Morphology, ploidy and molecular phylogenetics reveal a new diploid species from Africa in the baobab genus *Adansonia* (Malvaceae: Bombacoideae)

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Abstract The genus *Adansonia* has a disjunct geographical distribution: six species are endemic in Madagascar, one in Africa, and one in Australia. The well-known African baobab (*Adansonia digitata*) is an iconic tree with considerable ethnobotanical significance. In contrast to the other seven species, which are diploid, *A. digitata* is tetraploid. A common ancestor of *A. digitata* and the other diploid baobab species would be diploid; however, there are no diploid species recorded on the African mainland. Examining variation in floral and pollen characters and chromosome number in specimens from Africa identified a new diploid baobab species, *Adansonia kilima* sp. nov., which co-exists with *A. digitata* in Africa. *Adansonia kilima* is restricted to moderate elevations (650–1500 m), in contrast to *A. digitata*, which is widespread throughout Africa but prefers elevations below 800 m. *Adansonia kilima* is superficially similar to *A. digitata*, but can be differentiated on the basis of floral morphology, pollen, and chromosome number. We used two chloroplast DNA markers and the nuclear ITS to examine phylogenetic relationships within *Adansonia*. Three lineages were observed: one containing the Malagasy species, one containing the Australian species, and one containing the African species. The relationships between these clades were difficult to resolve, but a link between the African and Australian clades emerged when the analysis used fewer replicate samples of individual Malagasy taxa, included indel characters and included fewer outgroup taxa. The ITS phylogeny demonstrated that *A. digitata* and *A. kilima* are genetically similar, suggesting that tetraploidy evolved relatively recently.

Keywords *Adansonia kilima*; Africa; baobab; diploid; molecular phylogeny

Supplementary Material Figures S1–S7 are available in the Electronic Supplement to the online version of this article (http://www.ingentaconnect.com/content/iapt/tax).

INTRODUCTION

The African baobab tree, *Adansonia digitata* L., sometimes known as the Tree of Life, has myriad uses in many different cultures, supplying a wide variety of foodstuffs, as well as water, fibers for weaving and rope-making, vessels, artistic materials, etc. (Wickens, 1982; Gebauer & al., 2002; Sidibe & Williams, 2002; Wickens & Lowe, 2008; De Caluwé & al., 2009). Baobabs also supply raw materials for hunting and fishing equipment, for making musical instruments, and as a source of many herbal remedies. Hollow baobabs are used for shelter and to store water; also as prisons, burial sites, stables, storage rooms, watchtowers, etc. Virtually all parts of the plant can be used, and the tree is indelibly implanted in the folk traditions and mythology of many cultures. Their iconic shape, extraordinary longevity (individual trees can live for well over thousand years; Adanson, 1761; Swart, 1963; Patrut & al., 2007), economic importance, ethnobotanical significance, and intriguing natural history have captured popular imagination for many centuries. They were known to the Ancient Egyptians and have been mentioned in travel accounts with relative frequency since the 1300s (Wickens, 1982; Baum, 1995; Blench, 2007).

Michel Adanson described the baobab in 1761 (Adanson, 1761) and Linneaus memorialized him as the tree's namesake (Linneaus, 1762). Darwin documented baobab trees on St Jago in the Cape Verde Islands in 1832, commenting on their size and longevity (Armstrong, 2004). Livingstone mentions baobab trees several times during his account of his travels in southern Africa during the 1840s and 1850s (Livingstone, 1861).

The genus *Adansonia* consists of eight known species: *A. digitata*, which is native to mainland Africa but which has been widely distributed throughout the tropics by humans;

A. gregorii F. Muell., endemic to the Kimberley region of north-western Australia; and six species (*A. grandidieri* Baill., *A. suarezensis* H. Perrier, *A. rubrostipa* Jum. & H. Perrier, *A. za* Baill., *A. madagascariensis* Baill., *A. perrieri* Capuron) which are endemic to Madagascar (Baum, 1995; Brummitt, 2004; Wickens & Lowe, 2008). Baobabs are divided into three sections according to floral morphology: *A.* sect. *Adansonia* (*A. digitata* L.), sect. *Brevitubae* Hochreutiner (two Malagasy species: *A. grandidieri*, *A. suarezensis*) and sect. *Longitubae* Hochreutiner (five species: the remaining four Malagasy species, and the Australian species *A. gregorii*) (Hochreutiner, 1908; Baum, 1995; Brummitt, 2004).

The curious biogeography of *Adansonia* has caused much speculation (Armstrong, 1983; Wickens & Lowe, 2008). The disjunct distribution—in particular, the presence of *A. gregorii* in Australia, so far from the other species—is unusual. It was originally presumed that the genus represents a relict of the Gondwanan land mass; however, genetic analyses have demonstrated the genus probably arose only 10 million years ago (Ma), long after Gondwana fragmented (Baum & al., 1998). Long-distance dispersal (via humans and transoceanic currents; Armstrong, 1977; Wickens, 1982; Armstrong, 1983; Bowman, 1997; Baum & al., 1998; Blench, 2007) is now thought to be responsible for the modern distribution.

The African species, *A. digitata*, presents an interesting problem concerning *Adansonia* origins because it is tetraploid—unlike the other seven species, all of which are diploid (Baum & Oginuma, 1994). Africa could be the centre of origin for *Adansonia*, following direct or indirect dispersal of the progenitor from the New World, where the remainder of subfamily Bombacoideae (with a small number of exceptions) is found (Baum & al., 2004; Duarte & al., 2011). In this case, ancestors of *A. digitata* living in Africa must have been diploid, with tetraploidy evolving only after the divergence of the Australian and Malagasy lineages. Alternatively, *A. digitata* may have had a diploid progenitor that had its origins outside of Africa (e.g., in Madagascar, which is the centre of diversity), with tetraploidy evolving during or after dispersal to Africa. This hypothesis is shown in Fig. 1, and raised the possibility that a diploid representing the progenitor of the tetraploid is still extant in Africa. A recent field search in Africa has revealed a new diploid species that might represent the postulated diploid progenitor line, thus providing empirical evidence in support of this hypothesis. In the present paper we describe this new species, *A. kilima*, and examine the phylogenetic relationships within *Adansonia* in light of the new species.

■ MATERIALS AND METHODS

Sampling. — Leaves, flowers, and seeds were collected from baobab trees in a number of locations in Africa. The survey region ranged from the eastern slopes of Mt. Kilimanjaro (Tsavo West National Park) in Kenya to the south Kenyan coast, the central plateau of Tanzania and Zambia, west to Botswana and Namibia and south to the limits of baobab distribution in the Limpopo region of South Africa. A few samples from Senegal in West Africa were also provided by Alain Coache (OCIS). Leaf material from the Australian species *A. gregorii* was collected from trees growing wild in the remote Kimberley region of Western Australia. Leaf material from Malagasy species was kindly provided by Pascal Danthu (CIRAD, Madagascar) and Christian Kull and Haripriya Rangan (Monash University, Australia).

