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RESEARCH PAPER

Molecular phylogeny of genus *Guizotia* (Asteraceae) using DNA sequences derived from ITS

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Abstract Complete sequences for the internal transcribed spacers of the 18s–26s nuclear ribosomal DNA were generated to establish phylogenetic relationships among five species of the genus *Guizotia*. Parsimony analysis and pairwise distance data produced a single tree with four clearly distinguished clades that accord with previously reported chromosomal data. The clades produced here have been discussed with reference to existing taxonomic treatments. It appears that *Guizotia scabra*, ssp. *scabra* *G. scabra* ssp. *schimperi* and *Guizotia villosa* have contributed to the origin of *Guizotia abyssinica*, the cultivated species of the genus. The present composition of the species of genus *Guizotia* and the subtribe the genus presently placed in are suggested to be redefined.

Keywords ITS · Phylogeny · *Guizotia*

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Introduction

The genus *Guizotia* Cass. (nom. cons.) is a small but economically important genus that belongs to family Asteraceae, tribe Heliantheae and subtribe Coreopsidinae. Although Baagøe (1974) suggested transferring the genus to subtribe Verbesininae within the same tribe, Stuessy (1977) after revising the tribe Heliantheae maintained the genus in Coreopsidinae. On the other hand, Karis (1993) asserts that Robinson's (1981) placement of *Guizotia* in the Milleriinae seems more acceptable, even though the delimitation of this subtribe has to be emended. In addition to this, some African *Sigesbeckia* species treated by Humbles (1972) were transferred to genus *Guizotia* by Schulz (1990). According to Baagøe (1974), the distribution of *Guizotia* is typical of an afromontane, endemic genus. The genus is native to tropical Africa with most of the taxa restricted to East Africa, and with the highest concentration of species in Ethiopia.

Baagøe (1974) circumscribed the genus to six species viz.: *Guizotia abyssinica* (L. F) Cass., *Guizotia arborescens* I. Friis, *Guizotia jacksonii* (S. Moore) J. Baagøe *Guizotia scabra* (Vis.) Chiov. ssp. *scabra*, *G. scabra* (Vis.) Chiov. ssp. *schimperi* (Sch. Bip. in Walp.) J. Baagøe *Guizotia villosa* Sch. Bip. in Walp., and *Guizotia zavattarii* Lanza in Chiov. et al. Except *G. jacksonii* all species of *Guizotia* have been recorded in

Ethiopia. Baagøe (1974) gives an account on the endemism of *Guizotia* species and indicates that except for *G. scabra* ssp. *scabra* which has wider distribution, endemism is shown by *G. arborescens* (to the south west of Ethiopia and to the boarders of Sudan and Uganda), *G. villosa* (to the northern part of Ethiopian highlands), *G. scabra* ssp. *schimperi* as native to the Ethiopian highlands and *G. zavattarii* (to the Southern Ethiopia and North Kenya). *G. abyssinica*, *G. scabra* ssp. *schimperi* and *G. villosa* are annuals while the rest of the species of the genus are perennials.

Baagøe (1974), Hiremath and Murthy (1988) and Dagne and Heneen (1992) consider *G. scabra* ssp. *schimperi* to be the wild progenitor of *G. abyssinica*, the cultivated species. Hiremath and Murthy (1992) and Dagne (1995) concluded that *G. scabra* ssp. *scabra* and *G. scabra* ssp. *schimperi* reflect significant karyotypic differences, thus supporting the differences in morphology reported by Baagøe (1974). Hiremath et al. (1992) reported that *G. scabra* ssp. *scabra* and *G. scabra* ssp. *schimperi* differ distinctly in their genome size and based on this advised to treat these two taxa as independent species.

