Genetic Diversity, Phylogenetics and Molecular Systematics of *Guizotia* Cass. (Asteraceae)

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Abstract

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The genus *Guizotia* belongs to the tribe Heliantheae in the family Asteraceae. It has been placed under different subtribes. The genus has its center of origin, distribution and genetic diversity in Ethiopia, where *G. abyssinica* (niger) has been domesticated. Amplified Fragment Length Polymorphism (AFLP), Random Amplified Polymorphic DNA (RAPD) and DNA sequencing were applied to study the genetic diversity, phylogenetics, and molecular systematics of this genus.

A large number of niger populations, representing all regions in Ethiopia where this crop is grown, was investigated using AFLP and RAPD molecular marker techniques. The extent of genetic variation in niger is distributed throughout its growing regions, regardless of the extent and altitude of cultivation. Despite the fact that most of the variation was within populations, significant population differentiation was obtained (AMOVA; $P < 0.001$) in all guizotias. It is concluded that both *G. abyssinica* and its wild and/or weedy relatives have wide genetic bases that need to be conserved and utilized for the improvement of *G. abyssinica*. Further collection of niger germplasm and exploration and conservation of highly localized guizotias are recommended.

Most of the diagnostic markers generated from AFLPs and RAPDs in this study were specific to *G. arborescens* and *G. zavattarii*. Phylogenetic analyses of the genus *Guizotia* were undertaken based on molecular sequence data from the internal transcribed spacers (ITS) and five chloroplast DNA regions. The analyses revealed a close phylogenetic relationship between *G. abyssinica* and *G. scabra* ssp. *schimperi* and support the previous suggestion that the latter is the progenitor of the former. According to this study, *G. scabra* ssp. *scabra*, *G. scabra* ssp. *schimperi*, and the Chelelu and Ketcha populations are best viewed at present as separate species within the genus *Guizotia*. Those perennial guizotias with highly localized geographic distribution appears to have evolved first during the evolutionary history of the genus. This study supports the placement of the genus *Guizotia* within the subtribe Milleriinae. It is suggested that the present species composition of *Guizotia* and the subtribal placement of the genus need to be redefined.

Key words: AFLP, cpDNA, DNA sequencing, genetic diversity, *Guizotia*, ITS, niger, phylogenetics, RAPD, systematics.

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Dedicated to my parents: Geleta and Leakie

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List of Appendices

Paper I-VI

The present thesis is based on the following papers, which will be referred to by their Roman numerals:

I. Geleta, M., Bryngelsson, T., Bekele, E. & Dagne, K. 2006. Genetic diversity of *Guizotia abyssinica* (L. f.) Cass. (Asteraceae) from Ethiopia as revealed by Random Amplified Polymorphic DNA (RAPD). *Genetic Resources and Crop Evolution* DOI 10.1007/s10722-006-0018-0.

II. Geleta, M., Bryngelsson, T., Bekele, E. & Dagne, K. Assessment of genetic diversity of *Guizotia abyssinica* (L.F.) Cass. (Asteraceae) from Ethiopia using Amplified Fragment Length Polymorphism (AFLP). *Plant Genetic Resources*. In press.

III. Geleta, M., Bryngelsson, T., Bekele, E. & Dagne, K. AFLP and RAPD analyses of genetic diversity of wild and/or weedy *Guizotia* (Asteraceae) from Ethiopia. *Hereditas* DOI:10.1111/j.2007.0018-0661.01983.x.

IV. Geleta, M., Bryngelsson, T., Bekele, E. & Dagne, K. 2007. Comparative analysis of genetic relationship and diagnostic markers of several taxa of *Guizotia* Cass. (Asteraceae) as revealed by AFLPs and RAPDs. *Plant Systematics and Evolution* DOI 10.1007/s00606-007-0521-6. In press.

V. Bekele, E., Geleta, M., Dagne, K., Jones, A. L., Barnes, I., Bradman, N. & Thomas, M.G. 2006. Molecular phylogeny of genus *Guizotia* (Asteraceae) using DNA sequences derived from ITS. *Genetic Resources and Crop Evolution* DOI 10.1007/s10722-006-9126-0

VI. Geleta, M., Bryngelsson, T., Bekele, E. & Dagne, K. Molecular Phylogeny of the genus *Guizotia* (Asteraceae) based on sequences derived from the *trn*T/*trn*L and *trn*L/*trn*F intergenic spacers, *trn*L and 3'*trn*K/*mat*K introns and *mat*K gene. Submitted.

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Introduction

History of the genus *Guizotia*

Guizotia Cass. is a small Afromontane endemic genus that belongs to the tribe Heliantheae in the family Asteraceae. The early history of the genus *Guizotia* is the history of its type species, *Guizotia abyssinica* (L. f.) Cass. (Baagøe, 1974), which is the only cultivated species of the genus. *G. abyssinica* (Fig. 1), commonly known as 'noug' (in Amharic), ramtil (in Hindi) and 'niger' (in English) is known in Ethiopia and India earlier than anywhere else. The Ethiopian and Indian niger had been described separately until 1877. On the Ethiopian side, the first valid name given to niger was *Polymnia abyssinica* L. f. in 1781 (Baagøe, 1974). According to Baagøe (1974), the name *Polymnia frondosa* was also given to this species in 1805. In 1821, a French botanist and naturalist, Count Alexandre Henri Gabriel de Cassini (1781-1832), described this species under the name of *Heliopsis platyglosa*; however, in 1929 he found that his *H. platyglosa* is the same as *P. abyssinica*. He then described a new genus, *Guizotia*, named after the French historian, orator and statesman Francois Pierre Guillaume Guizot (1787-1874; Seegeler, 1983). Cassini changed his *H. platyglosa* to *Guizotia abyssinica* L. f. and described it as a typical species of the genus. On the Indian side, the name *Verbesina sativa* was given to this species and published in 1807 (Baagøe, 1974). In 1834, the famous botanist Augustin Pyramus de Candolle (1778-1841) described a new genus *Ramtilla*, where he placed the Indian *V. sativa* under the new name of *Ramtilla olifera*. De Candolle then recognized that his *Ramtilla* and Cassini's *Guizotia* are the same and consequently changed the name *Ramtilla olifera* to *Guizotia olifera* (Baagøe, 1974). However, in 1877, the epithet "*abyssinica*" was maintained instead of "*olifera*" by Oliver and Hiern (Baagøe, 1974). The name *Guizotia* Cass. was conserved for the genus by the Vienna Congress in 1905, and *G. abyssinica* has been considered as the typical species of the genus *Guizotia* since 1930 (Briquet, 1935).

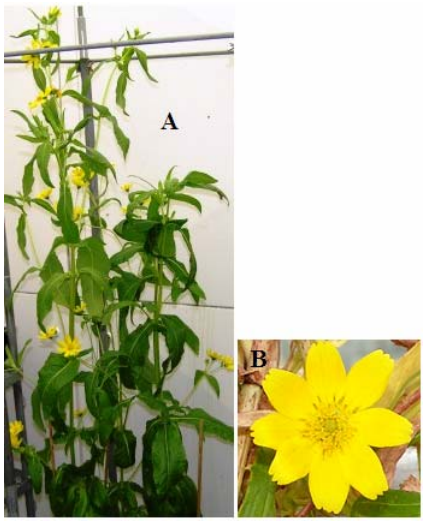

Figure 1. *Guizotia abyssinica* (A) plants and (B) flower.

Systematics of *Guizotia* Cass.

The genus *Guizotia* has been placed under different subtribes of tribe Heliantheae by different authors. Robinson (1981) stated that the restriction of the genus to the African region and its uncertain relationship to the western plant species make its position less certain in the tribe Heliantheae. Bentham (1873) placed the genus under the subtribe Coreopsidinae, where it had been treated until Baagøe (1974) suggested its transfer to the subtribe Verbesininae. Three years later, Stuessy (1977), after revising the tribe Heliantheae, maintained the genus within the Coreopsidinae, to which Baagøe later agreed (Baagøe, 1977). However, Robinson (1981) indicated that the terete, striate achenes, the ornamented seed coats, and the glanduliferous anther appendages are evidences against the placement of the genus within the Coreopsidinae. Consequently, he placed the genus under the subtribe Milleriinae based on close approximation of technical characters despite differences in habit and flower color. The placement of the genus under the subtribe Milleriinae was also asserted by Karis (1993). The transfer of an African *Sigesbeckia* species (*S. somalensis* S. Moore) that belong to the subtribe Milleriinae (Humbles, 1972) to the genus *Guizotia* by Schulz (1990) indirectly supports the placement of the genus *Guizotia* under the subtribe Milleriinae.

Figure 2. A young plant of *Guizotia arborescens*.

In her taxonomic revision of the genus *Guizotia*, Baagøe (1974) reduced the number of species considerably through merging two or more taxa, reducing the taxonomic status to subspecies level and excluding some species. Baagøe excluded *Guizotia bidentoides* Oliv. & Hiern and *Guizotia discoidea* Sch. Bip. in Schweinf. nom. nud., as they were found to be synonyms of *Bidens pinnatipartita* (O. Hoffm) Wild and *Sigesbeckia discoidea* (Vatke) Blake, respectively. *S. discoidea* was later moved to the genus *Micractis* by Schulz (1990). Baagøe circumscribed the genus to six species viz.: *G. abyssinica* (L. f) Cass., *G. arborescens* I. Friis, *G. jacksonii* (S. Moore) J. Baagøe comb. nov., *G. scabra* (Vis.) Chiov. ssp. *scabra*, *G. scabra* (Vis.) Chiov. ssp. *schimperi* (Sch. Bip. In Walp) J. Baagøe stat. nov., *G. villosa* Sch. Bip. in Walp, *G. zavattarii* Lanza in Chiov. & al. var. *zavattarii*, and

G. zavattarii Lanza in Chiov. & al. var. *angustata* Cuf. After Baagøe's (1974) taxonomic revision, two new populations of *Guizotia* were discovered in Ethiopia by K. Dagne who called them "Chelelu" and "Ketcha" (Dagne, 1995). These populations are distinct both from each other and from the recognized taxa of the genus (Dagne, 1995, 2001).

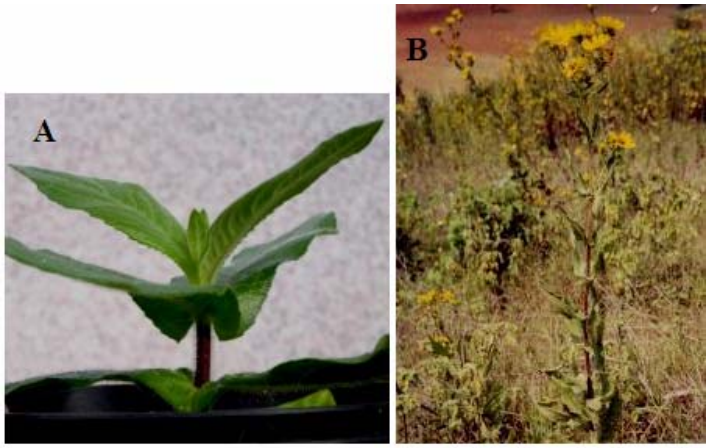

Figure 3. *Guizotia scabra* ssp. *scabra* (A) young plant and (B) mature plant in its natural habitat.

Some characteristics and geographical distribution of *Guizotia* species

The genus *Guizotia* 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). Ethiopia is a country in the horn of Africa located approximately between 3°N and 15°N latitude and 33°E and 48°E longitude. *G. abyssinica* is an annual herb with capitulum that consists of six to eight fertile female ray florets and 40-60 hermaphroditic disk florets (Getinet & Sharma, 1996). It is distinguished from other guizotias mainly by its corymbous cymes of heads, 5 broadly ovate-obovate outer involucral leaves, 5-nerved paleae and bigger achenes (Baagøe, 1974). Being a crop, niger has the widest geographic distribution among the guizotias. It has also been collected as a weed and as a wild plant in Ethiopia (Baagøe, 1974; Weiss, 1983). However, it is not clear whether this is a result of seeds dispersed from farmers' fields or a true wild/weedy form. Since we have not encountered any *G. abyssinica* in the wild during our extensive survey and collection of *Guizotia* species, it is possible that the reported *G. abyssinica* in the wild was just an escape from cultivation that died out.

