

Cytinaceae are sister to Muntingiaceae (Malvales)

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The holoparasitic family Cytinaceae (*Cytinus* and *Bdallophyton*) was previously shown to be allied with Malvales, however its closest photosynthetic ancestor was not determined. A molecular phylogenetic study was conducted using nuclear small-subunit rDNA and three chloroplast genes (the latter missing from the holoparasites but included to stabilize the overall tree topology) using Cytinaceae and exemplars from all recognized families from an expanded Malvales. Using maximum parsimony and maximum likelihood methods, Cytinaceae were resolved with strong support as sister to the newly described Muntingiaceae. Potential morphological synapomorphies include trichome types, mature pollen released as tetrads, inferior ovaries, intrusive parietal placentation with many ovules, and fruit type. Given that all Muntingiaceae are neotropical, it is likely that the ancestor of Cytinaceae arose in the New World and migrated to continental Africa prior to the separation of Africa from South America.

KEYWORDS: *Bdallophyton*, *Cytinus*, holoparasite, Malvales, phylogeny, Rafflesiales

INTRODUCTION

Over the past two years molecular phylogenetics has required a radical revision of the classification of plants traditionally placed together in Rafflesiales. The major questions associated with these holoparasitic angiosperms involve determining their nearest photosynthetic relatives and ascertaining relationships among the component genera. The nine genera have either been classified as members of one family (Rafflesiaceae s.l.) or as four families in the order Rafflesiales (Takhtajan, 1997). For the latter, one can recognize (1) the “hypogynous clade” (Mitrastemonaceae with *Mitrastema*); (2) the “large-flowered clade” (Rafflesiaceae s.str. with *Rafflesia*, *Rhizanthus*, and *Sapria*); (3) the “small-flowered clade” (Apodanthaceae with *Apodanthes*, *Berlinianche*, and *Pilostyles*); and (4) the “inflorescence clade” (Cytinaceae with *Bdallophyton* and *Cytinus*). Molecular phylogenetic analyses (Nickrent & al., 2004) supported the latter view, i.e., the presence of four separate lineages with at least three independent origins. Specifically, Mitrastemonaceae are a component of Ericales, Rafflesiaceae s.str. is part of Malpighiales, and Cytinaceae is a member of Malvales. Strong support was obtained for these three relationships but such was not the case for Apodanthaceae which varied between Malvales and Cucurbitales depending upon the data partition used.

Previous phylogenetic analyses using chloroplast *rbcL* (Chase & al., 1993) and nuclear small-subunit rDNA sequences (Soltis & al., 1997) provided the first evidence that placed malvales taxa in the rosoid clade, but these studies did not include all putative members of Malvales.

This was rectified by Fay & al. (1998) and Alverson & al. (1998) who used *rbcL* to examine relationships in core Malvales (= Malvaceae sensu Bayer & al. [1999] and APG II [2003]) as well as representatives of other orders that previously had been shown (via molecular analyses) to have malvales affinities such as Cistales, Violales, Geraniales, and Theales. These studies documented the presence of four major clades in an expanded Malvales: core Malvales, a thymelaealean clade, a bixalean clade, and a dipterocarpacean clade (including *Muntingia* and *Neurada*), however the topological relationships among these four groups were not resolved. A more focused analysis showed that *Neurada* (Neuradaceae) was basalmost in the malvales clade but this relationship lacked bootstrap support (Alverson & al., 1998). The affinity of *Neurada* with Malvales (as opposed to Rosaceae) is supported by morphological features including exotegmic seed coats with a palisade layer and the presence of cyclopropenoid fatty acids in the seeds. The genus *Muntingia*, previously allied with Elaeocarpaceae or Tiliaceae, emerged as sister (with weak support) to a clade composed of Cistaceae, Dipterocarpaceae, and Sarcocaulaceae. This taxon was later used as outgroup in a study of core Malvales using chloroplast *ndhF* sequences (Alverson & al., 1999). Molecular data as well as discriminating morphological features prompted Bayer & al. (1998) to circumscribe a new family, Muntingiaceae, which included the neotropical monotypic genera *Muntingia*, *Dicraspidia*, and *Neotessmannia*.

