

## Towards a Phylogeny for *Coffea* (Rubiaceae): Identifying Well-supported Lineages Based on Nuclear and Plastid DNA Sequences

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Received: 28 June 2007 Returned for revision: 25 July 2007 Accepted: 30 August 2007 Published electronically: 22 October 2007

- **Background and Aims** The phylogenetic relationships between species of *Coffea* and *Psilanthus* remain poorly understood, owing to low levels of sequence variation recovered in previous studies, coupled with relatively limited species sampling. In this study, the relationships between *Coffea* and *Psilanthus* species are assessed based on substantially increased molecular sequence data and greatly improved species sampling.
- **Methods** Phylogenetic relationships are assessed using parsimony, with sequence data from four plastid regions [*trnL-F* intron, *trnL-F* intergenic spacer (IGS), *rpl16* intron and *accD-psaI* IGS], and the internal transcribed spacer (ITS) region of nuclear rDNA (ITS 1/5.8S/ITS 2). Supported lineages in *Coffea* are discussed within the context of geographical correspondence, biogeography, morphology and systematics.
- **Key Results** Several major lineages with geographical coherence, as identified in previous studies based on smaller data sets, are supported. Other lineages with either geographical or ecological correspondence are recognized for the first time. *Coffea* subgenus *Baracoffea* is shown to be monophyletic, but *Coffea* subgenus *Coffea* is paraphyletic. Sequence data do not substantiate the monophyly of either *Coffea* or *Psilanthus*. Low levels of sequence divergence do not allow detailed resolution of relationships within *Coffea*, most notably for species of *Coffea* subgenus *Coffea* occurring in Madagascar. The origin of *C. arabica* by recent hybridization between *C. canephora* and *C. eugenioides* is supported. Phylogenetic separation resulting from the presence of the Dahomey Gap is inferred based on sequence data from *Coffea*.

**Key words:** Africa, *accD-psaI* IGS, *Coffea*, coffee, Indian Ocean Islands, ITS, Madagascar, molecular phylogeny, *rpl16* intron, Rubiaceae, *trnL-F* intron, *trnL-F* IGS.

### INTRODUCTION

The genus *Coffea* L. comprises 103 species (Davis *et al.*, 2006) and occurs naturally in tropical Africa, Madagascar, the Comoros and the Mascarenes (Mauritius and Reunion). *Coffea* species are mostly restricted to humid evergreen forest, although some species are found in seasonally dry deciduous forest and/or bushland. The most recent classifications of *Coffea* (Bridson, 1988a, b, 2003; Davis *et al.*, 2005, 2006) divide the genus into two subgenera: subgenus *Coffea* (95 spp.) and subgenus *Baracoffea* (J.-F. Leroy) J.-F. Leroy (eight spp.). *Coffea* subgenus *Coffea* occurs throughout the range of the genus, whereas *Coffea* subgenus *Baracoffea* is restricted to the seasonally dry forest and scrubland of western Madagascar (Davis *et al.*, 2005) and, according to Leroy (1982), NE Kenya and SE Somalia. *Coffea* subgenus *Coffea* includes the species used in the production of coffee, i.e. *C. arabica* (Arabica coffee), *C. canephora* (robusta coffee) and *C. liberica* (Liberian coffee). *Coffea arabica* is by far the most important traded species, and provides at least 65% of commercial production.

*Coffea arabica* is the only allotetraploid *Coffea* species ( $2n = 4x = 44$ ; Carvalho, 1952; Grassias and Kammacher, 1975); all other *Coffea* species are diploid ( $2n = 2x = 22$ ). *Coffea arabica* is also self-compatible (Carvalho *et al.*, 1991), thus far only reported in two other species: *C. heterocalyx* (Coulibaly *et al.*, 2002) and *C. anthonyi* ined. (P. Stoffelen, pers. comm.).

It is now well established that *Psilanthus* Hook.f. is the closest relative of *Coffea*. Davis *et al.* (2007) showed that these genera form a well supported monophyletic group within tribe Coffeae, based on molecular (BP [bootstrap percentage] 100; *b* [Bremer support value/decay value] = 9) and combined molecular–morphological data (BP 100; *b* = 13), for example. *Coffea* and *Psilanthus* share a unique carpel morphology: the endocarp (pyrene shell) is hard (horny/crustaceous), the pyrene (and seed) has a deep ventral invagination (i.e. ‘coffee bean’ morphology) and the seed coat consists of crushed endotestal cells with  $\pm$  isolated fibres (Robbrecht and Puff, 1986). *Psilanthus* comprises 22 species and occurs in tropical Africa, southern and SE Asia, and as far east as tropical northern Australia (Davis, 2003). *Psilanthus* is also divided into two subgenera (Bridson, 1988b): *Psilanthus*

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subgenus *Psilanthus* (two spp.), and *Psilanthus* subgenus *Afrocoffea* (Moens) Bridson (20 spp.). *Psilanthus* subgenus *Psilanthus* is restricted to tropical West and Central Africa, whereas subgenus *Afrocoffea* occurs throughout the range of the genus. The morphological characterization of *Coffea* and *Psilanthus* and their subgenera is reported in detail by Davis *et al.* (2005).

*Coffea* and *Psilanthus* have been the focus of several recent phylogenetic studies, using systematic data from various sources, including morphology (Stoffelen, 1998; Davis *et al.*, 2005), random amplified polymorphic DNA (RAPD) (Lashermes *et al.*, 1993), sequences from plastid DNA (Cros, 1994; Lashermes *et al.*, 1996; Cros *et al.*, 1998) and internal transcribed spacer (ITS) sequences of nuclear rDNA (Lashermes *et al.*, 1997). At the species level, the studies of Lashermes *et al.* (1997) and Cros *et al.* (1998) provided the most useful data.

On the basis of ITS2 data, Lashermes *et al.* (1997: 953, fig. 4) separated *Coffea* into four main geographical groups, although the bootstrap support for three of these groups was negligible to weak, i.e. Madagascar (BP 53), East Africa (BP 22) and Central Africa (BP 67), and West and Central Africa formed an unresolved group. Cros *et al.* (1998) used sequence data from the *trnL-trnF* intergenic spacer (IGS) region, and separated *Coffea* into five area groupings, four the same as Lashermes *et al.* (1997), but with better support values, i.e. Madagascar (BP 82), East Africa (BP 64), Central Africa (BP 100) and West and Central Africa (BP 41), plus a west Africa clade (BP 100). Cros *et al.* (1998) concluded that there was good agreement between their *trnL-trnF* data analysis and the ITS study of Lashermes *et al.* (1997), including the separation of *Coffea* species into the four major geographical groups as given above, but also noted that there was some conspicuous incongruence between the two data sets. The most notable incongruity was the position of *C. arabica* within a clade of central African taxa [i.e. *C. eugenioides*, and *C. sp.* 'Moloundou' (= *C. anthonyi* ined.); see Davis *et al.*, 2006] in their plastid DNA analysis (BP 100), compared with placement within a 'canephoroid' species group (*C. canephora*, *C. brevipes*, *C. congensis*; BP 53) with ITS2 (Lashermes *et al.*, 1997). Further to this observation (Cros *et al.*, 1998), Raina *et al.* (1998) used genomic *in situ* hybridization (GISH) and fluorescent *in situ* hybridization (FISH) to study the genome organization and evolution of *C. arabica*, and they concluded that *C. congensis* and *C. eugenioides* are the diploid progenitors of *C. arabica*. Using restriction fragment length polymorphism (RFLP) markers in combination with GISH data, Lashermes *et al.* (1999) suggested that *C. arabica* is an amphidiploid formed by hybridization between *C. eugenioides* and *C. canephora*, or ecotypes related to these diploid species. Although Raina *et al.* (1998) did not take into account the diversity of *C. canephora*, *C. congensis* and *C. arabica* (they used only one sample per species), their result is not in any real conflict given that *C. congensis* and *C. canephora* are genetically very similar (Lashermes *et al.*, 1997; Prakesh *et al.*, 2005).

The other topological inconsistencies identified by Cros *et al.* (1998) involved two taxa from West Africa,

*C. stenophylla* and *C. humilis*, and *C. sp.* 'X' (= *C. heterocalyx*; Coulibaly *et al.*, 2002, 2003), which were placed in different African groups for ITS and *trnL-trnF*. The plastid analysis of Cros *et al.* (1998) placed *C. stenophylla* as sister to *C. humilis*, and with the Central African, East African and Madagascan species (BP 35). *Coffea sp.* 'X' (= *C. heterocalyx*) was placed with species from West and Central Africa (BP 32; unresolved in relation to two accessions of *C. liberica*). The ITS2 analysis of Lashermes *et al.* (1997) placed *C. stenophylla* with species from West and Central Africa (BP 4), and *C. heterocalyx* with their Central Africa clade (BP = 67; unresolved in relation to *C. eugenioides* and *C. sp.* 'Moloundou' (= *C. anthonyi* ined.). Cros *et al.* (1998) suggested that these could be interpreted as the result of interspecies transfer of plastid DNA mediated by hybridization. However, with generally low levels of support, such discussions are speculative.

Owing to limited sequence divergence between *Coffea* and *Psilanthus* and the nested position of one *Psilanthus* species [*P. travancorensis* (Wight & Arn.) J.-F. Leroy] within *Coffea*, Lashermes *et al.* (1997) concluded that their ITS data did not support the recognition of two genera. On the basis of their *trnL-trnF* data, Cros *et al.* (1998) concurred with Lashermes *et al.* (1997) on this matter, although their tree topology shows an unresolved relationship between the two species of *Psilanthus* sampled (*P. mannii* and *P. ebracteolatus*) and *Coffea*. Cros *et al.* (1998) and Lashermes *et al.* (1997) did not include other representatives of Coffeae (cf. Davis *et al.*, 2007) as outgroups.

Despite recent advances in *Coffea* systematics, the phylogenetic relationships between species of *Coffea* and *Psilanthus* are still poorly understood. This is mostly due to low levels of sequence variation so far recovered, coupled with a relatively small taxon sample size. Lashermes *et al.* (1997) used 37 samples, including 22 species of *Coffea* (approx. 21 % of known species diversity) and three species of *Psilanthus* (approx. 14 % of species diversity); the number of variable characters (nuclear substitutions and indels) was not given. In the study of Cros *et al.* (1998), 26 samples were used, covering 18 species of *Coffea* (approx. 17 % of known species diversity) and two species of *Psilanthus* (approx. 9 % of species diversity); only 32 variable characters (26 nucleotide substitutions and six indels) were found within their study sample. The studies of Lashermes *et al.* (1997) and Cros *et al.* (1998) did not include any samples of *Coffea* subgenus *Baraccoffea* (eight spp.), Mascarene *Coffea* (at least three spp.) or the morphologically and geographically isolated (Bridson, 1979, 1983; Davis *et al.*, 2005) *C. rhannifolia*. Small sample size is mostly a problem of logistics, as wild *Coffea* and *Psilanthus* species are not well represented in cultivation, are often difficult to find in the wild and DNA suitable for PCR and sequencing is not easily isolated from herbarium specimens. In addition, numerous new species of *Coffea* have been discovered and/or described since 1998, including seven species from west and central tropical Africa (Stoffelen *et al.*, 1996a, b, 1997a, b; Cheek *et al.*, 2002; Sonké and Stoffelen,

2004; Sonké *et al.*, 2006b), two from East Africa (Davis and Mvungi, 2004) and 13 from Madagascar (Davis and Rakotonasolo, 2000, 2001a, b, 2003; Davis, 2001). Further new species are in the process of formal description and publication (Davis *et al.*, 2006; Table 1).

In the investigation reported here, plastid sequence data from the *trnL-F* intron, *trnL-F* IGS, *rpl16* intron and *accD-psaI* IGS, and ITS sequences of nuclear rDNA were analysed in an attempt to increase the number of molecular characters available for phylogenetic reconstruction. Better sampling of *Coffea* has been made possible due to recent collecting activities, and it was possible to examine approx. 83 % of total species diversity for the genus, and 32 % of total species diversity for *Psilanthus* (see Materials and Methods). The main objective of the study was to identify well-supported lineages within *Coffea*, and to discuss these within the contexts of geographical coherence, morphology, systematics and biogeography. Secondly, the aim was to elucidate consistently retrieved lineages within *Coffea* and well-supported lineages within *Psilanthus*, and to assess the relationship between *Coffea* and *Psilanthus*.

## MATERIALS AND METHODS

### *Taxon sampling and plant material*

As this study is concerned with assessing relationships above the rank of species, multiple species samples or infraspecific taxa were not used. Only two exceptions were made: a second sample of *C. heterocalyx* (IRD-Montpellier JC 66; voucher K) was included, which it is believed is the same as (or very similar to) that used by Lashermes *et al.* (1997) and Cros *et al.* (1998), and *C. liberica* var. *dewevrei*. *Coffea heterocalyx* is of considerable interest because it is reported to be self-compatible (Coulibaly *et al.*, 2002). N'Diaye *et al.* (2005) report that *C. liberica* var. *liberica* and *C. liberica* var. *dewevrei* have a high genetic differentiation ( $G_{st}$  0.25) with AFLP markers, and the pollen viability of  $F_1$  hybrids between them is low (44.2 %) and similar to interspecific hybrids, indicating that there are marked reproductive barriers between the two varieties. For these reasons, one sample of each variety of *C. liberica* was included.