Figure 4. A young plant of *Guizotia scabra* ssp. *schimperi*.

G. arborescens (Fig. 2) is a shrubby perennial endemic to southwest Ethiopia and mountain Imatongs in Sudan and Uganda within an altitudinal range of 1800-2600 m (Friis, 1971). Its arborescent habit, petiolate leaves, densely corymbous cymes and shrubby habit make it distinct from the rest of the taxa (Baagøe, 1974). *G. jacksonni* is a creeping perennial herb with sparsely branching habit grown within an altitudinal range of 2200-3700 m, endemic to Aberdares, Mt. Kenya and Mt. Elgon in Kenya and Uganda (Baagøe, 1974). Its creeping habit and solitary heads make it distinct from the other taxa. *G. scabra* ssp. *scabra* (Fig. 3) is a perennial, course, densely scabrous plant with stiff leaves, distributed, as part of natural vegetations, in East Africa, Cameron and Nigerian highlands, commonly within an altitudinal range of 1100-2700 m (Baagøe, 1974; Hiremath & Murthy, 1986). According to Baagøe (1974), it is the most variable taxon of the genus morphologically. *G. scabra* ssp. *schimperi* (Fig. 4) is an annual plant with foliaceous leaves native to Ethiopia and is a common weed of crops in mid and high altitude areas (1600-2300 m). *G. scabra* ssp. *scabra* and *G. scabra* ssp. *schimperi* are distinct from each other, for example, in having 50-120 and 35-50 disk florets and 8-15 and 6-8 ray florets, respectively (Baagøe, 1974).

Figure 5. *Guizotia villosa* (A) young plant and (B) flower.

G. villosa (Fig. 5) is an annual herb restricted in distribution to the northern and north-western part of the Ethiopian highlands, and can be easily distinguished from other taxa by densely pilose indumentum, small heads and nearly square ray florets (Baagøe, 1974). *G. zavattarii* is a predominantly woody perennial plant restricted to southern Ethiopia (around mount Mega, Arero and Yabelo) and the Hurri Hills in northern Kenya, growing within the altitudinal range of 1500-2200 m. This species can be distinguished from the other taxa by its suffrutescent habit (Baagøe, 1974). *G. zavattarii* var. *angustata* was reported to be collected only in the vicinity of mount Mega (Baagøe, 1974) though we could not find it during our germplasm collections. The pandurate lacerate leaves are unique to *G. zavattarii* var. *zavattarii* (Fig. 6) within the genus. The geographic distribution of Chelelu and Ketcha is not fully explored; and they have not been botanically described. Chelelu (Fig. 7) is a riverine perennial plant that can be distinguished from other guizotias by its rhizomatous-like vegetative propagation and seed color (Dagne, 1995). It has been collected from the Chelelu river about 20 km north of Addis Ababa. Ketcha (Fig. 8) is a perennial plant that has been collected from Ketcha locality about 64 km from Bale-Goba to Delo-Mena in southeast Ethiopia.

Figure 6. A young plant of *Guizotia zavattarii*.

Cytogenetics of *Guizotia* taxa (guizotias)

All guizotias are diploids with 2n = 30 chromosomes and characterized by relatively small chromosomes with small differentiation between each complement (Dagne & Heneen, 1992; Hiremath & Murthy, 1992; Dagne, 1995). The number of satellite chromosomes in *Guizotia* species reported by Hiremath & Murthy (1992) was different from that of Dagne (1995). Hiremath & Murthy (1992) reported two satellite chromosomes in all *Guizotia* species they studied, while Dagne (1995) reported four to eight satellite chromosomes in these taxa. According to Dagne (1995), the discrepancy is likely due to chromosome preparation techniques and stages at which the satellite chromosomes were analyzed. A single B-chromosome was reported in some Ethiopian *G. scabra* ssp. *scabra* populations, a case that was not observed in populations of this taxon from other countries (Hiremath & Murthy, 1986) and in other guizotias. Later, Dagne

(1994b) reported three types of B-chromosomes in one population of this taxon in Ethiopia. About threefold variation in genome size was reported within the genus *Guizotia* (Hiremath, Murthy & Salimath, 1992) despite their similarity in number of chromosomes. The genome size of *G. abyssinica* was reported to be larger than that of its more closely related *Guizotia* taxa, including the suggested progenitor, *G. scabra* ssp. s*chimperi*. The implication is that the genome size of *G. abyssinica* has increased over its evolutionary and domestication period without a change in number of chromosomes. Gene duplication (*e.g* Ohta, 1994; Meyerowitz, 1999) and retrotransposition (*e.g*. Vicient *et al*., 1999; Bennetzen, Ma & Devos, 2005) are the likely mechanisms through which genome size variations have occurred within the genus *Guizotia*. The karyotypes of *G. abyssinica* and *G. scabra* ssp. *schimperi* are similar although the latter has relatively smaller chromosomes (Dagne & Heneen, 1992; Dagne, 1995). The difference in size of their chromosomes explains the variation in their genome size reported by Hiremath, Murthy & Salimath (1992). *G. abyssinica*, *G. scabra* ssp. *schimperi* and Chelelu are characterized by predominantly m type (symmetrical) chromosomes (Dagne, 1995). *G. arborescens* and *G. zavattarii* have relatively large chromosomes, which are predominantly asymmetrical (Hiremath & Murthy, 1992; Dagne, 1995). Although the genome size of *G. arborescens* has not been reported, the similarity with *G. zavattarii* in terms of chromosome size may indicate its relatively large genome size, as the genome size of the latter was reported to be the largest among the guizotias studied (Hiremath, Murthy & Salimath, 1992). The chromosomes of *G. scabra* ssp. *scabra*, *G. villosa* and Ketcha are more asymmetrical than those of *G. arborescens* and *G. zavattarii* (Dagne, 1995). The karyotype of *G. villosa* was first reported as symmetrical (Hiremath & Murthy, 1992); however, it was later reported that it is even more asymmetrical than that of the other guizotias (Dagne, 1995).

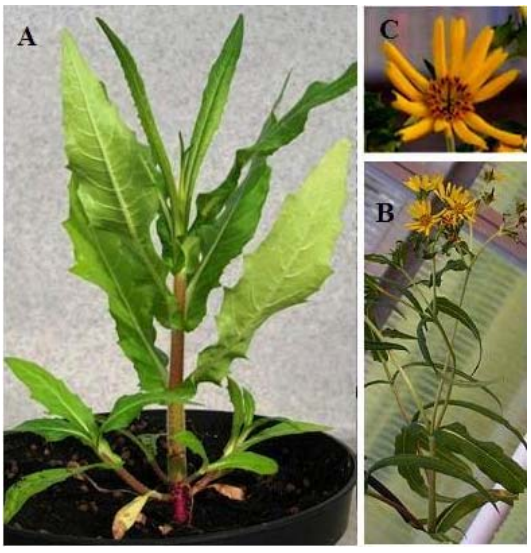

Figure 7. Plants of Chelelu at different growing stages (A &B) and its flower (C).

Figure 8. A young plant and flower of Ketcha.

Reproductive mechanism and crossability among taxa

Murthy, Hiremath & Salimath (1993) mentioned that *Guizotia* species in general are highly cross-pollinated and self-incompatible. The outcrossing nature of *Guizotia* species can be inferred from the cross-compatibility between various taxa that produces viable and fertile hybrids (Murthy, Hiremath & Salimath, 1993; Dagne, 1994a, 2001). However, direct evidence regarding self-incompatibility is only available for *G. abyssinica* (Ramachandran & Menon, 1979; Riley & Belayneh, 1989; Nemomissa, Bekele & Dagne, 1999). Self-incompatibility in niger is of the sporophytic type (Prasad, 1990; Nemomissa, Bekele & Dagne, 1999) that causes inhibition of pollen germination or twisting of pollen tube over the surface of the papillae (Prasad, 1990). Self-compatible niger genotypes were also reported in low frequencies within the Ethiopian gene pool, which can be as high as 5% in some populations (Getinet & Sharma, 1996; Nemomissa, Bekele & Dagne, 1999). Niger is mainly pollinated by insects (Fig. 9), particularly by bees (Ramachandran & Menon, 1979; Geleta *et al*., 2002; Kandel & Porter, 2002), which are also the most likely agent of pollination in other guizotias.

Figure 9. Pollinators of *Guizotia abyssinica*.

Sexual hybrids can easily be obtained from crosses between *G. abyssinica, G. scabra* ssp. *schimperi, G. scabra* ssp. *scabra* and *G. villosa* (Dagne, 1994a). The crossing of these taxa with *G. zavattarii* was not successful, as the shriveled seeds

produced from these crosses failed to germinate (Murthy, Hiremath & Salimath, 1993; Dagne, 1994a). According to Dagne (1994a), the failure of the crosses involving *G. zavattarii* might be due to a postzygotic cross-incompatibility. Pollen viability of the F1 hybrid between *G. abyssinica* and *G. scabra* ssp. *schimperi* was about 80%, while that of the other F1 hybrids was less than 50% (Dagne, 1994a). Chelelu is cross compatible with *G. scabra* and *G. zavattarii* while *G. arborescens* is cross-compatible with *G. zavattarii* (Dagne, 2001). Therefore, almost all *Guizotia* taxa do not fulfill the definition of "biological/reproductive species", as viable and fertile hybrids can be formed between them at least in some combinations. Generally, hybrids are intermediate between their parents in terms of overall morphological appearance (Dagne, 1994a).

Domestication of *G. abyssinica*

Plant domestication is a gradual process that involves selective breeding through which plants are adapted to human needs. Plant domestication has been conducted for about 10,000 years in several regions of the world independently (Gepts, 2002). Ethiopia is one of the world centers where crop plant diversity is strikingly high and where some crop species were domesticated *de novo* (Vavilov, 1951; Harlan, 1969). The country has a unique position in the crop domestication history of a wide range of species, including oil crops (Harlan, 1969; Zeven & de Wet, 1982). Evidences regarding the origin and domestication of crop plants can be generated from various sources, such as history, linguistics, archeobotany, comparative morphology, phytogeography, cytogenetics and molecular biology. Although archeobotanical evidence regarding the origin and domestication of niger is lacking, based on morphological, phytogeographical, and cytological evidences, it was reported that *G. abyssinica* might have originated from *G. scabra* ssp. *schimperi* through selection and further cultivation (Baagøe, 1974; Hiremath & Murthy, 1988; Murthy, Hiremath & Salimath, 1993; Dagne, 1994a, 1995, 2001). Again based on these evidences, Ethiopia, where the crop has been under cultivation for much longer time than in any other place, has been considered as the center of origin, domestication and diversity of *G. abyssinica* (Baagøe, 1974; Weiss, 1983; Hiremath & Murthy, 1988). According to these authors, niger was domesticated in Ethiopia as early as 3,000 BC and taken to India by Ethiopian immigrants and/or through trade routes along with other crops. However, the magnitude of genetic diversity and use value of this crop imply a longer date of domestication than has been suggested.

Uses of niger and other guizotias

Niger seed is the major source of edible oil in Ethiopia. It also accounts for a considerable proportion of edible oil production in several African and Asian countries, including India. In India, it is frequently used as a substitute for sesame oil (Weiss, 1983). The seed contains 17-20% protein (Abebe, Yermanos & Bingham, 1978; Kandel & Porter, 2002) that offers an important source of protein and significantly contributes to the human dietary protein intake. It also contains 34-40% carbohydrate and 13.5% fiber and is an important source of thiamine,

riboflavin, and niacin (Kandel & Porter, 2002). Niger seed is consumed after being processed in various forms in Ethiopia and India (Seegeler, 1983; Getinet & Sharma, 1996). It is also used as a component of birdseed in USA and Europe (Kandel & Porter, 2002) and for cultural and medicinal purposes in Ethiopia (Geleta *et al*., 2002). The press-cake left after oil extraction is an excellent poultry and livestock feed, as it contains 33-37% protein and is rich in inorganic constituents and crude fibers (Seegeler, 1983; Kandel & Porter, 2002). The whole plant is used as fodder and green manure (Weiss, 1983).