Baagøe also considered that *G. villosa* is derived from *G. scabra* and that *G. zavattarii*, derived from that of the same species or from an unknown ancestor common to the two. Later studies based on Karyology by Hiremath et al. (1992) suggested that *G. villosa* originated from species like *G. scabra* ssp. *schimperi*. The study on chromosome morphology of Dagne (1995) placed *G. villosa* to *G. scabra* ssp. *scabra* partly supporting Baagøe (1974) but still differing from her in the position and origin of *G. zavattarii*. Using chromosome morphology and giemsa C-banding Dagne and Heneen (1992) grouped the five *Guizotia* species into three:

Group I: *G. abyssinica* and *G. scabra* ssp. *schimperi;*
Group II: *G. zavattarii* and *G. arborescens*;
Group III:*G. villosa* and *G. scabra* ssp. *scabra*.

Baagøe (1974), using six presumed primitive and advanced morphological characters with in genus *Guizotia* concluded that *G. scabra* ssp. *scabra* has the highest number of primitive morphological characters while *G. arborescens* and *G. jacksonii* have advanced characters, the rest of the species were considered to be in the intermediate states.

Molecular techniques are being widely used in systematic and phylogenetic studies to provide a measure of genetic relatedness based on DNA sequences variation (Soltis et al. 1998). The internal transcribed spacers (ITS) of the nuclear ribosomal RNA genes have been among the most widely used sequences for DNA sequence variation studies. However, in spite of the small number of species in the genus *Guizotia*, there are no DNA sequences available for systematic purposes. The objective of this report is to make phylogenetic inferences using the information from ITS sequence generated for all species of *Guizotia* except for that of *G. jacksonii*. Here we report the results of comparison of their ITS sequences and relate this to the previous understanding and unresolved problems of the genus.

Materials and methods

Five *Guizotia* species, out of a total six (Baagøe 1974), were available for analysis from the samples collected (Table 1). The origin of these samples is given in Table 1. *Axiniphyllum durangense* B. L. Turner and *Sigesbeckia orientalis* L. were used as outgroups based on their ready availability of ITS sequences and their placement within the tribe Heliantheae. *Bidens setigera* (Sch. Bip.) Sherff, *Crepis aurea* (L.) Willd., *Helianthus annuus* L., *Senecio vulgaris* L. and *Vernonia noveboracensis* Cass. (L.) were also used as additional outgroups.

DNA was extracted from homogenized 300 mg of fine powder of leaf material in liquid nitrogen using 750 µl extraction buffer (pH 7.5) (consisting of 0.1 M Tris, 50 mM EDTA, 500 mM NaCl) and 100 µl of 10% SDS. The whole mix was incubated at 65°C for 20 min. Two hundred and fifty microliters of 5 M KAc was added to the mix and the samples were kept on ice for at least 30 min. The samples were later centrifuged at 14,000 rpm for 15 min and the supernatant was transferred to a new Eppendorf tube. To the supernatant equal volume of cold isopropanol was added and centrifuged for 10 min at 14,000 rpm. The supernatant

Table 1 *Guizotia* taxa used for ITS sequence analysis. Voucher specimens with their code are deposited in the National Herbarium of Addis Ababa University

	Species	Region	Altitude (m)	Location
1	*G. abyssinica*H15	Tigray	1,400	86 km from Shire to Adiarky
2	*G. abyssinica*J2	Illubabor	1,865	3 km from Metu to Gore
3	*G. arborescens*D170	Keficho	2,110	14 km from Chida to Ameya
4	*G. arborescens*B200	Jimma	2,372	27 km from the Jimma-Addis Ababa road to Omo Nadda town
5	*G. arborescens*7	Keficho	2,200	24 km from Chida to Ameya
6	*G. arborescens*8	Keficho	2,200	24 km from Chida to Ameya
7	*G. scabra* ssp. *scabra*2	Sidamo	1,770	3.8 km from Kibre Mengist to Shakisso
8	*G. scabra* ssp. *schimperi*A23	Hararge	2,375	44 km from Kobo to Hirna
9	*G. villosa*A20	Tigray	2,410	102 km from Maychew to Mekele
10	*G. villosa*J15	Gondar	2,220	6 km from Gondar to Azezo
11	*G. zavattarii*K	Sidamo	1,820	2 km from Arero to Babila
12	*G. zavattarii*P	Sidamo	1,974	3 km from Yabelo to Konso