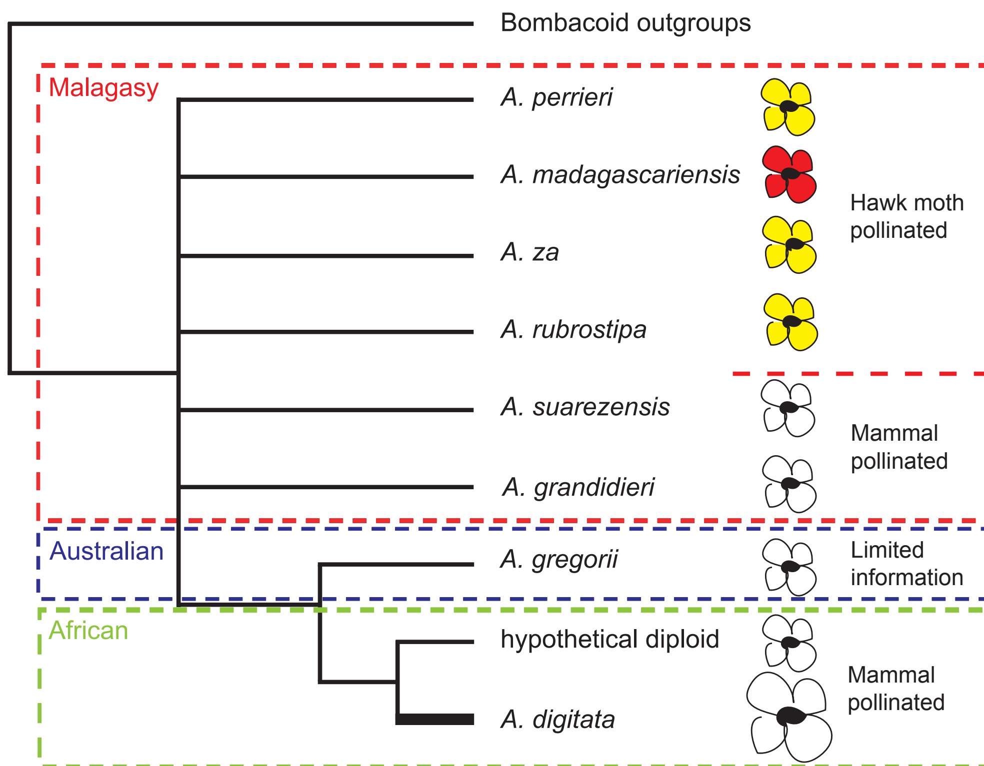


Fig. 1. A hypothetical phylogeny that guided the search for an African diploid baobab. The tetraploid lineage is represented by a thicker line. Flower colour and size, and pollinators are noted for each species. The possibility of an African diploid was raised by the fact that all extant baobab species are diploid, with the exception of the very successful, widespread, African tetraploid *A. digitata*. In the hypothetical phylogeny shown, tetraploidy is derived, as it is in most plants, and has evolved at the crown of the phylogeny from an unknown diploid progenitor. Since there is no evidence of any tetraploid baobab in Madagascar or Australia, a scenario involving the evolution of a tetraploid baobab in Africa from an African diploid progenitor may be expected.

DNA sequencing. — Leaves were dehydrated by adding them to an excess (20 : 1) volume of silica-gel granules and storing in a sealed bag. DNA was isolated using the DNeasy Plant Kit (Qiagen, Doncaster, Victoria, Australia), with the following modifications: approximately 10 mg of polyvinylpyrrolidone was added to each sample prior to grinding in a mortar and pestle; the volume of buffer AP1 was increased to 650 µl; the volume of buffer AP2 was increased to 195 µl; after precipitation on ice the samples were centrifuged at 20,000 *g* at 4°C for 10 min; DNA was eluted from the spin columns three times, each with 100 µl of buffer AE.

Two chloroplast spacers (*psbA-trnH*, *trnL-trnF*) and the nuclear ribosomal DNA region including 5.8S rRNA and the internal transcribed spacers ITS-1 and ITS-2 were amplified using the primers of Kress & al. (2005), Taberlet & al. (1991) and Baum & al. (1998), respectively. The PCR reactions contained 1× Qiagen PCR buffer (1.5 mM $MgCl_2$), 8 pmol of each primer, and approximately 0.5–2.0 ng template DNA, in a total volume of 20 µl. Typical PCR cycles for all loci consisted of an initial denaturation at 95°C for 15 min, followed by 35 cycles of 94°C for 30 s, 59°C for 30 s, and 72°C for 1 min, followed by a final extension at 72°C for 5 min. PCR products were sequenced by Macrogen Inc. (Seoul, South Korea) or AGRF (Brisbane, Australia), using Applied Biosystems Big-Dye sequencing chemistry. DNA sequences were edited, and forward and reverse strands aligned in Geneious Pro v.5.3.6 (Drummond & al., 2011). Outgroup sequences were obtained via GenBank, and included the most closely related species, according to previously published phylogenetic studies, for which the three loci of interest were available (Kress & al., 2009; Duarte & al., 2011).

Phylogenetic analysis. — Sequences used in the analysis are shown in the Appendix. Analyses were carried out using all sequences for which voucher specimens were available. Further analyses were carried out with a more complete dataset, including at least one representative of all species in the genus, but for which not all sequences had voucher specimens. Multiple sequence alignments were carried out using MUSCLE v.3.8, through the Geneious interface, with eight iterations. Regions where the alignment was ambiguous were removed, indels of more than one nucleotide were re-coded as a single character, and then analyses were run with gaps treated as a new character state, and with gaps treated as unknown. Parsimony analyses were carried out in PAUP* v.4.0β10 (Swofford, 2003). For each DNA sequence region, a heuristic search with 1000 replicates, random addition of taxa, and tree bisection reconnection (TBR) branch swapping was used. Bootstrap analyses were carried out with 1000 bootstrap replicates, each with 100 heuristic search replicates, with random addition of taxa and TBR branch swapping. Uncertainty about the topology of the basal Australian, African and Malagasy lineages was reduced by treating gaps as a new character state (as described above), reducing the number of sequences in the large Malagasy clade, and including different outgroup taxa. We continued with *Cavanillesia platanifolia* (Bonpl.) Kunth as outgroup, as this gave the lowest number of equally most parsimonious trees, all of which were consistent in their resolution of the basal node, and did not conflict with consensus trees derived using other outgroup taxa.

Bayesian analysis was carried out using the MrBayes (Huelsenbeck & Ronquist, 2001; Ronquist & Huelsenbeck, 2003) plugin v.2.0.3 for Geneious. The optimal models of sequence evolution for each gene region were determined with the Akaike information criterion (AIC) using Modeltest, through the Geneious interface. Using the selected model, two simultaneous searches were performed, each with four MCMC chains (one cold and three hot), with a sample frequency of 1000. Every 1,000,000 generations, the standard deviation of split frequencies between the two simultaneous analyses was calculated and when this decreased to less than 0.01 the search was stopped. This process was repeated with two further simultaneous runs of the MCMC, and the results were compared to confirm that apparent convergence of the first two runs was not due to both reaching the same local optimum. Plots of the likelihoods of sampled trees were examined to determine when the MCMC chains had reached stationarity, and the sampled trees prior to this were discarded as burn-in. A majority-rule consensus tree was obtained from the remaining trees.

Morphological analyses. — Stomatal dimensions were determined by using a small, portable field microscope (Pyser Evolution, Pyser-SGI Ltd, Edenbridge, U.K.). Clear nail varnish lifts of the adaxial leaf surface were examined under ×600 magnification. Images were captured through the eyepiece using an iPhone (Apple) and stomatal aperture measured with calipers at the maximum magnification on the iPhone screen. Trials on diploid (*A. gregorii*, *A. madagascariensis*) and tetraploid (*A. digitata*) baobabs from the Brisbane Botanical Gardens (Mt. Coot-tha, Brisbane) demonstrated the utility of this method for differentiating between diploid and tetraploid species. Mean stomatal length was measured and stomatal density was calculated for leaves sampled in Africa. Trees were assigned to either diploid *A. kilima* or tetraploid *A. digitata* based on size of stomatal aperture. Pollen measurements using the portable microscope were available in many cases and were used to support the diagnosis because of the much larger pollen size of the tetraploid (see below). Measurements of androecium length and stalk diameter, maximum calyx diameter, and staminal corolla width were taken using a ruler. The number of free staminal filaments was counted.