Refined niger seed oil is used for the preparation of soaps, paints, illuminants and lubricants and for cleaning machinery (Baagøe, 1974; Riley & Belayneh, 1989; Dutta *et al*., 1994; Kandel & Porter, 2002). It is also used in perfumes as a carrier of the scents and fragrances (Kandel & Porter, 2002). The Ethiopian seed is superior to the Indian seed in its use for paints due to its relatively higher linoleic acid content. Niger seed oil can also be used as biodiesel through *trans* esterification of its long chain fatty acids with methanol that can partially substitute diesel oil and perform better with lower emission levels (Devi *et al*., 2006).

The economic use of the wild and/or weedy guizotias is not well known. However, *G. scabra* ssp. *scabra* was reported to be used for human consumption (seeds) in Nigeria, as a medicine (seeds) and to make fishing nets (stem) in Congo (Baagøe, 1974). It has also been used as a treatment for hepatotoxicity and stomach disorders in Rwanda and neighboring countries (Fujimoto *et al*., 1990). *G. scabra* ssp. *schimperi* was reported to be used as a substitute for *G. abyssinica* (Baagøe, 1974) and also as herbal medicine in some places in Ethiopia. The similarity in fatty acid composition between niger and the other guizotias (Dagne & Jonsson, 1997) may indicate the possibility of using wild and/or weedy guizotias for human consumption though further content analysis is required.

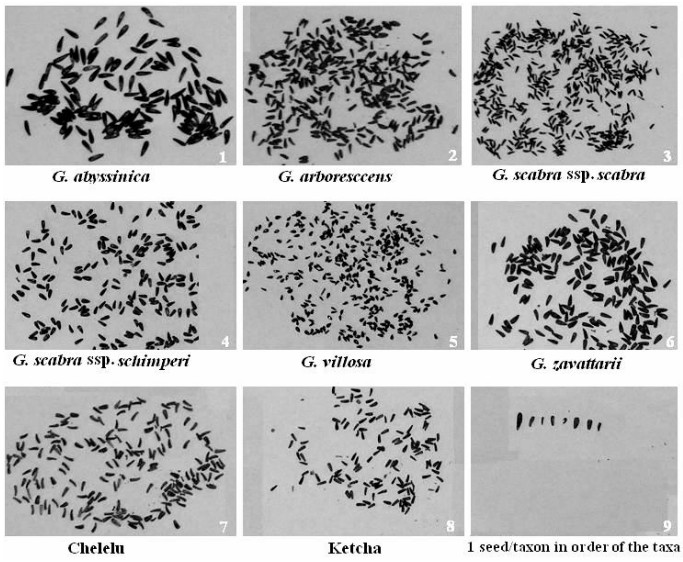

Figure 10. Seeds from eight *Guizotia* taxa.

Distribution, ecology and agronomy of *G. abyssinica*

Niger is mainly cultivated in Ethiopia and India. It is also cultivated in small scale in several other African (Sudan, Uganda, Zaire, Tanzania, Malawi and Zimbabwe) and Asian (Nepal, Bangladesh and Bhutan) countries, and in USA and West Indies (Weiss, 1983; Murthy, Hiremath & Salimath, 1993; Getinet & Sharma, 1996; Kandel & Porter, 2002). Niger has also been grown in Germany, Switzerland, France, Liechtenstein and the former USSR in the nineteenth and/or early twentieth century (Weiss, 1983).

In Ethiopia, niger is grown mainly from 1,600 masl to 2,200 masl, where the range in temperature is 15°C to 23°C, and where the annual rainfall is 500-1,000 mm (Getinet & Sharma, 1996). However, it can be cultivated at altitudes as low as 500 m and as high as 3000 m in the availability of enough rainfall (Weiss, 1983; Getinet & Sharma, 1996). The major niger producing regions in Ethiopia are Gojam, Gonder Shewa and Welega, which are followed by Tigray and Welo. The production of niger in Arsi, Bale, Harerge, Jimma and Illubabor is low and restricted to few districts. Ethiopian niger normally matures within 120-150 days after emergence, but varieties with a shorter maturation period of 90-120 days also exist (Weiss, 1983). Niger occupies about 50% of the total oil crop area and production volume in Ethiopia (Getinet and Sharma, 1996). It is grown either as a sole crop or inter/border crop in fields of other crops (Geleta *et al*., 2002) usually without the application of fertilizer or herbicide. Fertilizer application promotes vegetative growth rather than increasing seed yield (Getinet & Sharma, 1996). Niger can grow on a wide variety of soils, but appears to thrive best on clayey loams or sandy clays (Weiss, 1983) within a pH range of 5.2-7.3 (Getinet & Sharma, 1996). It can grow on waterlogged, marginal and poor soils where most other crops fail to grow, as it is able to withstand salinity and low oxygen levels (Abebe, Yermanos & Bingham, 1978).

Despite its long cultivation history, niger has low seed yield due to several factors, including an indeterminate growth habit, lodging and shattering. Its low yield makes niger unsuccessful in Europe, the former USSR and Canada as a commercial crop (Weiss, 1983). On average, both Ethiopian and Indian niger yields 300–400 kg/ha when grown in pure stands although higher yield was reported for some varieties from Kenya (600 kg/ha; Kandel & Porter, 2002) and India (1,000 kg/ha; Weiss, 1983).

Pests and diseases of *G. abyssinica*

A large number of niger pests and diseases have been recorded though they are fewer than those recorded in most other oilseed crops (see Getinet & Sharma, 1996). Niger pests and diseases do not cause serious damage to the crop (Weiss, 1983; Kandel & Porter, 2002) due to its apparently considerable level of tolerance/resistance, which needs to be well investigated. Niger fly (*Dioxyna sororcula* and *Eutretosoma* spp.), black pollen beetles (*Meligethes* spp.) and dodder (*Cuscuta campestris*; a parasitic weed) are the most important pests of niger while niger blight (*Alternaria* sp.) and bacterial leaf spot (*Xanthomonas* sp.)

are the most serious diseases of niger (Gebre-Medhin & Mulatu, 1992; Getinet & Sharma, 1996).

Breeding and biotechnology in niger

Ethiopian and Indian niger are different in several characteristics, including plant height, days to maturity, and fatty acid composition (Weiss, 1983; Getinet & Sharma, 1996), which is the result of separate evolution since the separation of the two gene pools. Niger production in Ethiopia is mainly based on local landrace populations, which makes it more variable than Indian niger. According to Getinet & Sharma (1996), three landraces of niger are known in Ethiopia. These are 'abat' 'bungne' and 'mesno', which are medium to late maturing, early maturing and late maturing, respectively. Abat is the common landrace with higher yield than the other two varieties and with higher oil content than bungne (Getinet & Sharma, 1996). Lodging, shattering, indeterminate growth habit and self-incompatibility have been reported as major factors that contribute to low yield in *G. abyssinica*.

The low yield in niger makes it less competitive with other oil crops and hampers its improvement through breeding, as the interest of breeders has been low. In India, it was possible to improve seed yield by about three-fold (Weiss, 1983). Unlike the case in India, only few improved varieties have been released in Ethiopia. These varieties are not that much better than the landraces, in terms of seed yield, oil content and days to maturity (Getinet & Sharma, 1996). In this regard, niger breeding in Ethiopia is still in its relative infancy. In niger, seed yield is positively correlated with seed size, number of seeds/capitula, number of capitula/plant and number of primary branches in niger (*e.g.* Singh & Patra, 1989). Our unpublished agromorphological data showed the existence of several-fold variation in number of seeds/capitula, capitula/plant, and primary branches/plant both within and between Ethiopian niger populations. Some degree of variation in achene size also exists in the Ethiopian niger gene pool. Therefore, great effort is needed to use the existing genetic diversity for the improvement of the crop.

Biotechnology might be considered as an alternative approach for niger improvement. Several cell tissue culture studies have been conducted on niger. Regeneration has been reported from leaves (Sujatha, 1997; Jadimath *et al*., 1998; Kumar *et al*., 2000), cotyledons (Adda, Reddy & Kishor, 1993b, 1994b; Nikam & Shitole, 1997), and anthers (Adda, Reddy & Kishor, 1993a, 1994b). In India, dwarfs and large headed self-compatible doubled haploid niger genotypes were obtained from anther culture (Adda, Reddy & Kishor, 1994a). The protocol for *in vitro* plant regeneration for large-scale propagation of male sterile niger was published by Sujatha (1997). This protocol may facilitate the efficient use of pollen grains carrying desirable traits to pollinate the male sterile genotypes (Sujatha, 1997). The various regeneration protocols reported so far and the *Agrobacterium* mediated transformation protocol developed by Murthy *et al*. (2003) are useful for the future improvement of niger.

Oil content and fatty acid composition of guizotias

Seed lipids usually contain over 95% neutral storage lipids in the form of triacylglycerol (TAG) (Ohlrogge & Jaworski, 1997). The oil content of the niger seed is commonly within the range of 27-50%, as reported by various authors (*e.g.* Seegeler, 1983; Dutta *et al*., 1994; Getinet & Teklewold, 1995; Dagne & Jonsson, 1997; Marini *et al*., 2003; Ramadan & Mörsel, 2003; Asilbekova *et al*., 2005), which is higher than that of wild and/or weedy guizotias (Dagne & Jonsson, 1997). However, up to 60% oil has been obtained from niger seed of improved varieties (Kandel & Porter, 2002).

The fatty acid composition of wild and/or weedy guizotias is similar to that of *G. abyssinica* apart from some degree of variation in their proportion (Dagne & Jonsson, 1997). The predominant fatty acid in niger seed oil is linoleic acid (LA) regardless of the differences among reports in terms of its proportion within the range of 54-85% (*e.g.* Weiss, 1983; Getinet & Teklewold, 1995; Dagne & Jonsson, 1997; Marini *et al*., 2003; Ramadan & Mörsel, 2003). Niger seed oil has a higher proportion of LA and a lower proportion of oleic acid (OA) as compared to that of wild and/or weedy guizotias (Dagne & Jonsson, 1997), and sunflower and safflower (Dutta *et al*., 1994). OA is the second major unsaturated fatty acid in niger seed oil, which commonly accounts for 5-13% of the fatty acids. This fatty acid is higher in the Indian than in the Ethiopian niger (Riley & Belayneh, 1989; Marini, 2003). Generally, LA, OA and the two major saturated fatty acids in niger seed oil (palmitic acid and stearic acid) make up more than 90% of the fatty acids in niger seed oil (*e.g.* Dutta *et al*., 1994; Dagne & Jonsson, 1997; Ramadan & Morsel, 2003).

The high content of LA (an essential fatty acid) in niger seed oil is nutritionally highly valuable, as it is known to prevent cardiovascular disorders such as coronary heart diseases, arteriosclerosis and high blood pressure (Vles & Gottenbos, 1989). Niger seed oil is also a good source of vitamin E, as almost all of the tocopherols are α-tocopherol (94-96%; Dutta *et al*., 1994; Ramadan & Mörsel, 2004). The oxidative stability of niger seed oil was reported to be lower than that of black cumin and coriander seed oils (Ramadan & Mörsel, 2004), which may limit its utilization in processed and fortified foods as well as nutritional supplement, as lipid oxidation negatively affects the flavor, odor, color and nutritional value of foods during storage. The lower oxidative stability of niger seed oil is partly explained by its high LA content that overcome the antioxidant activity expected from tocopherols and partly by its relatively lower level of phenolics and polar lipids that have anti-oxidant activity (Ramadan & Mörsel, 2004).