was poured off and the pellet was air-dried. Two hundred and fifty microliters of TE buffer (pH 7.6) consisting of 10 mM Tris and 1 mM EDTA was added to dissolve the pellet. After the pellet was dissolved, 250 µl of CTAB buffer (pH 7.5) composed of 0.2 M Tris, 50 mM EDTA, 2 M NaCl and 2% CTAB was added and the mix was incubated for 15 min at 65°C. Equal volume of chloroform was added to the mix and centrifuged for 5 min. The supernatant was recovered and chloroform extraction was repeated. The supernatant was taken into new Eppendorf tube and precipitated with equal volume of cold isopropanol and centrifuged for 15 min. The supernatant was poured off and the pellet was air-dried. One hundred microliters of TE buffer was added to dissolve the pellet overnight. Five microliters of RNase (1 mg/ml) was added the next morning and incubated at 37°C for 30 min. DNA quality and DNA concentration was determined using Shimadzu UV-240 Graphicord UV–visible light recording spectrophotometer.

The following primers were used:

18F 5′-GGAAGGAGAAGTCGTAACAAGG-3′
26R 5′-GCCGTTACTAAGGGAATCCTTGTTAG-3′.

Conditions for the thermal cycler were 95°C 4′, 95°C 30″, 54°C 30″, 72°C 1′ for 38 cycles. After 38 cycles, the PCR reactions were incubated at 72°C for 6 min. The PCR products were cleaned using Qiagen MinElute kit according to the manufacturer's instruction.

Automated sequencing of the purified double-stranded PCR products was carried out in both directions with sequencing conditions of 95°C 10 s, 54°C 1 s and 60°C 4 min for 25 cycles. The sequencing reaction was cleaned with 80 and 70% isopropanol at two different stages by centrifuging and the pellet was dried at 65°C for 5 min. To each of this pellet, 10 µl of high purity formamide was added and the two are mixed and incubated at 65°C for 5 min. The incubated samples were centrifuged at 1,000 rpm for 1 min. The samples were heated for 4 min at 96°C and then cooled on ice before it was loaded to 3700 genetic analyzer for sequencing.

Analysis of the ITS sequences were performed using PAUP 4.0 Beta 10 (Dave Swofford Sinauer Associates). Boundaries of the coding and spacer sequences were determined by comparison with published sequences in EMBL database. Sequences were aligned using Sequencher and manually edited. Forward and reverse sequences were compared, to check for consistency.

A pairwise distance value was calculated between taxa based on Kimura's 2-parameter correction method (Kimura 1980) with the estimated number of substitution per 1,000 bases. Parsimony analysis of the aligned sequences was

performed using PAUP 4.0 Beta 10. Trees were generated using the 'branch-and-bound' options. Bootstrap values were computed using PAUP. The consistency and homoplasy indices were calculated for all trees. A BLAST search was performed to search for homologous sequences of Asteraceae to compare with the sequences of *Guizotia* species reported here.

Results

Sequence analysis

Complete sequences of the ITS region were obtained for the five *Guizotia* taxa and the two outgroup species *S. orientalis* and *A. durangense* (GenBank accession number AF465890.1 and AF465846.1). The length of the entire ITS region was 645 alignment length of both the ingroup and *S. orientalis*, 644 for *G. villosa*A20 and 647 for *A. durangense*. The size of ITS1 is 263 bp while that of 5.8 s rRNA and ITS2 is 159 and 226, respectively. Forty parsimony informative characters (ingroups only) are present both in the whole fragment and ITS1 and ITS2 (excluding 5.8 s). There are additional four characters which are variable but not informative. Transition versus transversion ratios seem to be 1.3:1 excluding outgroup and 1.9:1 with the outgroup. These values are lower than the accepted 2:1 ratio required for Kimura's 2-parameter test when the outgroups are excluded. A total of four gaps were introduced into the alignment. Three gaps were introduced in the ingroup to improve fit with the outgroup. One gap was introduced into *S. orientalis* and *G. villosa*A20 to improve fit with the other taxa. The ratio of pyrimidines is slightly higher than purines (Table 2).

