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Olaf Werner, Mahmoud Magdy & Rosa María Ros

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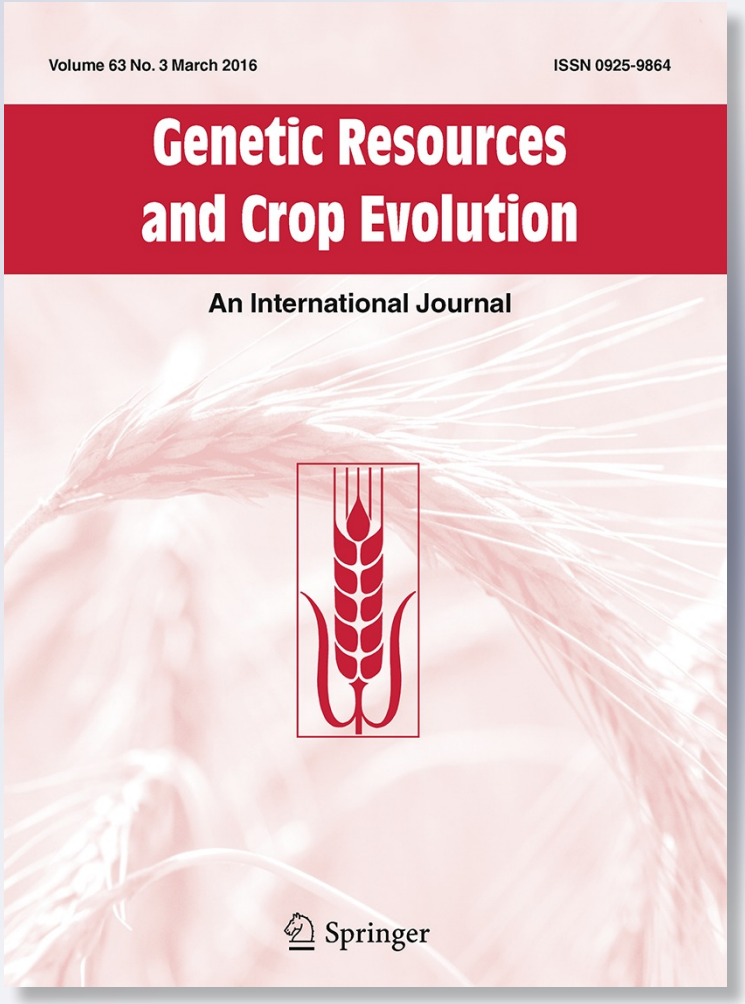

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RESEARCH ARTICLE

Molecular systematics of *Abelmoschus* (Malvaceae) and genetic diversity within the cultivated species of this genus based on nuclear ITS and chloroplast *rpL16* sequence data

Olaf Werner · Mahmoud Magdy · Rosa María Ros

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Abstract The genus *Abelmoschus* includes several crop plants which are especially important in SE Asia and several African countries. However, the systematic treatment of this genus is difficult, in part because hybridization between different forms seems to be frequent. In this study we present nuclear internal transcribed spacer ITS and chloroplast *rpL16* sequences with the aim of reconstructing phylogenetic relationships within *Abelmoschus*, and its relationship with the genus *Hibiscus* and other related Malvaceae. Based on our analysis of nuclear ITS and chloroplast *rpL16* sequence data, *Abelmoschus* is resolved as a monophyletic clade. *Abelmoschus tetraphyllus* is clearly separated from *A. manihot* but closely related to *A. ficulneus* and should not be treated as a subspecies of *A. manihot*. None of the wild species included in this study can be confirmed as an ancestor of *A. esculentus* or *A. caillei*. Neither *A. esculentus* nor *A. caillei* can be distinguished from each other by the markers used for this study, although the evidence does not exclude the possibility of a hybrid origin of *A. caillei* involving *A. esculentus* and an unknown species. The genetic diversity within *A. esculentus* and *A. caillei* is low if compared with *A. manihot*. The evidence presented here does not allow us to draw any conclusions about the geographic origin (Africa vs. Asia) of *A. esculentus*.

Keywords *Abelmoschus caillei* · *Abelmoschus esculentus* · *Abelmoschus tetraphyllus* · Crop plants · Phylogenetic relationships · Taxonomy

O. Werner (✉) · R. M. Ros
Departamento de Biología Vegetal (Botánica), Universidad de Murcia, Campus de Espinardo, 30100 Murcia, Spain
e-mail: werner@um.es

M. Magdy
Genetic Department, Faculty of Agriculture, Ain Shams University, 68 Hadayek Shubra, 11241 Cairo, Egypt

Introduction

Depending on the treatment, the genus *Abelmoschus* Medik. may include from 6 (van Borssum-Waalkes 1966) to 14 (Hochreutiner 1924) species. The center of its distribution lies in South East Asia, but *A. ficulneus* (L.) Wight and Arn. is found in Africa, Asia and Australia (Siemonsma 1982; Charrier 1984; Lamont 1999), and *A. moschatus* Medik. subsp. *tuberosus* (Span.) Borss.-Waalk. is found in the tropical regions of Northern Australia. Although Medikus established the genus in 1787, it was not until the work of Hochreutiner (1924), which clearly defined key morphological characteristics (essentially the caducous calyx), that the genus was generally accepted. Within the genus, there are still several unresolved taxonomic problems (Hamon and van Sloten 1995); e.g., the nature of some infraspecific categories of *A. moschatus* and *A. manihot* (L.) Medik., the position of *A.*

caillei (A. Chev.) Stevels within the genus, and the relationship of *A. esculentus* (L.) Moench to the suggested wild forms, *A. tuberculatus* Pal et Singh and *A. ficulneus*.

Four species, *A. caillei*, *A. esculentus, A. manihot* and *A. moschatus*, are cultivated and are now distributed through tropical and subtropical regions of the world, with the exception of the first, which is restricted to West Africa (Siemonsma 1982). According to Mansfelds World Database of Agricultural and Horticultural Crops (http://mansfeld.ipk-gatersleben.de/apex/f?p=185:3:9797333538778; Ochsmann et al. 1999) other species like *A. angulosus* Wight et Arn., *A. crinitus* Wall., and *A. ficulneus* are also used as crop plants. There are several uses for the cultivated species. For example, *A. moschatus* is used as a vegetable (leaves, unripe seed pods) (Facciola 2001; Manandhar 2002; National Research Council of the National Academies 2006). The seeds possess a musky odor, and perfumers know them as ambrette ("abelmoschus" from the Arabic "father of musk", with "moschatus" also referring to a musky smell) (National Research Council of the National Academies 2006) and use them as a perfume ingredient (Singh et al. 1996). The plant has also a wide variety of medical uses (Agharkar 1991; Bown 1995), including the treatment of depression and anxiety, stomatitis and gonorrhea.

Abelmoschus manihot is cultivated for its leaves, but its immature pods are too prickly to be consumed (Hamon and van Sloten 1995). Pharmacological effects, including anti-inflammatory, anti-viral, anti-bacterial, wound healing, and anti-fungal activities, have been confirmed for this species (Todarwal et al. 2011). Several infraspecific taxa have been described, one of the most important being *A. manihot* var. *tetraphyllus* Hochr. (Hochreutiner 1924) but the taxonomic status of this entity remains unclear. For example, van Borssum-Waalkes (1966) considered it as a subspecies and Hamon and van Sloten (1995) recommended giving it species rank.