Genetic diversity and conservation of crops and their wild relatives

Crop genetic diversity is the genetic variation within and between individuals, populations and varieties of the cultivated species, which has occurred through mutation, introgression, recombination, adaptation to new environments and

continuous selection. In most cases, crop's genetic diversity is greatest in regions where it was originally domesticated and where its evolution has the longest record (Hawkes, 1983). The genetic diversity within crop species is an invaluable genetic resource for farmers, scientists and consumers elsewhere. The significance of such diversity can be measured, in part, by its role in increasing crop resistance to pests, diseases, and abiotic stresses, which in turn contribute to the production of higher, more stable and good quality yields (*e.g.* Bekele, 1985; Wood & Lenne, 1997; Jana, 1999). In other words, genetic diversity of crops is of paramount importance in maintaining and increasing agricultural productivity and product quality and helps to withstand newly emerging pests and pathogens, as it is a defense against the uncertain future. Genetic diversity in the crops' wild relatives is also very important to tackle various problems associated with crop failure. Crop plants have been improved through using wild relatives especially those within the primary gene pools of crops (Harlan & de Wet, 1971; Hawkes, 1977). Since the 1980's, crop improvement has been made by transferring desirable genes from distantly related and even non-related taxa through genetic engineering, thereby broadening the value of crops' wild relatives by expanding their usefulness into secondary and tertiary gene pools (Meilleur & Hodgkin, 2004).

There has been an increasing concern over the loss of genetic diversity in crops and their wild relatives in areas of crop domestication. Deforestation and habitat loss due to agricultural expansion, soil erosion and degradation are among the most serious environmental problems threatening the genetic diversity of crops' wild relatives (Jana, 1999), which is perceived as threats to the genetic base of world agriculture (Meilleur & Hodgkin, 2004). Unless genetic variation in crop plants and their wild relatives is properly conserved, they may be lost forever, to the great detriment of agriculture and human food security. Since several decades, there have been attempts to conserve crop genetic variation both *in situ* and *ex situ* (*e.g.* Brush, 1995; Bellon, 1996, Heal *et al*., 2004). For example, 668 niger accessions and 510 accessions of wild and/or weedy guizotias are conserved *ex situ* at the Institute of Biodiversity Conservation (IBC) of Ethiopia (Adugna Abdi, personal communication). The *in situ* conservation of agricultural crops and their wild relatives requires conservation of their respective original habitats in their major centers of diversity (Jana, 1999). Therefore, identifying the genetic diversity center for a crop and its wild relatives is important for proper conservation.

Analyses of plant genetic diversity may lead to the identification of diverse parental combinations to create segregating progenies with maximum variability for further selection (*e.g.* Barrett & Kidwell, 1998; Brush & Meng, 1998), to introgression of desirable genes from diverse germplasms into the variety of interest (*e.g.* Thompson & Nelson, 1998) and to a reliable classification of accessions of germplasm collections (*e.g*. Ma *et al*., 2006). Such an assessment of levels and patterns of plant genetic variation can be carried out based on various types of data, such as pedigree (*e.g.* Barata & Carena, 2006; Soleiman, Baum & Johnson, 2007), morphological (*e.g.* Ayana & Bekele, 1999; Talhinhas, Leitao & Neves-Martins, 2006), isozymes (*e.g.* Hamrick & Godt, 1997; Ayana, Bryngelsson & Bekele, 2001) storage proteins (*e.g.* Bekele *et al*., 1995; Alvarez, Moral & Martin, 2006) and DNA markers (*e.g.* Assefa, Merker & Hailu, 2003; Cavagnaro *et al*., 2006).

AFLP and RAPD

Amplified Fragment Length Polymorphism (AFLP) and Random Amplified Polymorphic DNA (RAPD) are among the most commonly used PCR based molecular marker techniques and they have various applications in plants, including genetic diversity (*e.g.* Nybom & Bartish, 2000; Raina *et al*., 2001; Nybom, 2004), phenetic and phylogenetic analyses (*e.g.* Landry & Lapointe, 1996; Blattner *et al*., 2001; Després *et al*., 2003; Bänfer, Fiala & Weising, 2004; Kadereit & Kadereit, 2005) and species/cultivar identification (*e.g.* Johnson *et al*., 2003; Kelly & Miklas, 1998; Boukar *et al*., 2004).

The RAPD method makes use of single short oligomers (usually 10-mer) of arbitrary sequence which anneal to random homologous target sites within the genome. It allows the generation of a product only when two copies of a primer are annealed in an inverted fashion to each other within an amplifiable distance (Welsh & McClelland, 1990; Williams *et al*., 1990). RAPD polymorphisms result from DNA sequence variation at primer binding sites and from DNA length differences between primer binding sites, which disrupt or displace homologous target sites and results in the loss of a product. This technique largely generates dominant markers, although co-dominant markers that are produced due to length polymorphisms caused by small insertions/deletions can occur at low frequencies (2–5%; Milbourne *et al.*, 1997). RAPD is specially suited to analyze large number of samples as it is relatively simple, fast and cheap. Problems associated with reproducibility (Jones *et al*., 1997) and co-migration of some non-homologous fragments (Rieseberg, 1996) are drawbacks of the RAPD technique. However, reproducibility can be considerably improved by making the reaction conditions uniform across samples, and by thorough screening of primers for reproducibility (Skroch & Nienhuis, 1995). The problem associated with co-migration of non-homologous fragments is minimal when RAPD is applied to study closely related populations or species (Rieseberg, 1996).

AFLP is a molecular marker technique that involves digestion of target DNA by restriction endonucleases, ligation of adaptors to the restricted fragments at both ends and amplification of fragments (Vos *et al.*, 1995). In higher plants, fragment amplification is usually conducted in two steps: preamplification and selective amplification. AFLP primers possess complementary sequences to the adaptors and the adjacent restriction site that serve as primer binding sites for subsequent amplification of the restriction fragments. Additionally, these primers possess one (preamplification) or three (selective amplification) selective nucleotides at their 3'-end (Vos *et al.*, 1995). The presence or absence of these selective nucleotides in the genomic fragments being amplified and the restriction fragment size variation provide the basis for revealing polymorphism in AFLPs. Despite the fact that AFLPs are generally treated as dominant markers, codominance seems to be more common in AFLPs than in RAPDs, as 10% codominant AFLPs were reported in *Populus* (Yin *et al*., 2002) and 14% in *Arabidopsis thaliana* (Alonso-Blanco *et al*., 1998). The AFLP technique has the capacity to detect a higher number of polymorphic loci in a single assay than RFLPs or RAPDs (Powell *et al.*, 1996), has the highest discrimination efficiency in comparison to RAPD and ISSR

(Archak *et al*., 2003), and produces highly reproducible results (Jones *et al*., 1997).

The use of RAPD and AFLP techniques to assess genetic relationship between different groups of taxa, to assess genetic variability within and among populations and to screen for diagnostic markers is promising because many polymorphic loci can be obtained fairly easy, in a relatively short time and without any prior knowledge of the genome of the species under study (*e.g.* Vos *et al*., 1995, Gupta *et al*., 1999, Nybom & Bartish, 2000; Nybom, 2004). These marker techniques were reported to work well for phylogenetic studies in closely related species (Landry & Lapointe, 1996; Després *et al*., 2003) and can be used especially when DNA sequencing data fails to resolve the phylogeny (Weising *et al*., 2005).

cpDNA and nDNA sequence data for phylogenetics and systematics

DNA sequencing is the most direct method of detecting genetic variation at the DNA level. The conserved regions of the DNA are the bases for designing PCR and sequencing primers. The PCR-amplified target regions can be either directly sequenced or sequenced after cloning (Hillis, Moritz & Mable, 1996). Comparative DNA sequencing has become a widespread tool for systematic and phylogenetic studies as it is relatively fast and convenient, and provides highly informative, robust and reproducible data sets. It can be applied for comparing organisms at different taxonomic levels by choosing appropriate genomic target regions (Weising *et al*., 2005). The majority of data used in plant molecular phylogenetic and systematic studies derive from chloroplast DNA (cpDNA) and nuclear ribosomal DNA (nrDNA) (Small, Cronn & Wendel, 2004). Slowly evolving DNA regions such as the chloroplast *rbc*L gene (Clegg, 1993; Soltis, Soltis & Chase, 1999) and the nrDNA genes (Kuzoff *et al*., 1998) have been used at higher taxonomic levels.

For systematic and phylogenetic studies at infrafamilial taxonomic levels, the internal transcribed spacers (ITS1 and ITS2) of nrDNA (Baldwin, 1992; Baldwin *et al*., 1995; Crawford & Mort, 2005), and various cpDNA regions such as non-coding regions of tRNA genes (*e.g. trn*T/*trn*L and *trn*L/*trn*F intergenic spacers and *trn*L intron) and *mat*K gene have been widely used (*e.g.* Taberlet *et al*., 1991; Johnson & Soltis, 1994; Liang & Hilu, 1996; Bayer, Greber & Bagnall., 2002; Winkworth *et al*., 2002; Crawford & Mort, 2005; Shaw & Small, 2005). DNA sequence data based phylogenetic analyses can be conducted by a variety of approaches and algorithms (*e.g.* Saitou & Nei, 1987; Archibald, Mort & Crawford, 2003; Huelsenbeck & Crandall, 1997; Hall, 2001).

The relatively simple genetics of cpDNA makes it a primary target for molecular phylogenetic analyses in plants (Small, Cronn & Wendel, 2004). A typical DNA sample contains a relatively high copy number of cpDNAs, as multiple copies of chloroplasts per leaf cell and multiple copies of cpDNAs per chloroplast exist. This facilitates PCR amplification of specific cpDNA regions, as high copy-number sequences are more readily accessible. Moreover, cpDNA is generally

stable, haploid, non-recombinant and uniparentally inherited, which facilitates its use in systematic studies (*e.g.* Birky, 1995; Small, Cronn & Wendel, 2004). However, the haploid nature and uniparental inheritance of cpDNA limit its use in phylogenetic studies of hybrids and polyploids, as it may incorrectly identify them as belonging to the clade of one of the two parents without revealing the hybrid history (Small, Cronn & Wendel, 2004). Ribosomal genes (including ITS) exist in tandem arrays of genes composed of hundreds to thousands of copies per array, which facilitates DNA sequencing directly from a PCR product (Baldwin *et al*., 1995; Kuzoff *et al*., 1998). At lower taxonomic level, ITS sequences generally provide greater levels of divergence and thus greater resolution and stronger support than an equivalent sample of non-coding cpDNA (Sang, Crawford & Stuessy, 1997; Whitten, Williams & Chase, 2000).

Objectives

The major objective of this doctoral thesis was to study the genetic diversity, phylogenetics and systematics of the genus *Guizotia* with the ultimate goal of conserving and utilizing its genetic diversity. The following are the specific objectives of the study:

1. to determine the genetic structure and the extent of genetic variation within and between populations of *G. abyssinica* and its wild and/or weedy relatives using AFLP and RAPD for their conservation and use value
2. to resolve the phenetic and phylogenetic relationship between *Guizotia* taxa and to identify the closest relatives to *G. abyssinica* using molecular markers and DNA sequence data
3. to determine the taxonomic status of two yet taxonomically untreated *Guizotia* populations (Chelelu and Ketcha)
4. to evaluate the taxonomic status of the two subspecies of *G. scabra*
5. to provide data and suggest the appropriate subtribal placement of the genus *Guizotia* within the tribe Heliantheae

Material and methods

Plant material and DNA extraction

The germplasm of all guizotias used in this study was collected in Ethiopia from early November to the end of December 2003. *G. abyssinica* populations (Table 1) were collected directly from farmers' fields and the crop on a single field was considered as a population. Individuals were sampled at an equidistant along the longest line found across the field. The sampled populations represent the altitudinal range and geographic regions where niger is currently grown within the country. In the case of wild and/or weedy guizotias, plants of the same taxon

found in their habitat without large gaps in between were considered as a population. Samples were collected randomly and systematically depending on the size of the populations. Different number of populations were studied per taxon based on the geographic range and abundance of the species. Seeds were grown in a greenhouse and fresh leaves from 15-30 days old plants were used for genomic DNA extraction. DNA was extracted by a modified CTAB procedure. The detailed procedure is given in paper V.