The phylogenetic study by Nickrent & al. (2004) provided a broad view of the relationships among the holoparasites and nonparasites, however, it was not designed to test their exact placements within these orders.

To accomplish this, greater taxon sampling is required wherein representatives of all families within these orders are included. For Rafflesiaceae, this has recently been accomplished through extensive sampling within the large and diverse order Malpighiales (Davis & al., 2007). To date, no further work identifying the photosynthetic sister groups to the other three families has been published. Specifying the green plant relatives of these enigmatic parasites is important because this is the first step toward understanding the development and evolution of various vegetative and floral organs whose homology has been poorly understood for over a century. Here, results are presented of phylogenetic analyses that included representatives of both genera of Cytinaceae (*Bdallophyton* and *Cytinus*) as well as all families of the expanded Malvales. The goal of this study is to determine the closest photosynthetic relative to Cytinaceae.

MATERIALS AND METHODS

Taxon and gene sampling. — Cytinaceae were strongly supported as members of Malvales in a broad-scale study using nuclear and mitochondrial genes. Moreover, previous molecular work focused on Malvales suggested it is most closely related to Capparales and Sapindales. For these reasons, representatives of these two orders were included and used as outgroup to root the trees. The multigene alignment was constructed using sequences available from GenBank (Appendix). The four genes used were nuclear small-subunit (SSU) rDNA and chloroplast *rbcL*, *atpB*, and *ndhF*. Although the chloroplast genes are missing from the holoparasites, they were included to stabilize the overall tree topology. With 21 taxa representing Malvales, three species of *Cytinus* and one species of *Bdallophyton*, and six outgroup taxa, the matrix contained 107 sequences. Five SSU rDNA sequences from autotrophic plants and all twelve sequences of chloroplast genes from the holoparasites were unavailable. To maximize the number of filled cells in the matrix, placeholder taxa were used which were generally a different species of the same genus.

Alignment and phylogenetic analysis. — Sequences were aligned by eye in Se-Al (Rambaut, 2004). Alignment of protein-coding genes was optimized using inferred amino acid sequences. This alignment is available on the Parasitic Plant Connection web site (Nickrent, 1997 onwards). Maximum parsimony (MP) and maximum likelihood (ML) analyses were performed for two partitions (nuclear SSU rDNA and the three chloroplast genes concatenated) and a combined nuclear plus chloroplast gene matrix. Parsimony and bootstrap analyses (1,000 bootstrap replicates) were conducted in PAUP* (Swofford, 2002) using heuristic search strategies. In all analyses

gaps were coded as missing. Maximum likelihood analysis was conducted on the combined nuclear plus chloroplast matrix using RAxML-III (Stamatakis & al., 2005) which implements the GTR (General Time Reversible) model of sequence evolution. In that program, the initial random parsimony tree was determined using Dnapars (Felsenstein, 1981). Bootstrap nodal support was assigned by analyzing 100 replicates. The alignment is available on the Parasitic Plant Connection at: www.parasiticplants.siu.edu/Alignments/Alignments.html.

RESULTS

The four-gene alignment was composed of the following genes and sites: SSU rDNA (1,748), *rbcL* (1,402), *atpB* (1,440), and *ndhF* (2,201). Heuristic MP searches of the SSU rDNA partition alone resulted in 160 parsimony informative sites that yielded one tree of length 587 (consistency index [CI] minus uninformative sites 0.4968). For the three-gene chloroplast partition (excluding the four holoparasites), MP searches gave 1,061 parsimony informative sites that produced 12 trees of length 4,289 (CI minus uninformative sites 0.4902). The topologies of the SSU rDNA tree and the strict consensus tree from the chloroplast partition were largely congruent (data not shown), thus they were combined for a total evidence analysis. This MP analysis included all 31 taxa, had 1,221 parsimony informative sites, and produced six trees of length 4,885. The strict consensus of these six trees yielded a strongly supported Malvales clade with *Neurada* sister to the remaining taxa (Fig. 1). Five additional clades also received strong support: core Malvales, *Muntingia* plus Cytinaceae, a thymelaeacean clade, a dipterocarpacean clade, and a bixalean clade. Maximum likelihood generated a bootstrap tree with nearly identical topology and support for these clades (Fig. 1). A sister relationship between *Rhopalocarpus* (Sphaerosepalaceae) and the bixalean clade was recovered using ML (bootstrap support [BS] = 65%) but not with MP which placed this genus as sister to the dipterocarpacean and bixalean clades. Although support with both MP and ML for the five major clades was high, resolution of relationships among these clades was not obtained.