Sequence divergence comparison

Distances generated using Kimura's 2-parameter method are shown in Table 3. Divergence based on this method ranged from 0 to 5.5% between *Guizotia* species with the highest distance occurring between *G. villosa*J15 and *G. zavattarii*P. The divergence values between *Guizotia* species and outgroups vary from 2.7 to 7.2%. This shows that the sequences of both outgroups are not too different from those of *Guizotia*. Because of this they seem ideal as outgroup species due to the absence of hidden changes. Thus, exclusion of both outgroups can alter the topology of the trees produced. This is further noticed (Fig. 1) when seven species are used as outgroups. The neighbour-joining tree (Fig. 2), which shows branch lengths that are proportional to distances calculated with the method of Kimura (1980), indicates that *Guizotia* species are grouped into four groups and that there are two forms of *G. abyssinica* namely *G. abyssinica*J2 and *G. abyssinica*H15. The neighbour-joining tree (Fig. 3) produced similar results with that of Kimura's method except for the slight differences in the placement of the outgroups. On the other

Table 2 The proportions of the various bases and the number of ITS sites in the *Guizotia* species studied and the two outgroups considered

Taxon	A	C	G	T	No. of sites
*G. abyssinica*J2	0.22481	0.24651	0.27442	0.25426	645
G. scabra ssp. *schimperi*A23	0.22551	0.24496	0.27372	0.25581	645
*G. villosa*J15	0.22171	0.24883	0.27442	0.25505	645
*G. arborescens*B200	0.22016	0.25193	0.27287	0.25504	645
*G. arborescens*8	0.22016	0.25349	0.27287	0.25349	645
*G. arborescens*7	0.22016	0.25349	0.27287	0.25349	645
*G. arborescens*D170	0.22016	0.25271	0.27287	0.25426	645
G. scabra ssp. *scabra*2	0.22171	0.24883	0.26597	0.25350	645
*G. zavattarii*K	0.22171	0.25193	0.26822	0.25815	645
*G. zavattarii*P	0.22171	0.25116	0.26977	0.25736	645
G. orientalis—AF465890.1	0.21705	0.25581	0.27752	0.24961	645
*G. abyssinica*H15	0.22240	0.24492	0.27528	0.25740	645
A. durangense—AF465846.1	0.21638	0.26584	0.27666	0.24111	647
*G. villosa*A20	0.22205	0.24996	0.27566	0.25233	644
Mean	0.22112	0.25146	0.27379	0.25363	645.07

Table 3 Pairwise distances of ITS sequences in species of *Guizotia*, *Axiniphyllum durangense* and *Sigesbeckia orientalis*

	1	2	3	4	5	6	7	8	9	10	11	12	13	14
1														
2	0.0353													
3	0.0467	0.0270												
4	0.0467	0.0270	0.0000											
5	0.0450	0.0254	0.0000	0.0000										
6	0.0433	0.0254	0.0015	0.0016	0.0000									
7	**0.0720**	**0.0532**	0.0464	0.0466	0.4480	0.0448								
8	0.070	0.0482	0.0482	0.0483	0.0465	0.0465	0.0031							
9	0.0468	0.0253	0.0174	0.0173	0.0158	0.0158	**0.0550**	**0.0500**						
10	0.0467	0.0254	0.0142	0.0142	0.0126	0.0126	0.0515	**0.0499**	0.0031					
11	0.0636	0.0449	0.0383	0.0398	0.0383	0.0383	0.0351	0.0303	0.0382	0.0383				
12	0.0725	0.0533	**0.0500**	**0.0498**	**0.0484**	**0.0484**	0.0063	0.0032	**0.0515**	0.0400	**0.0383**			
13	0.0686	0.0499	0.0498	**0.0499**	0.0481	0.0481	0.0032	0.0000	**0.0516**	**0.0515**	0.0319	0.0032		
14	0.0654	0.0466	0.0417	0.0415	0.0400	0.0400	0.0319	0.0270	0.0399	0.0400	0.0078	0.0286	0.0286	