Okra (*A. esculentus*) is a widely used plant in tropical and subtropical countries all around the world. *Abelmoschus caillei*, the West African okra or atypical okra (Martin et al. 1981), in contrast, is restricted to western Africa. The pods, leaves, and seeds of these two species are edible. Among their useful non-food products are mucilage, industrial fiber, and medicines (National Research Council of the National Academies 2006). The total world area occupied by okra culture in 2012 rose to 1,085,146 ha and production reached 8,359,944 tonnes (Food and Agriculture Organization of the United Nations 2014), India and Nigeria being the main producers. Two recent reviews (Benchasri 2012; Kumar et al. 2010) provide detailed descriptions of the chemical composition of okra leaves, unripe pods and seeds and possible uses for different plant parts. One of the most important aspects is the amino acid composition of the seeds, which are rich in tryptophan and sulfur-containing amino acids, and, unlike the proteins of cereals and pulses, are balanced in both lysine and tryptophan amino acids (National Research Council of the National Academies 2006).

While wild and cultivated forms coexist within both *A. manihot* and *A. moschatus*, the origins of *A. esculentus* and *A. caillei* are not well established. Cytogenetic data (Siemonsma 1982) suggest that *A. esculentus* is an amphidiploid possessing a genome in common with *A. tuberculatus* and a complementary genome, whose origin has not yet been established. This would imply an Asian origin of *A. esculentus* because *A. tuberculatus* is native to Uttar Pradesh, north India (Hamon and van Sloten 1995). Another hypothesis (Hamon and van Sloten 1995) suggests that African populations of *A. ficulneus* are a possible ancestor of *A. esculentus*, therefore making it of African origin.

Although *Abelmoschus* includes important crop species and offers interesting pharmaceutical uses, there are very few data available related to its molecular systematics. A search of accessions available at GenBank made on 19 November 2014 returned only 52 nucleotide sequences. Pfeil et al. (2002) and Small (2004) added isolated DNA sequences of *Abelmoschus* specimens when they studied aspects of the molecular systematic of *Hibiscus* L. Other researchers working with molecular markers have centered their attention on variability found among *Abelmoschus* cultivars of specific geographical regions. Gulsen et al. (2007) used SRAP to investigate diversity and relationships within Turkish germplasm, and Sawadogo et al. (2009) used SSR markers to study the genetic diversity of okra from Burkina Faso. Salameh (2014) studied genetic relationships among 48 okra genotypes (mainly of *A. esculentus*) from different agro-ecological regions with AFLP markers. In a RAPD-marker study by Prakash et al. (2011), two

samples of *A. caillei* were deeply nested within *A. esculentus* samples. Interestingly, in a similar study also using RAPD markers, Aladele et al. (2008) found that these two species were clearly separated.

More recently, Schafleitner et al. (2013) used transcriptome data to develop SSR markers. Their study included three species, mainly *A. esculentus* from a vast geographical range alongside two samples of *A. manihot* and *A. moschatus* each. The clustering of their resulting phylogram corresponded well both to species and geographic origin of the samples. Although the work based on DNA fingerprinting does not provide strong evidence for limited genetic diversity, Hamon (1988) found a very low degree of genetic diversity within cultivated species based on isoenzymic data, but Hamon and Yapo (1985) were able to distinguish easily between *A. esculentus* and *A. caillei* based on three enzyme systems, in agreement with the work of Aladele et al. (2008) based on RAPD markers.

The relative scarcity of data for *Abelmoschus* may in part be due to the fact that the main production areas are situated in tropical regions where economic resources for research are more limited than in highly industrialized countries. Although many plant species have already undergone conscious selection for food production, most still fall outside the ambit of modern research and economic development (National Research Council of the National Academies 2006). But genetic data are the bases of modern breeding technology and can help greatly in directing the efforts of breeding programs. It is important to know the variability within species and also relationships with related species to improve our understanding of the current genetic status of crop species (Salamini et al. 2002), identify useful genes in wild relatives and introduce them into the cultivated gene pool (Septiningsih et al. 2003), and also identify genes involved in the domestication process or in subsequent selection events (Wright et al. 2005).

Correns (1909) was the first to establish that chloroplasts are inherited from the female parent in angiosperms (*Mirabilis jalapa* L.). It was later confirmed that chloroplast inheritance in angiosperms is mostly maternal, although there is evidence that other patterns of inheritance occur (Xu 2005). The maternal inheritance of chloroplast DNA—contrasting with the biparental inheritance of nuclear DNA—allows valuable insights into population Genetics and biogeography of angiosperms. For example, with maternal inheritance chloroplast DNA can only migrate with the seed while nuclear genes may migrate twice (in the pollen and the seed) (Petit et al. 1993). As a consequence, this can lead to a higher differentiation between populations when analyses are based on chloroplast DNA markers as compared to nuclear markers, because of the higher levels of pollen flow. Incongruence of chloroplast and nuclear markers is also often used as indication of hybridization and introgression (reviewed in Wendel and Doyle 2000).

In this work, we apply nrITS and chloroplast *rpL16* sequence data in an attempt to elucidate relationships of the genus *Abelmoschus* with other genera of Malvaceae, phylogenetic relationships within *Abelmoschus*, and the genetic diversity of important crop species within the genus, with special attention to *A. esculentus*.

Materials and methods

Seed samples sequenced in this study were donated by the United States Department of Agriculture, Agricultural Research Service (USDA, ARS), J. K. Ahiakpa (University of Ghana-Legon, Ghana), and D. Achel (Ghana Atomic Energy Commission) or obtained from the commercial seed suppliers, Baker Creek Heirloom Seeds (Mansfield, Missouri, USA), Sunshine Seeds (Ahlen, Germany), and Exotische Nutz- und Zierpflanzen (Seeheim-Jugenheim, Germany). The University Ain Shams (Cairo, Egypt) maintains seeds of the samples "Egypt green" and "Egypt red".

The sampling includes four samples of *A. caillei* from West Africa, one of *A. crinitus* from Nepal, 27 of *A. esculentus* from Africa, America, Asia and Europe, one of *A. ficulneus* from India, four of *A. manihot*, of which one was from Japan, another from Thailand and the remainder of unknown origin, two of *A. tetraphyllus* from India, three of *A. moschatus* from Costa Rica, Maldives and Togo, and one of *A. tuberculatus* from India. We also included one Indian specimen that was not identified to species. In addition, five DNA sequences were retrieved from GenBank (Benson et al. 2005): one attributed to *A. esculentus*, one to *A. ficulneus*, two to *A. manihot* and one to *A. moschatus*.

To study relationships with other genera of the Malvaceae, we included an accession of *Hibiscus sabdariffa* L., and 12 ITS sequences and 29 *rpL16*

sequences from GenBank, mainly to represent related genera, especially *Hibiscus*. Details on voucher information, geographical origin of the samples, and GenBank accession numbers are given in Table 1.