DISCUSSION

These phylogenetic analyses provide the first specific hypothesis regarding the closest photosynthetic relatives for Cytinaceae, a family formerly considered closely related to Rafflesiaceae. Although the combination of four genes used here did not provide clear resolution of the topological relationships among the five major malval-ean clades, such a result was not required to address the

primary question of this paper. Strong bootstrap support (MP = 90, ML = 76) was obtained for a clade composed of *Cytinaceae* plus *Muntingia*. *Cytinaceae* was monophyletic with strong bootstrap support (MP = 97, ML = 72) with *Bdallophyton* occurring sister to the clade composed of three *Cytinus* species.

When viewing the SSU rDNA, two branches of the *Cytinaceae* clade have longer branches than most autotrophic plants: the branch connecting the common ancestor of *Cytinus* to the ancestral *Cytinaceae* node (53 steps) and the equivalent branch from *Bdallophyton* (36 steps).

However, the branch connecting the ancestral *Cytinaceae* node with *Muntingia* is 23 steps long, comparable to or shorter than other branches on the tree (such as those connecting genera in *Capparales*). Thus, long branches appears to be mainly confined to the *Cytinaceae* clade. In general, model-based methods have been shown to provide superior results when long-branch attraction is an issue (Nickrent & al., 2004), yet here the MP and ML results are highly congruent. Taken together, these data suggest that the association of the holoparasites with *Muntingia* is not an artifact resulting from long-branch attraction.

