieties. The Chinese cultivar, Red flower and Big Dollar, had the least percentage of necrotic spots.

Hierarchical cluster analysis showed that the varieties from the four different countries had genetic similarities ranging from 0.92- 0.99 (Figure 1). Clustering at the 94.5% level of similarity produced ten groups comprised of four major groups and six minor groups. The cultivars Zhongyan 90, H80A, Yunyan 97 and Juicaiping 2 (each of Chinese origin) as well as KM10 and T66 (from Zimbabwe) were isolated from the rest of the cultivars at about a 93.8% degree of similarity. Of the major groups at the 0.94 similarity level, the first cluster contained the two South African cultivars and one Brazilian variety, DVH 2101. Of the South African varieties, LK43/34 was closely associated with DVH 2101. The second cluster contained all the Brazilian varieties except DVH 2101 and also contained the local yield check, KRK 26, which was closely associated with the Brazilian variety AOV 911. These varieties also clustered together with four Chinese varieties (one originating from Guizhou Province and the other from Liangshan Tobacco Company in Sichuan Province). The fourth major group consisted of three Chinese varieties from the Liangshan Tobacco Company (Nanjiang 3, Yungyan 85 and Zhongyan 100) and one from the Guizhou Province (Nanjiang-3 Hao).

Analysis based on microsatellite markers

Of the sixteen primer pairs tested on the subset of 15 cultivars, four produced monomorphic bands and were not used for genetic diversity analysis. Complete information for the primer pairs producing polymorphisms is provided in Table 3.

Genetic distance (GD) which was found between the Zimbabwean variety T66 and the South African variety LK 43/34 was; GD = 1-2(17) / (33+23) = 0.39 while the genetic distance (GD) which was noted between the Zimbabwean varieties, KRK 26 and T66, was; GD = 1-2(33) / (33+36) = 0.04.

Based on the microsatellite marker data, seven clusters with only three major groups were produced at the 0.82 level of similarity (Figure 2). The first group was comprised of the Zimbabwean varieties (KRK 26 and T66). The two South African varieties were clustered together in the second group. The third cluster consisted of the Zimbabwean variety KM10 and all Brazilian varieties as well as three Chinese varieties from the Liangshan Tobacco Company and one variety, H80A, from the Guizhou Province. The other three varieties (the Zimbabwean cultivar, KE1, and two Chinese varieties, Zhongyan 90 and Guiyan 11) joined the rest of the flue-cured tobacco varieties to make one major cluster at the 0.8 level of similarity. Brondal was distantly attached to the flue-cured tobacco varieties at about a 0.5 level of similarity.

Combined analysis of phenotypic and microsatellite marker data

The UPGMA similarity matrix based on combined molecular and phenotypic data (Figure 3) indicated the cultivars T66 and LK 43/34 to be the most distantly to each other (similarity = 0.65), while cultivars T66 and KRK 26 were found to be the most similar (similarity = 0.94).

Results from a combined analysis of the molecular and phenotypic data revealed the tobacco varieties from the four different countries to be closely related with genetic similarities ranging from 0.80 to 0.94 (Figure 3). At these levels of similarity, clustering based on country of origin was more evident.

DISCUSSION

The significant phenotypic trait differences that were observed indicated that all the measured characters