The samples used in this study, with accepted taxon names, voucher information and GenBank accession numbers for the sequences, are given in Table 1. Most samples are of wild origin, with many collected during recent field expeditions to Madagascar (1997–2004), Tanzania (2001–2003) and Cameroon (in 2002). To complete the sampling, living material held in botanical gardens and coffee research stations, and some herbarium material (leaf samples or single seeds) taken from specimens held at K and BR (abbreviations after Holmgren *et al.*, 1990) were included. In total, 88 samples (86 spp.) of *Coffea* and seven samples (seven spp.) of *Psilanthus* were analysed. The sample includes representatives of all subgenera of *Coffea* and *Psilanthus*. Two species of the genus *Tricalysia* A. Rich ex DC. were used as outgroups, one from Madagascar and the other from Tanzania.

*Tricalysia* is a close relative of *Coffea* and a member of Coffeae (Andreasen and Bremer, 2000; Davis *et al.*, 2007).

The present *Coffea* taxon sampling includes a number of undescribed taxa, although these entities are well documented (Davis *et al.*, 2006) and appear to represent clearly defined species based on morphological data. Inclusion of undescribed species belonging to *Coffea* subgenus *Baracoffea* (Davis *et al.*, 2005) was necessary for testing the monophyly and systematic placement of the subgenus. The floristic study of Mascarene *Coffea* species by Leroy (1989) enumerated three native species of *Coffea*, *C. macrocarpa*, *C. myrtifolia* and *C. mauritiana*. A sample of *C. campaniensis* was also included, which Leroy (1989) placed in the synonymy of *C. mauritiana*.

Taxonomic details for all taxa (below generic rank) mentioned herein (such as author, place and date of publication, synonyms, distribution) follow the *World Rubiaceae Checklist* ([www.kew.org/wcsp/rubiaceae](http://www.kew.org/wcsp/rubiaceae)). More detailed information for *Coffea* species is given in Davis *et al.* (2006).

### *DNA extraction, amplification and sequencing*

Most of the DNA samples were obtained from silica-dried leaf material (Chase and Hills, 1991). The other DNA samples were extracted from seeds obtained from herbarium specimens; a single seed was used for each sample. DNA extraction was performed from a maximum of 0.3 g of silica-dried leaf material (or from one seed) using the  $2 \times$  CTAB method of Doyle and Doyle (1987). The DNA was purified on caesium chloride/ethidium bromide gradients ( $1.55 \text{ g mL}^{-1}$  density) and dialysed before inclusion in the DNA Bank at the Royal Botanic Gardens, Kew (<http://www.kew.org/data/dnaBank/homepage.html>). To avoid problems of PCR inhibition, all DNA samples were further purified using QIAquick purification columns (QIAGEN) following the manufacturer's protocol.

Amplification of the *trnL-F* region (the *trnL* intron and the *trnL-trnF* IGS), the *rpl16* intron, the *accD-psaI* (plastid DNA) IGS and ITS (nuclear encoded internal transcribed spacer) was performed using the primers listed in Table 2. Any ITS trace files showing evidence of heterogeneous ITS copies were cloned to isolate single sequences using the Promega pGem-T Easy Vector kit (catalogue no. A1360). The ITS region was then re-amplified from the transformed bacterial colonies using the M13 primers contained in the kit and a small portion of the colony as the DNA template. Amplification of *trnL-F* was carried out using primers c and f of Taberlet *et al.* (1991). For many taxa, the internal primers d and e also had to be used because of difficulty in amplifying the region as a single piece. Amplification of *rpl16* was carried out using primers 71F and 1661R of Jordan *et al.* (1996). For many taxa, the amplification of DNA using these primers was not satisfactory and so internal primers were designed on the basis of the first sequences in a conserved and GC-rich region suitable for amplification of *rpl16* in two fragments for Rubiaceae. The *accD-psaI* region was amplified using the primers ACCD-769 forward and PSA1-75 reverse from Mendenhall (1994). Two internal



TABLE 1. *Taxon accession data*

| Taxon  | Voucher                         | Source                   | <i>accD-psaI</i> | <i>rpl16</i> | <i>trnL-F</i> | <i>ITS</i>                         |
|--|---------------------------------|--------------------------|------------------|--------------|---------------|------------------------------------|
| <i>Coffea abbayesii</i> J.-F. Leroy                                      | Davis 2334 (K)                  | Madagascar               | DQ153438         | DQ153687     | DQ153805      | DQ153566                           |
| <i>Coffea ambongensis</i> J.-F. Leroy ex A.P. Davis & Rakotonas., ined.  | Davis 2509 (K)                  | Madagascar               | DQ153419         | DQ153668     | DQ153786      | DQ153539/<br>DQ153540/<br>DQ153541 |
| <i>Coffea andrambovatensis</i> J.-F. Leroy                               | Davis 2322 (K)                  | Madagascar               | DQ153422         | DQ153671     | DQ153789      | DQ153545                           |
| <i>Coffea anthonyi</i> Stoff. & F. Anthony, ined. (C. sp. 'Moloundou')   | IRD-Montpellier OE 53 (K)       | DR Congo                 | DQ153489         | DQ153738     | DQ153856      | DQ153620                           |
| <i>Coffea ankaranensis</i> J.-F. Leroy ex A.P. Davis & Rakotonas.        | Davis 2331 (K)                  | Madagascar               | DQ153407         | DQ153656     | DQ153774      | DQ153527                           |
| <i>Coffea arabica</i> L.   | Jaufeeraly-Fakim 29 (K)         | Mascarenes (Introduced)  | DQ153478         | DQ153727     | DQ153845      | DQ153609                           |
| <i>Coffea arenesiana</i> J.-F. Leroy                                     | Davis 2207 (K)                  | Madagascar               | DQ153440         | DQ153689     | DQ153807      | DQ153568                           |
| <i>Coffea augagneuri</i> Dubard  | Davis 2220 (K)                  | Madagascar               | DQ153433         | DQ153682     | DQ153800      | DQ153561                           |
| <i>Coffea bakossii</i> Cheek & Bridson                                   | Lane 361 (K)                    | Cameroon                 | DQ153468         | DQ153717     | DQ153835      | DQ153599                           |
| <i>Coffea bertrandii</i> A. Chev.  | Davis 2348 (K)                  | Madagascar               | DQ153424         | DQ153673     | DQ153791      | DQ153549                           |
| <i>Coffea betamponensis</i> Portères & J.-F. Leroy                       | Davis 2300 (K)                  | Madagascar               | DQ153421         | DQ153670     | DQ153788      | DQ153543/<br>DQ153544              |
| <i>Coffea boinensis</i> A.P. Davis & Rakotonas., ined.                   | Davis 2502 (K)                  | Madagascar               | DQ153408         | DQ153657     | DQ153775      | DQ153528                           |
| <i>Coffea boiviniana</i> (Baill.) Drake                                  | Davis 2231 (K)                  | Madagascar               | DQ153426         | DQ153675     | DQ153793      | DQ153551/<br>DQ153552/<br>DQ153553 |
| <i>Coffea brevipes</i> Heirn   | Maurin 8 (K)                    | Cameroon                 | DQ153460         | DQ153709     | DQ153827      | DQ153591                           |
| <i>Coffea bridsoniae</i> A.P. Davis & Mvungi                             | Davis 2904 (K)                  | Tanzania                 | DQ153455         | DQ153704     | DQ153822      | DQ153584/<br>DQ153585/<br>DQ153586 |
| <i>Coffea buxifolia</i> A. Chev.   | Rakotonasolo 69 (K, TAN)        | Madagascar               | DQ153442         | DQ153691     | DQ153809      | DQ153570                           |
| <i>Coffea campaniensis</i> J.-F. Leroy                                   | Leroy 55 (K)                    | Mascarenes (Mauritius)   | DQ153470         | DQ153719     | DQ153837      | DQ153601                           |
| <i>Coffea canephora</i> Pierre ex Froehn.                                | Maurin 21 (K)                   | Cameroon (cultivated)    | DQ153462         | DQ153711     | DQ153829      | DQ153593                           |
| <i>Coffea commersoniana</i> (Baill.) A. Chev.                            | Davis 2715 (K)                  | Madagascar               | DQ153432         | DQ153681     | DQ153799      | DQ153560                           |
| <i>Coffea congensis</i> A. Froehn.                                       | Harris & Fay 1507 (BR, K, MO)   | Cameroon                 | DQ153467         | DQ153716     | DQ153834      | DQ153598                           |
| <i>Coffea costatifructa</i> Bridson                                      | ORSTOM 08 117 (K)               | Tanzania                 | DQ153473         | DQ153722     | DQ153840      | DQ153604                           |
| <i>Coffea coursiana</i> J.-F. Leroy                                      | Davis 2278 (K)                  | Madagascar               | DQ153417         | DQ153666     | DQ153784      | DQ153537                           |
| <i>Coffea decaryana</i> J.-F. Leroy                                      | Davis 1537 (K)                  | Madagascar               | DQ153429         | DQ153678     | DQ153796      | DQ153556                           |
| <i>Coffea dubardii</i> Jum.  | Davis 2216 (K)                  | Madagascar               | DQ153435         | DQ153684     | DQ153802      | DQ153563                           |
| <i>Coffea eugenioides</i> S.Moore  | Harley 9332 (BR, K)             | Tanzania                 | DQ153457         | DQ153706     | DQ153824      | DQ153588                           |
| <i>Coffea fadenii</i> Bridson  | Mvungi 9 (DSM, K)               | Tanzania                 | DQ153446         | DQ153695     | DQ153813      | DQ153574                           |
| <i>Coffea farafanganensis</i> J.-F. Leroy                                | Davis 2317 (K)                  | Madagascar               | DQ153405         | DQ153654     | DQ153772      | DQ153525                           |
| <i>Coffea grevei</i> Drake ex A. Chev.                                   | Davis 2566 (K)                  | Madagascar               | DQ153414         | DQ153663     | DQ153781      | DQ153534                           |
| <i>Coffea heimii</i> J.-F. Leroy   | Davis 2241 (K)                  | Madagascar               | DQ153431         | DQ153680     | DQ153798      | DQ153558/<br>DQ153559              |
| <i>Coffea heterocalyx</i> Stoff.   | Maurin 23 (K)                   | Cameroon                 | DQ153463         | DQ153712     | DQ153830      | DQ153594                           |
| <i>Coffea heterocalyx</i> Stoff.   | IRD-Montpellier JC 66 (K)       | ?Cameroon/ DR Congo)     | DQ153492         | DQ153741     | DQ153859      | DQ153623                           |
| <i>Coffea homollei</i> J.-F. Leroy                                       | Davis 2305 (K)                  | Madagascar               | DQ153402         | DQ153651     | DQ153769      | DQ153521                           |
| <i>Coffea humbertii</i> J.-F. Leroy                                      | Rakotonasolo 50 (K, TAN)        | Madagascar               | DQ153437         | DQ153686     | DQ153804      | DQ153565                           |
| <i>Coffea humblotiana</i> Baill.   | Davis 2327 (K)                  | Madagascar               | DQ153411         | DQ153660     | DQ153778      | DQ153531                           |
| <i>Coffea humilis</i> A. Chev.   | Bamps 1967 (BR)                 | Ivory Coast              | DQ153480         | DQ153729     | DQ153847      | DQ153611                           |
| <i>Coffea kapakata</i> (A. Chev.) Bridson                                | Hepper & Maley 7723 (K)         | Angola                   | DQ153465         | DQ153714     | DQ153832      | DQ153596                           |
| <i>Coffea kianjavatensis</i> J.-F. Leroy                                 | Davis 2313 (K)                  | Madagascar               | DQ153482         | DQ153731     | DQ153849      | DQ153613                           |
| <i>Coffea kihansiensis</i> A.P. Davis & Mvungi                           | Mvungi 21 (DSM, K)              | Tanzania                 | DQ153454         | DQ153703     | DQ153821      | DQ153583                           |
| <i>Coffea kimbozensis</i> Bridson  | Mvungi 6 (DSM, K)               | Tanzania                 | DQ153447         | DQ153696     | DQ153814      | DQ153575                           |
| <i>Coffea kivuensis</i> Lebrun   | Lebrun 5539 (BR)                | Zaire                    | DQ153481         | DQ153730     | DQ153848      | DQ153612                           |
| <i>Coffea pterocarpa</i> A.P. Davis & Rakotonas., ined.                  | Davis 2519 (K)                  | Madagascar               | DQ153425         | DQ153674     | DQ153792      | DQ153550                           |
| <i>Coffea labatii</i> A.P. Davis & Rakotonas., ined.                     | Davis 3069 (K)                  | Madagascar               | DQ153499         | DQ153748     | DQ153866      | DQ153630                           |
| <i>Coffea lancifolia</i> A. Chev.  | Davis 2307 (K)                  | Madagascar               | DQ153403         | DQ153652     | DQ153770      | DQ153522                           |
| <i>Coffea leroyi</i> A.P. Davis  | Davis 2311 (K)                  | Madagascar               | DQ153404         | DQ153653     | DQ153771      | DQ153523/<br>DQ153524              |
| <i>Coffea liaudii</i> J.-F. Leroy ex A.P. Davis                          | Rakotonasolo 61 (K, TAN)        | Madagascar               | DQ153434         | DQ153683     | DQ153801      | DQ153562                           |
| <i>Coffea liberica</i> var. <i>liberica</i> Bull. ex Hiern               | Billiet 19370062 (BR)           | DR Congo                 | DQ153479         | DQ153728     | DQ153846      | DQ153610                           |
| <i>Coffea liberica</i> var. <i>dewerei</i> (De Wild. & T. Durand) Lebrun | Hepper & Maley 7729 (BR, K, MO) | Central African Republic | DQ153472         | DQ153721     | DQ153839      | DQ153603                           |
| <i>Coffea littoralis</i> A.P. Davis & Rakotonas.                         | Rakotonasolo 261 (K)            | Madagascar               | DQ153441         | DQ153690     | DQ153808      | DQ153569                           |