The highlighted values in the table are those with higher distance values in each column. Numbers *1–12* on first column/row are *Guizotia* taxa given in Table 1 in that order, while *13* and *14* are *S. orientalis* and *A. durangense*, respectively

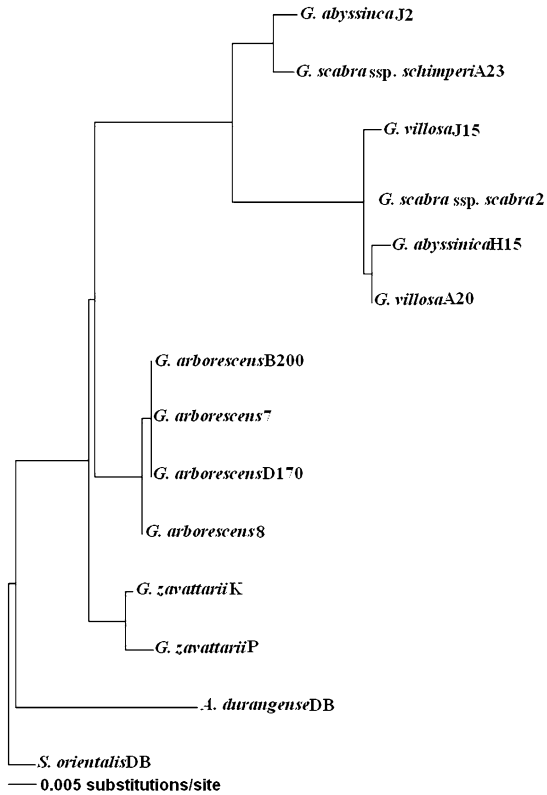

Fig. 1 Neighbour-joining tree obtained with ITS for five *Guizotia* species and five additional outgroups produced from pairwise distances calculated using Kimura's 2-parameter method

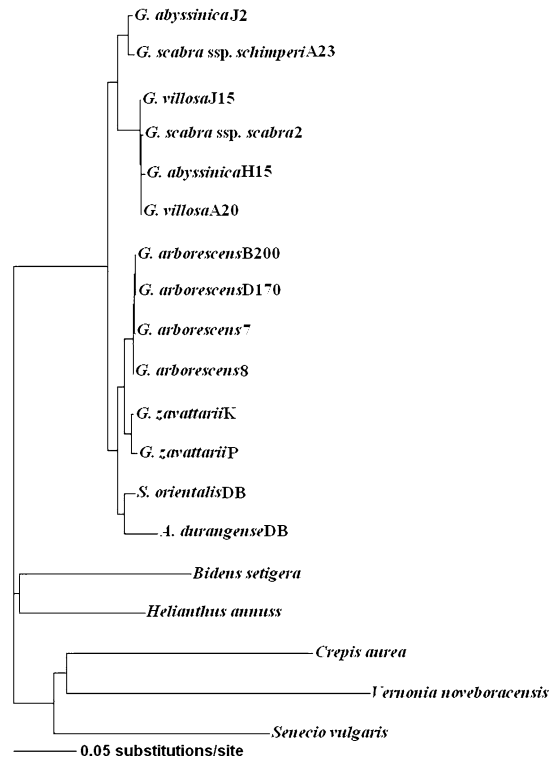

Fig. 2 Neighbour-joining tree obtained with ITS for five *Guizotia* species and two outgroups produced from pairwise distances calculated using Kimura's 2-parameter method

hand, the neighbour-joining tree (Fig. 4) constructed using ML-4KY-I-G resulted in similar clusters with that of the above two trees except for the slight differences in the placement of *G. abyssinica*H15 in the *G. villosa* and *G. scabra* ssp. *scabra* group.

