Short Communication

Testing the monophyly of *Aesculus* L. and *Billia* Peyr, woody genera of tribe

Hippocastaneae of the Sapindaceae

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Abstract

Hippocastaneae is a well-supported clade in Sapindaceae that comprises 15+ species; 12+ in *Aesculus*, two in *Billia*, and one in *Handeliodendron* Rehder. The monophyly of *Aesculus* and *Billia* were widely assumed, but a recent molecular phylogenetic study of Sapindaceae used seven species of *Aesculus* and one each of *Billia* and *Handeliodendron* and showed that *Billia* and *Handeliodendron* were nested within *Aesculus*. Here, we tested the hypothesis that *Aesculus* and *Billia* are mutually monophyletic using phylogenetic analyses of seven molecular markers and 31 accessions of Hippocastaneae representing 14 species. We performed phylogenetic analyses using a dataset of concatenated genes as well as with coalescent method for constructing a species tree from individual gene trees. The analysis of seven concatenated markers and the species tree strongly supported the mutual monophyly of *Aesculus* and *Billia*. We also recovered support for the traditional arrangement of genera within Hippocastaneae: *Aesculus* and *Billia* comprising a clade that is sister to *Handeliodendron*. However, the relationships among the genera remain incompletely resolved.

Keywords: *Aesculus*, *Billia*, *Handeliodendron*, Hippocastaneae, phylogeny, Sapindaceae

1. Introduction

Morphological and molecular evolutionary studies show that the woody plant genera, *Aesculus* L. (i.e., buckeyes and horsechestnuts), *Billia* Peyr., and *Handeliodendron* Rehder should be regarded as a distinct tribe, Hippocastaneae (Acevedo-Rodriguez et al., 2011), within a broadly circumscribed Sapindaceae (Buerki et al., 2009; Buerki et al., 2010; Gadek et al., 1996; Harrington et al., 2009; Harrington et al., 2005; Judd et al., 1994). *Aesculus*, (12+ spp.) *Billia* (2 spp.) and the monotypic *Handeliodendron* formerly comprised the family Hippocastanaceae (Fang, 1960, 1981; Hardin, 1957a, b, c, 1960; Turland and Xia, 2005, 2007). *Handeliodendron* was originally circumscribed within the tribe Harpullieae of Sapindaceae (Rehder, 1935) but was later resolved in clade with *Aesculus* and *Billia* based on phylogenetic analyses of morphological characters (Judd et al., 1994). The three genera of Hippocastaneae are distinguished within Sapindaceae by having opposite leaves, connate sepals (tubular or campanulate), and seven stamens (individuals rarely with more or less) (Hardin, 1957c; Jussieu, 1789; Lawrence, 1951; Rehder, 1935). However, they share 4- and 5-merous flowers, pollen in monads, and the lack of endosperm in common with other sapindaceous taxa (Acevedo-Rodriguez et al., 2011). Hippocastaneae is sister to Acereae, which comprises *Acer* L. (i.e., maples) and *Dipteronia* Oliv. (Acevedo-Rodriguez et al., 2011)

The polytypic genera in Hippocastaneae, *Aesculus* and *Billia*, are traditionally assumed to be mutually monophyletic (Acevedo-Rodriguez et al., 2011; Hardin, 1957a, b, c, 1960; Takhtajan, 2009; Turland and Gadek, 2007; Xia et al., 2007) and to comprise a clade that is sister to *Handeliodendron* (Judd et al., 1994; Xiang et al., 1998). However, a recent molecular phylogenetic study (Buerki et al., 2010) used eight sparsely sampled markers for nine species of Hippocastaeae and showed *Billia* and *Handeliodendron* nested within *Aesculus*. Nevertheless,

that study and other prior molecular studies on Hippocastaneae have either included too few species of *Aesculus* and *Billia* to evaluate their monophyly (Buerki et al., 2009; Buerki et al., 2011; Harrington et al., 2005) or have hardwired *Billia* and *Handeliodendron* as monophyletic outgroups (Harris et al., 2009; Xiang et al., 1998).

The primary objective of this study was to test the hypothesis that *Aesculus* and *Billia* are mutually monophyletic. We tested our hypothesis using phylogenetic reconstructions based on rigorous taxonomic sampling and seven molecular markers including chloroplast and nuclear genes. We also used our data to assess the relationships among *Aesculus*, *Billia*, and *Handeliodendron*.