After removing seed coats from the samples, DNA was extracted from them with the SDS-Potassium acetate method of Dellaporta et al. (1983). We found that it was important to add RNase A; otherwise, the amount of RNA present in the seeds inhibited PCR reactions. The nuclear ITS region was amplified with the primers ITS4 and ITS5 of White et al. (1990). PCR reactions were performed in 25 μL volume by using Thermo Scientific (Madrid, Spain) DreamTaq (1μ), 200 μM of each dNTP, 2 mM $MgCl_2$ and the buffer system supplied by the manufacturer of the enzyme. Reaction conditions were an initial denaturation step of 3 min at 95 °C, followed by 35 cycles at 30 s at 95 °C, 30 s annealing at 55 °C, and 1 min extension at 72 °C, with a final extension of 10 min at 72 °C. The amplified fragments were maintained at 4 °C and analyzed by agarose gel electrophoresis. Successful amplifications were cleaned up by an enzymatic reaction using FAST AP and ExoI (Thermo Scientific), followed by thermal inactivation (15 min at 85 °C) of the enzymes. The amplification of the *rpL16* region followed the protocol of Small (2004), with the exception that we used DreamTaq (Thermo Scientific) instead of ExTaq. Again, successful amplifications were cleaned up with FAST AP and ExoI. The amplified fragments were sequenced by Macrogen (Amsterdam, Netherlands) by using the amplification primers and additional internal primers ITS2 and ITS3 (White et al. 1990), in the case of ITS sequences, and 627F and 699R (Pfeil et al. 2002) for the chloroplast *rpL16* region.

The sequences were aligned by applying MAFFT 7 (Katoh and Standley 2013) with the default settings, except that we set the offset value for the *rpL16* region to 0.5. Minor evident alignment errors were corrected manually in BioEdit (Hall 1999).

The number of haplotypes, haplotype diversity, and the Waterson estimator Theta (Θ_W) = $4N_e\mu$ (were N_e is the effective population size and μ the mutation rate) were calculated in DnaSP v5 (Librado and Rozas 2009). For calculating genetic distances, ambiguous positions for each sequence pair were removed, and the number of base differences *per* sequence was calculated with the help of MEGA6 (Tamura et al. 2013). SplitsTree 4 (Huson and Bryant 2006) was used to look for evidence of recombination in the combined ITS-*rpL16* data file. This program uses a Phi-test of recombination (Bruen et al. 2006) to find evidence supporting recombination. An analysis by jModelTest 2 (Darriba et al. 2012) showed that models with gamma distribution and invariant sites offered the best fit to the data under both the Akaike and the Bayesian information criteria for the nrITS and the *rpL16* regions. MrBayes v3.2 (Ronquist et al. 2012) was used for the phylogenetic analyses. First both regions were analyzed separately. The possibility of incongruent data between chloroplast and nuclear DNA makes it necessary to test for the compatibility before using combined data sets, although the combination of datasets may minimize sampling error and therefore facilitate the retrieval of "true" clades (reviewed in Johnson and Soltis 2000 and Wendel and Doyle 2000). The congruence of the nuclear and the chloroplast data sets was tested in two ways. First we tested for reassortment between the nuclear and chloroplast sequences in the combined dataset using a phi test of recombination (Bruen et al. 2006) as implemented in SplitsTree 4 (Huson and Bryant 2006). Second we performed a partition homogeneity test as implemented in PAUP* 4.0b10 (Swofford 2003) with 100 replicates and maxtrees set to 1000.

Trees were sampled across the substitution model space in the Bayesian MCMC analysis (Huelsenbeck et al. 2004) by using the option nst = mixed, removing the need for a priori model testing. The present version of MrBayes does not allow reversible jumping for different models of rate variation across sites. Therefore, based on the results of the jModelTest 2, rate variation was set to rates = gamma. In a second Bayesian analysis, the indels coded by simple indel coding (SIC, Simmons and Ochoterena 2000) using SeqState (Müller 2004) were included, with sequence and indel data treated as separate and unlinked partitions, given the restriction site model ('F81') for the indel matrix. In the combined analysis of nr and cp regions, unlinked partitions were defined to allow the overall rate to be different across partitions. The a priori probabilities supplied were those specified in the default settings of the program. Posterior probability (pp) distributions of trees were created with the Metropolis-coupled Markov Chain Monte Carlo (MCMCMC) method. Two runs with four chains (2×10^7 generations each) were run simultaneously, with the temperature of the single heated chain set to

Table 1 Plant material

Species	Accession code or reference	Seed supplier	Country of origin	GenBank ITS accession number	GenBank *rpL16* accession number
Abelmoschus caillei (A. Chev.) Stevels	PI489858	USDA, ARS	Cote D'Ivoire 1	KP222401	KP222323
Abelmoschus caillei	PI490015	USDA, ARS	Cote D'Ivoire 2	KP222402	KP222324
Abelmoschus caillei	PI497027	USDA, ARS	Ghana	KP222400	KP222322
Abelmoschus caillei	PI497129	USDA, ARS	Togo	KP222399	KP222321
Abelmoschus crinitus Wall.	PI592390	USDA, ARS	Nepal	KP222434	KP222356
Abelmoschus esculentus (L.) Moench	Grif13541	USDA, ARS	Nepal	KP222412	KP222334
Abelmoschus esculentus	Grif16450	USDA, ARS	Argentina	KP222414	KP222336
Abelmoschus esculentus	PI117095	USDA, ARS	Turkey	KP222413	KP222335
Abelmoschus esculentus	PI123451	USDA, ARS	India	KP222406	KP222328
Abelmoschus esculentus	PI124977	USDA, ARS	Mexico	KP222453	KP222375
Abelmoschus esculentus	PI249007	USDA, ARS	Nigeria	KP222396	KP222318
Abelmoschus esculentus	PI256068	USDA, ARS	Afghanistan	KP222394	KP222316
Abelmoschus esculentus	PI274341	USDA, ARS	China	KP222405	KP222327
Abelmoschus esculentus	PI378631	USDA, ARS	Zaire	KP222403	KP222325
Abelmoschus esculentus	PI379352	USDA, ARS	Macedonia	KP222454	KP222376
Abelmoschus esculentus	PI390580	USDA, ARS	Peru	KP222411	KP222333
Abelmoschus esculentus	PI441494	USDA, ARS	Brazil	KP222456	KP222378
Abelmoschus esculentus	PI482039	USDA, ARS	Zimbabwe	KP222446	KP222368
Abelmoschus esculentus	PI496618	USDA, ARS	Ghana	KP222404	KP222326
Abelmoschus esculentus	PI496695	USDA, ARS	Philippines	KP222398	KP222320
Abelmoschus esculentus	PI496863	USDA, ARS	USA	KP222409	KP222331
Abelmoschus esculentus	PI505487	USDA, ARS	Burkina Faso	KP222415	KP222337
Abelmoschus esculentus	PI538061	USDA, ARS	Zambia	KP222460	KP222382
Abelmoschus esculentus	PI538063	USDA, ARS	Algeria	KP222416	KP222338
Abelmoschus esculentus	PI538122	USDA, ARS	Sudan	KP222397	KP222319
Abelmoschus esculentus	PI538160	USDA, ARS	Egypt	KP222407	KP222329
Abelmoschus esculentus	PI639676	USDA, ARS	Sri Lanka	KP222417	KP222339
Abelmoschus esculentus	Lot 888RB	Baker Creek Heirloom Seeds, Mansfield, Missouri. USA	Burma	KP222442	KP222364
Abelmoschus esculentus	Egypt green	M. Magdy, Ain Shams University, Egypt	Egypt	KP222390	KP222312