Pollen was collected onto double-sided adhesive tape that was attached to a microscope slide. Field measurements of pollen were carried out using the portable field microscope at ×400 magnification. Images were captured and grains measured on the iPhone screen as described above. Collected pollen was coated with platinum and imaged with a JEOL 6300F, Field Emission Scanning Electron Microscope (SEM) at 6 kV. Pollen grain diameter was measured and pollen spines were counted. Pollen volume and pollen spine density were calculated from these data.

Chromosome number. — Seeds were soaked in concentrated hydrochloric acid for 24 hr to break dormancy and then germinated in commercial seed raising mix (Searles, Kilcoy, Australia) until the cotyledons were expanded. Seedlings were then removed from the soil, rinsed, and placed in test-tubes filled with aqueous 0.1% colchicine. Tubes were wrapped in aluminium foil and incubated at 4°C for 10 hr. Root tips and

apical meristems were finely macerated and stained with propionic acid-ethanol-carmine using the method of Snow (1963). Chromosomes were counted directly using the field microscope with a ×100 objective.

RESULTS

Morphological differentiation between the diploid and the tetraploid. — We performed a survey of baobabs from several different locations in Africa and were able to identify two stomatal classes (Fig. S1). The class with a larger stomatal aperture and lower stomatal density (38.3 μm and 1.6 per 100 μm^2, respectively) had a chromosome count of $2n = 160$ (Table 1). This is in agreement with previous findings for tetraploid *A. digitata* (Baum & Oginuma, 1994). Numerous trees were found with a smaller stomatal aperture (mean length = 26.1 μm), higher stomatal density (5 per 100 μm^2) and much

Table 1. Morphological measurements and chromosome numbers for *A. kilima* and *A. digitata*. Numbers are averages or ranges from n = 6 trees per species (*A. kilima* samples from the Central Highlands of Tanzania; five *A. digitata* samples from the coastal region around Mombasa, Kenya and one from Brisbane Botanic Gardens) for all measurements except free staminal filaments (n = 4). Where averages are used, errors are given as standard deviation (S.D.).

Characteristic	*A. kilima* (diploid)	*A. digitata* (tetraploid)
Diameter of staminal corolla [mm]	38–42	95–106
Number of free staminal filaments	270–640	700–1600
Pollen spine density [per 1000 μm^2]	142–158	51–100
Pollen grain diameter [μm]	43.7 ± 3.4 S.D.	63.4 ± 7.7 S.D
Pollen volume [$\mu m^3 \times 10^3$]	42.8–44.6	118.8–133.0
Mean stomatal length [μm]	26.1 ± 5.7 S.D.	38.3 ± 4.5 S.D.
Stomatal density [per 100 μm]	5.0 ± 2.1 S.D.	1.6 ± 0.7 S.D.
Chromosome number ($2n$)	88	160

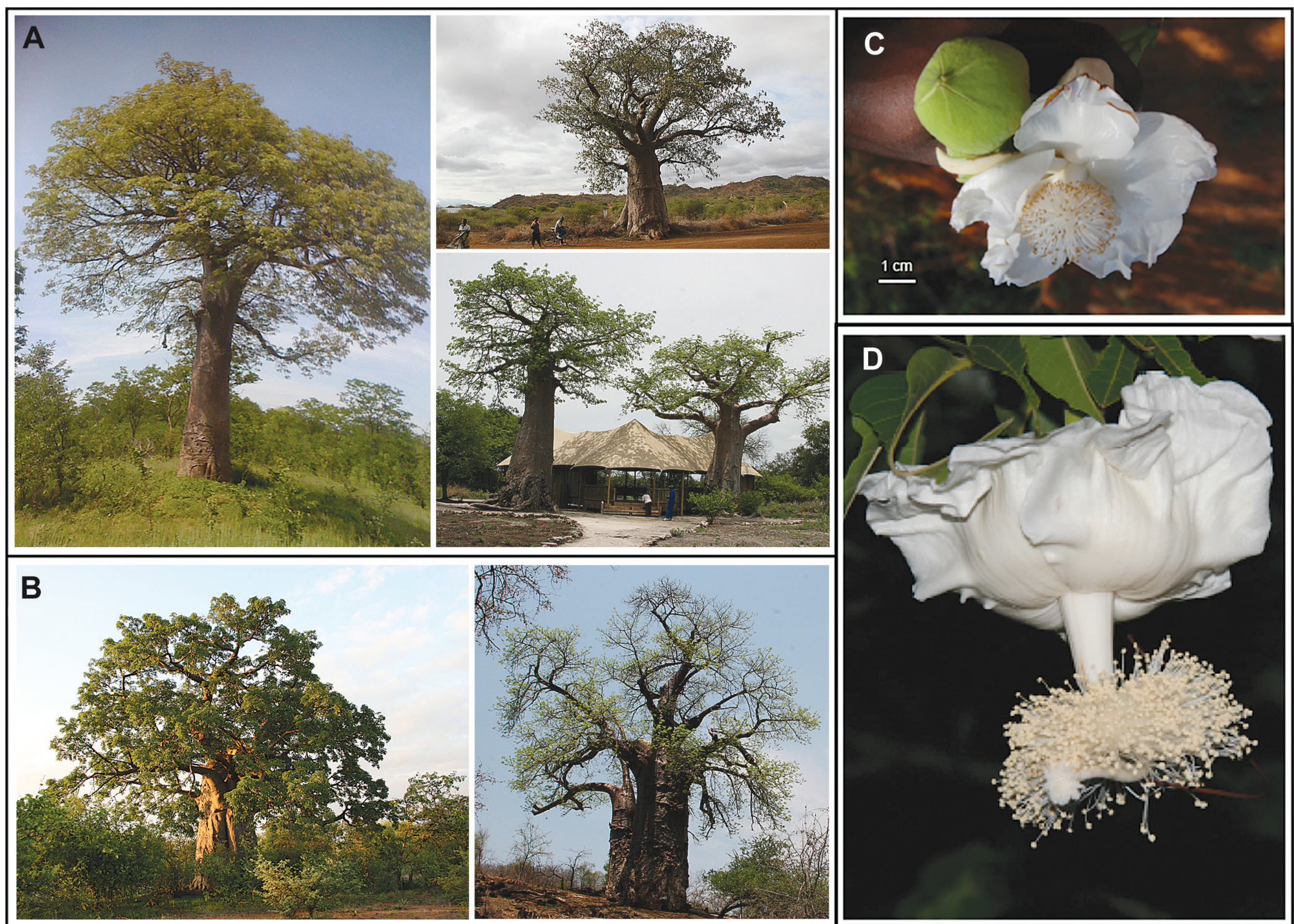


Fig. 2. Trees and flowers of the diploid *Adansonia kilima* and tetraploid *A. digitata*. **A,** *A. kilima*; locations are, clockwise from left to right, Botswana, Tanzania near Kondoa, and the highlands of north-eastern Botswana at 1000 m. **B,** *A. digitata*; both trees are from Limpopo, R.S.A.; the first is at 650 m and the second is at 400 m. **C,** *A. kilima* flower. **D,** *A. digitata* flower. Particular defining features between *A. kilima* and *A. digitata* include the size of the flowers, the length of the pistil, the number of anthers and the position of the petals. *Adansonia kilima* has shorter petals than *A. digitata*; anthers are consequently exposed beyond the petals. In *A. digitata*, the petals are strongly reflexed (see Figs. S4–S5). This difference in the relative length and posture of the petals makes it easy to distinguish from a distance the smaller, partly closed calyx and partially exposed anthers of *A. kilima* from the larger calyx with fully exposed anthers and effaced petals of open *A. digitata* flowers.

smaller pollen diameter (44 µm vs. 63 µm; Table 1). These trees had 88 chromosomes, the same as reported for the other diploid *Adansonia* species (Baum & Oginuma, 1994). We named this diploid species *A. kilima* (see below for species description). In our limited survey, *A. kilima* was found from the eastern slopes of Mt. Kilimanjaro, south to southern Tanzania and west to northern Namibia, as well as in the Venda of South Africa (Fig. S2). Although *A. kilima* and *A. digitata* were sometimes found in the same area, overlap was limited because the diploid was restricted to elevations between 650 and 1500 m, while the tetraploid was rarely found above 800 m (Fig. S3).