Continued

TABLE 1. *Continued*

| Taxon  | Voucher                                | Source                    | <i>accD-psal</i> | <i>rpl16</i> | <i>trnL-F</i> | <i>ITS</i>                         |
|--|--|---------------------------|------------------|--------------|---------------|------------------------------------|
| <i>Coffea lulandoensis</i> Bridson                                 | Mvungi 2 (DSM, K)                      | Tanzania                  | DQ153452         | DQ153701     | DQ153819      | DQ153580                           |
| <i>Coffea macrocarpa</i> A. Rich.                                  | Gueho 18555 (K)                        | Mascarenes<br>(Mauritius) | DQ153471         | DQ153720     | DQ153838      | DQ153602                           |
| <i>Coffea mapiana</i> Sonké, Nguembou & A.P. Davis                 | Sonké 3694 (K, YA)                     | Cameroon                  | DQ153509         | DQ153758     | DQ153876      | DQ153640                           |
| <i>Coffea mangoroensis</i> Portères                                | Rakotonasolo 41 (K, TAN)               | Madagascar                | DQ153503         | DQ153752     | DQ153870      | DQ153634                           |
| <i>Coffea manombensis</i> A.P. Davis                               | Davis 2141 (K)                         | Madagascar                | DQ153445         | DQ153694     | DQ153812      | DQ153573                           |
| <i>Coffea mauritiana</i> Lam.                                      | Friedmann 1267 (K)                     | Mascarenes<br>(Reunion)   | DQ153469         | DQ153718     | DQ153836      | DQ153600                           |
| <i>Coffea mayombensis</i> A. Chev.                                 | Maurin 16 (K)                          | Cameroon                  | DQ153461         | DQ153710     | DQ153828      | DQ153592                           |
| <i>Coffea mcphersonii</i> A.P. Davis & Rakotonas.                  | Davis 2339 (K)                         | Madagascar                | DQ153423         | DQ153672     | DQ153790      | DQ153546/<br>DQ153547/<br>DQ153548 |
| <i>Coffea millotii</i> J.-F. Leroy                                 | Davis 2306 (K)                         | Madagascar                | DQ153409         | DQ153658     | DQ153776      | DQ153529                           |
| <i>Coffea mongensis</i> Bridson                                    | Mvungi 11 (DSM, K)                     | Tanzania                  | DQ153448         | DQ153697     | DQ153815      | DQ153576                           |
| <i>Coffea montekupensis</i> Stoff.                                 | Davis 3010 (K)                         | Cameroon                  | DQ153459         | DQ153708     | DQ153826      | DQ153590                           |
| <i>Coffea montis-sacri</i> A.P. Davis                              | Davis 2308 (K)                         | Madagascar                | DQ153430         | DQ153679     | DQ153797      | DQ153557                           |
| <i>Coffea moratii</i> J.-F. Leroy ex A.P. Davis & Rakotonas.       | Davis 2326 (K)                         | Madagascar                | DQ153502         | DQ153751     | DQ153869      | DQ153633                           |
| <i>Coffea mufindiensis</i> Hutch. ex Bridson                       | Mvungi 19 (DSM, K)                     | Tanzania                  | DQ153449         | DQ153698     | DQ153816      | DQ153577                           |
| <i>Coffea myrtifolia</i> (A. Rich. ex DC.) J.-F. Leroy             | Jaufeerally-Fakim 22 (K)               | Mascarenes<br>(Mauritius) | DQ153477         | DQ153726     | DQ153844      | DQ153608                           |
| <i>Coffea perrieri</i> Drake ex Jum. & H.Perrier                   | Davis 1174 (K)                         | Madagascar                | DQ153500         | DQ153749     | DQ153794      | DQ153631                           |
| <i>Coffea pervillenana</i> (Baill.) Drake                          | Davis 2328 (K)                         | Madagascar                | DQ153412         | DQ153661     | DQ153779      | DQ153532                           |
| <i>Coffea pocsii</i> Bridson                                       | Mvungi 7 (DSM, K)                      | Tanzania                  | DQ153453         | DQ153702     | DQ153820      | DQ153581/<br>DQ153582              |
| <i>Coffea pseudozanguebariae</i> Bridson                           | Mvungi 16 (DSM, K)                     | Tanzania                  | DQ153450         | DQ153699     | DQ153817      | DQ153578                           |
| <i>Coffea racemosa</i> Lour.                                       | Hepper & Maley 7717<br>(BR, K)         | Mozambique                | DQ153464         | DQ153713     | DQ153831      | DQ153595                           |
| <i>Coffea rakotonasoloi</i> A.P. Davis                             | Davis 2265 (K)                         | Madagascar                | DQ153416         | DQ153665     | DQ153783      | DQ153536                           |
| <i>Coffea ratsimamangae</i> J.-F. Leroy ex A.P. Davis & Rakotonas. | Davis 2240 (K)                         | Madagascar                | DQ153444         | DQ153693     | DQ153811      | DQ153572                           |
| <i>Coffea resinosa</i> (Hook.f.) Radlk.                            | Davis 1103 (K)                         | Madagascar                | DQ153428         | DQ153677     | DQ153795      | DQ153555                           |
| <i>Coffea rhamnifolia</i> (Chiov.) Bridson                         | Friis <i>et al.</i> 4908<br>(K, BR, P) | Somalia                   | DQ153458         | DQ153707     | DQ153825      | DQ153589                           |
| <i>Coffea richardii</i> J.-F. Leroy                                | Davis 2253 (K)                         | Madagascar                | DQ153415         | DQ153664     | DQ153782      | DQ153535                           |
| <i>Coffea sahafaryensis</i> J.-F. Leroy                            | Davis 2345 (K)                         | Madagascar                | DQ153413         | DQ153662     | DQ153780      | DQ153533                           |
| <i>Coffea sakarahae</i> J.-F. Leroy                                | Davis 2167 (K)                         | Madagascar                | DQ153439         | DQ153688     | DQ153806      | DQ153567                           |
| <i>Coffea salvatrix</i> Swynn. & Philipson                         | IRD-Montpellier LA 51 (K)              | Mozambique                | DQ153491         | DQ153740     | DQ153858      | DQ153622                           |
| <i>Coffea sambavensis</i> J.-F. Leroy ex A.P. Davis & Rakotonas.   | Davis 2323 (K)                         | Madagascar                | DQ153418         | DQ153667     | DQ153785      | DQ153538                           |
| <i>Coffea schliebenii</i> Bridson                                  | Mbago 2256 (DSM)                       | Tanzania                  | DQ153456         | DQ153705     | DQ153823      | DQ153587                           |
| <i>Coffea sessiliflora</i> Bridson                                 | Mvungi 25 (DSM, K)                     | Tanzania                  | DQ153451         | DQ153700     | DQ153818      | DQ153579                           |
| <i>Coffea anthonyi</i> Stoff. & F. Anthony, ined.                  | IRD-Montpellier OE 53 (K)              | DR Congo                  | DQ153489         | DQ153738     | DQ153856      | DQ153620                           |
| <i>Coffea</i> sp. 'G' (FTEA)                                       | Mabberley 1417 (K)                     | Tanzania                  | DQ153474         | DQ153723     | DQ153841      | DQ153605                           |
| <i>Coffea stenophylla</i> G.Don                                    | Hepper & Maley 7723 (K)                | Ivory Coast               | DQ153466         | DQ153715     | DQ153833      | DQ153597                           |
| <i>Coffea tetragona</i> Jum. & H.Perrier                           | Davis 2318 (K)                         | Madagascar                | DQ153406         | DQ153655     | DQ153773      | DQ153526                           |
| <i>Coffea togoensis</i> Jum. & H.Perrier                           | Hall & Abbins 43367 (K)                | Togo                      | DQ153476         | DQ153725     | DQ153843      | DQ153607                           |
| <i>Coffea tsirananae</i> J.-F. Leroy                               | Davis 2215 (K)                         | Madagascar                | DQ153443         | DQ153692     | DQ153810      | DQ153571                           |
| <i>Coffea vianneyi</i> J.-F. Leroy                                 | Davis 2320 (K)                         | Madagascar                | DQ153436         | DQ153685     | DQ153803      | DQ153564                           |
| <i>Coffea vatovavyensis</i> J.-F. Leroy                            | Davis 2316 (K)                         | Madagascar                | DQ153410         | DQ153659     | DQ153777      | DQ153530                           |
| <i>Coffea zanguebariae</i> Lour.                                   | Groenendijk 884 (K)                    | Mozambique                | DQ153475         | DQ153724     | DQ153842      | DQ153606                           |
| <i>Psilanthus bridsoniae</i> Sivar., Biju & P.Mathew               | Biju & Sasi 44800 (K)                  | India                     | DQ153397         | DQ153646     | DQ153764      | DQ153516                           |
| <i>Psilanthus ebracteolatus</i> Heirn                              | Davis 3008 (K)                         | Cameroon                  | DQ153392         | DQ153641     | DQ153759      | DQ153510                           |
| <i>Psilanthus mannii</i> Hook.f.                                   | Maurin 1 (K)                           | Cameroon                  | DQ153393         | DQ153642     | DQ153760      | DQ153511                           |
| <i>Psilanthus sapinii</i> De Wild.                                 | Sapin s.n. (BR 0856914)                | Congo-Kinshasa            | DQ153394         | DQ153643     | DQ153761      | DQ153512                           |
| <i>Psilanthus semsei</i> Bridson                                   | Kisera 1473 (K)                        | Tanzania                  | DQ153395         | DQ153644     | DQ153762      | DQ153513                           |
| <i>Psilanthus</i> sp. 'A' (FTEA)                                   | Luke 10197 (K)                         | Tanzania                  | DQ153399         | DQ153648     | DQ153766      | DQ153518                           |
| <i>Psilanthus travancorensis</i> (Wight & Arn.) J.-F. Leroy        | Biju s.n. (K)                          | India                     | DQ153398         | DQ153647     | DQ153765      | DQ153517                           |
| <i>Tricalysia</i> sp.  | Davis 2173 (K)                         | Madagascar                | DQ153400         | DQ153649     | DQ153767      | DQ153519                           |
| <i>Tricalysia verdcourtiana</i> Robbr.                             | Mvungi 43 (DSM, K)                     | Tanzania                  | DQ153401         | DQ153650     | DQ153768      | DQ153520                           |

Herbarium abbreviations after Holmgren *et al.* (1990). Where several ITS types were isolated these are listed below with multiple GenBank accession numbers.

TABLE 2. Amplification primers for *trnL-F*, ITS, *rpl16* and *accD-psa1*

| Locus                | Primer                   | Primer sequence                     | Reference                     |
|----------------------|--------------------------|-------------------------------------|-------------------------------|
| <i>trnL</i> intron   | Forward (c)              | 5'-CGAAATCGGTAGACGCTACG-3'          | Taberlet <i>et al.</i> (1991) |
|                      | Reverse (d)              | 5'-GGGGATAGAGGGACTTGAAC-3'          |                               |
| <i>trnL-F</i> IGS    | Forward (e)              | 5'-GGTTCAAGTCCCTCTATCCC-3'          | Taberlet <i>et al.</i> (1991) |
|                      | Reverse (f)              | 5'-ATTTGAACCTGGTGACACGAG-3'         |                               |
| ITS                  | Forward (17SE or 101)    | 5'-ACGAATTCATGGTCCGGTGAAGTGTTCG-3'  | Sun <i>et al.</i> (1994)      |
|                      | Reverse (26SE or 102)    | 5'-TAGAATTCCTCCGGTTCGCTCGCCGTTAC-3' |                               |
|                      | Internal reverse (ITS 2) | 5'-GCTGCGTTCCTCATCGATGC-3'          | White (1990)                  |
|                      | Internal forward (ITS 3) | 5'-GCATCGATGAAGAACGCAGC-3'          |                               |
| <i>rpl16</i>         | Forward (F71)            | 5'-GCTATGCTTAGTGTGTGACTCGTTG-3'     | Jordan <i>et al.</i> (1996)   |
|                      | Reverse (R1661)          | 5'-CGTACCCATATTTTTCCACCECGAC-3'     |                               |
|                      | Internal forward         | 5'-GTAAGAAGTGTGAGGAAACGA-3'         | Designed at Kew               |
|                      | Internal reverse         | 5'-TCGTTCCCATCACTTCTTAC-3'          |                               |
| <i>accD-psa1</i> IGS | Forward (ACCD 769 F)     | 5'-GGAAGTTTGAGCTTTATGCAAATGG-3'     | Mendenhall (1994)             |
|                      | Reverse (PSA1 75 R)      | 5'-AGAAGCCATTGCAATTGCCGAAA-3'       |                               |
|                      | Internal forward Coffeae | 5'-GCTAAAAATCTCTSTTGGTTCGG-3'       | Designed at Kew               |
|                      | Internal reverse Coffeae | 5'-CCGAACCAASAGAGATTTTTAGC-3'       |                               |

primers were again designed to obtain satisfactory PCR products for the recalcitrant specimens.