Table 1 continued

Species	Accession code or reference	Seed supplier	Country of origin	GenBank ITS accession number	GenBank *rpL16* accession number
Abelmoschus esculentus	Egypt red	M. Magdy, Ain Shams University, Egypt	Egypt	KP222391	KP222313
Abelmoschus esculentus	Red Burgundy	Sunshine Seeds, Germany	NA	KP222393	KP222315
Abelmoschus esculentus	Asontem ASR	J.K. Ahiakpa, University of Ghana-Legon, Ghana	Ghana	KP222445	KP222367
Abelmoschus ficulneus (L.) Wight et Arn.	PI639668	USDA, ARS	India	KP222466	KP222388
Abelmoschus manihot (L.) Medik.	PI379584	USDA, ARS	Japan	KP222463	KP222385
Abelmoschus manihot	PI538178	USDA, ARS	Thailand	KP222438	KP222360
Abelmoschus manihot	NA	Sunshine Seeds, Germany	NA	KP222465	KP222387
Abelmoschus manihot	NA	Exotische Nutz- und Zierpflanzen, Germany	NA	KP222464	KP222386
Abelmoschus moschatus Medik.	PI338900	USDA, ARS	Costa Rica	KP222462	KP222384
Abelmoschus moschatus	PI496941	USDA, ARS	Togo	KP222427	KP222349
Abelmoschus moschatus	PI536548	USDA, ARS	Maldives	KP222439	KP222361
Abelmoschus sp.	PI639741	USDA, ARS	India	KP222440	KP222362
Abelmoschus tetraphyllus (Roxb. ex Hornem.) Borss.	Grif2789	USDA, ARS	India	KP222433	KP222355
Abelmoschus tetraphyllus	PI639674	USDA, ARS	India	KP222436	KP222358
Abelmoschus tuberculatus Pal et Singh	Grif12671	USDA, ARS	India	KP222441	KP222363
Hibiscus sabdariffa L.	PI496938	USDA, ARS	Sudan	KP222467	KP222389
Abelmoschus esculentus	GenBank unpublished	–	–	JN115011	–
Abelmoschus ficulneus	Pfeil et al. (2002)	–	–	–	AF384560
Abelmoschus manihot	GenBank unpublished	–	–	KC488173	–
Abelmoschus manihot	Pfeil et al. (2002)	–	–	–	AF384561
Abelmoschus moschatus	GenBank unpublished	–	–	JQ230968	–
Alyogyne pinoniana (Gaudich.) Fryxell	Pfeil et al. (2002)	–	–	–	AF384566
Bombax buonopozense Beauv.	Duarte et al. (2011)	–	–	HQ658376	–
Bombax buonopozense	Baum et al. (1998)	–	–	–	AF025541
Fioria vitifolia (L.) Mattei	Pfeil et al. (2002)	–	–	–	AF384570
Hibiscus apodus Juswara and Craven	Pfeil et al. (2002)	–	–	–	AF384574
Hibiscus calyphyllus Cav.	Pfeil et al. (2002)	–	–	–	AF384577
Hibiscus coatesii F. Muell.	Pfeil et al. (2002)	–	–	–	AF384578
Hibiscus coccineus Walter	Small (2004)	–	–	AY341386	AY341407
Hibiscus dasycalyx Blake et Shiller	Small (2004)	–	–	AY341388	AY341406
Hibiscus engleri K. Schum.	Pfeil et al. (2002)	–	–		AF384582
Hibiscus grandiflorus Michaux	Small (2004)	–	–	AY341389	AY384400

Table 1 continued

Species	Accession code or reference	Seed supplier	Country of origin	GenBank ITS accession number	GenBank *rpL16* accession number
Hibiscus laevis Allioni	Small (2004)	–	–	AY341387	AY341405
Hibiscus macrophyllus Roxb.	GenBank unpublished	–	–	EU188898	–
Hibiscus macrophyllus	Pfeil et al. (2002)	–	–	–	AF384589
Hibiscus moscheutus L.	Small (2004)	–	–	AY341390	AY341402
Hibiscus pentaphyllus F. Muell.	Pfeil et al. (2002)	–	–	–	AF384597
Hibiscus peralbus Fryxell	Pfeil et al. (2002)	–	–	–	AF384598
Hibiscus physaloides Guill. et Perr.	Pfeil et al. (2002)	–	–	–	AF384599
Hibiscus schinzii Hochr.	Pfeil et al. (2002)	–	–	–	AF384604
Hibiscus striatus Cav.	Pfeil et al. (2002)	–	–	–	AF384607
Hibiscus surattensis L.	GenBank unpublished	–	–	EU188876	–
Hibiscus surattensis	Pfeil et al. (2002)	–	–	–	AF384609
Hibiscus syriacus L.	GenBank unpublished	–	–	AF460188	–
Hibiscus syriacus	Pfeil et al. (2002)	–	–	–	AF384610
Hibiscus trionum L.	Small (2004)	–	–	AY3341385	–
Hibiscus trionum	Pfeil et al. (2002)	–	–	–	AF384612
Hibiscus waimeae A. Heller	Pfeil et al. (2002)	–	–	–	AF384613
Howittia trilocularis F. Muell.	Tate et al. (2005)	–	–	AY591832	–
Howittia trilocularis	Pfeil et al. (2002)	–	–	–	AF384615
Macrostelia grandiflora Fryxell	Pfeil et al. (2002)	–	–	–	AF384618
Malvaviscus arboreus Cav.	Pfeil et al. (2002)	–	–	–	AF384621
Pavonia hastata Cav.	Pfeil et al. (2002)	–	–	–	AF384622
Radyera farragei (F. Muell.) Fryxell et S.H. Hashmi	Pfeil et al. (2002)	–	–	–	AF384623
Sida hookeriana Miq.	Pfeil et al. (2002)	–	–	–	AF384624
Thespesia thespesioides (R. Br. ex Benth.) Fryxell	Seelanan et al. (1997)	–	–	U56780	–
Thespesia thespesioides	Pfeil et al. (2002)	–	–	–	AF384625

NA not available

Species name, voucher information (reference for sequences taken from GenBank), seed supplier, country of origin for newly sequenced samples and GenBank accession numbers are given

0.2. In the case of analyses involving the *rpL16* region with SIC the temperature of the single heated chain was set to 0.5. Chains were sampled every 10,000 generations, and the respective trees were written into a tree file. Consensus trees and posterior probabilities of clades were calculated by combining the two runs and using the trees sampled after the chains converged. The inspection of the sump file created by MrBayes showed that (1) the chains had converged and that there was no tendency for the log likelihood values to decrease or increase over time, (2) that the standard deviation of split frequencies was below 0.01 upon completion of the analyses, (3) that the potential scale reduction factor for each of the parameters was in the range 0.999–1.001, and (4) that the effective sample size was above 500 for all parameters. These values guarantee that the number of generations was sufficient and that a good sample from the posterior probability distribution was obtained.