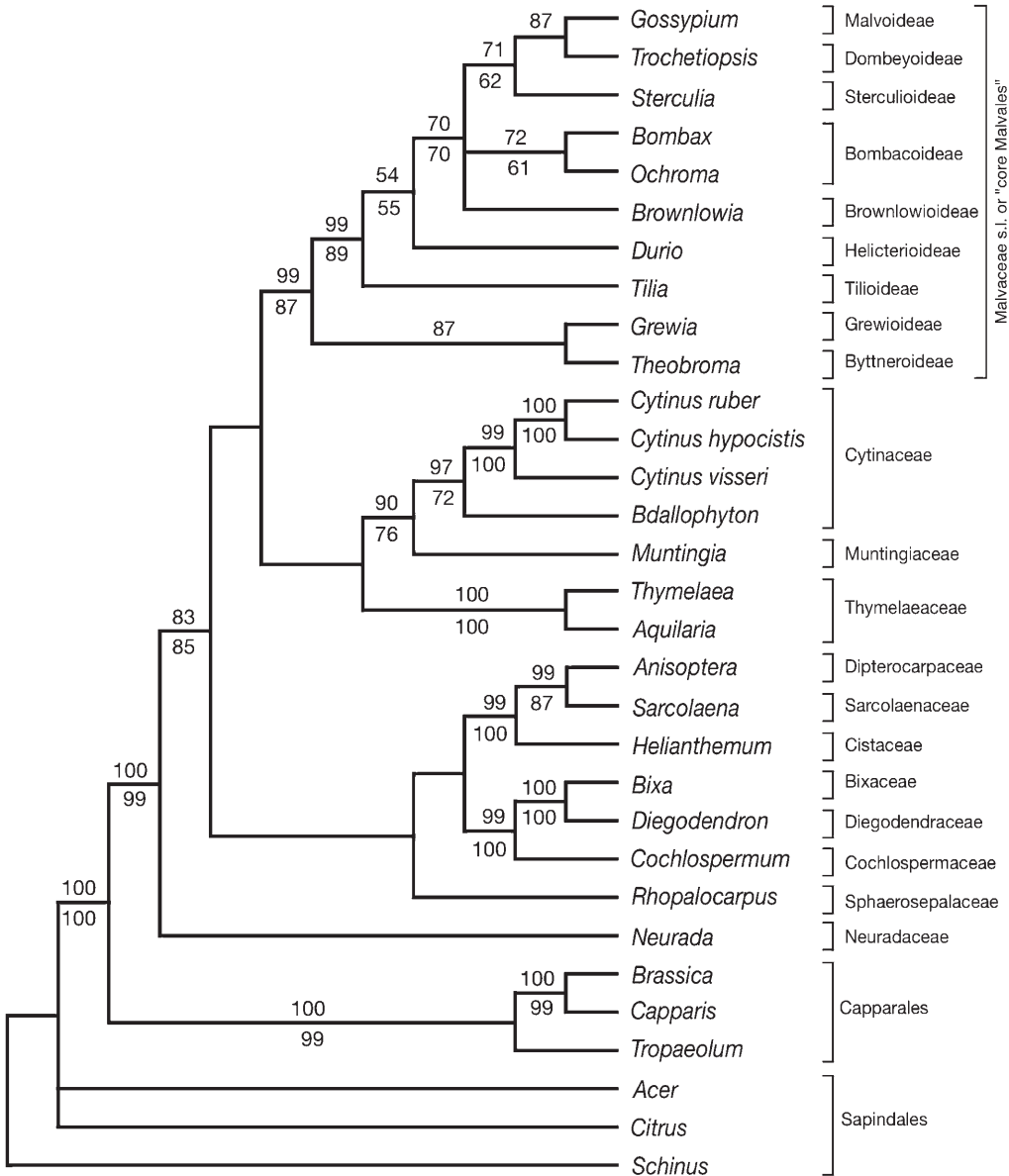


Fig. 1. Strict consensus (of 6) MP tree obtained from analysis of a combined SSU rDNA, *rbcL*, *atpB*, *ndhF* dataset for Malvales and outgroups (*Capparales* and *Sapindales*). Maximum parsimony bootstrap support given above branches and maximum likelihood bootstrap support below. Consistency index minus uninformative sites 0.4898, retention index 0.5646, rescaled consistency index 0.3360.

Another genus in Muntingiaceae, *Dicraspidia*, is currently represented in GenBank only by an *rbcL* sequence. This sequence was added to the four-gene alignment (coding the other genes as missing) and MP analysis showed this genus to be part of a polytomy with Cytinaceae with *Muntingia* sister to that clade (data not shown). No DNA sequences are available from the rare *Neotessmannia*.

Because some northern hemisphere species of *Cytinus* are parasitic on Cistaceae, and because this family is also in Malvales, suggestions have been made (Barkman & al., 2000, 2001) that Cytinaceae may have evolved via adelphoparasitism (i.e., that the parasite evolved directly from its host). The molecular phylogeny presented here argues strongly against this being the case, as Cistaceae are sister to Dipterocarpaceae and Sarcolaenaceae, and distinct from the Cytinaceae clade. Moreover, the host range for *Cytinus* is underestimated when one considers only those species centered around the Mediterranean. African and Malagasy species parasitize a number of other families (e.g., Asteraceae, Rutaceae, Euphorbiaceae) and the New World *Bdallophyton* occurs on Burseraceae.

The following discussion is an attempt to summarize existing knowledge on the morphology of Cytinaceae and Muntingiaceae. The task of comparing these two groups is complicated by the fact that relatively few vegetative characters exist in the achlorophyllous holoparasites that can be used for this exercise. Inflorescences and flowers provide the greatest potential for discovering phylogenetic affinities, thus they are used here.