Table 1. *(i) Number of populations used for genetic diversity (GD) and genetic relationship (GR) study using AFLP and RAPD techniques, and (ii) number of individuals sequenced for nrDNA and cpDNA based phylogenetic analyses of eight Guizotia taxa*

Taxa	AFLP[a]		RAPD[a]		Sequencing[b]	
	GD	GR	GD	GR	nrDNA	cpDNA
G. abyssinica	17	11	70	11	2	4
G. arborescens	3	2	4	2	4	3
G. scabra ssp. *scabra*	4	3	9	3	1	3
G. scabra ssp. *schimperi*	4	3	9	3	1	3
G. villosa	3	2	6	2	2	3
G. zavattarii[c]	3	2	6	2	2	3
Chelelu	1	1	1	1	-	2
Ketcha	1	1	1	1	-	2

[a]Seven AFLP primer combinations (PCs) and ten RAPD primers were used for each taxon. [b]Five cpDNA regions (*trn*T/*trn*L intergenic spacer, *trn*L intron, *trn*L/*trn*F intergenic spacer, 3'*trn*K/*mat*K portion of the *trn*K intron and *mat*K gene) and two nrDNA regions (ITS1 and ITS2) were used for this study. [c]Only *Guizotia zavattarii* var. *zavattarii* was used.

AFLP and RAPD

DNA amplification, electrophoresis, staining and data scoring

After optimizing all procedures, 150 RAPD primers and 56 AFLP primer combinations (PCs) were screened, out of which ten RAPD primers and seven AFLP PCs were used for final analyses. RAPD based DNA amplification, electrophoresis and staining of the amplified product were described in paper I. The detailed descriptions of DNA enzyme restriction, adaptor ligation, preamplification, selective amplification and silver staining procedures of AFLP are given in papers II & IV. Each RAPD and AFLP band was considered as a single bi-allelic locus with one amplifiable and one null allele. Data was scored manually as 1 for the presence and 0 for the absence of a DNA band for each locus across the genotypes of each taxon. As a strategy to minimize scoring error, both within and among gels, data scoring was performed twice separately and discrepancy was reconsidered and corrected.

Sequencing

The entire region of internal transcribed spacers (ITS1 and ITS2) of nrDNA, and the *trn*T/*trn*L intergenic spacer, the *trn*L intron, the *trn*L/*trn*F intergenic spacer, the 3'*trn*K/*mat*K portion of the *trn*K intron and the *mat*K gene of cpDNA were amplified using specific primer-pairs and sequenced using corresponding sequencing primers. Details for cleaning amplified products, sequencing and sequence processing were described in papers V (ITS) and VI (cpDNA regions).

Data analysis

Data from the AFLP and RAPD studies was analyzed using various statistical programs. The details of Shannon diversity estimates and gene diversity parameters (Nei, 1973, 1978; Lynch & Milligan, 1994) were described in paper I. NTSYSpc (Rohlf, 2000) was used for genetic similarity and distance estimates (Jaccard, 1908; Nei, 1972, 1978), cluster analysis, principal coordinate analysis (PCoA) and comparison of matrices. POPGENE version 1.31 (Yeh & Boyle, 1997) was used for analysis of percentage of polymorphic loci while Arlequin version 2 (Schneider, Roessli & Excoffier, 2000) was used for analysis of molecular variance (AMOVA). FreeTree-Freeware (Pavlicek, Hrda & Flegr, 1999) and TreeView (Win32) 1.6.6 (Page, 1996) were used for bootstrap analysis and to view trees, respectively. MINITAB release 14 was used for further analyses of some of the outputs from other programs. DNA sequencing data was handled and manipulated using BIOEDIT version 7.0.5 (Hall, 2005) and SEQUENCE SCANNER version 1.0 (Applied Biosystems®), and aligned using CLUSTAL X version 1.81 (Thompson *et al*., 1997) and SEQUENCHER (Gene Codes Corporation). Phylogenetic analysis of DNA sequence data was carried out using PAUP* 4.0 Beta 10 (Swofford, 2000).

Summary of results and discussions

Genetic diversity of *G. abyssinica* (papers I & II)

A total of 539 clear and unambiguous loci generated by seven AFLP PCs applied to 170 individual plants were scored, of which 483 (90%) loci were polymorphic. Similarly, a total of 194 loci that were consistently amplified by ten RAPD primers applied to 700 individuals were scored, of which 188 (97%) were polymorphic. Both studies revealed that, on average, about half of the loci in each population are polymorphic (Table 2). The high percentage of polymorphic loci revealed by both marker techniques suggests the existence of high genetic polymorphism in Ethiopian niger and was proven to be useful in distinguishing niger populations and even individuals within populations. The polymorphism detected in niger was higher than, for example, the RAPD based polymorphism (64%) reported by Sivolap, Solodenko & Burlov (1998) in sunflower (*Helianthus annuus* L.). The RAPD based study revealed an average of 19 polymorphic loci per primer while an average of 69 polymorphic loci per AFLP PC was obtained. Thus, the multiplex ratio (the number of loci analyzed simultaneously per experiment) of AFLPs is more than threefold when compared with that of RAPDs. The overall utility of AFLP was also higher than RAPD, as measured by the marker index (product of multiplex ratio and diversity index), which is in agreement with the report of Milbourne *et al*. (1997).

The overall gene diversity estimate (H_T; Nei, 1978) with the modification of Lynch & Milligan (1994) was 0.320 (AFLP) and 0.248 (RAPD). Similarly, the overall within population variation (H_S) was 0.205 (AFLP) and 0.176 (RAPD) and thus AFLP revealed relatively higher overall total and within population diversity

as compared to RAPD in *G. abyssinica*. The extent of genetic diversity of each population was calculated using gene diversity estimates as H_j, which ranged from 0.183 to 0.241 (AFLP) and from 0.112 to 0.245 (RAPD). It is interesting to note that the highest within population genetic variation obtained by using AFLP or RAPD refers to the same population (Wl-1), which shows an agreement between the two marker techniques. Niger populations from some regions such as Illubabor (Table 2) where niger cultivation seems to be declining (local informants), appears to have higher genetic variation within populations.

Table 2. *Mean percent polymorphic loci (PPL) and mean gene diversity estimates (H_j) generated from AFLP and RAPD data for eight Guizotia taxa*

Taxa	Regions (code)	*PPL*		H_j	
		AFLP	RAPD	AFLP	RAPD
G. abyssinica	Arsi (A)	49.7	54.6	0.19	0.19
G. abyssinica	Bale (B)	50.5	53.6	0.21	0.19
G. abyssinica	Gojam (Gj)	53.6	47.0	0.22	0.16
G. abyssinica	Gonder (Gr)	49.7	46.8	0.19	0.14
G. abyssinica	Harerge (H)	50.3	48.5	0.20	0.16
G. abyssinica	Illubabor (I)	55.1	55.0	0.27	0.19
G. abyssinica	Jimma (J)	54.9	55.9	0.22	0.19
G. abyssinica	Shewa (Sh)	50.0	51.8	0.19	0.18
G. abyssinica	Tigray (T)	46.2	54.1	0.18	0.18
G. abyssinica	Welega (Wg)	50.6	49.2	0.20	0.17
G. abyssinica	Welo (Wl)	54.2	57.7	0.21	0.21
G. abyssinica	Mean	51.3	52.2	0.20	0.16
G. arborescens		17.38	37.57	0.24	0.17
G. scabra ssp. *scabra*		61.09	51.20	0.24	0.17
G. scabra ssp. *schimperi*		64.57	51.13	0.24	0.17
G. villosa		62.63	56.85	0.24	0.19
G. zavattarii		23.69	34.84	0.17	0.16
Chelelu		40.82	74.68	0.37	0.31
Ketcha		80.64	91.67	0.33	0.30

Analysis of molecular variance of AFLP and RAPD data revealed a highly significant variation between populations ($P < 0.001$) with 23% and 35% of the total variation differentiating the populations, respectively. The RAPD based AMOVA calculated by grouping the populations into regions of origin revealed a highly significant variation between regions (13.9%; $P < 0.001$). Similarly, highly significant variation (7.5%; $P < 0.001$) was obtained when AFLP data was analyzed by grouping the populations based on geographic proximity and better access of gene flow. The result suggests considerable degree of regional differentiation of *G. abyssinica* populations. The RAPD based AMOVA calculated by grouping the populations into populations from major niger producing regions (MaNPRs) and populations from minor niger producing regions (MiNPRs) revealed significant variation ($P < 0.001$) between the groups, with the latter possessing higher diversity. Unlike RAPD, AFLP analysis did not result in significant variation between these groups. Thus, the results from both analyses do not agree with the report of Genet & Belete (2000) who concluded, based on phenological and morphological data, that niger populations from MaNPRs have higher genetic diversity. Rather, the result suggests that the extent of cultivation of landraces does not always result in higher genetic diversity. Both AFLPs and

RAPDs revealed that the level of genetic variation in populations from the higher altitude group (> 2000 masl) and the lower altitude group (< 2000 masl) are similar and that altitude and level of genetic diversity did not correlate. Based on these results, it is concluded that the extent of the existing genetic diversity in Ethiopian niger is distributed over all its growing regions regardless of altitude and extent of cultivation. These characteristics make niger suitable for adaptation to diverse environmental conditions as the chances of finding more adaptive genotypes are high.

Genetic differentiation of populations may occur for any genetically variable trait that is favored under the existing selection conditions (Bossdorf *et al*., 2005) as well as due to random stochastic processes, mutation and migration and the degree of such population differentiation could be estimated using different parameters. The overall degree of population differentiation estimated as G_{ST} (Nei, 1973) was 0.27 and 0.24 for AFLPs and RAPDs, respectively (Table 3). The RAPD based G_{ST} (0.24) revealed in this study was almost similar to the RAPD based mean G_{ST} (0.23) obtained for 18 outcrossing species (Nybom & Bartish, 2000). Nybom (2004) analyzed eight AFLP-based studies of outcrossing species and obtained a mean G_{ST} of 0.24. Therefore, this study demonstrates an average level of population differentiation with significant variation among niger populations. Partitioning of the total genetic diversity into within and among population components showed that 65% (RAPD) and 77% (AFLP) of the total variation was due to genetic variation within populations (AMOVA). Despite the relatively higher value obtained from AFLPs as compared to RAPDs, both marker systems demonstrated that a higher proportion of the total genetic variation is within populations, in *G. abyssinica.* Generally, at least 20% of the total genetic variation in niger is found among populations, implying that each population has unique genetic properties which makes it a significant unit for conservation efforts and breeding purposes. Therefore, it is recommended that as many populations as possible should be conserved *ex situ*, as it reduces the risk of losing unique genetic variants due to shifting of cultivation practices and many other factors.

Genetic distance and cluster analyses

The AFLP and RAPD based Nei's standard genetic distance (Nei, 1972) between niger populations ranged from 0.040 to 0.175 (mean = 0.118) and from 0.050 to 0.300 (mean = 0.176), respectively. The lowest and the highest genetic distance were recorded between populations from the same region and different regions, respectively, both with AFLP and RAPD. The comparison of matrices of Nei's standard genetic distance (from combined data set of AFLP and RAPD) and geographic distance between populations using normalized Mantel statistics (Mantel, 1967) revealed a significant positive correlation ($r = 0.266$; $P < 0.01$). Such positive correlation between geographic distance and genetic distance in outcrossing species is not uncommon (*e.g.* Ayres & Ryan, 1997; Shim & Jørgensen, 1999). The RAPD based UPGMA cluster analysis for the 70 niger populations revealed that the majority of the populations from the same region were clustered together. Populations from adjacent regions were also clustered to a considerable degree. Almost similar results were obtained when AFLP based

cluster analysis and PCoA (Fig. 11) were calculated, despite the low correlation (r = 0.37) between the similarity matrices of AFLP and RAPD.