Results

The sequences for the ITS region had lengths between 626 bp for *A. esculentus* and *A. caillei* and 682–684 bp for the remaining specimens of *Abelmoschus*. This large difference in length is essentially due to a major deletion in the ITS1 region of *A. esculentus* and *A. caillei*, which affected base pairs 104–168 of the alignment. The *rpL16* region had lengths that varied between 1087 and 1122 bp due to several smaller indels. The final alignments had a length of 743 characters (789 with indel coding) for the ITS region and 1463 characters (1575 with indel coding) for the *rpL16* region. We found three different haplotypes for the 28 accessions of *A. esculentus* for the ITS region and three haplotypes for the *rpL16* region. The four accessions of *A. caillei* had two ITS haplotypes, but both haplotypes were shared with *A. esculentus* and the only *rpL16* haplotype of *A. caillei* was also shared with the majority of *A. esculentus* samples. *Abelmoschus manihot* and *A. moschatus* showed greater genetic and haplotype diversity than did *A. esculentus* for the ITS region, and *A. manihot* did as well for the *rpL16* region. Details regarding haplotype diversity and genetic distances are given in Table 2. The Phi-test did not find statistically significant evidence for reassortment in the combined ITS-*rpL16* dataset ($p = 1.0$) and the partition-homogeneity test did not show significant values for incongruence between the nuclear and the chloroplast dataset ($p = 0.25$). Based on these results we proceeded with a combined Bayesian analysis of the two regions in addition to analyses of the individual regions.

Table 2 Data on haplotype diversity and genetic distances for the three species of *Abelmoschus* based on at least four samples

Species	*A. caillei*	*A. esculentus*	*A. manihot*	*A. moschatus*
ITS Data				
ITS sample number	4	39	5	4
Haplotype number	2	3	4	3
Haplotype diversity	0.5	0.177	0.9	0.833
Θ_W	0.00084	0.00097	0.01648	0.00542
Genetic distance min–max	0–1	0–3	0–18	0–5
Genetic distance mean	0.5	0.4	10.3	3.8
rpL16 Data				
rpL16 sample number	4	39	5	3
Haplotype number	1	3	5	1
Haplotype diversity	0	0.141	1	0
Θ_W	0	0.00085	0.00263	0.0000
Genetic distance min–max	0	0–4	1–4	0
Genetic distance mean	0	0.24	2.8	0

The number of haplotypes, haplotype diversity, Θ_W, the minimum and maximum number of differences between sequences of each species and the mean number of differences between the samples of each species are given for ITS and *rpL16* separately. *A. moschatus* and, especially, *A. manihot* generally show higher diversity indices than *A. esculentus* regarding the ITS sequences. All accessions of *A. moschatus* had identical *rpL16* sequences

The Bayesian analyses of the sequence data all resolved *Abelmoschus* as a monophyletic group within the "*Hibiscus*"-clade with high confidence (pp = 1.00; Figs. 1, 2, 3). As can be suspected from the haplotype data, *A. caillei* is not separable based on our data from *A. esculentus*. Phylogenetic trees based on our analysis of ITS data indicate that *A. tuberculatus* is the sister-clade of *A. esculentus–A. caillei*, but analysis of the chloroplast data suggests a more complex scenario. From the maternal perspective, *A. tuberculatus* is sister to all other *Abelmoschus* taxa included in this study. *Abelmoschus tetraphyllus* is clearly separated from *A. manihot* but closely related to *A. ficulneus*. According to the ITS tree *A. crinitus* is sister to the *A. manihot–A. moschatus* clade but more isolated according to the *rpL16* tree. The relationship between *A. manihot* and *A. moschatus* is not fully resolved. The analyses suggest a sister-clade relationship (ITS tree, Fig. 1) or even that *A. manihot* is paraphyletic, with *A. moschatus* nested within (*rpL16* tree, Fig. 2). However, the pp values of this part of the *rpL16* tree are partly below 0.50 and therefore inconclusive. One sample, available as PI 639741 in the Germplasm Resources Information Network (GRIN) Database of the United States Department of Agriculture (2015) (http://www.ars-grin.gov/) and identified there as *A. moschatus* (but given here as *Abelmoschus* sp) is closely related to the *A. ficulneus–A. tetraphyllus* complex. This atypical sample had a much higher 1000-seed-weight (27.2 g) than the remaining samples originally identified as *A. moschatus* (8.2–17.8 g, Table 3), but within the range of values for *A. tetraphyllus* (26.8–31.0 g). In contrast, *A. ficulneus*, had a lower 1000-seed weight (17.6 g). All *A. esculentus* (48.2–72.6 g) and *A. caillei* (45.4–61.6 g) samples had high 1000-seed weights. *Abelmoschus manihot* seeds were in the range of 14.8–17.6 g, while the *A. tuberculatus* sample weighed 30.4 g, and, finally, *A. crinitus* weighed 14.4 g.

Chromosome numbers (Table 2) taken from the recompilations of Benchasri (2012), Hamon and van Sloten (1995) and Siemonsma (1982) tend to correlate with the seed weights, but the correlation is not perfect: for example, *A. tuberculatus*, the species with the lowest reported chromosome count, shows an intermediate value for 1000-seed weight.