Mature trees of *A. kilima* and *A. digitata* look similar (Fig. 2A–B). However, *A. kilima* can be easily distinguished from *A. digitata* at a gross morphological level based on floral characteristics (Fig. 2C–D; Fig. S4; Table 1). Flowers of *A. kilima* are superficially similar to flowers of *A. digitata*: both have pendent buds, flowers and fruits. Flowers of *A. kilima* are smaller (about half the size of *A. digitata*), and often found in large numbers per tree compared with the two or three flowers that *A. digitata* typically presents nightly. The flowers of *A. kilima* have short petals in comparison with the length of the androecium, whereas petals of *A. digitata* flowers are longer than the androecium, and are reflexed in mature flowers (Fig. 2C–D; Figs. S4–S5). *Adansonia digitata* flowers have a remarkable kinesis which is absent in *A. kilima* flowers: the petals evert rapidly at night time to reveal the androecium, which is initially hidden by the long petals (Figs. S4–S5). Flowers of *A. digitata* also have roughly twice as many free stamen filaments as those of *A. kilima* (Table 1). Fruit, buds and leaves of *A. kilima* tend to be smaller than those of *A. digitata*, but dimensions overlap too much for these features to be diagnostic.

If a tree is not in leaf or in flower, the dried flowers underneath can yield pollen that can be used for microscopic identification (the size of dried flowers themselves cannot be used as a reliable distinguishing character because the shrinkage of drying is too variable). The pollen grains are again superficially similar, being globose with broad, short, irregularly sized spines (Fig. 3). However, they are clearly distinguishable by size in the field and by spine density in the laboratory: *A. kilima* pollen grains are two-thirds of the diameter of those of *A. digitata*, and show a higher density of spines (Table 1; Fig. 3; Fig. S6). The smaller size of *A. kilima* pollen resembles other diploid *Adansonia* species (Fig. 3; Fig. S6).

Phylogenetic analysis. — We obtained new *psbA-trnH* spacer sequences for 14 individuals and *trnL-trnF* spacer sequences for 24 individuals, of which four had herbarium vouchers; in addition, we obtained the ITS region for 18 individuals, of which three had vouchers. Sequences were also retrieved from GenBank for inclusion in the analysis. When combined with outgroup sequences, and with the indels recoded as described in Materials and Methods, this gave a total aligned DNA sequence length of 1538, of which 186 characters were variable, and 88 of these were parsimony-informative. The strict consensus trees of the most parsimonious trees (MPTs) for each gene region, using only those samples with vouchers, are shown in Fig. 4A–B, and the majority-rule consensus trees

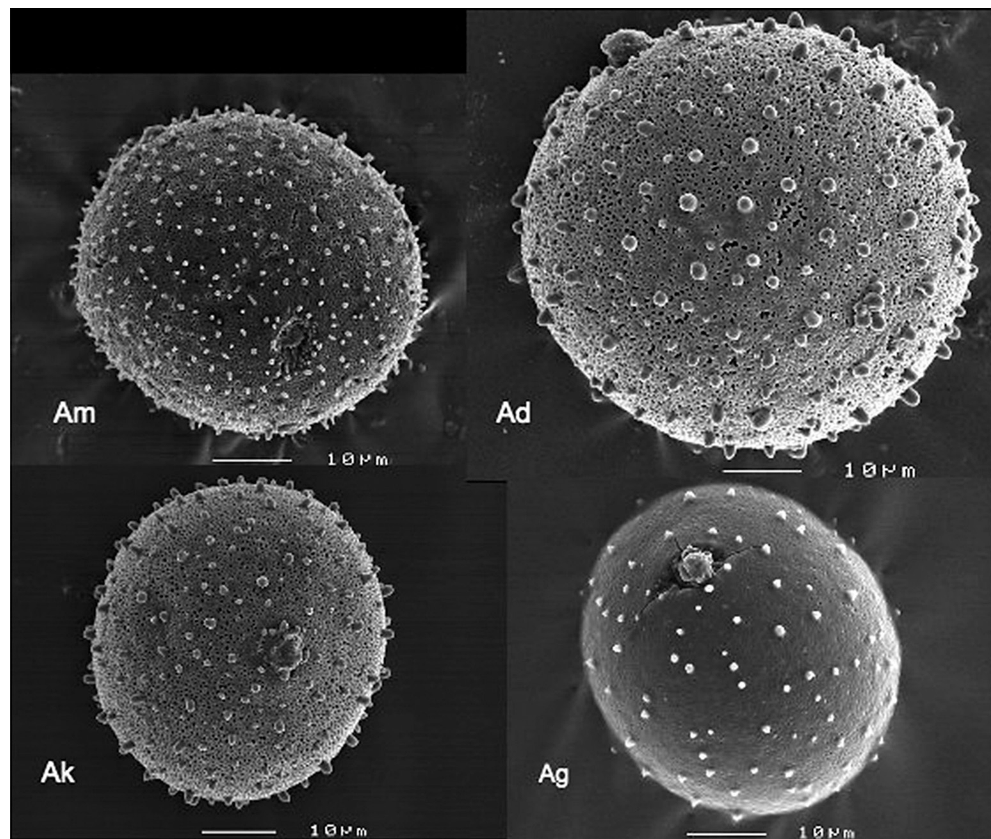
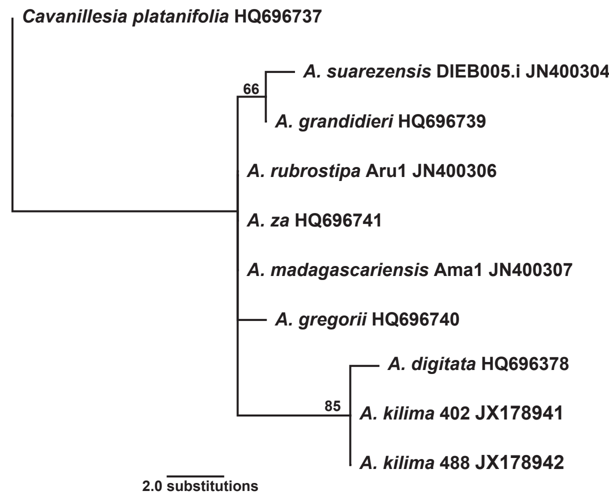


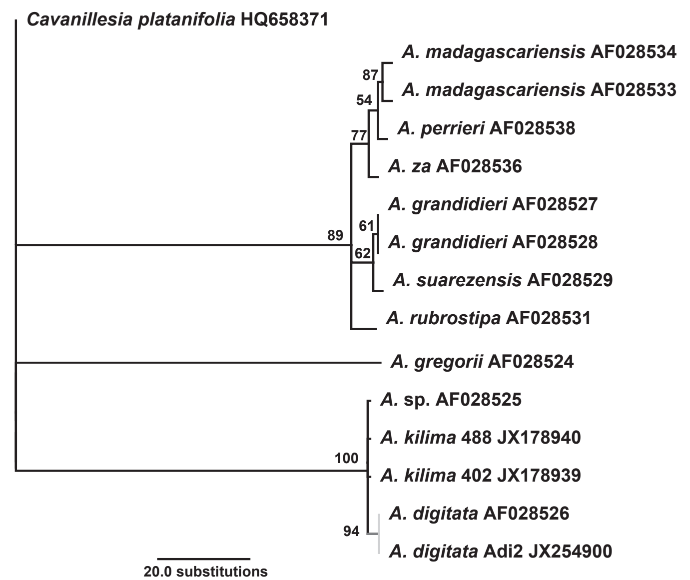
Fig. 3. Pollen from diploid and tetraploid species of *Adansonia*. Pollen from tetraploid *A. digitata* **(Ad)** has twice the volume of pollen from diploid *A. kilima* **(Ak)** (Table 1), making identification straightforward using a field microscope. This, and the lower density of spines, differentiates it from *A. kilima* pollen. *Adansonia digitata* spines are also larger and stouter than *A. kilima* spines. Pollen from *A. madagascariensis* (Am) has much greater spine density than that of *A. kilima*, while pollen from *A. gregorii* (Ag) has a much lower spine density. Quantitative analysis of pollen diameter and spines on the pollen face (Fig. S6) clearly distinguishes all four species. There are also some similarities between the pollen of the African tetraploid (Ad) and the African diploid *A. kilima* (Ak), such as the larger perforations in the exine and broad spines, but note that these species are clearly separated by pollen size and spine number.