Table 3. *Summary of gene diversity and AMOVA for eight Guizotia taxa*

Taxon-marker technique	Gene diversity			AMOVA		
	H_T	H_S	G_{ST}	PVWP[a]	PVAP[b]	F_{ST}
G. abyssinica-AFLP	0.32	0.20	0.27	76.56	23.44	0.23
G. abyssinica-RAPD	0.25	0.18	0.24	64.58	35.42	0.35
G. arborescens-AFLP	0.37	0.24	0.30	70.69	29.31	0.29
G. arborescens-RAPD	0.28	0.15	0.34	54.56	45.44	0.45
G. scabra ssp. *scabra*-AFLP	0.32	0.24	0.18	77.51	22.49	0.22
G. scabra ssp. *scabra*-RAPD	0.23	0.17	0.19	63.70	36.30	0.36
G. scabra ssp. *schimperi*-AFLP	0.32	0.24	0.19	82.67	17.33	0.17
G. scabra ssp. *schimperi*-RAPD	0.22	0.17	0.20	62.20	37.80	0.38
G. villosa-AFLP	0.33	0.24	0.19	74.10	25.90	0.26
G. villosa-RAPD	0.23	0.19	0.15	68.83	31.17	0.31
G. zavattarii-AFLP	0.37	0.17	0.41	49.88	50.12	0.50
G. zavattarii-RAPD	0.28	0.14	0.40	42.62	57.38	0.57
Chelelu-AFLP	-	0.37	-	-	-	-
Chelelu-RAPD	-	0.31	-	-	-	-
Ketcha-AFLP	-	0.33	-	-	-	-
Ketcha-RAPD	-	0.30	-	-	-	-
Mean-AFLP	0.34	0.25	0.26	63.05	36.95	0.28
Mean-RAPD	0.25	0.20	0.25	61.88	38.13	0.40

[a]Percent variation within populations. [b]Percent variation among populations.

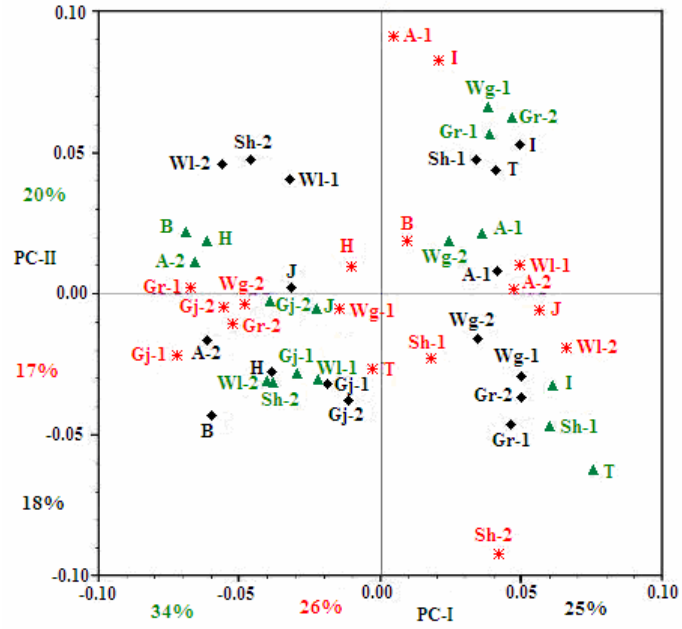

Figure 11. Two-dimensional plot for the 17 populations of *Guizotia abyssinica* generated by PCoA from (i) AFLP (green), RAPD (red) and combined data (black). Note: the codes of the populations refer to their regions of origin (see Table 2).

The general trend in Ethiopian niger is that genetic similarity between populations increases with geographic proximity due to the corresponding increase in rate of gene flow. Therefore, germplasm collections should consider geographic distance between populations as one major criterion of sampling. However, this is not always the case as populations from geographically distant regions were also clustered in some cases, which may be explained by long distance movement of niger populations along with the movement of humans into new settlement areas in the past. Out of the total of 733 loci generated by AFLP and RAPD, no population specific monomorphic marker was found which may indicate continuous gene flow between populations. Generally, this study generated comprehensive information regarding genetic diversity in *G. abyssinica*.

Genetic diversity of wild and/or weedy guizotias (paper III)

Various parameters were used to analyze the genetic diversity in seven wild and/or weedy guizotias. The mean percentage of polymorphic loci per population (P_p) for *G. arborescens*, *G. scabra* ssp. *scabra*, *G. scabra* ssp. *schimperi*, *G. villosa* and *G. zavattarii* was 17%, 61%, 65%, 62% and 24% in the case of AFLP and 38%, 51%, 51%, 57% and 35% in the case of RAPD, respectively. When all loci were considered for each taxon as a whole, the percentage of polymorphic loci (P_S) was 28.5%, 84.5%, 90.0%, 83.9% and 50.1% for AFLP and 86.6%, 99.6%, 99.6%, 98.5% and 92.7% for RAPD in that order for the above taxa. The percentage of polymorphic loci (P_P) for Chelelu and Ketcha was 41% and 81% in the case of AFLP and 75% and 92% in the case of RAPD, respectively. Both AFLPs and RAPDs revealed a relatively higher level of genetic polymorphism in those *Guizotia* taxa with relatively wider geographic distribution in Ethiopia (*G. scabra* ssp. *scabra* and *G. scabra* ssp. *schimperi*), which might be partly due to introgressive hybridization between them. The mean AFLP based estimate of the Jaccard's similarity coefficient between populations of each taxon ranged from 0.24 (*G. scabra* ssp. *schimperi)* to 0.51 (*G. arborescens*) while that of RAPD ranged from 0.51 (*G. villosa*) to 0.89 (*G. arborescens*). Despite higher similarity obtained using AFLPs, both marker systems revealed that the similarity between populations of *G. arborescens* and *G. zavattarii* is higher as compared to that of the other guizotias.

Considering AFLPs and RAPDs together, the extent of genetic similarity between populations of *G. scabra* ssp. *scabra*, *G. scabra* ssp. *schimperi* and *G. villosa* was almost similar. These guizotias have a similar level of total genetic diversity as estimated by using gene diversity estimate (H_T), which is also similar to that of *G. abyssinica* (Table 3). The G_{ST} and F_{ST} values generated from AFLPs and RAPDs indicated that all guizotias are more diverse within with less genetic differentiation between populations with the exception of *G. zavattarii* (Table 3), which is in agreement with the general understanding that outcrossing species tend to be more diverse within, with less genetic differentiation between populations (Hamrick & Godt, 1996; Nybom, 2004). Significant genetic variation between populations was obtained in all guizotias (AMOVA; $P < 0.001$). The estimate of the total genetic diversity was higher in *G. arborescens* and *G. zavattarii* as

compared to the other guizotias mainly due to their higher population differentiation (Table 3; Fig. 12).

A lower percentage of polymorphic loci and higher population differentiation in *G. arborescens* and *G. zavattarii* as compared to their relatively common congeners might be best explained by the fact that these species are rare with small population size and highly localized; as species with such ecological characteristics tend to have this type of population genetic structure (Loveless & Hamrick, 1984; Slatkin, 1987). The counter-example to this trend is the relatively high polymorphism and genetic variation revealed within the populations of Chelelu and Ketcha, regardless of their small population size and seemingly being isolated populations. The implication is that genetic drift could be counterbalanced by other factors that promote genetic variation (*e.g.* a high rate of mutation and interspecific hybridization) even in small populations. The relatively higher within population diversity in Chelelu and Ketcha (H_S; Table 3) might also be explained by the fact that they are perennials. The general trend is that perennials have higher within population diversity as compared to annuals, provided that they have similar mating systems (Hamrick & Godt, 1989; Nybom & Bartish, 2000). However, this does not explain the low within population variation in the other two perennial species (*G. arborescens* and *G. zavattarii*). Therefore, different factors such as life forms and geographic distribution with differential intensity of selection contributed differently to the population genetic structure of different taxa of the genus *Guizotia*.

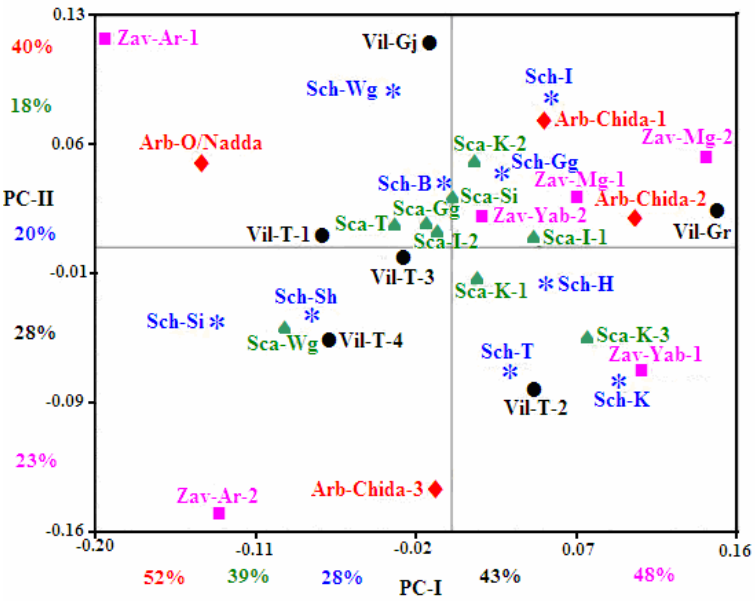

Figure 12. Two-dimensional plot for the populations of (i) *Guizotia arborescens* (red), *Guizotia scabra* ssp. *scabra (*green), *Guizotia scabra* ssp. *schimperi* (blue), *Guizotia villosa* (black) and *Guizotia zavattarii* (pink) generated by PCoA from RAPD band profiles. Note: The code for each population is derived and combined from taxon name-region/place-number. For example, Vil-T-4 refers to population number 4 of *G. villosa* collected from Tigray (See Table 1 of paper III).

Analysis of the extent of genetic diversity in each population and genetic similarity between populations is important to suggest genetic diversity centers for each taxon. The abundance of *G. arborescens* is relatively better in Chida than in Omo Nadda. Correspondingly, this study revealed that the genetic diversity of Chida populations is slightly higher than that of Omo Nadda (see Table 3 of paper III). This may suggest Chida as a micro genetic diversity center of this species and thus should be considered as a priority site for *in situ* conservation. On the other hand, the extent of genetic variability of the two subspecies of *G. scabra* seems to be distributed regardless of their geographic locations within the country. *G. scabra* ssp. *scabra* is relatively frequent in southwestern Ethiopia (*e.g.* in Jimma and Keficho). In this region, its distribution overlaps with that of *G. scabra* ssp. *schimperi* and *G. arborescens*. Therefore, this region might be considered for *in situ* conservation of the two subspecies of *G. scabra*. Although *G. villosa* is relatively common in Tigray, populations from Gonder and Gojam seem to possess higher genetic variation as compared to those of Tigray. Thus, future conservation activities, including germplasm collection, should also consider populations located at the periphery of the species distribution range. In the case of *G. zavattarii*, populations from Arero are genetically less similar to the other populations and seem to have slightly higher diversity. The fact that *G. zavattarii* populations are highly differentiated emphasizes the importance of conservation of populations in all the three sites (Mega, Yabelo and Arero). However, if priority needs to be given, Arero should be preferred, as it is a relatively stable community.

Analysis of population differentiation based on combined data of AFLPs and RAPDs revealed a significantly higher population differentiation ($P < 0.001$) in perennials ($G_{ST} = 0.32$; $F_{ST} = 0.40$) as compared to annuals ($G_{ST} = 0.18$; $F_{ST} = 0.33$). The mean G_{ST} for *Guizotia* taxa was higher than the average values reported for several outcrossing species (*e.g.* Bussell, 1999; Nybom, 2004), suggesting the relatively high population differentiation in wild and/or weedy guizotias, specifically in *G. arborescens* and in *G. zavattarii*. Since they are Afromontane species, they are restricted to Afromontane archipelago and some populations of each taxon are separated by lowlands, which contributed to the significant differentiation between populations. Generally, although the extent of within and between populations genetic variation varies among taxa, a substantial amount of overall genetic diversity was revealed in all guizotias. Acknowledging the existing genetic variation within and between populations is important for conservation and breeding purposes. The combination of a significant level of genetic variation and the cross-compatibility of *G. scabra* ssp. *schimperi*, *G. scabra* ssp. *scabra* and *G. villosa* with *G. abyssinica* is an opportunity for improvement of *G. abyssinica*. This study highlights the importance of molecular analysis in understanding the genetic diversity and population structure of various guizotias and contributes to the knowledge of conservation of genetic resources in the Ethiopian flora.