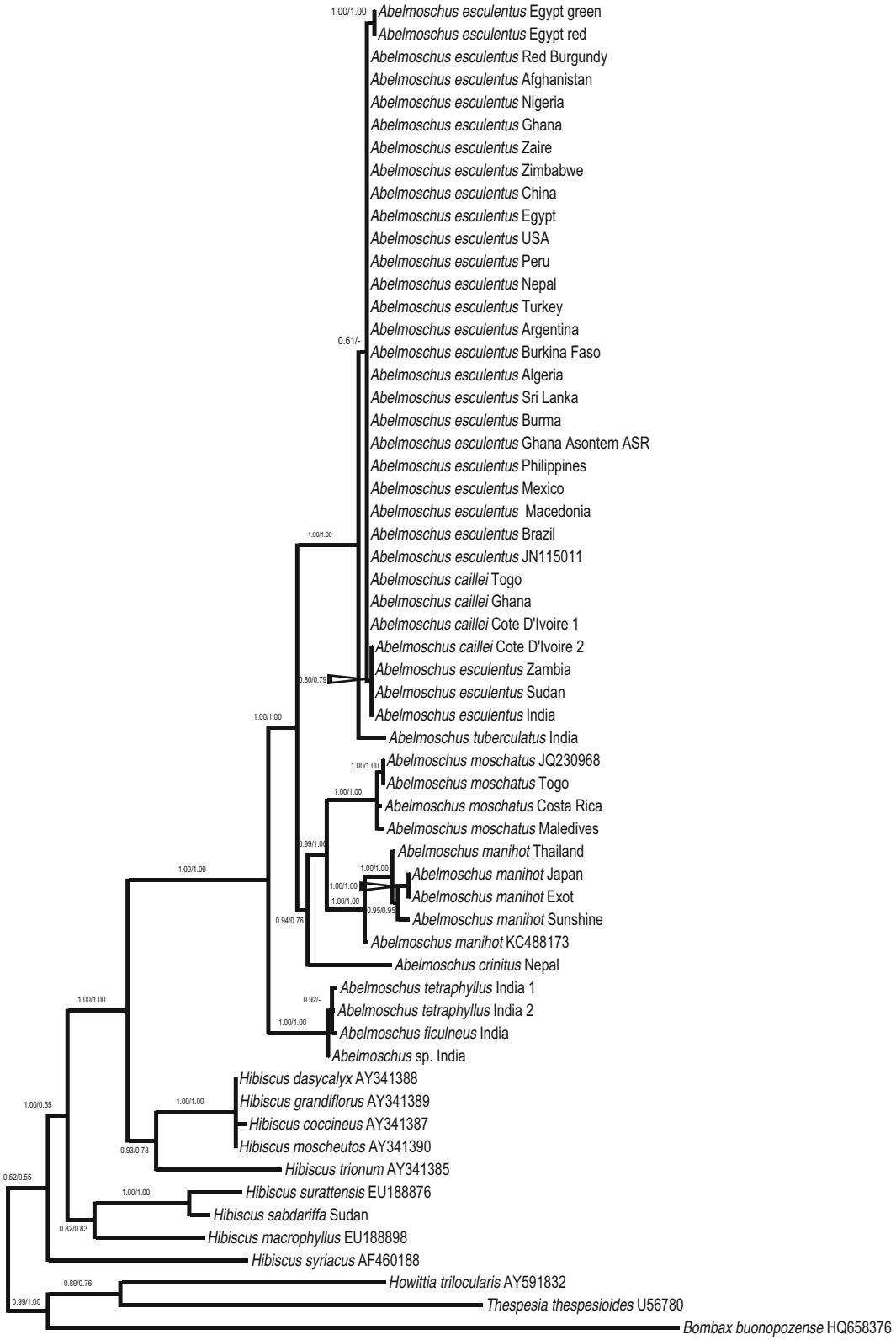
Discussion

The taxonomic position of *Abelmoschus*

All analyses resolve *Abelmoschus* as a well-supported clade within *Hibiscus*. But this still leaves open the question of the taxonomic treatment of *Abelmoschus*. If genera are to be monophyletic, one possibility is to maintain *Abelmoschus*, but in this case *Hibiscus* would need major taxonomic changes. Alternatively, *Abelmoschus* could be included within a broad *Hibiscus* genus. Pfeil et al. (2002) were already aware of this problem when they studied the molecular systematics of *Hibiscus* based on chloroplast sequence markers, but did not propose a solution. Unfortunately, both alternatives involve numerous name changes for many well-known and commonly cultivated plants (Pfeil et al. 2002; Pfeil and Crisp 2005). In such a situation, we think that changes should only be made when clear evidence makes it possible to draw very solid conclusions for the whole complex of taxa. Therefore, we believe that it is premature to propose generic name changes at this time.

The taxonomic position of *Abelmoschus tetraphyllus*

In the past, different authors treated this taxon as a variety or subspecies of *A. manihot* (Hochreutiner 1924; Pal et al. 1952; van Borssum-Waalkes 1966) or as a distinct species (Hamon and van Sloten 1995; Siemonsma 1982). Our data clearly indicate that this taxon should be treated at the species level. In addition, our phylogenetic analyses also suggest that *A. tetraphyllus* is more closely related to *ficulneus* than it is to *A. manihot*. Based solely on molecular data, it would be difficult to separate the species pair, *A. tetraphyllus-A. ficulneus*, but *A. tetraphyllus* is reported to have 130–138 chromosomes (Ugale et al. 1976; Joshi and Hardas 1976) whereas *A. ficulneus* has fewer (72–78; Hardas and Joshi 1954; Gadwal et al. 1968; Joshi et al. 1974), which argues in favor of two distinct species. Clearly, additional data from more samples and markers are needed to establish clear genetic relationships within the *A. tetraphyllus–A. ficulneus* clade.



◀**Fig. 1** Phylogram based on the Bayesian analysis of the nuclear ITS sequences including codified indels. Posterior probability values (PP) are given for the data with indel information and after the slash for the same data without indel information. *Abelmoschus* forms a monophyletic clade within the paraphyletic genus *Hibiscus*. *Bombax buonopozense*, *Howittia trilocularis* and *Thespesia thespesioides* were used to root the tree