The PCR program for *trnL-F* consisted of 2 min at 94 °C followed by 28 cycles of 1 min at 94 °C (denaturation), 1 min at 50 °C (annealing) and 1 min at 72 °C (extension), followed by a final 7 min extension (72 °C). For *rpl16*, the PCR program used was 2 min at 94 °C followed by 30 cycles of 1 min 94 °C, 1 min at 52 °C and 3 min at 72 °C, with a final extension at 72 °C for 7 min. The *accD-psa1* region was amplified using the PCR program: a denaturation phase of 3 min (94 °C), followed by 30 cycles of 1 min at 94 °C, 1 min at 51 °C and 1 min at 72 °C, and a final extension of 72 °C for 5 min. The ITS region was amplified using primer 17SE forward and 26SE reverse from Sun *et al.* (1994). Dimethylsulfoxide (DMSO; 4.5 %) was used to reduce secondary structure problems common with nuclear DNA.

A PCR mastermix containing 2.5 mM MgCl<sub>2</sub> (Advanced Biotechnologies, Ltd) was used for *trnL-F* and *rpl16* amplifications. For *accD-psa1*, commercial mastermix did not give good amplifications, and so a pre-mix was prepared using Biotaq DNA polymerase (Bioline, UK), 10× NH<sub>4</sub> reaction buffer (Bioline, UK), 50 mM MgCl<sub>2</sub> and dNTPs (Promega, Maddison, WI, USA). Amplified products were purified using QIAquick purification columns (QIAGEN) as described in the manufacturer's protocol. Cycle sequencing reactions were carried out using BigDye™ Terminator Mix (Applied Biosystems, Inc., Warrington, Cheshire, UK). The program consisted of 26 cycles of: 10 s denaturation (96 °C), 5 s annealing (50 °C) and 4 min elongation (60 °C). PCR and sequencing reactions were run using a Perkin-Elmer GenAMP™ model 9600 or 9700 PCR system, and sequencing products were run on either an ABI 3100 Genetic Analyzer or an ABI 377 automated sequencer according to the manufacturer's protocols (Applied Biosystems, Inc.). Electropherograms were edited and assembled into contigs using Sequencher version 3.2.2. (Gene Codes Corp., Ann Arbor, MI, USA). The sequences generated were submitted to GenBank using the Sequin Application (version 5.26; available from <http://www.ncbi.nlm.nih.gov/Sequin/>).

#### Data matrix composition and parsimony analysis

All sequences were aligned manually in PAUP\* (version 4.0b10; Swofford, 2002) without difficulty due to low levels of sequence variation. Areas of ambiguous alignment were excluded from the analysis, as were regions with missing sequences, for example the beginning and end of sequences and around the internal primer-binding sites.

Maximum parsimony was implemented to analyse (a) *trnL-F*, (b) *rpl16*, (c) *accD-psa1*, (d) combined plastid data, (e) ITS and (f) combined sequence data, using PAUP\*. In all analyses, gaps were treated as missing data and characters were equally weighted and unordered (Fitch, 1971). All data sets were analysed separately and examined by eye in order to identify topological conflict, i.e. moderate to strong support for placement of a taxon in different clades. Thirteen insertions/deletions were identified and scored for the plastid DNA regions.

Tree searches were conducted using 10 000 replicates of random taxon sequence addition, retaining ten trees at each step, with tree-bisection-reconnection (TBR) branch swapping, delayed transformation (DELTRAN) optimization, MulTrees in effect, and saving a maximum of ten trees per replicate. Support for clades in all analyses was estimated using bootstrap analysis (Felsenstein, 1985), with 10 000 replicates of full heuristic search, simple sequence addition, TBR swapping, with MulTrees in effect and saving a maximum of ten trees per replicate. BPs are described as strongly/well supported (85–100 %), moderate (75–84 %) or low (50–74 %). Support was also estimated by calculating Bremer support values (b) (Bremer, 1988, 1994; Källersjö *et al.*, 1992), otherwise known as decay values. These were obtained using PAUP\* (Swofford, 2002), in conjunction with AutoDecay 4.0.2 (Eriksson, 1999), with 100 replicates of random addition for each constraint tree.

#### Map construction

Figure 1 is based on the distribution of individual species as recorded in an African *Coffea* specimen database

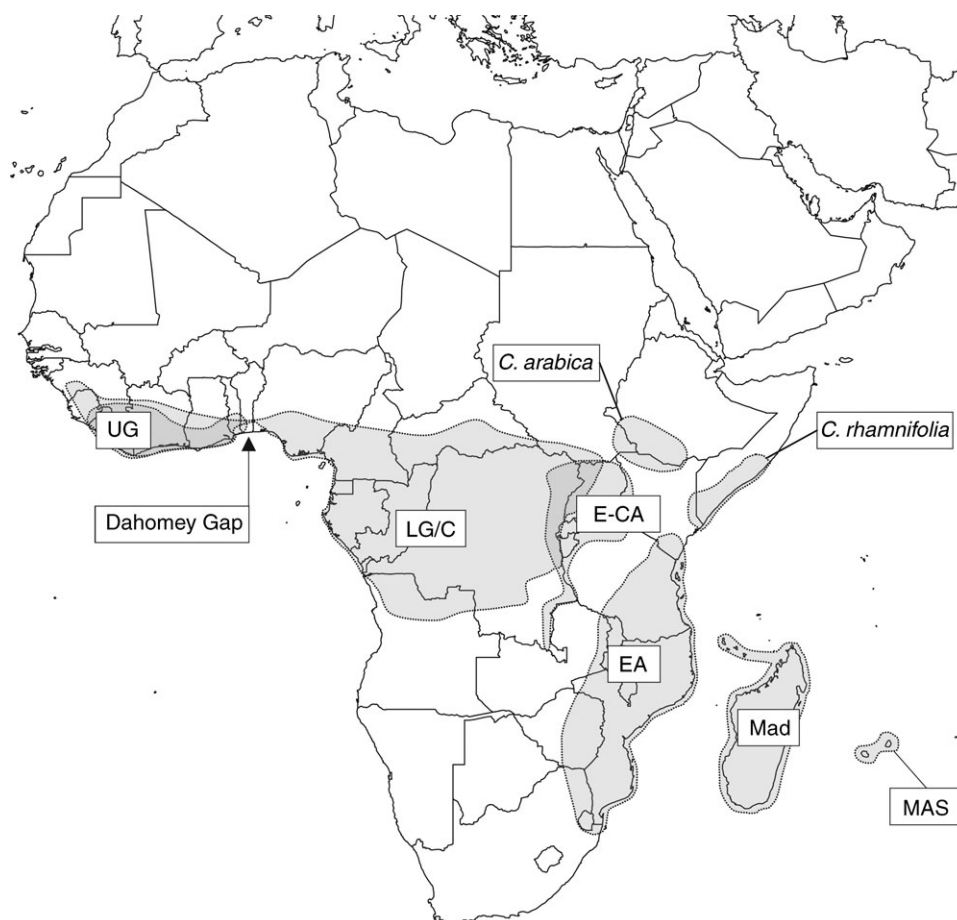


FIG. 1. Distribution map of *Coffea* showing location of clades and groups. UG = Upper Guinea clade; LG/C = Lower Guinea/Congolian clade; E-CA = East-Central Africa clade; EA = East Africa clade; Mad = Madagascan species; MAS = Mascarene clade. The map does not indicate the distribution of the poorly known *C. anthonyi* ined. (see Materials and Methods).

(approx. 2400 records; P. Stoffelen and A. Davis, unpubl. data) and Madagascan/Mascarene *Coffea* specimen database (approx. 1000 records; A. Davis and S. Dawson, unpubl. data). Species distributions maps were plotted and then a generalized map was drawn by hand.

## RESULTS

Tree data and statistics for individual and combined analyses using (a) *trnL-F*, (b) *rpl16*, (c) *accD-psa1*, (d) combined plastid data, (e) ITS and (f) combined sequence data are given in Table 3. Individual plastid sequence analyses were topologically consistent (negligible to zero incongruence) and for the purpose of the results and discussion were combined and treated as a single analysis. The combined plastid analysis is largely congruent with the ITS analysis, apart from the placement of *C. arabica*, an accession of *C. heterocalyx* [IRD-Montpellier JC 66 (K); see Table 1], and three species from the Upper Guinea region (*C. humilis*, *C. stenophylla*, *C. togoensis*). *Coffea arabica* and the accession of *C. heterocalyx* were the only strongly supported points of incongruence. It is now generally accepted that *C. arabica* is of hybrid origin, as discussed in the Introduction. On the basis of the present results, it

is believed that the accession of *C. heterocalyx* [IRD-Montpellier JC 66 (K)] used here is the same as that sampled by Lashermes *et al.* (1997) and Cros *et al.* (1998). The combined plastid data analysis places *C. heterocalyx* with *C. liberica* var. *dewevrei*, and the ITS analysis with *C. eugenioides*. Examination of the plastid sequences shows that there are only 3 bp differences and one 11 bp deletion (for *C. liberica* var. *dewevrei*) between this accession of *C. heterocalyx* and *C. liberica* var. *dewevrei* [Hepper & Maley 7729 (BR, K, MO)] across the three plastid regions. Cros *et al.* (1988) reported that their *trnL-trnF* sequence of *C. heterocalyx* (no accession data) was identical to one of their samples of *C. liberica*. The present ITS sequence data show that there is only 1 bp difference between this sample of *C. heterocalyx* and *C. eugenioides* [Harley 9332 (BR, K)]. The ITS2 data of Lashermes *et al.* (1997) placed *C. heterocalyx* in an unresolved position within a clade containing four samples of *C. eugenioides* and two of *C. sp.* Moloundou (= *C. anthonyi* ined.). Based on these data, it is believed that this accession of *C. heterocalyx* is a hybrid between *C. eugenioides* and *C. liberica*, resulting from either introgression in the wild or a chance crossing in cultivation. The natural distributions of these taxa overlap in the wild



TABLE 3. Description of trees for each plastid region, combined plastid region and combined molecular data sets

| Characteristics                    | <i>trnL-F</i> | <i>rpl16</i> | <i>accD-psal</i> | Combined plastid data | <i>ITS</i> | Combined molecular data |
|------------------------------------|---------------|--------------|------------------|-----------------------|------------|-------------------------|
| Number of taxa                     | 95            | 95           | 95               | 95                    | 107*       | 106†                    |
| Total number of characters         | 915           | 1120         | 1187             | 3222                  | 831        | 3901                    |
| Invariable characters              | 837           | 995          | 1052             | 2884                  | 652        | 3395                    |
| Parsimony uninformative characters | 43            | 73           | 82               | 198                   | 72         | 251                     |
| Parsimony informative characters   | 35            | 52           | 53               | 198                   | 107        | 255                     |
| Tree length                        | 90            | 106          | 199              | 499                   | 415        | 952                     |
| Consistency index (CI)‡            | 0.808         | 0.677        | 0.594            | 0.563                 | 0.438      | 0.467                   |
| Retention index (RI)               | 0.941         | 0.867        | 0.849            | 0.809                 | 0.764      | 0.761                   |
| Number of trees                    | 18 753        | 6860         | 6290             | 75 120                | 7760       | 11 520                  |

\* Including ITS clones.

† Including ITS clones but *C. arabica* removed (see Materials and Methods and Results).

‡ Calculated without uninformative sites.

[Democratic Republic of Congo, Sudan, Uganda (Davis *et al.*, 2006; Fig. 1)], but there do not appear to be field data indicating wild hybrids between *C. liberica* and *C. eugenioides*. The sequences of the sample of *C. heterocalyx* taken directly from the wild [Maurin 23 (K)] in Cameroon do not closely match those of the accession IRD-Montpellier JC 66 (K), as the former is consistently placed with species from West Africa (see Fig. 4). The sample IRD-Montpellier JC 66 (K) was originally held at the IRD coffee breeding station of Divo, Ivory Coast (ex IRD-IFCC station) and then transferred to IRD-Montpellier, France. The accession data imply that it was collected from either Cameroon or the Democratic Republic of Congo during the 1960s; it has been maintained in cultivation for >40 years (F. Anthony, pers. comm.).