Phylogenetic relationship between guizotias (papers IV, V & VI)

Phenetic and phylogenetic relationships between *Guizotia* taxa were investigated based on data generated using AFLP, RAPD and DNA sequencing (ITS of nrDNA

and various cpDNA regions). A total of 658 AFLP and 353 RAPD loci generated by seven AFLP PCs and ten RAPD primers were used for this analysis. The extent of genetic similarity between the taxa was investigated based on molecular variance, genetic distance and F_{ST} from AFLPs and RAPDs (paper IV). Both AFLP and RAPD based AMOVA revealed a highly significant variation ($P < 0.001$) between the taxa. Cluster analyses revealed that intraspecific populations were clearly clustered together with higher genetic similarity between them as compared to the genetic similarity between any pair of interspecific populations and thus proved a clear differentiation among *Guizotia* taxa.

Complete sequences of the ITS were obtained for *G. abyssinica*, *G. arborescens*, *G. scabra* ssp. *scabra*, *G. scabra* ssp. *schimperi*, *G. villosa* and *G. zavattarii* (paper V). The length of the entire ITS region was 645 bp in these guizotias, except that one sample of *G. villosa* was shorter by one nucleotide. Forty parsimony informative characters were obtained within the genus *Guizotia* for the entire ITS. A total aligned length of the sequences of the five cpDNA regions for all guizotias and three outgroups was 2997 bp, of which 45 characters were parsimony informative (paper VI). A significant length variation between taxa of the genus *Guizotia* was obtained in the entire *trn*T/*trn*L intergenic spacer, ranging from 582 (*G. zavattarii*) to 634 (Ketcha) nucleotides. The complete sequence of the *trn*L intron is 433 bp for all guizotias. The *trnL*/F intergenic spacer is shorter than both the *trn*T/*trn*L spacer and the *trn*L intron (ranging from 345-347 nucleotides long). Sequences of 1255 bp long 5'-most portion of the *mat*K gene and 301 bp long 3'*trn*K/*mat*K portion of the *trn*K intron were also available for analysis. Four variable sites were revealed in the *mat*K coding region between *Guizotia* taxa (Table 4), two of which resulting in synonymous and the other two in non-synonymous amino acid substitutions.

Table 4. *Part of the first 1254 nucleotide sequences and 408 amino acid (AA) sequences of matK coding region of Guizotia taxa showing variable regions*

	1	255[a]	588[a]	1171[a]	1209[a]
G. abyssinica-DNA	ATG --- AA**G** --- GG**T** TCT --- **G**TT --- CG**C** TTT --- TCT
G. abyssinica-AA	M --- **K** --- G S --- **V** --- R F --- S
G. arborescens-DNA	ATG --- AA**G** --- GG**T** TCT --- **A**TT --- CG**A** TTT --- TCT
G. arborescens-AA	M --- **K** --- G S --- **I** --- R F --- S
G. scabra ssp. *scabra*-DNA	ATG --- AA**G** --- GG**T** TCT --- **G**TT --- CG**C** TTT --- TCT
G. scabra ssp. *scabra*-AA	M --- **K** --- G S --- **V** --- R F --- S
G. scabra ssp. *schimperi*-DNA	ATG --- AA**G** --- GG**T** TCT --- **G**TT --- CG**C** TTT --- TCT
G. scabra ssp. *schimperi*-AA	M --- **K** --- G S --- **V** --- R F --- S
G. villosa-DNA	ATG --- AA**G** --- GG**T** TCT --- **G**TT --- CG**C** TTT --- TCT
G. villosa-AA	M --- **K** --- G S --- **V** --- R F --- S
G. zavattarii-DNA	ATG --- AA**G** --- GG**C** TCT --- **A**TT --- CG**C** TTT --- TCT
G. zavattarii-AA	M --- **K** --- G S --- **I** --- R F --- S
Chelelu-DNA	ATG --- AA**T** --- GG**T** TCT --- **A**TT --- CG**C** TTT --- TCT
Chelelu-AA	M --- **N** --- G S --- **I** --- R F --- S
Ketcha-DNA	ATG --- AA**T** --- GG**T** TCT --- **A**TT --- CG**C** TTT --- TCT
Ketcha-AA	M --- **N** --- G S --- **I** --- R F --- S
	1	85[b]		391b	

[a]Variable sites of DNA sequence. [b]Variable sites of amino acid sequence. Note: Variable nucleotides/amino acids are indicated in bold, and gaps are represented by **'---'**.

G. abyssinica versus other guizotias

The phylogenetic analysis of the cpDNA regions produced a clade that contains *G. abyssinica*, *G. scabra* ssp. *scabra*, *G. scabra* ssp. *schimperi* and *G. villosa*, which indicates their close phylogenetic relationship (Fig. 13C). These taxa are cross compatible and produce a viable and fertile hybrid (Dagne, 1994a). It is also interesting to note that these taxa possess identical sequences of the *mat*K coding region but differ at least at two of the four variable sites from the other taxa (Table 4). However, they appear to form two sub-clades when analyzed based on the combined data set of AFLP and RAPD (paper IV) and ITS data (paper V), in which *G. abyssinica* and *G. scabra* ssp. *schimperi* form one sub-cluster while *G. villosa* and *G. scabra* ssp. *scabra* form another (Fig. 13A, 13B). Although the bootstrap support at some branches of Figure 13A is low, a similar phenogram with high bootstrap support was generated when Unweighted Pair Group Method with Arithmetic mean (UPGMA) method was applied (see figure 2C of paper IV). This grouping is in agreement with the grouping based on karyotypes (Dagne, 1995) and meiotic behavior of their hybrids (Dagne, 1994a). The ITS data was different from the combined data of AFLP and RAPD in that it revealed two forms of *G. abyssinica* that fell into different sub-clades. Despite this difference, the two data sets agree in that at least one form of *G. abyssinica* is most closely related to *G. scabra* ssp. *schimperi* and that *G. scabra* ssp. *scabra* and *G. villosa* are closely related.

The lower genetic variation obtained in the cpDNA regions as compared to the ITS between *G. abyssinica*, *G. villosa* and the two subspecies of *G. scabra* is in line with the lower evolutionary rate of the chloroplast genome as compared to the nuclear genome (*e.g.* Small, Cronn & Wendel, 2004). Molecular marker techniques such as AFLP are useful tools to resolve phylogeny when DNA sequencing fails, as they sample a larger portion of the genome (Després *et al*., 2003). The general similarity of results obtained by AFLP and DNA sequence data in this study substantiates the use of AFLPs for phylogenetic inference at lower taxonomic levels as previously suggested (Després *et al*., 2003; Weising *et al*., 2005). Based on various evidences, several authors suggest that *G. abyssinica* might have originated from *G. scabra* ssp. *schimperi* through selection and cultivation (Baagøe, 1974; Hiremath & Murthy, 1988; Murthy, Hiremath & Salimath, 1993; Dagne, 1994a, 1995, 2001). Similarly, this study showed that *G. scabra* ssp. *schimperi* is the most likely progenitor of *G. abyssinica* although *G. scabra* ssp. *scabra* and *G. villosa* might also have some contribution successively albeit at various stages of evolution to the origin of *G. abyssinica*.

G. scabra ssp. scabra, G. scabra ssp. schimperi and G. villosa

The combined data set of AFLPs and RAPDs have shown that *G. scabra* ssp. *scabra* and *G. scabra* ssp. *schimperi* are more closely related to each other as compared to most of the pairs of taxa compared in this study. However, AFLP based analysis showed that *G. scabra* ssp. *scabra* vs. *G. villosa* are slightly more closely related to one another than *G. scabra* ssp. *scabra* vs. *G. scabra* ssp. *schimperi* (Paper IV). Sequence divergence analysis based on the cpDNA regions revealed a similar level of divergence between any pair of *G. scabra* ssp. *scabra*,

G. scabra ssp. *schimperi*, *G. abyssinica* and *G. villosa*. On the other hand, the ITS data placed these subspecies under different sub-clades (Fig. 13B). As a result, all sets of data generated in this study agree that the genetic similarity between *G. scabra* ssp. *scabra* and *G. scabra* ssp. *schimperi* is not more than the similarity revealed, for example, between *G. scabra* ssp. *scabra* and *G. villosa* and between *G. scabra* ssp. *schimperi* and *G. abyssinica*. The combined result of this study and the previously reported morphological and karyotypic differences (Hiremath & Murthy, 1992; Dagne, 1995) lead to the conclusion that *G. scabra* ssp. *scabra* and *G. scabra* ssp. *schimperi* are best viewed as separate species rather than as the subspecies of the same species. Based on morphological similarities, Baagøe (1974) suggested that *G. villosa* might have been derived from *G. scabra* (without mentioning the subspecies). The close similarity between these taxa was also reported by Dagne (1994a, 1995). The AFLP, RAPD and ITS data revealing that *G. villosa* is more closely related to *G. scabra* ssp. *scabra* than to *G. scabra* ssp. *schimperi* thus disagree with the speculation of Hiremath & Murthy (1992) that *G. villosa* has evolved from *G. scabra* ssp. *schimperi*.

Chelelu and Ketcha

The Chelelu and Ketcha populations were first reported to belong to the genus *Guizotia* based on their morphology and karyotypes (Dagne, 1995), which was further strengthened by the study of cross-compatibility with other guizotias and chromosome pairing of the hybrids (Dagne, 2001). In this study, there was no complete agreement between different sets of data regarding the degree of relatedness of these populations to each other and to the taxonomically recognized *Guizotia* taxa. For example, when the overall cpDNA regions and the combined data set of AFLP and RAPD were considered, Chelelu and Ketcha appeared to be more closely related to each other than to the other taxa (Fig. 13A); but was not supported when only the two intergenic spacers of cpDNA were considered (Fig. 13C). The grouping of Chelelu together with *G. abyssinica* and *G. scabra* ssp. *schimperi* and the grouping of Ketcha together with *G. villosa* and *G. scabra* ssp. *scabra* by Dagne (1995, 2001) were not supported by this study, except that the cluster analysis based on RAPD data groups Chelelu and *G. abyssinica* together. In spite of these differences, both Chelelu and Ketcha were clearly separated from the other taxa and from each other. The AFLP and RAPD based genetic distance between these populations and the other guizotias was equivalent to the genetic distance between the taxonomically recognized species. Additionally, the cpDNA sequence divergence between Chelelu and Ketcha was higher than the sequence divergence between any pair of taxa within the group of *G. abyssinica*, *G. scabra* ssp. *scabra*, *G. scabra* ssp. *schimperi* and *G. villosa.* Generally, the phenetic and phylogenetic analyses of cpDNA data and the data from the two marker techniques confirm that these populations certainly belong to the genus *Guizotia* and that they are distinct enough to be treated as separate species (Fig. 13). It will be of interest to see the relationship between *Sigesbeckia* species moved to the genus *Guizotia* by Schulz (1990) and the species excluded from the genus *Guizotia* by Baagøe (1974) as well as Chelelu and Ketcha in the revision of the genus by considering as many characters as possible.