In some taxa, such as *Bdallophyton* and some species of *Cytinus*, the branched or unbranched axis (stem or inflorescence) is elongate and contains numerous flowers in a raceme, spike, or capitulum. In other species of *Cytinus* (e.g., *C. baroni*), this axis is quite reduced (or absent) and the aerial portion of the plant is entirely represented by a flower emerging from the host tissues. In this regard such plants appear quite similar in habit to *Apodanthes*, *Berlinianche*, and *Pilostyles* (Apodanthaceae). The scale leaves of the axis may grade into floral bracts. Most Cytinaceae have flowers subtended by a floral bract and sometimes prophylls and these may form somewhat of an involucre around the cluster of flowers.

As discussed by Bayer & al. (1998), the combined presence of stellate hairs, long simple trichomes and glandular trichomes supports the view that Muntingiaceae are not closely related to Tiliaceae, Elaeocarpaceae, or Flacourtiaceae. Similar types of pubescence are present in Cytinaceae, these most commonly associated with floral as opposed to vegetative organs: long simple trichomes in *Cytinus capensis*, short obtuse trichomes in *C. hypocistis*, and glandular, sometimes branched trichomes in *C. glandulosus* (Harms, 1935). Trichomatic floral nectaries have been documented for core Malvales, but their presence in

Muntingiaceae is disputed. Venkata Rao (1952) indicates they are present in *Muntingia* but Stevens (2001 onwards) and Bayer & al. (1998) indicate that these trichomes are eglandular. For *Cytinus hypocistis*, nectaries are located at the base of the staminal and stylar columns and glandular trichomes line the inner surface of the petals (see Harms, 1935, Fig. 146G, H). Glandular trichomes also line the openings of axillary cavities formed by the petals (see below) in South African *C. capensis* (Visser, 1981) and *C. visseri* (Burgoyne, 2006). These trichomes appear to briefly produce nectar that collects in the floral cavities (P.M. Burgoyne, pers. comm.).

Flowers in Muntingiaceae are bisexual whereas unisexuality is most frequent in Cytinaceae, and the distribution of sexes is then monoecious or dioecious. Harms (1935) reports a description [by Heinricher] of an hermaphroditic flower in *Cytinus hypocistis*. *Muntingia* apparently has a flexible form of andromonoecy (Bayer, 2003). A valvate, basally connate calyx is seen in Muntingiaceae, but this floral whorl cannot be compared to Cytinaceae because their monochlamydous flowers show no vestige of a calyx. Here, the single floral whorl will be referred to as the perigone given that its homology with sepals or petals has not been determined. Petal number in Muntingiaceae is typically five but varies slightly around this value. Similarly, Cytinaceae typically have 4 to 6 perigone members that are fused into a tube (of various lengths) with free lobes. The situation in *Bdallophyton* is somewhat different in that the perigone lobes are initially fused and then at anthesis tear open giving from 5 to 9 lobes in a campanulate arrangement. In *Cytinus* (but not *Bdallophyton*) the base of the perigone tube produces flanges (lamellae in Harms, 1935) that connect to the staminal or stylar columns, thus forming the walls of axillary cavities which serve as nectar receptacles.

The malvalean androecium is often composed of “many” stamens, and the number for Muntingiaceae is ca. 15 to 100. Stamen number in Cytinaceae varies but involves lower numbers (ca. 7 to slightly more than 20). Only a tendency towards coherence of filaments is seen in Muntingiaceae, whereas in Cytinaceae all members are fully monadelphous. Indeed the connation extends to the longitudinally dehiscent anthers as well. Anther dehiscence is by short slits in *Dicraspidia* and *Neotessmannia* or by longitudinal slits in *Muntingia*. This latter type is most common in Cytinaceae. Possibly all Cytinaceae have an extension from the connective between the anther thecae. This extension may be large and pointed (as in *Bdallophyton americanum*), short and blunt (*B. andrieuxi*), or branched and antler-like (*Cytinus visseri*). In *Cytinus*, one vascular strand exists for every two thecae which are sessile on the monadelphous staminal tube (Harms, 1935; Watanabe, 1936). These are thus dithecal but derived from the condition seen in *Bdallophyton* (Eichler, 1872)

where the two thecae are clearly associated with a distinct stamen.