Based on the evidence given above, the Montpellier accession of *C. heterocalyx* was excluded from the analyses, and *C. arabica* was removed from the final combined analysis (combined plastid plus ITS). After the deletion of these species, any highly supported incongruence between the combined plastid analysis and the ITS analysis was removed, enabling these two data sets to be combined. The position of three species (*C. humilis*, *C. stenophylla* and *C. togoensis*) from the Upper Guinea region is different for the combined plastid analysis and ITS analysis. In the combined plastid analysis, they form a well-supported clade (the UG clade; BP 100,  $b = 5$ ; Fig. 2), which falls within a clade of species from East and East-Central Africa (BP 70,  $b = 1$ ; Fig. 2). In the ITS analysis, these three species do not form a clade, but are all positioned within a clade of species that contains species from the Lower Guinea/Congo region (the ‘canephora alliance’; see below) and *C. arabica* (BP 74,  $b = 1$ ; Fig. 3). Similar results were reported by Cros *et al.* (1998) based on their *trnL-trnF* data for *C. humilis* and *C. stenophylla*, and by comparison with the ITS data of Lashermes *et al.* (1987) for *C. stenophylla* (see Introduction). The combined molecular analysis places the UG clade in approximately the same position as the combined plastid analysis, although this relationship is very weakly supported (BP 23,  $b = 1$ ; Fig. 4). The incongruence identified in the present investigation is not well supported, and the above three species were retained in combined plastid and ITS analysis,

where their inclusion does not significantly influence either the topology of the tree or support values. In the combined molecular (plastid-ITS) analysis, the UG clade is in approximately the same position as the combined plastid analysis, although this relationship is only weakly supported (BP 23,  $b = 1$ ; Fig. 4).

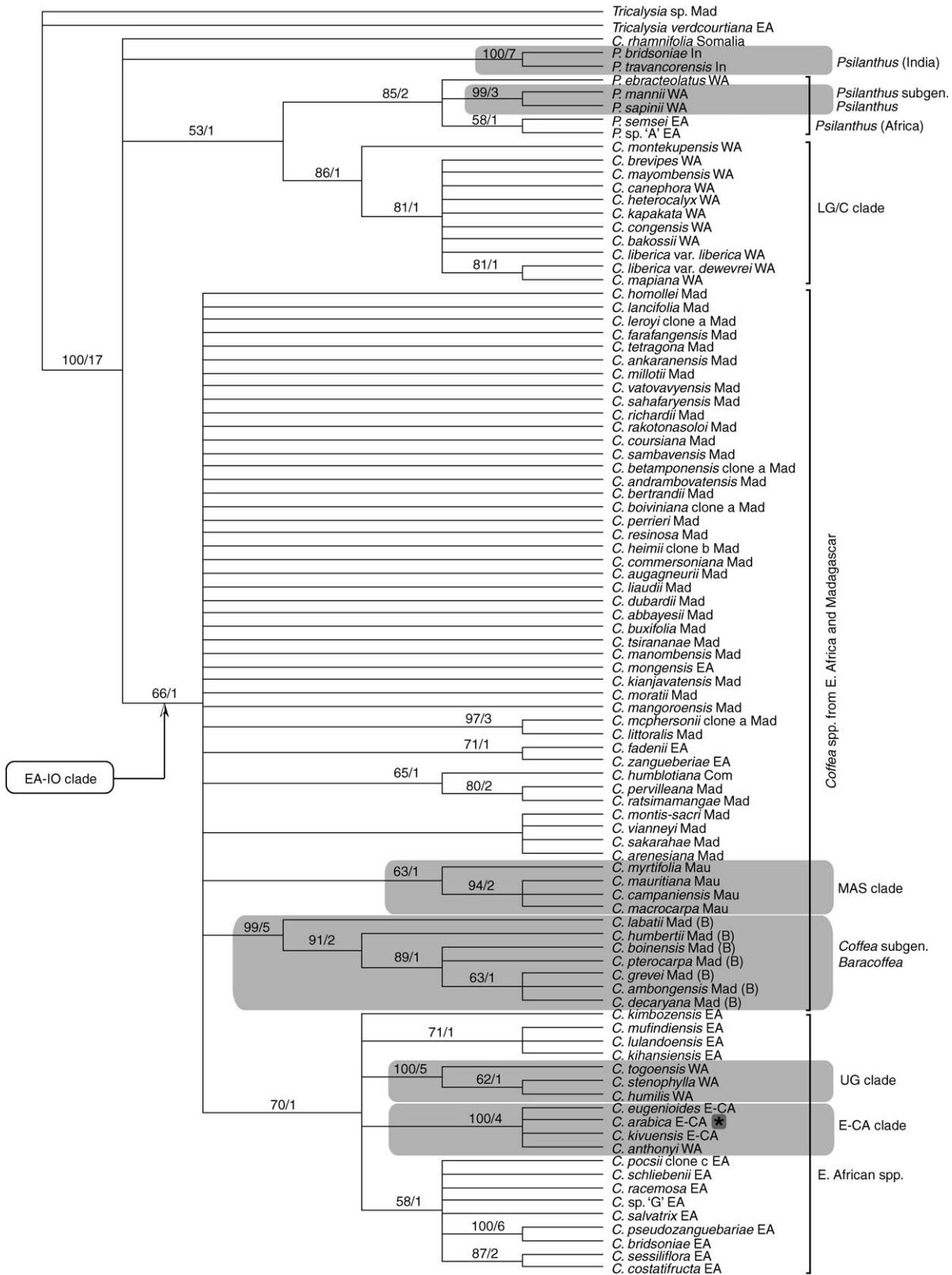
Negligible to moderate resolution was found in some parts of analyses, particularly for the Madagascan taxa. This lack of resolution was mostly due to low levels of sequence divergence, as indicated by the values for the consistency index (CI) and retention index (RI), and review of branch lengths (see Fig. 5).

#### Terminology of clades

Many of the species groups recovered in the present analyses are consistent, or nearly so, with geographical or phytogeographical regions, and geographical abbreviations have been used for clades (cf. Lashermes *et al.*, 1997; Cros *et al.*, 1998). Where the analyses recovered groups congruent with the most recent infrageneric classification of *Coffea* and *Psilanthus* (Davis *et al.*, 2005, 2006), these taxonomic groupings were retained. The following terminology was used for the geographical groupings: Upper Guinea (UG) clade, Lower Guinea/Congolian (LG/C) clade, East Africa-Indian Ocean (EA-IO) clade, East-Central Africa (E-CA) clade, East Africa (EA) clade, and Mascarenes (MAS) clade (see Fig. 1). The EA-IO clade includes the E-CA, EA and MAS clades, and species from Madagascar. The humid Central and West African forests are contained within the Guineo-Congolian Regional Centre of Endemism (White, 1983). Within this major region there are three subcentres of endemism for humid forest species: (1) Upper Guinea; (2) Lower Guinea; and (3) Congolian (White, 1979). For practical purposes, the subcentres (2) and (3) are often put together as the Lower Guinean/Congolian region, and this convention has been followed here. The distribution and systematic positions of *C. arabica* and *C. rhamnifolia* are isolated and are treated independently.

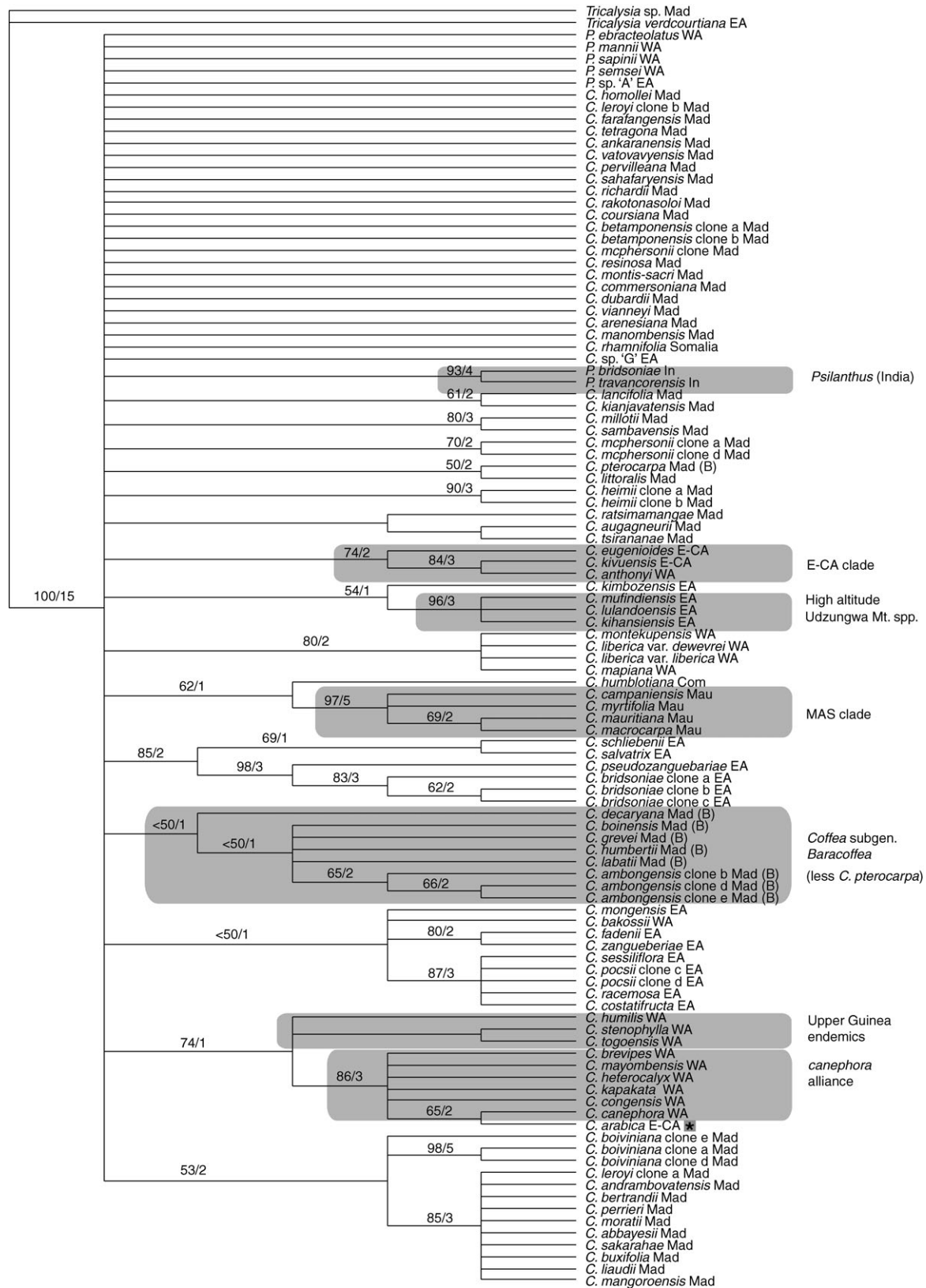
There is considerable agreement between the geographical distribution of species and their placement within clades or assumed species groupings. There is 100% endemism





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FIG. 2. Strict consensus tree generated from combined plastid analysis. Bootstrap values of >50 % are placed above the branches, followed by Bremer support (decay) values. See Table 1 for species authorities and provenance. EA-IO clade = East Africa-Indian Ocean clade; E-CA = East-Central Africa clade; LG/C clade = Lower Guinea/Congolian clade; UG clade = Upper Guinea clade; MAS clade = Mascarene clade. Regions, given after species names: EA = East Africa; E-CA = East Central Africa; Com = Comoros; In = India; Mad = Madagascar; Mau = Mauritius; WA = West Africa. \* = Reference symbol for *C. arabica*. (B) = species belonging to *Coffea* subgenus *Baracoffea*.



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FIG. 3. Strict consensus tree generated from ITS analysis. Bootstrap values of >50 % are placed above the branches, followed by Bremer support (decay) values. See Table 1 for species authorities and provenance. E-CA clade = East-Central Africa clade; MAS clade = Mascarene clade. Regions: EA = East Africa; E-CA = East Central Africa; Com = Comoros; In = India; Mad = Madagascar; Mau = Mauritius; WA = West Africa. \* = Reference symbol for *C. arabica*. (B) = species belonging to *Coffea* subgenus *Baracoffea*.