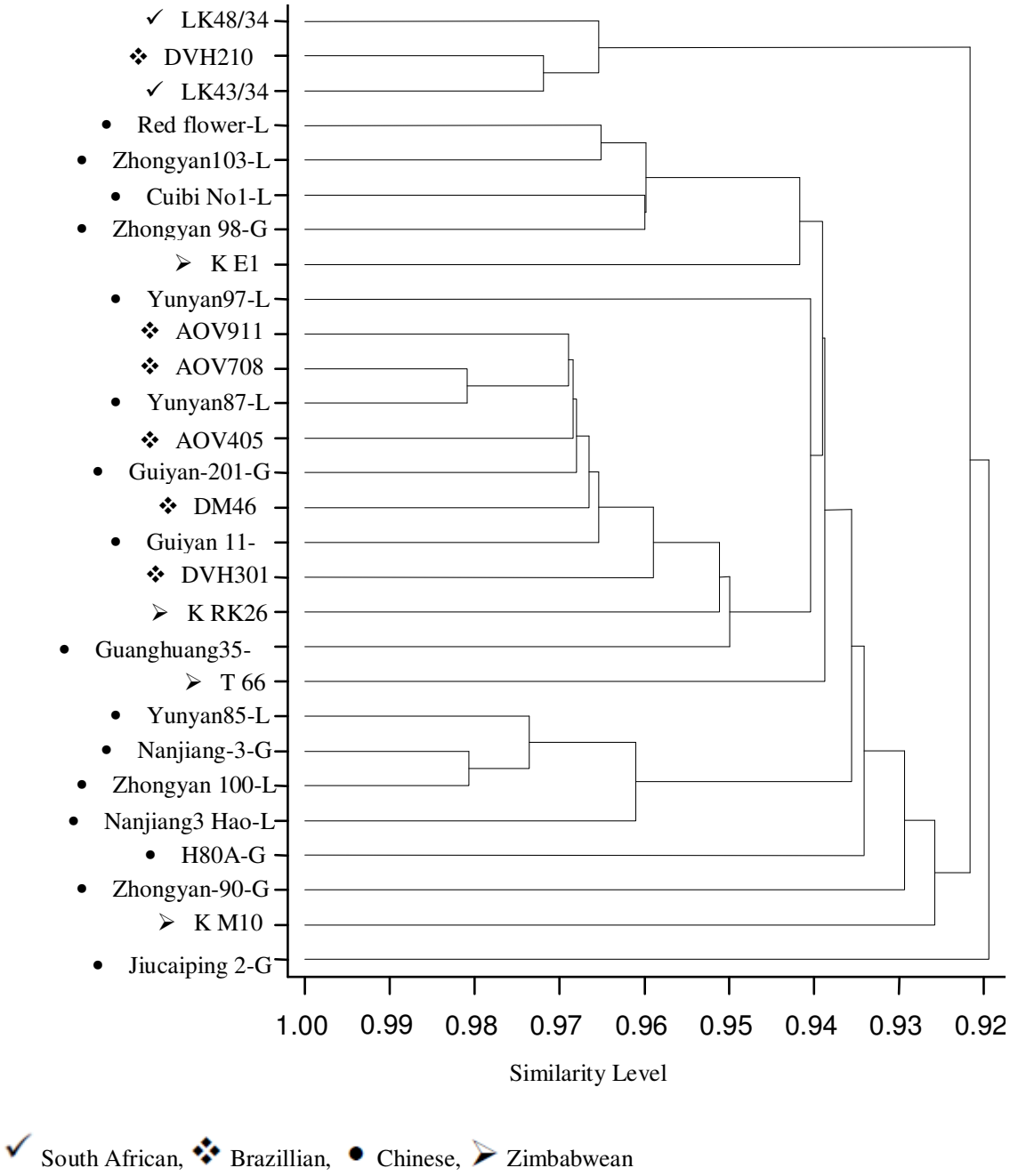

Figure 1. Dendrogram generated by the UPGMA method based on phenotypic characters measured on 28 tobacco varieties.

might be useful for genetic diversity studies. This was in agreement with the findings by Shah et al. (2008) who also reported significant phenotypic differences among different flue-cured tobacco varieties. Cultivars H80A and Guanghuang 35 might be useful as breeding materials for increasing percent nicotine since they exhibited the highest percentages for this alkaloid. The Zimbabwean varieties KRK 26 and T66 might be useful as sources of resistance to root-knot nematodes, as these were found to be the most resistant. The incidence of PVY-induced necrotic spots was lowest for the Chinese cultivar, Red Flower and Big Dollar, suggesting the potential value of this germplasm as a donor of alleles affecting tolerance to this virus. For yield, cured leaf quality, and other agronomic traits, the local varieties were generally superior to all other studied materials.

The observed phenotypic differences might be attributed to slight sequence dissimilarities in the relevant

Table 3. Data for 12 primer pair combinations that produced polymorphisms within the subset of fifteen studied cultivars.