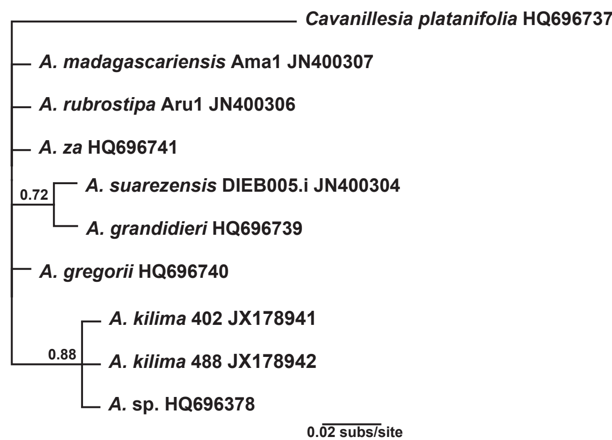
 Version of Record (identical to print version).

of the trees sampled through Bayesian analysis are shown in Fig. 4C–D. Trees derived following the same methods, with the larger dataset, including those samples for which vouchers were not obtained, are shown in Fig. S7.

The chloroplast (*psbA-trnH*, *trnL-trnF*) phylogenies were less informative than the nuclear (ITS) phylogeny. The *trnL-trnF* tree (Fig. 4A, C) suggested that more than one species might be present within the African clade, but the relationships between the remaining taxa were unresolved. When extra samples (without vouchers) were added (Fig. S7B, E), the analysis gave some support for the sister-species relationship of *A. perrieri* and *A. za*, and the sister-species relationship of *A. grandidieri* and *A. suarezensis* (i.e., *Adansonia* sect. *Brevitubae*). Insufficient voucher specimens were available for full







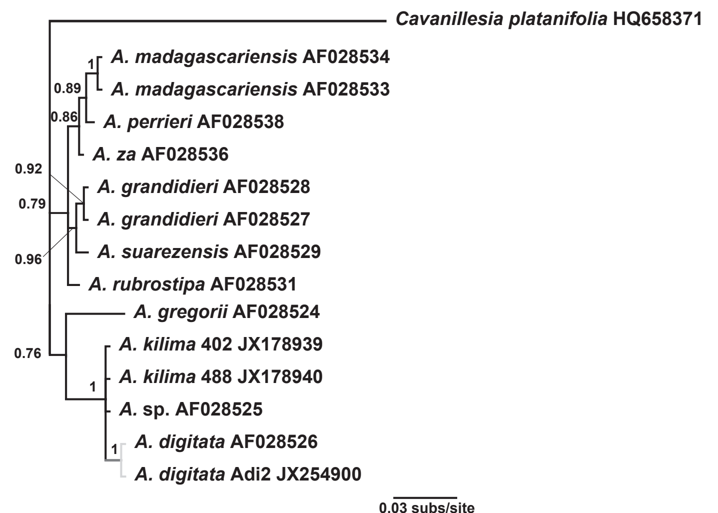


Fig. 4. Strict consensus of most parsimonious trees based on *trnL-trnF* **(A)** and ITS **(B)** sequences; Majority rule consensus of trees sampled post-burnin during Bayesian analysis of *trnL-trnF* **(C)** and ITS **(D)** sequences. Bayesian posterior probabilities are shown at all nodes supported by more than 0.5. Bootstrap values or Bayesian posterior probabilities are shown above branches, and branch lengths are scaled to number of mutations. Sequences from different taxa are identified by a sample number as well as a GenBank accession number (e.g., *A. kilima* 326 JN400288; 326 is the sample number) where they were obtained as part of this research, or by a GenBank number only where they were sourced from GenBank. Ploidy level is mapped onto the ITS phylogeny, with black being diploid, dark grey being equivocal, and light grey being tetraploid. The strict consensus of the most parsimonious trees for the ITS sequence **(B)** and the tree obtained by Bayesian analysis **(D)** render all three extant areas (Africa, Madagascar and Australia) equally parsimonious as the centre of origin of *Adansonia*.

analysis of the *psbA-trnH* spacer sequence; when unvouchered specimens were included (Fig. S7A, D) the trees were mostly unresolved, with low bootstrap support and Bayesian posterior probability, though there was some support for the sister-species relationship of *A. rubrostipa* and *A. madagascariensis* (BS = 59%; PP = 93%) and the monophyly of *A. gregorii* (BS = 64%; PP = 93%). While the topologies of the phylogenies of the two chloroplast regions differ from each other and from that derived by Baum & al. (1998) using the chloroplast *rpl16* gene, there are no strongly supported clades that are in conflict between the topologies. The lack of resolution in the chloroplast DNA phylogenies of both studies, and the differences in topologies, probably reflect the low number of parsimony-informative characters.

The ITS region showed better support for clade separations, although the number of equally most parsimonious trees varied depending on the treatment of indels, and the number of taxa included in the ingroup and outgroup. The number of equally most parsimonious trees could be reduced from 30 to 12 by including indel characters, retaining similar numbers of sequences in each of the three major clades, and using *Cavanillesia platanifolia* as outgroup (Fig. 4B, D; Fig. S7C, F). The vouchered samples of *A. digitata* form a monophyletic clade within a paraphyletic *A. kilima* under parsimony and Bayesian analysis (Fig. 4B, D). The ITS data from both our study and that of Baum &. al. (1998) show strong phylogenetic support for the monophyly of the African lineage and, when multiple samples are included (Fig. S7C, F), the monophyly of *A. gregorii*. Within the Malagasy lineage, there was support for the sister-species relationship of *A. grandidieri* and *A. suarezensis* (i.e., *Adansonia* sect. *Brevitubae*) and for the monophyly of those species with more than one individual included. Both analyses inferred the Australian lineage to be sister to the African lineage, with the Malagasy lineage sister to this clade. The sister-group relationship between the African and Australian clades is consistent with the analysis of Baum & al. (1998), based on a combined dataset of chloroplast DNA, nuclear DNA and morphology, but differs from their analysis of ITS only, which places *A. gregorii* as sister to the remaining *Adansonia* species. It is worth noting that the basal nodes had relatively low bootstrap support (less than 70) in both our study and that of Baum & al. (1998), and low Bayesian posterior probability in our analysis. To obtain a well-supported resolution of this polytomy would require more data, including the addition of more closely related outgroup taxa.