Phylogenetic analysis of sequences

The 50% majority rule most parsimonious search performed on the entire ITS region produced one maximally parsimonious tree (Fig. 5). Bootstrapping showed that all the major branches were highly supported. The organization of taxa into clades is as follows: the two outgroups (*A. durangense* and *S. orientalis*) were separated from ingroups taxa in all trees, and the species of the genus *Guizotia*. Within the ingroups, four clades are distinguished. The first clade consists of *G. zavattarii*. The second clade consists of *G. arborescens*. The third clade consists of *G. villosa*, *G. scabra* ssp. *scabra* and *G. abyssinica*H15 (collected from Tigray). The fourth clade consists of *G. scabra* ssp. *schimperi* and *G. abyssinica*J2 (collected from Illubabor).

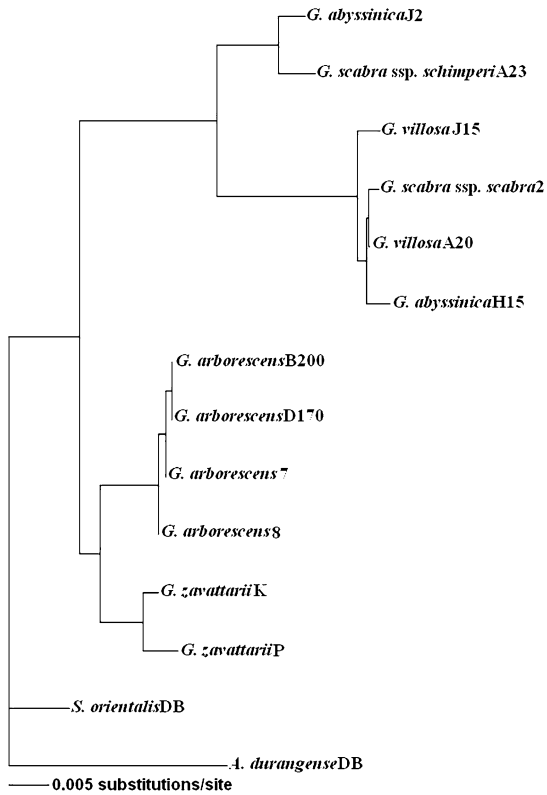

Fig. 3 Neighbour-joining tree obtained with ITS for five *Guizotia* species and two best outgroups produced from NJ-JC method

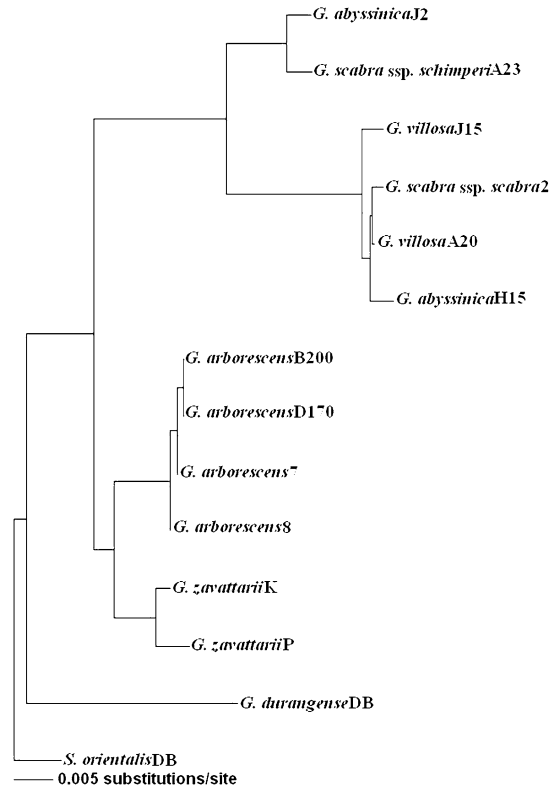

Fig. 4 Neighbour-joining tree obtained with ITS for five *Guizotia* species and two best outgroups produced from ML-Y-I-G method