Marker	No. of alleles	No. of polymorphic alleles	Proportion of polymorphic alleles	Rate of polymorphism (< 99%)	Size range (bp)	Average number of alleles
PT30021	10	10	100	Yes	50-1100	5
PT30480	3	2	67	Yes	150-900	5
PT30292	6	6	100	Yes	50-1000	5
PT50519	5	5	100	Yes	100-900	5
PT20378	4	4	100	Yes	150-1000	5
PT30005	2	2	100	Yes	100-800	5
PT54299	3	3	100	Yes	50-150	5
PT30077	6	4	67	Yes	100-120	5
PT30096	5	2	40	Yes	400-500	5
PT51635	2	2	100	Yes	100-900	5
PT20712	5	4	80	Yes	50-150	5
PT32072	1	1	100	Yes	100	5

genes and/or perhaps variation in gene expression. Most economically important traits are complex and influenced by multiple genes, however, and they can also be influenced by environmental conditions (Yang et al., 2007; Kumar et al., 2009). The identification of allelic polymorphism for the majority of the microsatellite primer pairs tested indicated their potential value for genetic diversity studies.

Multivariate cluster analysis indicated limited genetic diversity among Zimbabwean and exotic flue-cured tobacco varieties with genetic similarities ranging from 0.80 to 0.99 for the three cluster analyses performed. This was confirmed by the short genetic distances (< 0.4) obtained from the molecular data. The very narrow range of phenotypic and molecular diversity observed among the flue-cured tobacco varieties from the four different countries is possibly due to (1) a genetic bottleneck caused during the evolution of the amphidiploid *N. tabacum* species, and (2) conservative breeding strategies used by breeders of various market classes of tobacco. Strict quality requirements generally cause breeders to tend not to make crosses between lines of different market classes. A high degree of genetic similarity among flue-cured tobacco cultivars around the world has also been reported by other researchers. These include Yang et al. (2007) who detected >70% genetic similarity among different tobacco lines, and Yang et al. (2007) who reported low genetic diversity among flue-cured tobacco varieties in China. Arslan and Okumus (2006) also indicated limited genetic diversity among tobacco varieties in eastern Anatolia.

The possible cause of clustering of Chinese varieties together with Brazilian varieties and the Zimbabwean cultivar KRK 26 which was observed using phenotypic data is that most breeding materials that are used around the world are ultimately derived from materials originating from the United States. For example, many modern cultivars around the world are derivatives of popular U.S. flue-cured cultivar K326.

The two separate dendrograms produced using only phenotypic data or only marker data did not show any clear clustering of the flue-cured tobacco varieties according to their geographic origin. Similar patterns were also noted by Zhang et al. (2007) in a study which characterized flue-cured tobacco cultivars based on RAPD and AFLP markers. This may suggest that meeting stringent world market requirements is one of the major concerns of plant breeders, thus resulting in materials from different countries being directed towards common characteristics required by the market as was also suggested by Gepts (2006).

When the combined data set was analyzed, however, some increased congruency was observed with country of origin. This suggests that most varieties from the same country may be sharing common ancestry. This may also be due to both natural and artificial selection in favour of the prevailing environmental and climatic conditions of their regions of origin as was also noted by Sarala and Rao (2008). Liu and Zhang (2008) also suggested that different breeding efforts for specific biotic and abiotic conditions in a particular country can result in some materials from the same country being concentrated in the same cluster.

The restricted genetic diversity among cultivated tobacco varieties could imply that there could be merit in utilizing external material to improve some quantitative traits such as leaf yield and quality. Some genes coding for resistance to some biotic and abiotic stresses might be exploited (Lewis, 2007).

Some experimental factors, however, may have contributed to the narrow genetic diversity observed in the molecular diversity analysis. The narrow genetic diversity observed at the molecular level may possibly have been affected by the few number of molecular markers used. Increasing the number of primer pairs could help to identify additional variation existing