In the *trnL-trnF* analysis, the single vouchered sample of *A. digitata* differed from those identified as *A. kilima* by one nucleotide substitution. In the ITS analysis, the two vouchered samples differed from *A. kilima* by three nucleotide substitutions. Expanding the dataset to include several unvouchered *A. digitata* and *A. kilima* samples (Fig. S7C, F) it becomes clear that *A. digitata* is a monophyletic clade within a paraphyletic *A. kilima*. These relationships could not be resolved with chloroplast DNA. Three ITS sequences available in the literature and originally denoted as *A. digitata* were included in the ITS analysis: GenBank samples AF028525 and AF028526 (Baum & al., 1998) and AF460193 (Shi & Yuan, unpub.). AF028525 and AF460193 are labeled as *Adansonia* sp. in Fig. 4B and D, since they were collected prior to recognition of the diploid species. AF028526 was sampled from a tree in Burkina-Faso; its geographic location (hot, coastal) indicates that it is most likely *A. digitata*. Furthermore, the seed batch which provided this DNA sample was also used for chromosome counts in a subsequent study by Baum & Oginuma (1994; D. Baum, pers. comm.); the chromosome number indicates it is indeed tetraploid. This supports its position on the ITS tree, which places it with the *A. digitata* accessions. AF028525 was sampled from a cultivated tree in Iowa State University (seed source unknown); its placement in the ITS phylogeny suggests that it is in fact *A. kilima*. The accession of AF460193 is unknown, as there is no publication associated with this GenBank sequence. Its placement in the phylogeny suggests that it is *A. digitata.* If we consider *Adansonia* sp. AF028525 to be *A. kilima*, the phylogenetic analysis suggests that the tetraploid is monophyletic within a paraphyletic diploid, consistent with the two groups being separate species. Only a small number of substitutions separate them, approximately an order of magnitude less than the divergence between these two species and *A. gregorii.*

■ DISCUSSION

Ploidy level and morphology differentiate two African species. — Polyploidization is known to have several direct effects on plant morphology. Increases in the size of cells and organs (e.g., flowers, stomata, pollen, seeds) relative to diploids is common (Stebbins, 1971; Ramsey & Schemske, 2002). In particular, increasing ploidy level often results in decreased numbers of stomata and increased guard cell size (Evans, 1955; Bingham, 1968; Mishra, 1997; Aryavand & al., 2003; Chen & al., 2009) and these traits can sometimes be used to infer ploidy. We used this approach to successfully differentiate between diploid *A. kilima* and tetraploid *A. digitata* in the field, with the diagnosis confirmed by chromosome counts (Table 1). Further analysis revealed several morphological differences (Table 1; Figs. 2–3; Figs. S1, S4, S6). Figure S2 shows the discovered locations of the African diploid within the sampled regions.

Phylogenetic conclusions for *Adansonia*. — Phylogenies inferred from all three gene regions in our study were concordant in suggesting: a close relationship between *A. kilima* and *A. digitata*; the monophyly of *A. gregorii*; and close relationships among the Malagasy species. In addition, the existence of three major subclades within *Adansonia*, corresponding to the three geographical areas (Africa, Australia, Madagascar), is strongly supported by the ITS phylogeny. Previous studies based on a combination of chloroplast and nuclear DNA and morphological characters (Baum & al., 1998), and phylogenetic analysis of Bombacoideae based on ITS sequences (Duarte & al., 2011) has suggested, albeit weakly, that the Australian and African clades were sisters. Our data also tend to support this conclusion, but again support was low, indicating that additional data will be needed to resolve the deep relationships among the major lineages of *Adansonia*.

Adansonia digitata and *A. kilima* are differentiated by ploidy level, and by morphological characters, particularly in the flowers and pollen. Of the three markers used, only the ITS region (Fig. 4B, D; Fig. S7C, F) contained sufficient information to distinguish between the two species. Other studies have found the ITS region to be more informative than chloroplast markers in making correct DNA identifications to the species level (Gonzalez & al., 2009; Chen & al., 2010; Ren & al., 2010). The potential to diagnose these species with ITS sequences could be particularly useful since most of the diagnostic morphological characters occur in reproductive structures, which may not be available.

A recent divergence such as that between *A. digitata* and *A. kilima* cannot be calculated with high accuracy with the currently available data. Several clock calibrations are available for ITS (reviewed in Kay & al., 2006), and these vary by more than an order of magnitude (between 0.38×10^{-9} and 19×10^{-9} substitutions/site/year). This would correspond to an age of 13–0.17 Ma for the age of the divergence of *A. kilima* and *A. digitata* (ranging from the Miocene to the late Pleistocene). If we considered the age of the earliest divergence in *Adansonia* to be 15–5 Ma as inferred by Baum & al. (1998), and estimated the date of the divergence between *A. kilima* and *A. digitata* based on the number of ITS substitutions, it would be between 2.5 and 0.33 Ma (Pleistocene). Evidence that mutation rates are faster at higher temperatures (Gillooly & al., 2007; for example in the Holocene, relative to the Pleistocene), may lead to overestimates of divergence time for recent divergences, so the divergence between *A. digitata* and *A. kilima* could be more recent than we have estimated here. The fact that the ITS sequence but not the chloroplast sequences could be used to distinguish the two species supports a relatively recent divergence. Figure 1 shows a cladogram of the relationships within *Adansonia*, taking into account the new species *A. kilima* and the molecular work presented here.

Ploidy of *Adansonia digitata*. — *Adansonia digitata* is presumed to be an autotetraploid (Baum & Oginuma, 1994). We did not find a second African diploid during our survey, though we did not include a thorough survey of West Africa. We were provided with some samples from West Africa (see Materials and Methods) that were all *A. digitata*. It is unlikely that a second diploid will be found in West Africa if it has the same preference for a cooler climate as *A. kilima*. It is also possible that a second diploid may once have existed but is no longer extant. In either case, the potential exists that *A. digitata* is an allotetraploid. The most parsimonious character reconstruction for ploidy level infers a diploid ancestor for the genus *Adansonia*, with a recent transition to tetraploidy occurring during or shortly after the speciation event between *A. digitata* and *A. kilima*. This supports the idea that the two shared a common ancestor very recently. It seems likely that *A. digitata* is an autotetraploid of this common ancestor.

Heterosis in polyploids can confer on them greater vigour relative to their diploid ancestors, and gene duplication can allow them to be more resistant to the effects of deleterious mutations (Comai, 2005). However, over large geological time scales, there appears to be no strong benefit to polyploids over their diploid ancestors (Wood & al., 2009). Despite this it should be noted that the tetraploid *A. digitata* has several features which might favour artificial selection by humans relative to *A. kilima*, in particular, the larger fruits. This, coupled with the stomatal properties that may allow *A. digitata* to colonise dryer, hotter and coastal environments, might explain its broader distribution in Africa relative to *A. kilima*.

Altitudinal distribution of African species. — Although stomata in diploids are smaller than those of tetraploids, the much higher stomatal density in diploids leads to a net increase in water loss from closed stomata at night (Caird & al., 2007). The decreased stomatal density means that polyploids can be more tolerant of water deficit than diploids (Li & al., 1996). This may explain the preference of the diploid *A. kilima* for elevations above 800 m, where higher moisture is available. In the Limpopo region, there was more overlap between diploids and tetraploids at lower altitudes than we observed in Kenya and Tanzania. This is consistent with the idea that distribution is related to stomatal characteristics, since the climate is cooler and moister at this southern limit of baobab distribution. An altitude ceiling of ~1500 m could be related to the extreme sensitivity of the high-water-content baobab to frost (Wickens, 1982; Wickens & Lowe, 2008); frost is officially recorded down to 2000 m for many crops in the Kenyan and Tanzanian highlands, but probably reaches even lower at infrequent intervals.