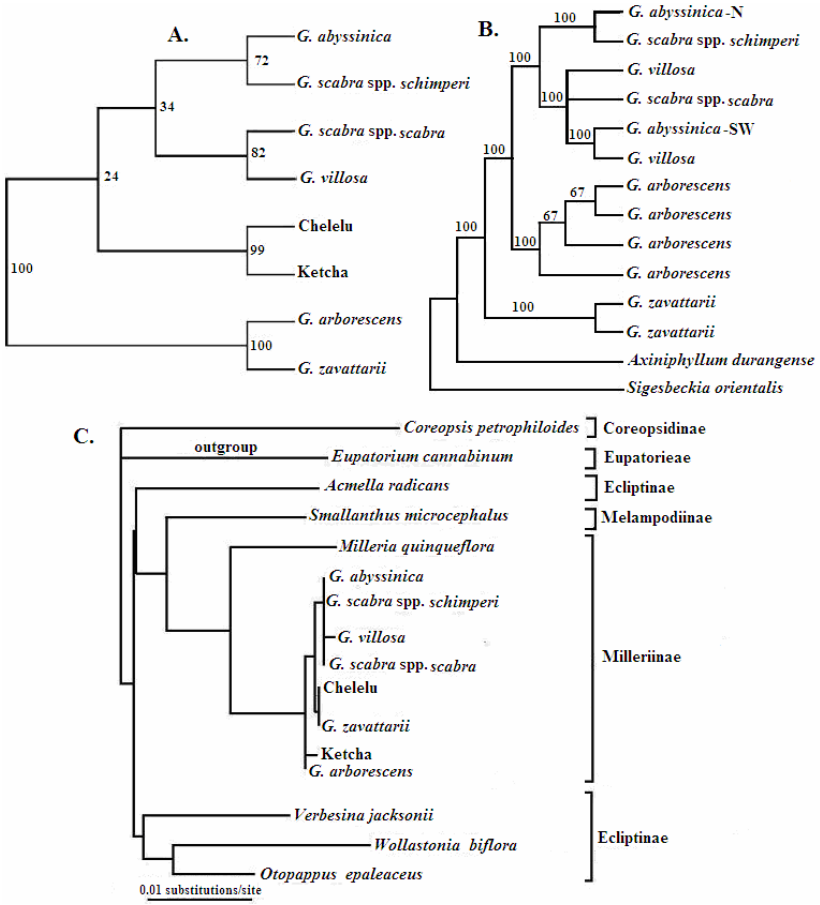

Figure 13. (A) Neighbor-joining tree from the combined data of AFLP and RAPD, (B) most parsimonious tree for the entire ITS region, and (C) neighbor-joining tree from the *trn*T/*trn*L and *trn*L/*trn*F intergenic spacers of cpDNA showing the phylogenetic relationship between guizotias and other closely related species.

G. arborescens and G. zavattarii

Dagne (1995, 2001), based on karyotype similarity and a high degree of chromosome pairing affinity in the F1 hybrid between *G. arborescens* and *G. zavattarii*, concluded that these species are more closely related to one another than to the other taxa. The degree of similarity between these species is relatively low when all data sets in this study are considered and thus do not support the previous grouping. Most putative taxon-specific markers generated both from AFLPs and RAPDs were specific to *G. arborescens* or *G. zavattarii* implying that these species are genetically distant from the other taxa and from each other. The phylogenetic analyses conducted in this study showed that *G. arborescens*, *G. zavattarii*, Ketcha and Chelelu evolved earlier than the rest of the taxa during the evolutionary history of the genus (Fig. 13A, 13C). Thus, Baagøe's (1974) suggestion regarding the derivation of *G. zavattarii* from *G. scabra* seems unlikely. The advancement versus primitiveness of morphological characters

suggested for *Guizotia* species by Baagøe (1974) is not in line with the results of phylogenetic analyses of this study.

Diagnostic markers

Twelve AFLP and four RAPD taxon specific markers were identified in this study. The identification of such taxon specific DNA markers in *Guizotia* has a potential use for conservation of these taxa and, most importantly, to improve *G. abyssinica*. In other words, these markers are potentially useful in *G. abyssinica* breeding, in species identification and determination of the degree of natural hybridization between *Guizotia* taxa that have overlapping geographic distribution. Such markers might be linked to desirable traits that are worth transferring to *G. abyssinica*, which needs to be one target of future studies. If the cross-incompatibility between *G. abyssinica* and *G. zavattarii* is postzygotic as suspected by Dagne (1994a), it can be overcome, for example, through ovule culture (*e.g*. Kumlehn & Nitzsche, 1996). Thus, *G. zavattarii* can be considered as a potential candidate as a source for some desirable traits, such as high OA seed oil (Dagne & Jonsson, 1997) to improve *G. abyssinica*. Conversion of such potential diagnostic markers to Sequence Characterized Amplified Region (SCAR) markers (Paran & Michelmore, 1993) improves the efficiency of their use as a molecular tool in marker assisted selection.

The position of *Guizotia* Cass. within Heliantheae (papers V &VI)

The genus *Guizotia* has been placed under different subtribes (Coreopsidinae, Verbesininae and Milleriinae) since 1873 (Bentham, 1873; Baagøe, 1974; Stuessy, 1977; Robinson, 1981; Karis, 1993). According to Stuessy's systematic review of Heliantheae (Stuessy, 1977), *Acmella radicans* (Jacq.) R. K. Jansen and *Verbesina jacksonii* B. L. Turner belong to subtribe Verbesininae, a subtribe that later was incorporated into Robinson's subtribe Ecliptinae (Robinson, 1981). *Coreopsis petrophiloides* belongs to the subtribe Coreopsidinae of the tribe Heliantheae, while *Eupatoria cannabinum* belongs to the tribe Eupatorieae (Stuessy, 1977; Robinson, 1981). The DNA sequence analysis in this study revealed that the nucleotide divergence between guizotias and *C. petrophiloides* was higher than the divergence between guizotias and *E. cannabinum*, which disagrees with the placement of the genus *Guizotia* under the subtribe Coreopsidinae. On the other hand, the ITS sequence of guizotias is more similar to various species of the subtribe Milleriinae (*e.g. Sigesbeckia* spp., *Axiniphyllum* spp. *Trigonospermum* spp.) than to species of other subtribes of the tribe Heliantheae (paper V). The phylogenetic analysis based on 32 parsimony informative characters from DNA sequence data of two intergenic spacers of cpDNA revealed that *Milleria quinqueflora* L. (subtribe Milleriinae; Fig. 13C) was the closest species to the genus *Guizotia*. The second closest species to the genus *Guizotia* was *Smallanthus microcephalus* (Hieron) H. Rob. Robinson (1981) placed *S. microcephalus* under the subtribe Melampodiinae. However, Panero *et al*. (1999) advised the transfer of this genus from this subtribe to the subtribe Milleriinae. Thus, this study strongly supports Robinson's (1981) placement of the genus *Guizotia* under the subtribe Milleriinae.

Conclusions

- *G. abyssinica* has a wide genetic basis that can be used for its improvement through breeding. The extent of niger genetic variation within populations is distributed regardless of the extent and altitude of cultivation, suggesting that all regions where niger is currently grown are equally important from a species conservation point of view. However, for the promotion of *in situ* conservation and enrichment of diversity through introgressive hybridization between both cultivated and wild forms some regions can be prioritized. Welo, where the highest within populations genetic variation was obtained and other regions such as parts of Jimma, Illubabor, Welega, Gojam and Tigray, where more than two *Guizotia* taxa overlap significantly, can be considered as priority regions.
- There is a significant "regional" differentiation between niger populations, and a significant positive correlation between genetic and geographic distance between populations.
- All wild and/or weedy guizotias possess a quite high level of genetic diversity.
- Higher genetic variation is found within populations than between populations in all guizotias, except in *G. zavattarii* that seems to have almost equal proportion. Significant population differentiation was obtained in all guizotias, with *G. zavattarii* exhibiting the highest differentiation.
- Chelelu and Ketcha acquired higher within-population genetic variation than the other guizotias while *G. arborescens* and *G. zavattarii* showed lower percentage of polymorphic loci.
- AFLP was superior to RAPD in various characteristics and thus has to be preferred to characterize accessions of niger held in gene bank. In comparison to RAPD, AFLP revealed genetic relationships between *Guizotia* taxa that is more inline with the DNA sequence data and the cytogenetic and hybridization studies and thus can be concluded that AFLPs give strong phylogenetic signals.
- *G. scabra* ssp. *schimperi* is the most likely progenitor of *G. abyssinica* although there are indications that *G. scabra* ssp. *scabra* and *G. villosa* might also have contributed successively at various stages to the evolution of *G. abyssinica*.
- The two subspecies of *G. scabra*, and Chelelu and Ketcha are best viewed at present as separate species of the genus *Guizotia*. However, a complete revision of the genus by considering those species taken into and out from the genus by different authors needs to be considered.
- Highly localized guizotias evolved first during the evolutionary history of the genus *Guizotia*.
- *Guizotia* Cass. belongs to the subtribe Milleriinae of tribe Heliantheae.

Recommendations and future prospects

G. abyssinica, just like many other domesticates (*e.g. Coffea arabica* L.), is Ethiopia's contribution to the world. Maintaining and promoting the genetic diversity of this crop and its wild relatives at their center of origin is highly valuable not only for Ethiopia but also for the rest of the world. This study assessed the level and pattern of genetic diversity in niger and its wild and/or weedy relatives and generated information of importance for conservation, breeding and utilization. In Ethiopia, niger germplasm collection has been conducted mainly in regions of its major cultivation and most of the accessions are from areas with better access to modern transportation and accordingly their representation of the existing genetic diversity of the crop within the country is questionable. The significant variation between populations and the regional differentiation of *G. abyssinica* populations obtained in this study has an important implication for its conservation and utilization. It indicates the need to conserve a large number of populations from all of its growing regions *ex situ* to prevent the loss of unique genetic variants. In other words, future germplasm collections should represent all growing regions by giving special emphasis to areas that are not yet represented or are underrepresented. In regions where niger production is not the priority, populations with unique genetic properties are at risk of being lost, as farmers may not continue growing them. Transferring representative samples of such populations to MaNPRs for *in situ* conservation, as a complementary approach to *ex situ* conservation, helps to maintain the existing genetic variation of the crop and increases the genetic variation within regions of major cultivation. Before characterizing gene bank collections at the molecular level, populations from remote and underrepresented areas should be collected first. This helps to increase the range of genetic variation among accessions and thus facilitates generation of a well represented core collection.

G. abyssinica has to be improved in order to make it a competitive oil crop worldwide. Niger breeding should focus primarily on increasing seed yield, as it is the major factor that hampers large-scale production and commercialization of this crop. Breeding for pest and disease resistance and oil content and quality is also of interest. The existing wide genetic basis in Ethiopian niger suggests that achieving these objectives may well be possible both through conventional breeding and marker assisted selection. Additionally, the considerable level of genetic variation in wild and/or weedy guizotias, especially in those that are cross-compatible with niger is a valuable resource to improve niger through interspecific hybridization in order to transfer desirable genes that may exist in these species into *G. abyssinica*. The diagnostic markers revealed in this study are useful in this regard. A loss of yield through shattering, for example, can be minimized by selecting non-shattering genotypes with determinate growth habit, provided that genes responsible for these traits exist in niger or in its wild and/or weedy relatives. Seed yield can be improved through stepwise selection of large seed size, a higher number of seeds/capitula, capitula/plant and branches/plant, as high variation in these traits exists in the Ethiopian niger gene pool. Problems associated to lodging can be overcome by looking for genotypes with short stature and strong stem.

Niger seed is well known for its high linoleic acid oil. Oleic acid accounts, commonly, for 5-13% of niger seed oil. However, there are indications that high OA (up to 40%) niger genotypes exist in the Ethiopian gene pool, which is a manifestation of the existing genetic diversity. OA has a higher oxidative stability than LA (Wanasundara & Shahidi, 1994), which makes high OA niger oil preferable to some applications in food industry. Therefore, developing niger varieties with a higher percentage of OA should be one direction of breeding in niger. The molecular marker techniques used in this study revealed high genetic polymorphism in guizotias. These and other molecular techniques could be extended to mapping and linkage analysis in *G. abyssinica*, as molecular markers linked to desirable traits are useful tools for the early generation selection of genotypes with desirable traits in niger breeding programs. Generally, in view of the existing genetic diversity, the potential for improving niger through breeding is promising.

G. zavattarii and *G. arborescens* are characterized by a highly localized geographic distribution, small population sizes, lower percentage of polymorphic loci and higher population differentiation in comparison with other taxonomically recognized guizotias. The phylogenetic evidence showed that they evolved earlier than most of the other guizotias. These conditions and the continuously increasing human activities in their natural habitats are signals of urgency to start their conservation immediately. Only single populations of Chelelu and Ketcha are known so far, which may indicate their localized distribution. It is of great interest to explore their geographic distribution and unravel the possible reasons behind their high genetic variation.

The cpDNA sequence data did not resolve the phylogenetic relationship between *G. abyssinica*, *G. villosa* and the two subspecies of *G. scabra*. It might be worthy to study more variable cpDNA regions and low-copy nuclear genes to resolve the phylogeny of this group and to further assess the results obtained based on the ITS data.

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