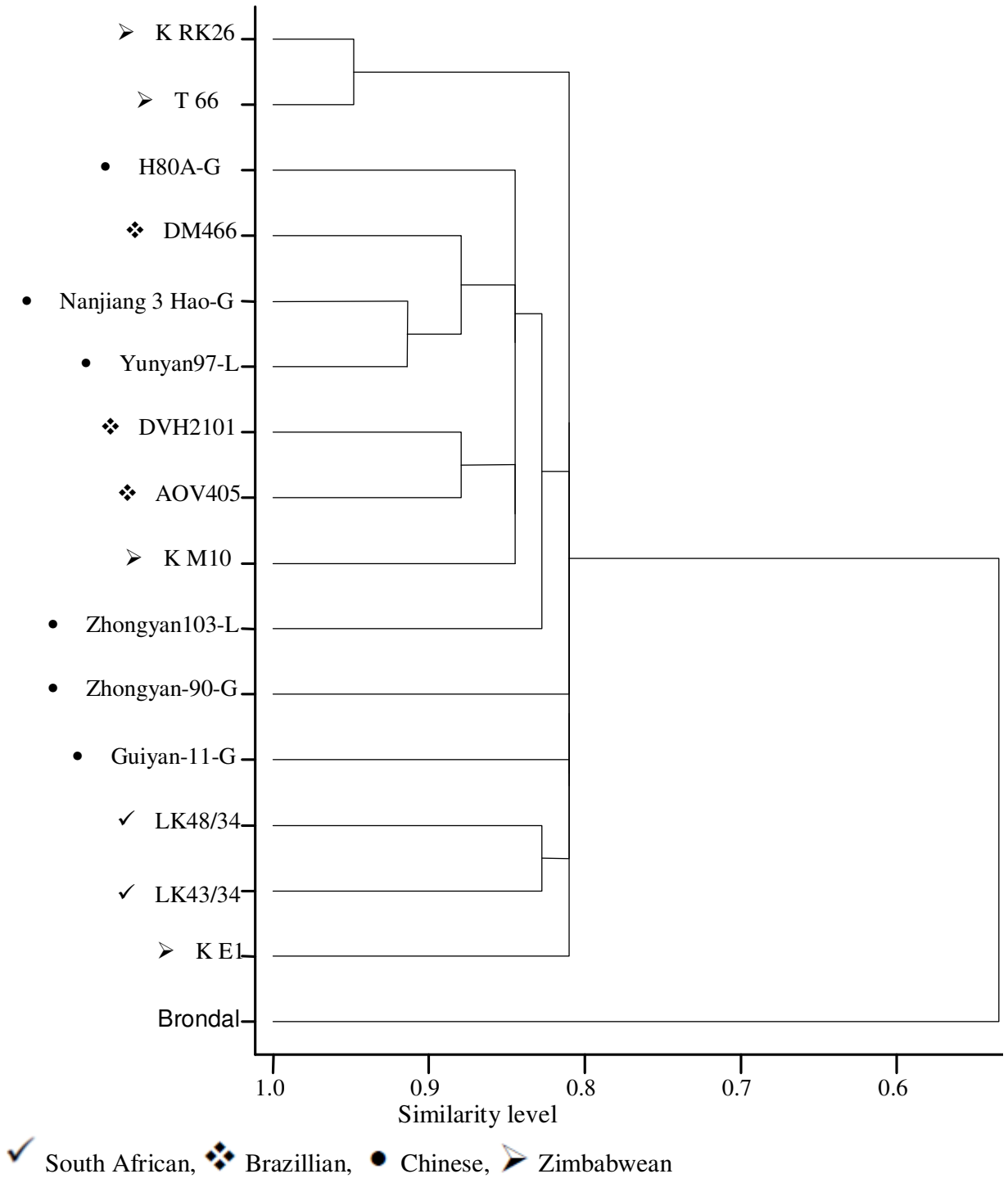

Figure 2. Dendrogram generated by UPGMA analysis of microsatellite marker polymorphisms detected for fifteen tobacco accessions.

between the studied cultivars.

Results from multivariate analysis using phenotypic and molecular marker data confirmed similar but not identical clustering. Clustering based on SSR markers proved to be more sensitive than based on phenotypic markers since the former resulted in clustering from a 0.8 level of similarity, while the later clustered varieties from about a 0.91 level of similarity. The molecular results also had much influence on the cluster analysis using the combined data. The clustering based on the combined