Concluding comments. — It is somewhat surprising that a separate baobab species has co-existed alongside *A. digitata* without detection when both are common and widely distributed. There have been occasional suggestions that there might be more than one species of baobab in Africa. In 1859, Burton identified inland and coastal varieties as well as northern and southern varieties, based on differing morphology (Burton, 1859). One of these was described as rare and found only in the Usagara mountains; it had smaller leaves than the lowland baobab. In 1906, Chevalier distinguished between two species, one in the west and one in the east; his distinction was based on differently sized fruits (Chevalier, 1906). None of these varieties were widely accepted. With the exception of Burton's Usagara mountain variety, none of the descriptions corresponds with that of *A. kilima*, and it seems likely that the other varieties are simply a manifestation of the great morphological and genetic diversity which is observed within the African baobab population (Wickens, 1982; Sidibe & Williams, 2002; Assogbadjo & al., 2006; Kyndt & al., 2009; Pock Tsy & al., 2009; Cuni Sanchez & al., 2010). Some of the observed variability, and possibly Burton's Usagara mountain variety, is likely explainable by the presence of *A. kilima* in Africa. The fact that variability is well known, in combination with the superficial similarity of the two species, may explain why *A. kilima* remained undetected for so long amongst the *A. digitata* population.

The presence of *A. kilima* within populations presumed to consist only of *A. digitata* might explain inconsistencies observed in past studies. Firstly, there has been a wide variety of chromosome counts reported for *A. digitata* (see Wickens, 1982 and references therein). A mixed population might easily

explain these discrepancies. In addition, it should be noted that previous studies of baobab genetics and population characteristics (Baum & al., 1998; Kyndt & al., 2009; Pock Tsy & al., 2009; Venter & Witkowski, 2010) may have unwittingly included *A. kilima* samples in their *A. digitata* collections. This may well affect interpretation of the datasets. Indeed, all phylogenetic comparisons to date within *Adansonia* using presumed *A. digitata* samples may be compromised. Studies localized in Benin (Assogbadjo & al., 2005, 2006; Cuni Sanchez & al., 2010) are unlikely to be compromised, since it is probable that the population in Benin consists entirely of *A. digitata* given the low elevation of the country (mostly below 400 m; reaching a peak of about 650 m at the highest point in the northern hills). In support of this presumption, guard cell measurements in trees from Benin were 35–45 µm (Cuni Sanchez & al., 2010), suggesting that the trees were all tetraploid *A. digitata*; this is consistent with the observed altitude preference of *A. kilima*.

■ TAXONOMIC CONCLUSIONS

We have identified a new diploid African species in *Adansonia*. We name this species *A. kilima* Pettigrew, Bell, Bhagwandin, Grinan, Jillani, Meyer, Wabuyele & Vickers. The epithet *kilima* is derived from Swahili, 'of the hills'. The floral characteristics of *A. kilima* place it with *A. digitata* in *A.* sect. *Adansonia*.

22.90159
22.90159S
29.99656E

Adansonia kilima Pettigrew, Bell, Bhagwandin, Grinan, Jillani, Meyer, Wabuyele & Vickers, **sp. nov.** – Holotype: South Africa, Limpopo, on south side of the road between Tshkuwi and Tshituni, 16 km East of the N1 Highway; 22.18004° S 28.0091° E, *Jack Pettigrew & Chloë Callistemon 1*, 12 Nov 2009 (PRE 0857831-0!)

Paratype: Namibia, Caprivi Strip, 17.6177° S 24.3991° E, *Jack Pettigrew & Chloë Callistemon s.n.*, 8 Dec 2009 (WIS v0263271WIS!)

Diagnosis. – *Adansoniae digitatae* tetraploideae affinis sed differt floribus dimidio minoribus, petalis androecio superantibus, foliis brevioribus, stomatibus minoribus densioribusque, polline spinosiore (spinis 142–158, in *A. digitata* 51–100), minore (44 µm, in *A. digitata* 63 µm diametro), filamentis liberis staminum paucioribus (270–640, in *A. digitata* 700–1600). Chromosomatum numerus diploideus 88.

Like the tetraploid *A. digitata*, but differs in having flowers half the size, petals shorter than the androecium with no kinesis, smaller leaves, smaller and denser stomata, smaller and more densely spinose pollen (44 µm in diameter compared with 63 µm in *A. digitata*), fewer free staminal filaments (270–640, compared with 700–1600 in *A. digitata*); diploid chromosome number $2n = 88$.

Description (modified and extended from the description of *A. digitata* by Baum, 1995 where appropriate). – Tree reaching 20 m in height and up to 3 m diam., usually with single cylindrical trunks and spreading, rounded crowns. Branches irregularly distributed. Bark dark grey, smooth to irregularly tuberculate. Leaves 5–7(–9)-foliolate; leaflets sessile to subsessile, varying greatly in size, median leaflet 3–10 × 1.5 × 5 cm, usually elliptic-obovate, with acuminate apex and decurrent base; glabrous, margins entire. Stomata: stomatal aperture 26 ± 5.7 µm; adaxial stomatal density 5 per 100 µm². Flower buds globose, solitary; flower stalk pendulous, 4–10 cm long; flowers produced after the end of the wet season; in contrast to *A. digitata*, there is no rapid kinesis during the opening of the floral buds. Calyx lobes 5, triangular, green and tomentose outside, cream and villous within, slightly reflexed, 3–5 × 2–3 cm, fused into a broad (4 cm diam.) disc below. Petals white, folded in bud, broadly obovate, approximately as long as broad, 25 × 25 mm, shorter than androecium, not reflexed. Androecium white, comprising a 12–20 mm long cylindrical or tapering staminal tube, surmounted by 270–640 free filaments, ± as long as the staminal tube. Ovary conical-ovoid or globose; style white, sinuous, bent over at right angles or rarely straight; densely villous below, glabrous above, fitting loosely into staminal tube and persisting after floral abscission. Stigma white with irregular lobes. Pollen globose with broad, short, irregularly sized spines (142–158 per 1000 µm²); 44 µm in diameter. Fruit variable; globose to ovoid to oblong-cylindrical (4–20 cm), calyx lobes caducous; pericarp up to 5–8 mm thick, woody, with few embedded longitudinal fibers, covered by a velvety indumentum of yellow-brown hairs. Seeds reniform, laterally flattened, 10–13 × 8–10 × 6–7 mm, embedded in cream-coloured pulp penetrated by fine strings. Germination phanerocotylar.

Distribution and ecology. – In our limited survey, *A. kilima* was found from the eastern slopes of Mt. Kilimanjaro, south to southern Tanzania and west to northern Namibia, as well as in the Venda of South Africa, restricted to elevations between 650 and 1500 m. *Adansonia digitata* but not *A. kilima* were found in coastal Namibia and Senegal.

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Appendix. GenBank accession numbers used in molecular analysis. Information is listed as follows: taxon name, collection locality (country, state/territory), collector and collection number, *psbA-trnH*, *trnL-trnF*, and ITS GenBank numbers, respectively. Newly generated sequences are noted with an asterisk.