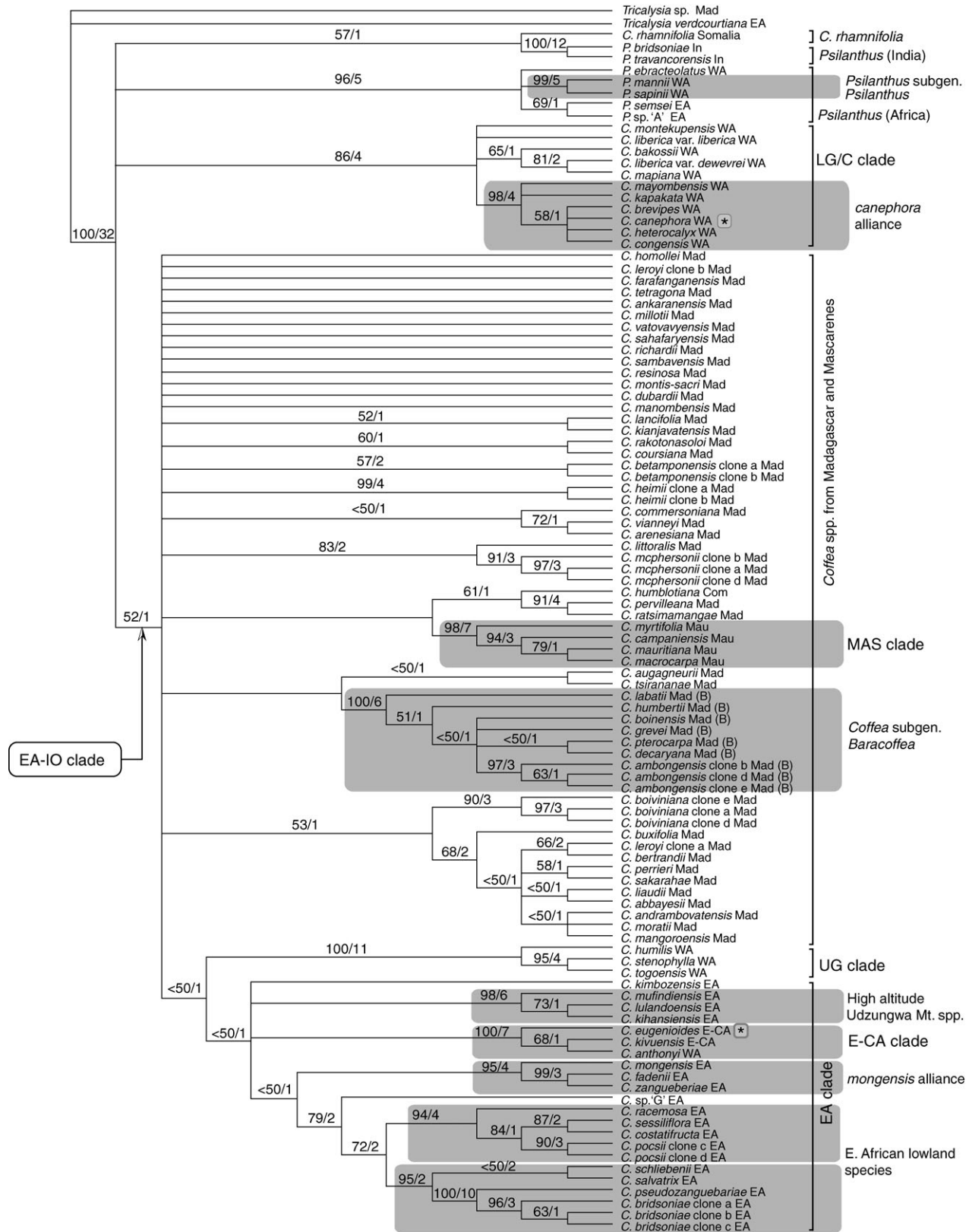
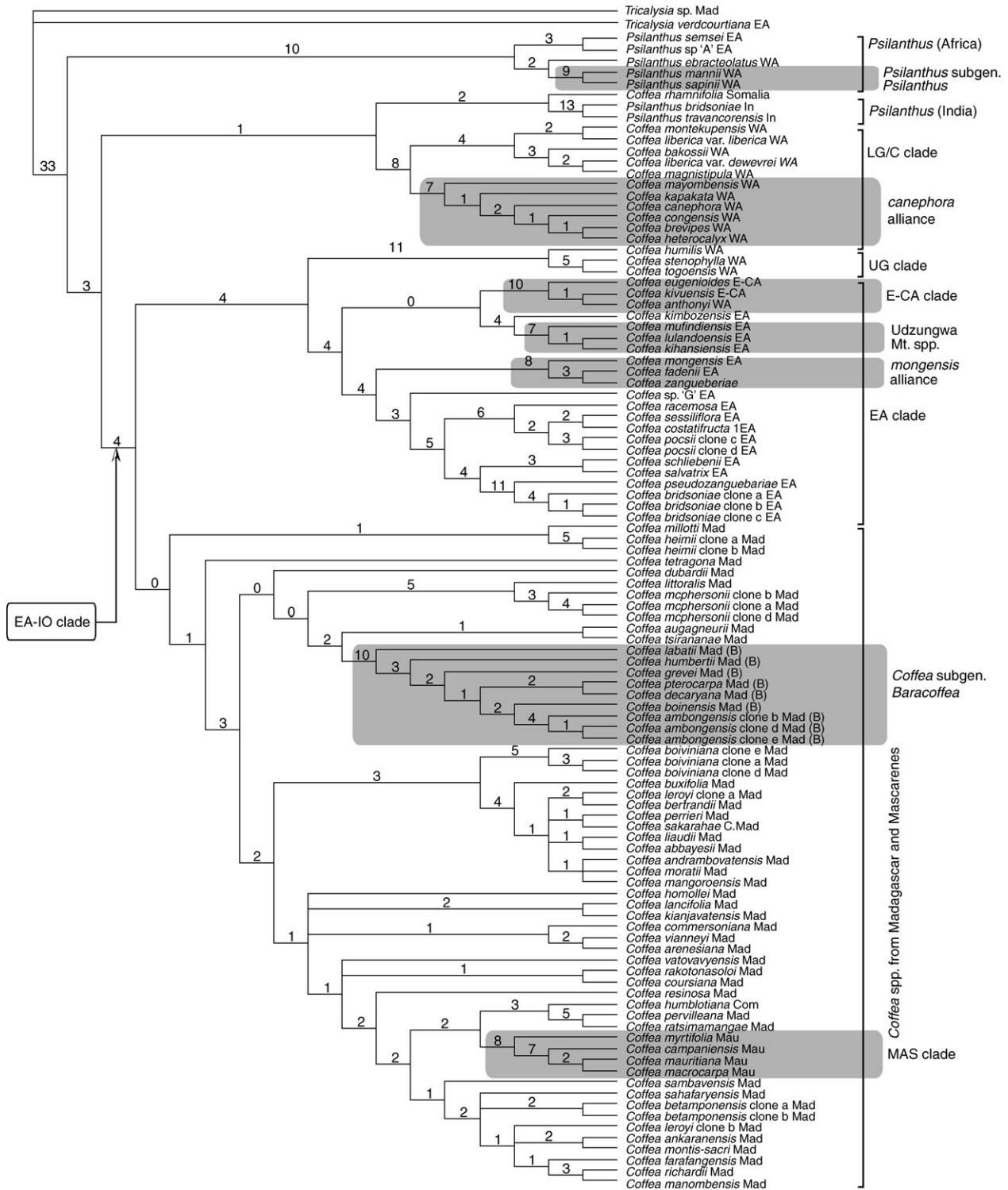


FIG. 4. Strict consensus tree generated from combined molecular (plastid-ITS) analysis. Bootstrap values of > 50 % are placed above the branches, followed by Bremer support (decay) values. See Table 1 for species authorities and provenance. EA-IO clade = East Africa-Indian Ocean clade; EA clade = East Africa clade; E-CA clade = East-Central Africa clade; LG/C clade = Lower Guinea/Congolian clade; UG clade = Upper Guinea clade; MAS clade = Mascarene clade. Regions: EA = East Africa; E-CA = East Central Africa; Com = Comoros; In = India; Mad = Madagascar; Mau = Mauritius; WA = West Africa. \*Denotes progenitor species for *C. arabica*. (B) = species belonging to *Coffea* subgenus *Baracoffea*.





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FIG. 5. One tree of 11 520 trees from the combined molecular (plastid-ITS) data analysis, with branch lengths. See Table 1 for species authorities and provenance. EA-IO clade = East Africa-Indian Ocean clade; E-CA clade = East-Central Africa clade; EA clade = East Africa clade; LG/C clade = Lower Guinea/Congolian clade; UG clade = Upper Guinea clade; MAS clade = Mascarene clade. Regions: EA = East Africa; E-CA = East Central Africa; Com = Comoros; In = India; Mad = Madagascar; Mau = Mauritius; WA = West Africa. (B) = species belonging to *Coffea* subgenus *Baracoffea*.

for the MAS, and EA clades. The UG, LG/C and E-CA clades do not have complete endemism owing to the inclusion of widespread species: *C. canephora* and

*C. liberica* occur in both the Upper Guinea (Portères, 1937) and Lower Guinea/Congolian regions and central-east Africa (see Fig. 1); *C. anthonyi* ined. occurs

in the East Central Africa and the Lower Guinea/Congolian regions. However, the natural range of *C. canephora* and *C. liberica* in West Africa has no doubt been obscured by introduction and naturalization, and the distribution of *C. anthonyi* is still poorly known. The Madagascan species do not form a clade, but there is 100% endemism for Madagascan *Coffea* species. Owing to the relatively low sample size for *Psilanthus*, and because this study is focused on *Coffea*, abbreviations have not been provided for well-supported clades within *Psilanthus*.

#### Single plastid analyses

The *accD-psaI* data set yielded the most potentially parsimony informative sites and *trnL-F* the least. Tree statistics for each separate plastid analysis are given in Table 3.

#### Combined plastid analysis (Fig. 2)

Several well-supported geographical groupings are revealed within *Coffea*, including the UG clade (BP 100;  $b = 5$ ), LG/C clade (BP 86;  $b = 1$ ), E-CA clade (including *C. arabica*) (BP 100;  $b = 4$ ), *Coffea* subgenus *Baracoffea* (BP 99;  $b = 5$ ) and clades within this subgenus. There are also several well-supported *Coffea* species pairs. The MAS clade is weakly supported (BP 63;  $b = 1$ ), although three species within the MAS clade receive better support (BP 94;  $b = 2$ ). The EA-IO clade is weakly supported (BP 66;  $b = 1$ ). *Coffea arabica* is placed within the E-CA clade (BP = 100;  $b = 4$ ), which is consistent with the placement in the Central Africa clade of Cros *et al.* (1998). The relationship of *C. arabica* to the other species in this clade (*C. eugenioides*, *C. kivuensis*, *C. anthonyi* ined.) is unresolved, although the sequences of *C. arabica* and *C. eugenioides* are identical. The monophyly of *Coffea* is not supported: *C. rhamnifolia* is unresolved in relation to other *Coffea* and *Psilanthus* species, and species from the Lower Guinea/Congolian region are sister to African *Psilanthus* (BP 53;  $b = 1$ ). *Psilanthus* is unresolved, although African *Psilanthus* (BP 85;  $b = 2$ ) and Indian *Psilanthus* (BP 100;  $b = 7$ ) are both well-supported.

#### ITS analysis (Fig. 3)

The cloned ITS sequences from each species showing evidence of heterogeneous copies grouped together, with the exception of the two cloned sequences from *C. leroyi*. The problems associated with direct sequencing in these taxa thus appear to be due to incomplete homogenization of the ITS copies, rather than hybridization or other causes. Groupings within *Coffea* receiving high support include a group of species from the Lower Guinea/Congolian region (*C. brevipes*, *C. mayombensis*, *C. heterocalyx*, *C. congensis* and *C. canephora*), *C. arabica* (originating from Ethiopia) and *C. kapakata* (Angola) (BP 86;  $b = 3$ ). These six species (less *C. arabica*) are here referred to as the canephora alliance. Other well-supported clades include a group of species from the Udzungwa Mountains of East Africa (BP 96;

$b = 3$ ) and the MAS clade (BP 97;  $b = 5$ ). There are also some well-supported species pairs and well-supported groups of ITS clones. The ITS analysis places *C. arabica* within the canephora alliance, sister to *C. canephora* (BP 65;  $b = 2$ ), and with only 2 bp differences between these species. The two species of Indian *Psilanthus* are well supported (BP 93;  $b = 4$ ). The relationship between *Coffea* and *Psilanthus* is unresolved.

#### Combined plastid and ITS analysis (Fig. 4)

Several well-supported groupings within *Coffea* are recovered, including the UG clade (BP 100;  $b = 11$ ), LG/C clade (BP 86;  $b = 4$ ), E-CA clade (BP 100;  $b = 7$ ), the MAS clade (BP 98;  $b = 7$ ), *Coffea* subgenus *Baracoffea* (BP 100;  $b = 6$ ) and the canephora alliance (BP 98;  $b = 4$ ). The EA-IO clade (BP 52;  $b = 1$ ) and EA clade (BP <50;  $b = 1$ ) are consistently recovered, but only weakly supported. There are a number of strongly supported lineages of East African species [e.g. Udzungwa Mountains (BP 98;  $b = 6$ ), and the mongensis alliance (BP 95;  $b = 4$ )], several well-supported species pairs and several strongly supported groupings of ITS clones. Indian *Psilanthus* (BP 100;  $b = 12$ ) and African *Psilanthus* (BP 96;  $b = 5$ ) form well-supported clades. Apart from *Coffea* subgenus *Baracoffea* (BP 100;  $b = 6$ ) and some small groupings of species, the relationships between most Madagascan species are unresolved. In contrast to the well-supported monophyly of *Coffea* subgenus *Baracoffea*, subgenus *Coffea* is paraphyletic. *Psilanthus* subgenus *Psilanthus* (BP 99;  $b = 5$ ) is well supported, but the monophyly of *Psilanthus* subgenus *Afrocoffea* was not substantiated. *Coffea rhamnifolia* is placed with the two species of Indian *Psilanthus*, but this is only weakly supported (BP 57;  $b = 1$ ). As in the combined plastid analysis and ITS analysis, the relationship between *Coffea* and *Psilanthus* is largely unresolved.