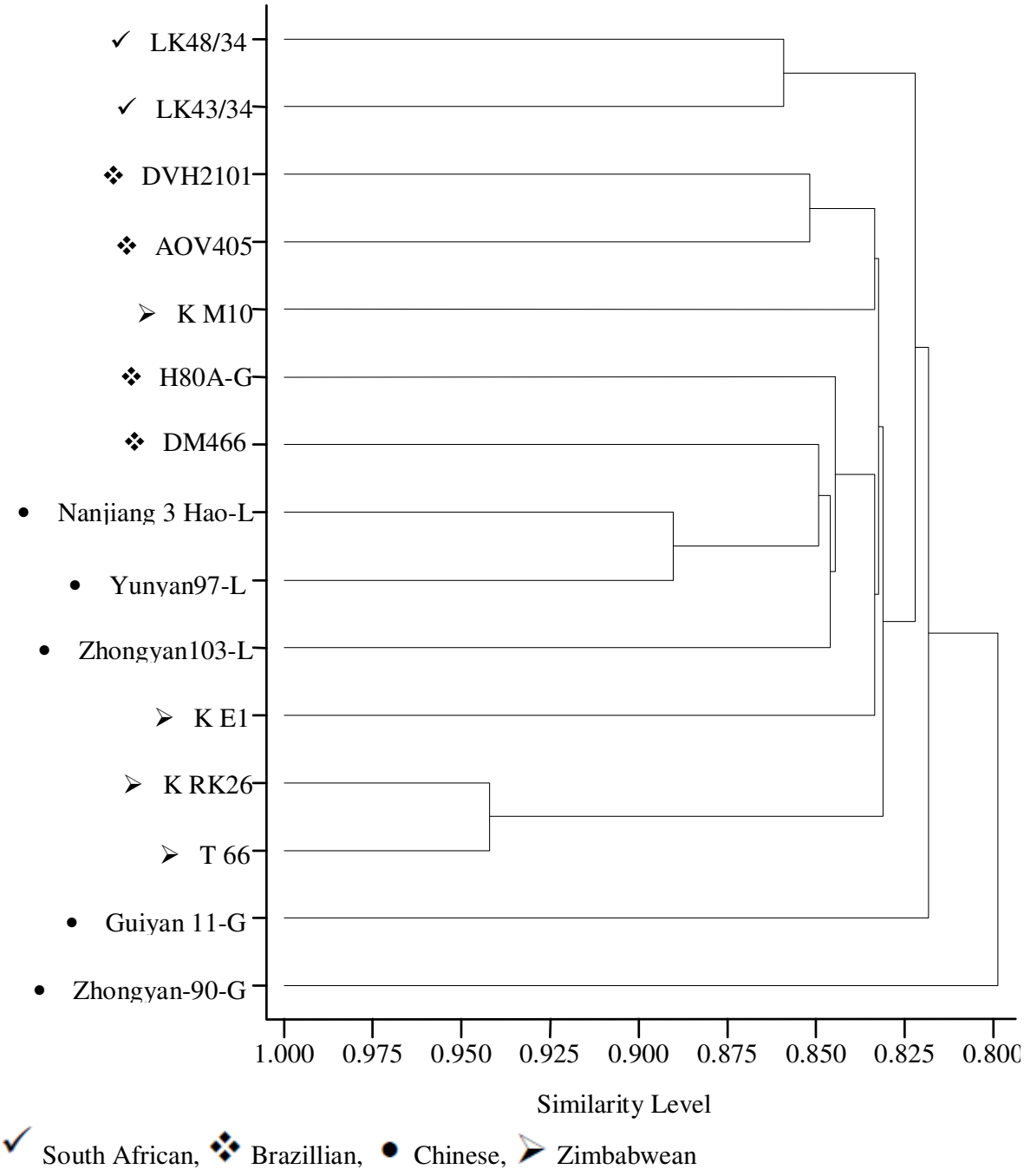

Figure 3. Dendrogram generated by UPGMA analysis of combined molecular and phenotypic data for 15 tobacco varieties.

data was more similar to that produced using the marker data only. Poehlman and Sleper (1987) ascribed slight differences between phenotypic and molecular clustering to environmental influence on the performance and phenotypes of quantitative traits. This might suggest the value of using of only molecular marker data for diversity analysis in future studies. Such data is free from environmental influence and is more economical than a combined analysis.

There is limited phenotypic and molecular diversity among Zimbabwean and exotic flue-cured tobacco varieties. Breeding programs are, therefore, recommended to rely on local varieties as primary sources of valuable alleles affecting economically important quantitative traits such as yield and cured leaf quality. Breeders are encouraged to conserve varieties

which are not of immediate usefulness since they may provide useful variation in the event of future pest or disease outbreaks. Researchers are recommended to supplement phenotypic markers with molecular markers for genotyping since the later proved to provide additional information to the genetic diversity clustering of tobacco varieties.

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