Pollen is dehisced as single grains in *Muntingia* and *Dicraspidia* but as tetrads in *Neotessmannia* and in members of the related family Sarcocaulaceae (Erdtman, 1952; Straka & Friedrich, 1983; Bayer & al., 1998). Pollen tetrads are also present in southern hemisphere species of *Cytinus* such as *C. baroni* (Baker, 1888; Watanabe, 1936) but apparently not in northern hemisphere species such as *C. hypocistis* nor the New World *Bdallophyton* (D.L. Nickrent, pers. obs.). The presence of mature pollen as tetrads in angiosperms is scattered among numerous families such as Monimiaceae (Sampson, 1977), Annonaceae (Tsou & Fu, 2002), Typhaceae (Skvarla & Larson, 1963), and Onagraceae (Skvarla & al., 1975), thus indicating that this feature has evolved multiple times independently. For this reason, and because the mode of binding together pollen grains in tetrads varies, it is not clear whether this feature is a synapomorphy between Cytinaceae and Muntingiaceae. Evolutionary-developmental approaches using the *quartet* mutant from *Arabidopsis* (Rhee & Somerville, 1998) may shed light on this question.

Ovary position in Muntingiaceae ranges from superior in *Muntingia*, to half inferior in *Dicraspidia* to fully inferior in *Neotessmannia*; the latter condition is found in all Cytinaceae. Both families have multilocular ovaries, but differ in placentation types. In *Muntingia* and *Dicraspidia* the placentae are bilobed axile-pendulous or axile-laminar (unknown for *Neotessmannia*), and false septa may lead to an increase in the number of locules (Robyns, 1964). For Cytinaceae, the final form of placentation appears to be intrusive parietal via gelatination of the central axis (Lutz, 1899). The ovary in Cytinaceae may have up to ca. 14 placentae that are densely covered with many orthotropic ovules that have tenuinucellate nucelli without parietal cells (Bouman & Meijer, 1994). Embryo sac development for *Muntingia* follows the Onagrad type (Venkata Rao, 1952) differing from most members of Malvales and *Cytinus* (Bouman & Meijer, 1994) that have the Polygonum type. Embryological features of *Dicraspidia* and *Neotessmannia* are unknown. During fruit development in Cytinaceae the seeds become embedded in a mucilaginous substance that is formed from the degeneration of the epidermal layer of the placentae and funicles. The fruit type for both families is a berry with numerous seeds embedded in pulp.

In summary, Cytinaceae may be linked to Muntingiaceae, particularly with the genus *Neotessmannia*, based on a several morphological features. These include trichome types, mature pollen released as tetrads, inferior ovaries with intrusive parietal placentation (the latter unknown in *Neotessmannia*), and fruits as pulpy berries with numerous small seeds. Other features that are best compared to Malvales in general include monadelphous stamens and the

Polygonum type of embryo sac development. Gallic and ellagic acids are present in *Muntingia* (Hegnauer, 1990) and have also been found in *Cytinus hypocistis* (Fürstenwerth & Schildknecht, 1976; Schildknecht & al., 1985).

The topology of the molecular tree has biogeographic implications for the evolution of Cytinaceae. All three genera of Muntingiaceae are neotropical whereas Cytinaceae are disjunct between the Old and New World. The distributions of *Cytinus hypocistis* and *C. ruber* center around the Mediterranean but extend from the Canary Islands to the Caucasus mountains of Eurasia. The newly named *C. visseri* (Burgoyne, 2006) occurs in South Africa (as do *C. capensis* and *C. sanguineus*, not sampled here). Three species have been named from Madagascar (*C. baroni*, *C. glandulosus*, *C. malagasicus*) but this region likely harbors several unnamed species—see Cytinaceae on the Parasitic Plant Connection (Nickrent, 1997 onwards). As expected given their similar morphologies, *C. hypocistis* and *C. ruber* are sister and this clade is sister to *C. visseri*. The genus *Bdallophyton* is sister to the entire *Cytinus* clade (Fig. 1). Given that all Muntingiaceae occur in the New World, it is most parsimonious to reconstruct an area cladogram that shows the common ancestor of all Cytinaceae evolving in the New World. A possible scenario explaining this would be that this ancestor was present in tropical regions of South America in the late Cretaceous or early Cenozoic. Following evolution of the parasitic habit, the Cytinaceae ancestor migrated to what is now continental Africa, possibly via island chains that later became Macaronesia. Further migration north and south and subsequent speciation resulted in a widely disjunct distribution for *Cytinus*. In the New World, *Bdallophyton* is presently restricted to tropical dry, deciduous forests from Mexico to Costa Rica (García-Franco & Rico-Gray, 1996). In comparison with *Cytinus*, *Bdallophyton* retains several plesiomorphies (e.g., inflorescence, anther morphology, etc.) that suggest it is less derived from the shared ancestor.