## DISCUSSION

#### West African *Coffea*: the Upper Guinea (UG) clade

The UG clade, comprising *C. humilis*, *C. stenophylla* and *C. togoensis*, is one of the most strongly supported groups within the combined analysis (BP 100,  $b = 11$ ; Fig. 4). Cros *et al.* (1998) found good support (BP 100) for *C. stenophylla* and *C. humilis*, which they recognized as the west Africa (W) clade. The convincing phylogenetic support for this clade may well be due to isolation of the Upper Guinea forests, as they are separated from those of the Lower Guinea/Congo region by the Dahomey Gap (Fig. 1), otherwise known as the Dahomey Interval (White, 1979, 1983). The gap is an extension of the woodland savannah of the Sahel to the Gulf of Guinea (Poorter *et al.*, 2004) presently some 250 km wide (White, 1979), which reaches the coast of southern eastern Ghana, Togo and Benin. Booth (1958) suggested that the Dahomey Gap was much wider during periods of glacial aridification, and this is generally supported by more recent studies (e.g. Maley, 1987). The present-day distribution of *Coffea*

species does not show complete separation between the Lower Guinea/Congo region and the Upper Guinea region, as *C. canephora* and *C. liberica* occur in both regions, and *C. togoensis* occurs in isolated humid forest patches in Ghana, Togo and Benin (Sonké *et al.*, 2006a). However, the Dahomey Gap has clearly played a role in the evolution of plant (White, 1983) and animal species (e.g. Booth, 1958; Murphy and Collier, 1997) and populations (Sehgal *et al.*, 2005) in the Upper Guinea region. It is proposed that this is first phylogenetic study to support the evolutionary influence of the Dahomey Gap within the flowering plants.

The incongruent position of the UG clade (see Results; Figs 2 and 3) is most probably due to (maternal) plastid genome transfer, which may have pre-dated speciation in this clade. The data imply plastid capture from a species or species lineage progenitor of East African origin or affiliation. Given the geographical location of the UG clade, the movement of a plastid genome from East to West Africa is most probable, perhaps either via long-distance dispersal or through dispersal via a once-continuous forest link between East and West Africa.

#### West Africa *Coffea*: Lower Guinea/Congo (LG/C) clade

The LG/C clade (BP 86,  $b = 1$ , Fig. 2; BP 86,  $b = 4$ , Fig. 4) is a group of ten predominantly lowland rainforest species, largely restricted to the Lower Guinea/Congolian region (Fig. 1). It does, however, include the widespread *C. canephora* and *C. liberica*, which also occur in the Upper Guinea region, and *C. kapakata* from Angola. *Coffea kapakata* occurs in the humid evergreen forests enclaves of the Guinea–Congolian/Zambezia Transition Zone (White, 1979), which is otherwise generally covered by non-forest vegetation. These enclaves are composed almost exclusively of humid forest Guineo-Congolian species (White, 1979), and thus the systematic position of *C. kapakata* within the present analysis is concomitant with humid forest distribution patterns in West Africa (White, 1979, 1983). The LG/C clade supports the findings of Cros *et al.* (1998, fig. 2), who recognized a west and central African (WC) clade (BP 41), which included five species and four provisional/unknown taxa from the Lower Guinea/Congolian region.

Within the Lower Guinea/Congo region, the canephora alliance [*C. brevipes*, *C. mayombensis*, *C. heterocalyx*, *C. congensis*, *C. canephora*, and *C. kapakata* (BP 98,  $b = 4$ ; Fig. 4)] represents an expansion of the ‘canephoroid group’ (*C. brevipes*, *C. canephora*, and *C. congensis*) as enumerated by Cros *et al.* (1998). Members of this alliance are all very similar morphologically, and (based on single species samples) appear closely related. Recognition of the canephora alliance is important as it provides a well-circumscribed group for further study of the economically important species *C. canephora* and *C. arabica*. Other Lower Guinea/Congo *Coffea* species (*C. carrisoi* A.Chev., *C. dactylifera* Robbr. & Stoff., *C. fotsoana* Stoff. & Sonké and *C. leonimontana* Stoff.) may belong in the canephora alliance; material of these species were not sampled in the present analyses.

Humid forest West African species and species groups have often been considered to represent the earliest diverging lineages within plant genera (Harris *et al.*, 2000; Davis *et al.*, 2002; Plana *et al.*, 2004) or to include species that are phylogenetically isolated (Malcomber, 2002), although systematic studies for African plants are relatively few (Plana *et al.*, 2004). On the basis of the present data, it is not possible to support these assumptions: the combined plastid analysis places the LG/C clade as sister to African *Psilanthus* (BP 53,  $b = 1$ ; Fig. 2) but in the combined molecular analysis (Fig. 4) its position is unresolved. Davis *et al.* (2002) posited that the response of ancient African plant communities to climate change should be detectable through phylogenetic analysis of plants that span both humid and lowland xeric regions of the African continent. This is based on the significant evidence that lowland rainforest dominated much of Africa in the late Cretaceous and was replaced by xeric vegetation as a response to continental uplift (and other Earth events) and consequent widespread aridification beginning in the late Palaeogene. They suggest that if aridification induced a relatively recent period of diversification, then species that inhabit the humid relict forests of West Africa should represent the earliest diverging lineages of these African radiations, whereas species restricted to arid regions of East Africa should be phylogenetically nested (Davis *et al.*, 2002). This pattern is not retrieved in the present analyses, although certain xeric species (*C. costatifructa*, *C. zanguebariae*, *C. racemosa*, *C. pocsii* and *C. schliebenii*) are convincingly nested within humid-dwelling East African lineages (Fig. 4). *Coffea rhamnifolia*, a species from SE Somalia and NE Kenya, and occurring in a xeric environment, is intriguing in this respect as it occupies an isolated and equivocal position within the analyses (Figs 2–5).

In the ITS analysis, *C. liberica* var. *dewevrei* is grouped with three other Lower Guinea/Congolian taxa (BP 80,  $b = 2$ ; Fig. 3): *C. liberica* var. *liberica*, *C. montekupensis* and *C. mapiana*, but the relationship among these taxa is unresolved. In the combined plastid analysis (BP 81,  $b = 1$ ; Fig. 2) and combined molecular analysis (BP 81,  $b = 2$ ; Fig. 4), it groups together with the morphologically unusual *C. mapiana* (Sonké *et al.*, 2006b). Thus, the results add further evidence in support of the findings of N’Diaye *et al.* (2005), indicating genetic differentiation between the two varieties of *C. liberica*. Further data and sampling are required to assess fully the relationships between the two varieties of *C. liberica* and related taxa in the LG/C clade.

#### East African–Indian Ocean *Coffea*: EA-IO clade

The EA-IO clade is consistently retrieved in the combined plastid analysis (BP 66,  $b = 1$ ; Fig. 2) and combined molecular analysis (BP 52,  $b = 1$ ; Fig. 4), although the support for this clade is weak. The West African UG clade is placed within the EA-IO clade in these analyses, but this position may be due to (presumably maternal) plastid genome transfer via either dispersal or diffusion (see above). The separation of West African *Coffea* species (the LG/C clades) and East African and Indian Ocean species (EA-IO clade) would be expected given



the geological history of Africa. The formation of the East Albertine African Rift Valley would have provided a climatic and physical obstruction to dispersal, separating a west/east humid forest belt that once spanned the African mainland (Maley, 1987; Davis *et al.*, 2002). In addition, further Neogene aridification would have cyclically separated and fragmented a continuous forest belt or larger forest blocks (Maley, 1987). On the basis of the current analysis, the separation of West Africa and East Africa *Coffea* would require a single vicariance event, such as a climatic incident or long-distance dispersal.

#### East Africa *Coffea*: EA clade

In the combined molecular analysis, all species from East Africa and East-Central Africa are placed within the EA clade (Fig. 4). There is negligible support for this group (BP 23;  $b = 1$ ), although deleting the UG clade from the combined molecular analysis gives the EA increased support (e.g. BP 52). Lashermes *et al.* (1997) and Cros *et al.* (1998) also recognized an East Africa (E) clade (see Introduction), although their analyses included only four and five taxa, respectively.

Within the EA clade there are other well-supported groups that have geographical/ecological or morphological correspondence. The clade formed by *C. mufindiensis*, *C. lulandoensis* and *C. kihansiensis* (BP 98,  $b = 6$ ; Fig. 4) represents a group of high altitude (800–2300 m) species from the Udzungwa Mountains, one of the mountain groups within the Eastern Arc Mountains (Lovett, 1985, 1988). *Coffea kimbozensis* is also found in the Udzungwa Mountains, but unlike the species above it is restricted to low elevation (300–450 m) on calcareous rocks (Bridson, 1988a; A. Davis and E. Mvungi, pers. observ.). The clade formed by *C. mongensis*, *C. fadenii* and *C. zanguebariae* (BP 95,  $b = 4$ ; Fig. 4) does not have a distinct geographical or geological delimitation within East Africa, although they are similar morphologically (Bridson, 1988a), and they are labelled here as the ‘mongensis group’. The E-CA clade (*C. eugenioides*, *C. kivuensis* and *C. anthonyi* ined.) is placed in an unresolved position at the base of the EA clade; further discussion of the E-CA clade is given below. The largest clade within the EA clade is a group of predominantly lowland species, labelled the ‘E. Africa lowland species’ (BP 72,  $b = 2$ ; Fig. 4), and includes two well-supported groups (BP 94,  $b = 4$ ; BP 95,  $b = 2$ ). This clade contains mostly species from low elevations (sea level to approx. 500 m, rarely up to 800 m), in seasonally dry forest (some species in xeric woodland). These species mainly occur within the Indian Ocean Coastal belt (White, 1979). One exception is *C. salvatrix*, which normally occurs at altitudes of 850–1650 m (Bridson, 1988a) within the ‘E. Africa lowland species’ clade (Fig. 4).

#### East-Central Africa *Coffea*: E-CA clade

*Coffea eugenioides*, *C. kivuensis* and *C. anthonyi* ined. (as *C. sp.* Moloundou, Lashermes *et al.*, 1997; Cros *et al.*, 1998) form the E-CA clade in the combined plastid analysis

(BP 100,  $b = 4$ ; Fig. 2) and combined molecular analysis (BP 100,  $b = 7$ ; Fig. 4). These results support the findings of Lashermes *et al.* (1997) and Cros *et al.* (1998), who received weak (BP 67) and strong (BP 100) support (respectively) for a central Africa (C) clade, based on *C. eugenioides* and *C. anthonyi*. The distribution of *C. eugenioides* and *C. kivuensis* falls mostly within the Lake Victoria Regional Mosaic (White, 1983), at elevations normally well above 1000 m, whereas *C. anthonyi* ined. occurs in a few isolated locations at low to mid-elevation [350–650(–900) m] in SE Cameroon and NW Congo. *Ex situ* material of *C. anthonyi* ined. was relied on for the DNA analysis, and further sampling would be desirable to test the close association of this species with *C. eugenioides* and *C. kivuensis*. The placement of the E-CA clade within the EA clade (Fig. 4) is consistent with the geographical proximity and the geological history of Africa (see above). Furthermore, clear associations between the Lake Victoria Regional Mosaic species and the Afromontane species of East Africa have been identified by White (1979); there are numerous species of *Coffea* occurring in the mountains of East Africa (Bridson, 1988a; Davis *et al.*, 2006).

#### Madagascan *Coffea* species

All Madagascan *Coffea* species are placed within a weakly supported EA-IO clade (BP 66,  $b = 1$ , Fig. 2; BP 52,  $b = 1$ , Fig. 4). The position of all Madagascan species and species groups is unresolved, due to low levels of sequence divergence (Fig. 4), which is a problem in species-level analysis of Madagascan Rubiaceae, as exemplified by the study of Malcomber (2002). *Coffea* subgenus *Baracoffea* (BP 100,  $b = 6$ ; Fig. 4) is the only well-supported Madagascan clade, apart from *C. pervilleana* and *C. ratsimamangae* (BP 91,  $b = 4$ ; Fig. 4), two closely allied species from northern Madagascar (Davis and Rakotonasolo, 2001a). When comparing morphological diversity of *Coffea* species occurring in Madagascar (Davis, 2001; Davis and Rakotonasolo, 2001a, b, 2003; Davis *et al.*, 2005) with those from Africa (Bridson, 1988a, 2003; Stoffelen, 1998), where sequence divergence is higher, the former exhibit far more interspecific differences, even excluding the unusual species of *Coffea* subgenus *Baracoffea* from western Madagascar (Davis *et al.*, 2005). The paucity of sequence divergence in Madagascan *Coffea* subgenus *Coffea* implies either a rapid evolutionary radiation or slow molecular evolution, or perhaps a combination of both.