Blast search to find homologous sequences

This search matched *G. abyssinica* sequences with those of other members of Asteraceae. Of these sequences, *G. abyssinica* was most similar to sequences generated from *S. orientalis*, *Sigesbeckia flosculosa* L'Her, *Sigesbeckia jorullensis* Kunth, *A. durangense*, *Trigonospermum annum* McVaugh et Lask., *Trigonospermum melampodioides* DC., *Milleria quinqueflora* L., *Coespeletia moritziana* (Sch. Bip. ex Wedd.) Cuatrec., *Rumfordia penninervis* S. F. Blake and *Espeletia pycnophylla* Cuatrec. On the other hand, the number of Asteraceae that showed similarity with *G. arborescens* and *G. zavattarii* is 27 and 23, respectively. The difference in number and extent of sequence similarities of species of various subtribes of tribe Heliantheae (Coreopsidinae, Milleriinae and Verbesininae) to various *Guizotia* species suggest that the present definition and

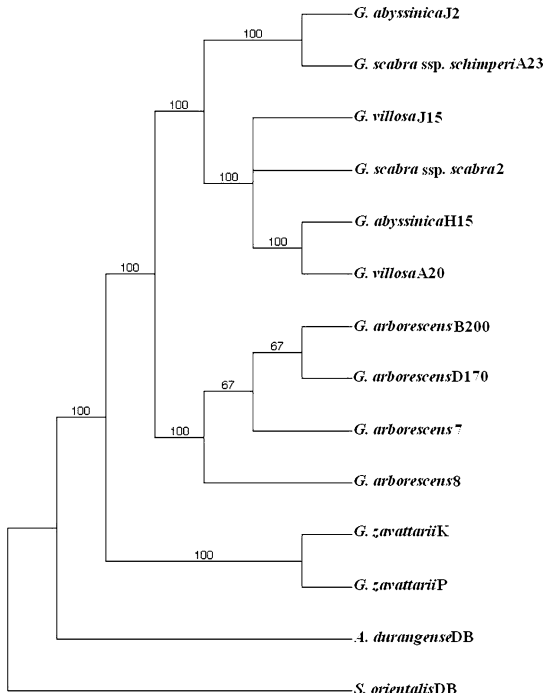

Fig. 5 Most parsimonious tree produced by the branch-and-bound search for the entire ITS region for five *Guizotia* species and two outgroups used. Bootstrap values are shown above the *line*

position of the genus *Guizotia* under subtribe Coreopsidinae has to be questioned, and need more data to resolve.

Discussion

A phylogeny tree has been constructed for genus *Guizotia* using ITS sequences. The total length of ITS region of *Guizotia* fall within the range reported for flowering plants (Baldwin et al. 1995). In all the species of *Guizotia* studied here, the length of ITS1 (159 bp) is higher than ITS2 (226 bp), a pattern which has also been found in other genera of Asteraceae (Baldwin 1992; Cerbah et al. 1998).

The most parsimonious tree produced separated the species of *Guizotia* into four clades. A truncated data set of ITS into ITS1 and ITS2 resulted retrieving the same topology (data not shown). The organization of taxa into clades produced by ITS is interestingly similar with that produced by Dagne and Heneen (1992) using chromosome morphology and Giemsa C-banding. The results of artificial inter-specific hybrids produced between *G. arborescens* and *G. zavattarii* through reciprocal crosses resulted in high percentage of seed setting and good pairing of chromosomes in the hybrids (Dagne 2001). Our ITS data and the clustering of *G. arborescens* and *G. zavattarii* corroborates with that of Dagne.