The origin of *Abelmoschus esculentus*

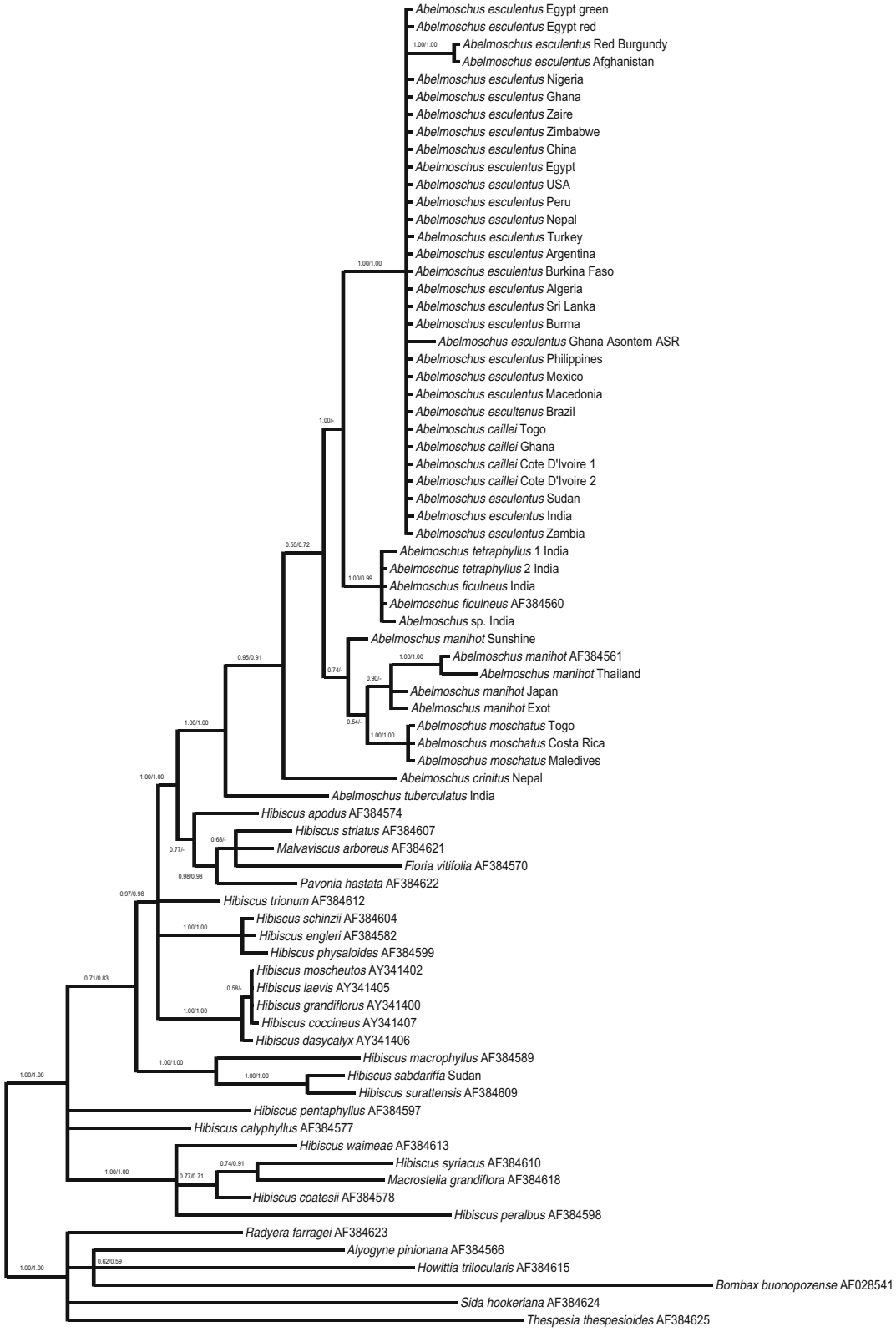
The origin of *A. esculentus* remains obscure in both senses, geographic and genetic. Our data do not elucidate a geographic origin for *A. esculentus* nor is much revealed about its initial parentage. One theory is that *A. esculentus* is of allopolyploid origin, whereby one parental species is close to *A. tuberculatus* and the other is unknown but similar to *A. ficulneus* (Siemonsma 1982; Hamon and van Sloten 1995). The ITS data (Fig. 1) suggest that *A. tuberculatus* is sister to the *A. esculentus–A. caillei* clade, but the phylogram derived from *rpL16* data (Fig. 2) present *A. tuberculatus* at a very distant position with respect to *A. esculentus–A. caillei*. This could be a consequence of hybridization, if an unknown female parent was responsible for the maternally inherited chloroplast genome that is now found in *A. esculentus*. At this point, there is no evidence that *A. ficulneus* (or any closely related species) was involved in the hypothetical allopolyploidization event. Interestingly, all ITS sequences were of good quality, and there was no need to use cloning procedures. At first sight, this may seem surprising in a putative allopolypoid, because the presence of both parental versions of the sequenced region would be expected, leading to complicated chromatograms (Soltis et al. 2008). But there are many well-documented cases where a rapid homogenization of sequence variants occurred after polyploidization events and where only one version of the region in question is now present or detectable by standard sequencing, although cloning may reveal a low number of copies that still retain an alternative version from the second parental contributor (reviewed in Soltis et al. 2008). One case treated in detail is the Malvacean genus *Gossypium* L. (cotton). Wendel et al. (1995) studied five allopolyploid species of this genus that carry the *A* and *D* genomes. In *Gossypium*, the rDNA is organized in arrays at four different *loci*, but interlocus-concerted evolution homogenized the sequences of the hybrids in both possible directions, and, as a result, some of the species derived from ancient hybridization have rDNA of type A and others of type D. This suggests that the apparent absence of two different versions of rDNA does not exclude a possible hybrid origin of *A. esculentus*.

Vavilov (1926) was the first to clearly link the origin of crop plants with (genetic) diversity, and, in recent decades, this concept has been much refined (reviewed in Engels et al. 2006). But the low degree of genetic diversity found among our samples of *A. esculentus* (despite the fact that we included all relevant regions where this species is cultivated) allowed us to make no conclusions regarding geographic origin. Martin et al. (1981) and Hamon and van Sloten (1989) reached similar conclusions based on morphological data when they compared collections of cultivars from different continents, because they could not find evidence for geographically correlated morphological variation.

We were to some degree surprised to find clearly higher levels of genetic diversity in *A. manihot* than in *A. esculentus.* To a lesser degree (only for ITS) this was also true for *A. moschatus*. However, this may be the consequence of the more advanced domestication process in *A. esculentus*; in *A. manihot* and *A. moschatus*, wild populations are widespread (Charrier 1984) and might exchange genetic material with cultivated plants as these species seem to be facultative outbreeders (Hamon and Koechlin 1991). It is known that the domestication process frequently leads to bottlenecks in genetic diversity (Buckler et al. 2001). Although diversity at the sequence level seems to be low in *A. esculentus*, fingerprinting techniques, like SRAP (Gulsen et al. 2007), RAPD (Aladele et al. 2008; Prakash et al. 2011) and AFLP (Salameh 2014), have been valuable in *A. esculentus* and *A. caillei* for showing sufficient variability to differentiate cultivars. The use of molecular techniques based on Next Generation Sequencing, recently used by Schafleitner et al. (2013) to develop simple sequence repeat markers form transcriptome data in *A. esculentus*, may also be productive as such techniques allow the sequencing of relevant parts of the genome with moderate financial investment.

The origin of *Abelmoschus caillei*

Siemonsma (1982) suggested that *A. caillei* is an amphidiploid, with *A. esculentus* and *A. manihot* as



◀**Fig. 2** Phylogram based on the Bayesian analysis of the chloroplast *rpL16* sequences including codified indels. PP are given for the data with indel information and after the slash for the same data without indel information. As in the case of the nuclear sequences, *Abelmoschus* forms a monophyletic clade within the paraphyletic genus *Hibiscus*. *Alyogyne pinionana, Bombax buonopozense*, *Howittia trilocularis, Radyera farragei, Sida hookeriana* and *Thespesia thespesioides* were used to root the tree

parental species. Kuwada (1957, 1961) produced a fertile amphidiploid resembling *A. caillei* after crossing these two putative parents. The F1 generation could be backcrossed with *A. esculentus* but the resulting plants had a reduced fertility. Backcrosses with *A. manihot* were more problematic. Siemonsma (1982) conducted similar crossing studies with *A. esculentus* (called Soudanien type) and *A. caillei* (called Guinéen type). Crosses could be produced readily, but hybrids had a strongly reduced fertility, with results generally in accordance with those obtained by Kuwada (1961). Nevertheless, other authors challenge this point of view; for example, Hamon and van Sloten (1995) stated that the origin of *A. caillei* through a hybridization involving *A. manihot* would be difficult to accept. While our data present clear evidence supporting the undisputed view that *A. esculentus* is very closely related to *A. caillei*, we found no evidence to suggest a close relation with *A. manihot*. But, again, this does not exclude such a relationship as *A. esculentus* might be the female parent contributing the chloroplast genome, and the rDNA of *A. esculentus* might have replaced by concerted evolution (Arnheim et al. 1980) almost all traces of the rDNA from the hypothetical male parent, *A. manihot*. As in the case of *A. esculentus*, the genetic diversity of *A. caillei* is very low (Θ_W 0.00084 for ITS and 0 for *rpl16*; low isoenzymic variability mentioned in Hamon and van Sloten 1995), indicating that the species may have passed through one or more genetic bottlenecks in the process of domestication.