***Adansonia digitata* L., Australia,** Queensland (cultivated specimen), 25.2003° S 146.5256° E, *J. Pettigrew 259* (no voucher), *JN400287, *JN400295, *JN400317**.** ***A. digitata*, Australia,** Northern Territory (cultivated specimen), 12.4425° S 130.8375° E, *J. Pettigrew 210* (no voucher), *JN400285, *JN400294, –. ***A. digitata*, Kenya,** Coast, 3.3322° S 40.0125° E, *J. Pettigrew 293* (no voucher), –, *JN400293, –. ***A. digitata*, Zimbabwe,** Masvingo, 20.9500° S 31.8809° E, *J. Pettigrew 444Z* (no voucher), –, *JN400302, –. ***A. digitata*, Tanzania,** Dodoma, 5.1035° S 35.8208° E, *J. Pettigrew 361* (no voucher), –, *JN400301, –. ***A. digitata*, South Africa,** Limpopo, 22.9011° S 29.9985° E, *J. Pettigrew 404* (no voucher), –, –, *JN400311. ***A. digitata*, Senegal,** *P. Danthu Adi2* (MEL), –, –, *JX254900. ***A. digitata*,[a,c] U.S.A.,** Iowa (cultivated specimen), *Small s.n.* (ISC), AF028525. ***A. digitata*,[b,c] Burkina Faso,** Pobé Mengao/Touflé, *Baum 349* (MO), –, –, AF028526. ***A. digitata*,[a,d]** unpublished, –, –, AF460193. ***A. digitata*,[e] Kenya,** collector unknown *770032002* (PTBG), –, HQ696738, –. ***A. digitata*,[f]** unpublished, –, AY328150, –. ***A. grandidieri* Baill.,[c,e] Madagascar,** Toliara, 20.0333° S 44.65° E, *Baum 345* (MO), –, HQ696739, AF028528. ***A. grandidieri*,[c] Madagascar,** Toliara, *Baum 347* (MO), –, –, AF028527. ***A. gregorii* Muell., Australia,** Western Australia, 14.0620° S 126.7392° E, *J. Pettigrew 81* (no voucher), *JN400281, *JN400305, –. ***A. gregorii*, Australia,** Western Australia, 13.9370° S 126.6927° E, *J. Pettigrew 76* (no voucher), *JN400282, –, –. ***A. gregorii*, Australia,** Western Australia, 15.7736° S 128.2959° E, *J. Pettigrew 37* (no voucher), *JN400283, *JN400310, –. ***A. gregorii*, Australia,** Western Australia, 15.9326° S 124.4221° E, *J. Pettigrew 233* (no voucher), –, *JN400309, –. ***A. gregorii*, South Africa,** Gauteng (cultivated specimen), 26.1800° S 28.0093° E, *J. Pettigrew 400* (no voucher), –, *JN400308, –. ***A. gregorii*, Australia,** Western Australia, 13.9096° S 127.0630° E, *J. Pettigrew 70* (no voucher), –, –, *JN400318. ***A. gregorii*, Australia,** Western Australia, 14.4483° S 125.987° E, *J. Pettigrew 220* (no voucher), –, –, *JN400319. ***A. gregorii*, Australia,** Western Australia, 14.1238° S 126.6837° E, *J. Pettigrew 85* (no voucher), –, –, *JN400321. ***A. gregorii*, Australia,** Western Australia, 13.8082° S 126.7685° E, *J. Pettigrew 73* (no voucher), –, –, *JN400320. ***A. gregorii*, Australia,** Western Australia, 15.9259° S 128.3824° E, *J. Pettigrew 201* (no voucher), –, –, *JN871592. ***A. gregorii*, Australia,** Western Australia, 15.1044° S 125.0014° E, *J. Pettigrew 222* (no voucher), –, –, *JN400323. ***A. gregorii*, Australia,** Western Australia, 14.4479° S 125.9867° E, *J. Pettigrew 219* (no voucher), –, –, *JN400322. ***A. gregorii*,[c,e] Australia,** Western Australia, *Wendell s.n.* (ISC), –, HQ696740, AF028524. ***A. kilima* Pettigrew & al., South Africa,** Limpopo, 26.18004° S 28.0091° E, *J. Pettigrew & C. Callistemon 1* (PRE), –, *JX178941, *JX178939. ***A. kilima*, Namibia,** Caprivi Strip, 17.6177° S 24.3991° E, *J. Pettigrew & C. Callistemon s.n.* (WIS), –, *JX178942, *JX178940. ***A. kilima*, Kenya,** Coast, 3.3524° S 37.7072° E, *J. Pettigrew 306* (no voucher), *JN400291, –, –. ***A. kilima*, Tanzania,** Tanga, 4.7908° S 38.1967° E, *J. Pettigrew 326* (no voucher), *JN400288, –, –. ***A. kilima*, Kenya,** Eastern, 2.3374° S 37.8836° E, *J. Pettigrew 313* (no voucher), *JN400290, –, –. ***A. kilima*, Tanzania,** Dodoma, 4.9830° S 35.7982° E, *J. Pettigrew 363* (no voucher), *JN400289, –, –. ***A. kilima*, Tanzania,** Kilimanjaro, 3.3520° S 37.3446° E, *J. Pettigrew 319* (no voucher), –, *JN400297, –. ***A. kilima*, Tanzania,** Morogoro, 7.5694° S 36.7134° E, *J. Pettigrew 331* (no voucher), –, *JN400298, *JN400324. ***A. kilima*, Kenya,** Coast, 3.3911° S 37.7175° E, *J. Pettigrew 304* (no voucher), –, *JN400296, –. ***A. kilima*, Tanzania,** Manyara, 3.7755° S 35.9678° E, *J. Pettigrew 371* (no voucher), –, *JN400300, –. ***A. kilima*, Tanzania,** Iringa, 7.1630° S 35.7626° E, *J. Pettigrew 350* (no voucher), –, *JN400299, *JN400327. ***A. kilima*, Tanzania,** Kilimanjaro, 4.0900° S 37.7576° E, *J. Pettigrew 325* (no voucher), –, –, *JN400326. ***A. kilima*, Tanzania,** Dodoma, 6.8311° S 36.0566° E, *J. Pettigrew 355* (no voucher), –, –, *JN400325. ***A. madagascariensis* Baill., Madagascar,** Mahamasin/Ankarana, 12.9522° S 49.1283° E, *P. Danthu Ama1* (MEL), *JN400280, *JN400307, –. ***A. madagascariensis*,[c] Madagascar,** Antsiranana, 14.20° S 48.08° E, *Baum 338* (MO), –, –, AF028533. ***A. madagascariensis*,[c] Madagascar,** Antsirinana, 14.20° S 48.08° E, *Baum 338* (MO), –, –, AF028534. ***A. perrieri* Capuron, Australia,** Northern Territory (cultivated specimen), 12.4427° S 130.8372° E, *J. Pettigrew 208* (no voucher), *JN400278, *JN400292, –. ***A. perrieri*,[c] Madagascar,** Antsiranana, *Baum 359* (MO), –, –, AF028538. ***A. rubrostipa* Jum. & H. Perrier, Madagascar,** Itampolo, 24.6516° S 43.9607° E, *P. Danthu Aru1* (MEL), *JN400284, *JN400306, –. ***A. rubrostipa*, Madagascar,** Toliara, *Baum 54* (MO), –, –, AF028531. ***A. suarezensis* H. Perrier, Madagascar,** Diego Suarez, 12.3° S 49.3° E, *C. Kull DIEB005.i* (MEL), *JN400286, *JN400304, –. ***A. suarezensis*,[c] Madagascar,** Antsiranana, *Baum 348* (MO), –, –, AF028529. ***A. za* Baill., Australia,** Northern Territory (cultivated specimen), 12.4427° S 130.8371° E, *J. Pettigrew 206* (no voucher), *JN400279, *JN400303, –. ***A. za*,[c,e] Madagascar,** Toliara, *Baum 357* (MO), –, HQ696741, AF028536. ***A. za*,[c]** Madagascar, Toliara, *Baum s.n.* (no voucher), –, –, AF028537. ***Cavanillesia platanifolia* (Bonpl.) Kunth,[g] Panama,** Barro Colorado Island, *R. Perez 445949* (SCZ), GQ982172, –, –. ***C. platanifolia*,[c] U.S.A.,** Florida, collector unknown *FG83343A* (FTG), –, HQ696737, HQ658371

[a] Identified as *A. digitata* in the original publication. Voucher not examined in the current study.
[b] Identified as *A. digitata* in the original publication. Identity confirmed by D. Baum (pers. comm.)
[c] Baum & al., 1998
[d] Shi & Yuan, unpub.
[e] Duarte & al., 2011
[f] Yuan & al., unpub.
[g] Kress & al., 2009