The advancement versus primitiveness of morphological characters suggested for species of genus *Guizotia* by Baagøe (1974) does not go with that of the ITS data clade formation and taxa organization.

Internal transcribed spacers data suggest that both *G. scabra* ssp. *scabra* and ssp. *schimperi* are the wild progenitor of *G. abyssinica*, the cultivated form. The cultivated *Guizotia* species collected from Northern Ethiopia seem to have evolved either from *G. scabra* ssp. *scabra* or *G. villosa* while that from the south might have evolved from *G. scabra* ssp. *schimperi*, thus suggesting that the significant karyotypic differences between *G. scabra* ssp. *scabra* and *G. scabra* ssp. *schimperi* and *G. villosa* reported by Dagne (2001) might exist between some of the cultivated forms. On the other hand, the karyotypes, C-banding and nucleolar numbers study made by Dagne (1995) agrees with the ITS data by placing *G. villosa* to *G. scabra* ssp. *scabra* than to *G. scabra* ssp. *schimperi* (see Figs. 3, 4, 5).

The diversity of plants, existence of complex home gardens and ethnic groups in Ethiopia has influenced the process of domestication (Bekele 1998). This is taken to promote diversification of many crops. Under such instances the two forms of *G. abyssinica* originating from two or even more wild progenitors of *Guizotia* species may take place.

The species of genus *Guizotia* seem to be natural. However, more data is required to suggest the ancestral species involved in the origin of the genus. The Ethiopian and Indian interface in the domestication processes is very interesting. For example, sesame (*Sesamum indicum* L.), which is the most ancient oil seed known and used by man (Purseglove 1976) is considered to have its primary centre of origin in Ethiopia or Sudan (Weiss 1971). It was taken at early date to India and eventually reached China. With

reference to *G. abyssinica* the case is clearer. Decandolle stated that the origin of cultivated plant could be found where it grows wild. This applies to the wild *Guizotia* species too. There is no need to think that these wild plants were brought from India as there are none. The presence of a wild progenitor of cultivated plants does not by itself bring about its domestication. For example, the wild African cotton [*Gossypium herbaceum* L. var. *africanum* (Watt) Mauer] which has remained in the wild state in South Africa has never been cultivated there at first. *Eragrostis pilosa* (L.) P. Beauv., which is a progenitor of *Eragrostis tef* (Zucc.) Trotter (Bekele and Lester 1981) is a cosmopolitan species but was domesticated only in Ethiopia. Since evolutionary events leading to man had occurred in and around Ethiopia, the hunter gatherer and the initial domestication stage must have been ancient in Ethiopia (Bekele 1998). The additional molecular data to probe such ancient period of domestication using model plants such as *Guizotia* species is highly desired.

Taxonomic considerations

So far there is no taxonomic treatment of Genus *Guizotia* into sections perhaps mainly because of the limited number of species in the genus. However, the distinct clades produced here imply such taxonomic delimitations. Dagne (2001) reported the successful production of artificial hybrids from all species within the genus although the chromosome behaviour of hybrids and degree of seed setting varies between the various species combinations used. Since species of *Guizotia* show self-incompatibility and are thus out breeders, the inter-specific hybrids production might also take place in nature provided the two species populations in the same locality flower at the same time.

Internal transcribed spacers data supports the group suggested by Dagne and Heneen (1992) and differ from that of Baagøe (1974) and Hiremath et al. (1992) on the origin of *G. villosa* and *G. zavattarii*. *G. villosa* is closer to *G. scabra* ssp. *scabra* than to *G. scabra* ssp. *schimperi*. *G. zavattarii* is quite different and distinct from all but closer to *G. arborescens*. The ITS data also supports the advice suggested by Hiremath et al. (1992) that the two subspecies of *G. scabra* be treated as independent species. In view of the unresolved sub-tribal definitions to accommodate *Guizotia*, more data is still required.

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