Suggestions concerning the origin of Madagascan *Coffea* species must be tentative with the present data at hand. A single dispersal event from Africa, followed by insular speciation, has been inferred for *Begonia* L. (Begoniaceae) by Plana *et al.* (2004) and for *Gaertnera* Lam. (Rubiaceae) by Malcomber (2002). Lavin *et al.* (2000) infer that the Madagascan species of the genus *Ormocarpum* P.Beauv. (Fabaceae) are the result of two dispersal events. Investigation of *Streptocarpus* Lindl. (Gesneriaceae) shows multiple colonization events for the Madagascan representatives of the genus (Möller and Cronk, 2001).

A single dispersal event from Africa seems the most likely scenario for Madagascan *Coffea*, and one that would not be in conflict with the present data (Figs 3–5).

In the combined plastid (BP 65,  $b = 1$ ; Fig. 2) and combined molecular (BP 61,  $b = 1$ ; Fig. 4) analyses there is weak support for a sister relationship between *C. humblotiana* (the only *Coffea* species from the Comoros), and two species from Madagascar [*C. pervilleana* and *C. ratsimamangae* (northern Madagascar)], providing an indication for a northern Madagascan origin for the Comorian species.

#### Mascarene *Coffea*: MAS clade

In all analyses, the four *Coffea* species from the Mascarene Islands (Fig. 1) are placed within the EA-IO clade and form the MAS clade (BP 63,  $b = 1$ , Fig. 2; BP 97,  $b = 5$ , Fig. 3; BP 98,  $b = 7$ , Fig. 4). Given that these islands are oceanic with a volcanic origin (the oldest approx. 8 million years old), the progenitor species must have arrived on the Mascarenes Islands via a long-distance dispersal event. The position of the MAS clade within the combined plastid and combined molecular analyses (Figs 2 and 5), coupled with the relative proximity of the Mascarenes to Madagascar, infers an ‘out of Madagascar’ origin for the MAS clade.

#### Taxonomic groups

The present study clearly shows that *Coffea* subgenus *Baracoffea* is a well-supported group (BP 100,  $b = 6$ ; Fig. 4), restricted to the seasonal drylands (including spiny/xerophytic deciduous forest) of western Madagascar (Davis *et al.*, 2005). *Coffea* subgenus *Baracoffea* is by far the most morphologically distinct group within *Coffea*, having evolved as a response to a seasonally dry environment. Morphological features include congested or shrubby (sympodial growth pattern) habit, indeterminate inflorescences, deciduous leaves and pubescent to densely pubescent leaves and corollas (Davis *et al.*, 2005). In comparison, *Coffea* subgenus *Coffea* are trees (monopodial growth pattern), with determinate inflorescences, evergreen leaves (all except three species, see Davis *et al.*, 2005), glabrous or rarely very sparsely puberulous leaves, and glabrous corollas (Davis *et al.*, 2005). If it can be convincingly demonstrated that *Coffea* subgenus *Baracoffea* has evolved from humid forest ancestors/progenitors, which is possible (Figs 2, 4 and 5), assumptions about the origin of dryland biomes in Madagascar could be posited.

*Coffea rhamnifolia* cannot be considered a member of *Coffea* subgenus *Baracoffea*, as supposed by Leroy (1982): the combined plastid analysis and ITS analysis (Figs 2 and 3) place *C. rhamnifolia* in an unresolved position at the base of the ingroup, and the combined molecular analysis places it as sister to the two Indian *Psilanthus* (BP 57,  $b = 1$ ; Fig. 4).

*Psilanthus* subgenus *Psilanthus* (*P. mannii* and *P. sapinii*) is well supported (BP 99,  $b = 5$ ; Fig. 4) and definable by a single morphological synapomorphy: the presence of accrescent calyx lobes. The monophyly of

the most species-rich group of *Psilanthus*, subgenus *Afrocoffea*, was not supported in the current analyses (Figs 2–4). Furthermore, the position and strong support for African (BP 85,  $b = 2$ , Fig. 2; BP 96,  $b = 5$ , Fig. 4) and Indian (BP 100,  $b = 7$ , Fig. 2; BP 93,  $b = 4$ , Fig. 3; BP 100,  $b = 12$ , Fig. 4) *Psilanthus* implies that the present subgeneric classification of this genus is not consistent with sequence data. Further sequence data and species sampling are required for *Psilanthus*.

#### The relationship between *Coffea* and *Psilanthus*

On the basis of ITS and *trnL-trnF* data, Lashermes *et al.* (1997) and Cros *et al.* (1998), respectively, concluded that the division of *Coffea* and *Psilanthus* into two genera was untenable. The present combined molecular analysis does not resolve the issues of monophyly for *Coffea* (including *C. rhamnifolia*) and *Psilanthus*, as the relationship between these genera is largely unresolved, mainly due to a lack of sequence divergence (Fig. 4). There is some support for inferring that *Coffea* and *Psilanthus* are not independent lineages: in the combined plastid analysis there is weak support (BP 53,  $b = 1$ , Fig. 2) for a sister relationship between African *Psilanthus* and West African *Coffea*, and in the combined molecular analysis *C. rhamnifolia* is weakly supported as the sister group to the two species of Indian *Psilanthus* (BP 57,  $b = 1$ ; Fig. 4).

Three morphological characters separate *Coffea* from *Psilanthus* (Davis *et al.*, 2005): short to long filaments ( $\pm$  absent to very short in *Psilanthus*); sub-medifixed anthers (supra-medifixed in *Psilanthus*); and (long) emergent style (short and included in *Psilanthus*). However, *P. melanocarpus* has short filaments and sub-medifixed anthers, as in *Coffea*, but an included style, as in *Psilanthus*. It was not possible to isolate DNA of *P. melanocarpus* from herbarium samples, and it was not included in the present analyses. If *P. melanocarpus* is indeed a species of *Psilanthus*, only one character would separate *Coffea* and *Psilanthus*: short vs. long style. If *P. melanocarpus* nested within *Coffea*, then the two anther characters would separate *Coffea* and *Psilanthus*. The number of pollen apertures (Lobreau-Callen and Leroy, 1980; Chinnappa and Warner, 1981; Stoffelen *et al.*, 1997c) has been used as additional evidence to separate *Coffea* and *Psilanthus* (Davis *et al.*, 2005), although considerable polymorphism is evident and there is overlap in the number of apertures between *Coffea* and *Psilanthus* and their subgenera (Stoffelen *et al.*, 1997c; Davis *et al.*, 2005). The morphological evidence for the separation of *Coffea* and *Psilanthus* is certainly not convincing, and if *P. melanocarpus* is found to nest within either of these genera there would seem negligible justification for the recognition of two genera on morphological grounds (see above). However, many other genera of Rubiaceae are separated on only one or two morphological characters. *Pagamea* Aubl. and *Gaertnera* are well-supported genera based on molecular data, but are separated by a single synapomorphy (pubescent vs. glabrous corolla lobes), for example (Malcomber, 2002).

The robust morphological (Robbrecht and Puff, 1986; Davis *et al.*, 2005) and molecular support for *Coffea* plus *Psilanthus* (Davis *et al.*, 2007), low sequence diversity between these genera (see above; Fig. 4) and indications of paraphyly (Figs 2 and 4), may be taken as evidence for accepting *Coffea* and *Psilanthus* as a single genus (Lashermes *et al.*, 1997; Cros *et al.*, 1998). However, it is believed that further molecular data are needed to resolve fully the relationship between *Coffea* and *Psilanthus*, and in particular sequence data are required for *P. melanocarpus* and other species of *Psilanthus*.

#### Origin of *Coffea arabica*

The present data support a hybrid origin for *C. arabica*, following the findings of Lashermes *et al.* (1997, 1999), Cros *et al.* (1998) and Raina *et al.* (1998). Examination of combined plastid and ITS analyses (Figs 2 and 3), and by pair-wise comparison of sequences (see Results), it is concluded that the progenitor species of *C. arabica* are *C. canephora* and *C. eugenioides*. The results concur with those of Lashermes *et al.* (1999), who found low genetic (RFLP) divergence between the two constituent genomes of *C. arabica* and those of its progenitor species, suggesting perhaps that the speciation of *C. arabica* took place relatively recently. The proposed hybrid origin of *C. arabica* is consistent with the potential recent sympatry of *C. canephora* and *C. eugenioides* based on present-day distribution (Davis *et al.*, 2006).

#### Conclusions

On the basis of sequences from four plastid regions and ITS, it has been possible to identify several well-supported lineages within *Coffea* that are consistent with major biogeographical regions and geographical areas, including the UG clade (BP 100;  $b = 11$ ), the LG/C clade (BP 86;  $b = 4$ ), the E-CA clade (BP 100;  $b = 7$ ) and the MAS clade (BP 98;  $b = 7$ ), although the distribution of widespread species (*C. canephora* and *C. liberica*) occurs across some of these regions in mainland Africa. The UG, LG/C and E-CA clades are consistent with those either retrieved or identified by Cros *et al.* (1998) and Lashermes *et al.* (1997). Within the LG/C clade, it has been possible to substantiate (Cros *et al.*, 1998) and expand on a group of species related to *C. canephora*, the canephora alliance (BP 98,  $b = 4$ ; Fig. 4). Other groups were consistently retrieved but received weak support, including the EA-IO clade (BP 52;  $b = 1$ ) and the EA clade (BP 23;  $b = 1$ ). Smaller biogeographical groupings were retrieved within the EA clade, including a group of high altitude Udzungwa Mountain species (BP 98;  $b = 6$ ), and the east African lowland species (BP 72;  $b = 2$ ). Groups corresponding to geographical distribution were also recovered in *Psilanthus*, including the two Indian representatives, *P. travancorensis* and *P. bridsoniae* (BP 100;  $b = 12$ ), and species of *Psilanthus* occurring in Africa (BP 96;  $b = 5$ ). Two formal taxonomic groups are well supported: *Coffea*

subgenus *Baracoffea* (BP 100;  $b = 6$ ) and *Psilanthus* subgenus *Psilanthus* (BP 99;  $b = 5$ ).

The combined sequence data do not substantiate the monophyly of either *Coffea* or *Psilanthus*, largely due a lack of resolution (Fig. 4), resulting from low levels of sequence divergence (e.g. see Fig. 5). This evidence, together with weak support for some intergeneric (*Coffea*–*Psilanthus*) relationships, and strong molecular and morphological support for a clade comprising *Coffea* plus *Psilanthus* [BP 100;  $b = 32$ ; see also Davis *et al.* (2007)], could be taken as justification for the recognition of a single genus (i.e. *Coffea*), although further data and critical sampling are required to resolve this matter fully. The subgeneric classification of *Coffea* and *Psilanthus* (Bridson 1988a, b; Davis *et al.*, 2005) is not consistent with phylogenetic data. Most notably, *Coffea* subgenus *Coffea* is paraphyletic, due to the nested position of *Coffea* subgenus *Baracoffea*.

A recent hybrid origin for *C. arabica*, with *C. canephora* and *C. eugenioides* as the likely progenitor species, is supported (Lashermes *et al.*, 1999). This study provides the first phylogenetic evidence for the influence of the Dahomey Gap (West Africa) on plant speciation.

#### ACKNOWLEDGEMENTS

We would like to thank the following organizations and individuals who provided plant material for this study, including National Centre for Applied Research on Rural Development (FOFIFA), P. De Block (BR), IRD-Montpellier, Q. Luke (EA), F. Mbago (DSM), F. Rakotonasolo (TAN), B. Sonké (Université de Yaoundé) and P. Stoffelen (BR). Permission to sample and study herbarium and/or living collections from DSM, K and BR is gratefully acknowledged. We also thank the DNA bank managers of the Jodrell Laboratory Royal Botanic Gardens, Kew: L. Csiba, E. Kapinos. In Madagascar fieldwork was carried out under collaborative agreements between the Parc Botanique et Zoologique de Tsimbazaza (PBZT), the University of Antananarivo, the Association Nationale de Gestion des Aires Protégées (ANGAP) and the Royal Botanic Gardens, Kew. We would like to thank J. Andriantiana (TAN), H. Ralimanana (K), and especially F. Rakotonasolo (TAN) for help with fieldwork in Madagascar. We would like to acknowledge the Tanzania Commission for Science and Technology (COSTECH) for providing research clearance to undertake fieldwork in Tanzania. In Tanzania, we are grateful to F. Mbago (DSM), S. Hall, A. Ntemi, R. Hind and Z. Bridger. In Cameroon, we thank the staff and Directors at the Université de Yaoundé, National Herbarium of Cameroon (YA) and Limbe Botanical and Zoological Gardens (SCA), and especially B. Sonké (Université de Yaoundé). At the Royal Botanic Gardens, Kew, we would like to thank W. Baker, D. Bridson, S. Dawson, F. Forest, E. Lucas and J. Moat. At IRD-Montpellier we would like to thank F. Anthony, P. Hamon, A. de Kochko, P. Lashermes and M. Noirot. A.D. would like to thank the staff at the Royal Botanic Gardens, Sydney (NSW) and in particular D. Crayn. E.M. gratefully acknowledges the



DANIDA-ENRECA biodiversity project, for their financial support. The funding for this study was largely provided by Kraft Foods (UK), and we are extremely grateful for their financial support for laboratory and herbarium research and the visits of E.M. and Y.J.-F. to Kew.

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