One vexing problem with *Abelmoschus* is the scarcity of pertinent taxonomic work. Twenty years ago, Hamon and van Sloten (1995) noted that the taxonomy of *Abelmoschus* needs clarification and that phylogenetic relationships among its species were unclear. This situation (still unrectified) has led to confusion when identifying samples. For example, one of our samples, originally identified as *A. moschatus*, seems to be closely related to *A. tetraphyllus*. Generally characters of the epicalyx and the capsule are used to separate the species of *Abelmoschus* (Hamon and van Sloten 1995), but our data suggest that seed weight might be a fast and easy to measure characteristic that could possibly help identify species. Partly, these problems are also due to taxonomic changes that are not always apparent when using material available in genebanks. For example, two samples of *A. caillei* are catalogued as *A. manihot* in the Germplasm Resources Information Network Database of the United States Department of Agriculture. The paper of Stevels (1988), which formally described *A. caillei* was published after these two samples were received by the National Plant Germplasm System in 1985. Such situations are not always so simple to detect; thus, we remind researchers to be diligent about searching for similar discrepancies when working with samples from genebanks.

Concluding remarks

Although this study throws some light on our understanding of the systematics of *Abelmoschus*, it is evident that much more data are necessary to fully understand relationships among its species, their delimitation, and the possible roles of past hybridization events. There are two promising (not mutually exclusive) ways to proceed. The first consists of using advanced molecular techniques based on next generation sequencing, which could generate large amounts of data representing entire genomes. Protocols like Genotyping by Sequencing (Elshire et al. 2011) and RADSeq (Davey and Blaxter 2010), which allow to parallel studies of numerous samples, thus reducing costs, are very promising, and can be used to resolve problems related to the genetic diversity of closely related samples. The second way ahead focuses on studying more samples, especially of those taxa not treated here and others represented only by a small number of samples. If, for example, *A. tuberculatus* is as variable as *A. manihot*, it is possible that the ITS sequences of *A. esculentus*, are nested within the diversity of *A. tuberculatus*, demonstrating the parental status.

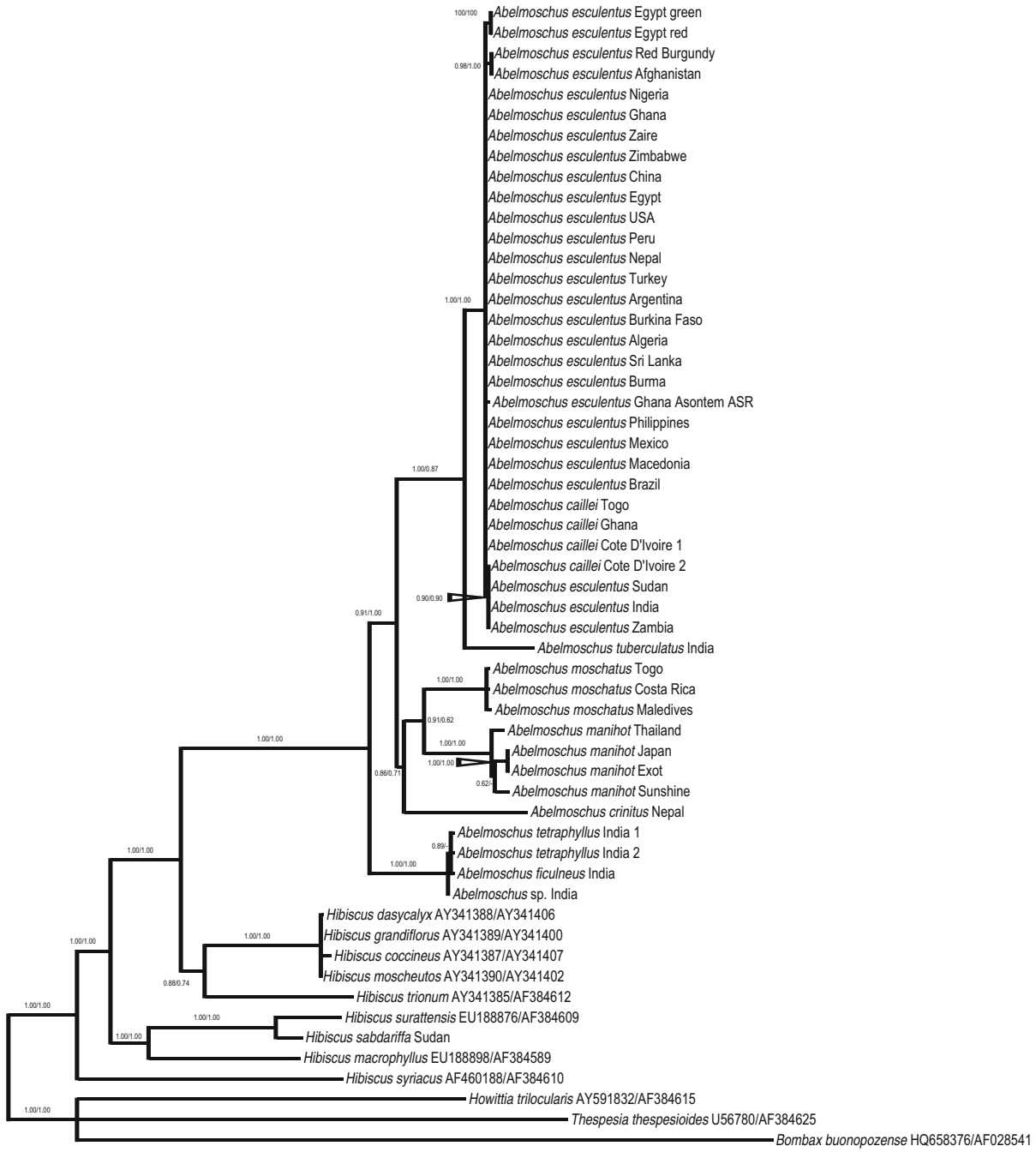


Fig. 3 Phylogram based on the Bayesian analysis of the combined nuclear ITS and chloroplast *rpL16* sequences including codified indels. The settings for the ITS and *rpL16* partitions were unlinked to allow rate differences across partitions. PP values are given for the data with indel information and after the slash for the same data without indel information. As in the case of the nuclear sequences, *Abelmoschus* forms a monophyletic clade within the paraphyletic genus *Hibiscus*. *Bombax buonopozense*, *Howittia trilocularis* and *Thespesia thespesioides* were used to root the tree

Table 3 1000 seed weight and chromosome numbers of *Abelmoschus* species

Species	1,000 seed weight (g)	Chromosome number
A. caillei	49.4–61.6	185–198
A. crinitus	14.4	NA
A. esculentus	48.2–72.6	62–144
A. ficulneus	17.6	72–78
A. manihot	14.8–17.6	60–68
A. moschatus	8.2–17.8	72
A. tetraphyllus	26.8–31.0	130–138
A. tuberculatus	30.4	58

A. crinitus, *A. ficulneus*, *A. manihot* and *A. moschatus* have a relatively low seed weight, *A. tetraphyllus* and *A. tuberculatus* have an intermediate seed weight and *A. caillei* and *A. esculentus* show the highest seed weight. Species with higher chromosome numbers tend to have higher seed weights, but *A. tuberculatus*, with a low chromosome number, shows an intermediate seed weight. The chromosome number of *A. crinitus* is not available

Modern, high-throughput techniques coupled with new interest in lost or orphan crops could drive rapid progress in the case of *Abelmoschus*. Hopefully in twenty years' time, there will be no need to repeat the statement made twenty years ago by Hamon and van Sloten (1995) that very little work has been done on okra.

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