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Appendix. Taxa sampled and GenBank Accession numbers (nuclear SSU rDNA, chloroplast *rbcL*, *atpB*, *ndhF*) with place-holder taxa in parenthesis.

Cytinaceae: *Bdallophyton americanum*, AY739089, –, –, –; *Cytinus hypocystis*, AY739092, –, –, –; *Cytinus ruber*, L24085, –, –, –; *Cytinus visseri*, AY739091, –, –, –.

Outgroups: *Acer saccharum*, U42494 (*A. rubrum*), L01881, AF035893, AF130227 (*A. negundo*); *Anisoptera marginata*, AF206849, Y15144, AF035918, EU077548 (*Monotes adenophyllus*); *Aquilaria beccariana*, EF116225, Y15149, AJ233079, EU077549 (*Octolepis casearia*); *Bixa orellana*, AF206868, Y15139, AF035897, EU077547; *Bombax buonopozense*, U42507 (*B. ceiba*), AF022118, AJ233051, AF111726; *Brassica oleracea*, X17062 (*B. hirta*), M88342, AF209545 (*B. balearica*), DQ288742; *Brownlowia elata*, –, AJ233147, AF035898, AF111756; *Capparis spinosus*, EU090942, M95755, AF035900, AY122363 (*C. amplissima*); *Citrus sinensis*, U38312 (*C. aurantium*), NC008334, NC008334, NC008334; *Cochlospermum intermedium*, –, Y15143, AJ233060, AF111779 (*C. vitifolium*); *Diegodendron humberti*, –, Y15138, AJ233061, EU077550; *Durio zibethinus*, AF206905, AF206764, AJ233053, AF111749; *Gossypium hirsutum*, U42827, X15886, AJ233063, GHU55340; *Grewia occidentalis*, AF206921, AJ233152, AJ233105, AF111769 (*G. biloba*); *Helianthemum grandiflorum*, AF206926, Y15141, AF035907, EU077551 (*H. nummularium*); *Muntingia calabura*, U42539, Y15146, AJ233068, AF111781; *Neurada procumbens*, AF206970, U06814, AJ233069, EU077552 (*Neuradopsis austro-africana*); *Ochroma pyramidale*, AF206975, AF022122, AF035910, AF111740; *Rhopalocarpus lucidus*, –, AF022130, AJ233071, AF111780; *Sarcolaena* sp., EF116224, Y15147, AJ233070, EU081848 (*S. eriophora*); *Schinus molle*, AF207015, U39270, AF035914, AY643123; *Sterculia apetala*, AF207029 (*S. recordiana*), AJ233140, AJ233089, AF111747 (*S. tragacantha*); *Theobroma cacao*, AF207040, AF022125, AJ233090, AF287916; *Thymelaea hirsuta*, AF20704, Y15151, AJ235626 & AJ233097, EU077553 (*Peddia involucrata*); *Tilia americana*, AF207042, AF022127, AJ233113 (*T. platyphyllos*), AF111760; *Trochetiopsis erythroxyton*, –, AJ233143, AJ233093, AY326477 (*T. ebenus*); *Tropaeolum majus*, L31796, L14706, AF035917 (*T. tricolor*), AJ236281.

– Sequence not available.