2. Materials and Methods

2.1 Taxonomic Sampling

We sampled 23 accessions of *Aesculus* representing 11 of 12+ species (including two subspecies each within *A. glabra* Willd. and *A. pavia* L.), six accessions of *Billia* representing 2 species and an unidentified individual, and two accessions of *Handeliodendron bodinieri* Rehder. Unfortunately, we were unable to sample *Aesculus assamica* Griff., which occurs in western China, Myanmar, and the Assam region in India (Hardin, 1960). However, *A. assamica* may be synonymous with *Aesculus wangii* Hu (Turland and Xia, 2007), which we did sample. We also sampled five species of *Acer* L. representing five traditional taxonomic sections (van Gelderen et al., 1994). We treated *Acer* within the ingroup in phylogenetic analyses to reaffirm the monophyly of Hippocastaneae. Our sampling comprised other species of Sapindaceae as outgroups: *Koelreuteria bipinnata* Franch., *Sapindus* L. sp. and *Ungnadia speciosa* Endl. All accessions are documented by herbarium collections, and we have provided voucher information in Table 1.

2.2 Molecular Data

2.2.1 Generating Molecular Data

We obtained DNAs from leaf materials that were either freshly collected and dried in silica gel or removed with permission from herbarium specimens at the United States National Herbarium (US). For the freshly collected and herbarium materials, we followed identical procedures for DNA extraction. We prepared the materials for extraction by freezing them in liquid nitrogen and grinding them into powder using a bead mill. We accomplished the extractions using manufacturer protocols for an Autogen Gene Prep robot (AutoGen, Inc., Holliston, MA, USA), which automates the CTAB extraction method (Doyle and Doyle, 1987).

We amplified DNA from seven molecular markers with priming sites broadly conserved in angiosperms and representing five chloroplast and two nuclear DNA (cpDNA and nDNA, respectively) regions. Our cpDNA markers comprised the open reading frame of *ycf1* (Dong et al., 2015) and the intergenic spacers *petN-trnC* (Lee and Wen, 2004), *trnS-trnG* (Shaw et al., 2005), *trnL-trnF* (Taberlet et al., 1991), and *psbA-trnH* (Sang et al., 1997). We amplified the chloroplast gene regions (or genes, hereafter) using the primers detailed in the cited studies. Our nDNA markers were composed of portions of the third exon and intron of *at103* and the second intron of *squd1* using primers reported by Li et al. (2008). Our PCR reactions for all primer pairs comprised 2.5μL of PCR Buffer, 2.0μL of 10mM dNTPs, 50mM of MgCl₂, 1.0μL each of forward and reverse primers, 0.5μL of BSA, 0.2μL of Taq Polymerase, and 14.05μL of nuclease free water; and we performed PCR reactions under thermocycler conditions of initiation at 95°C for 5min; 35 cycles of 94°C for 45s, 54°C for 45s (52°C for *trnL-trnF*), and 72°C for 45s; and a final extension at 72°C for 10min. We purified the amplified PCR products using the ExoSapIT

enzyme with activation at 37°C and deactivation at 95°C. We attempted to amplify and sequence each gene for all plant materials collected for this study.

We used the purified PCR products and PCR primers to perform cycle sequencing. Our PCR products were small, so internal primers were unnecessary. We prepared the sequencing reactions using standard Big Dye (Applied Biosystems, Foster City, USA) protocols except that we used 0.8µL of enzyme and brought the reaction to 10µL volume with water. The cycle sequencing comprised 30 thermocycles of 95°C for 30s, 50°C for 30s, and 60°C for 4 minutes. The products of cycle sequencing were read by an ABI 3730 automated sequencer (Applied Biosystems, Foster City, USA). All sequences were new to this study (Table 1).

2.2.2 Processing and Analyzing Molecular Sequence Data

We assembled DNA sequences using algorithms in Geneious version 7.1.5 (Kearse et al., 2012) and Mesquite version 2.75 (Maddison and Maddison, 2011). Specifically, we used Geneious to remove blocks of low quality bases ($\geq 5\%$ chance of error per base) at the 3' and 5' ends of sequencing results and, subsequently, to generate contigs of sequences for each marker for each accession. We edited base calls as needed by eye from chromatograms and allowed ambiguous nucleotide character states. We used the Geneious global alignment algorithm with a gap penalty of 12, extension cost of three, and free end gaps to align sequences of all taxa for each gene. We allowed the alignment algorithm to run for ten iterations beyond to the first one for refinement. We adjusted the alignments manually in Mesquite to minimize gaps.

For each aligned gene, we found the best models of nucleotide evolution using JModelTest version 2.1.4 (Posada, 2008). We compared a total of 203 models including those with a gamma distribution of rates (+G) approximated by ten categories and a proportion of

invariant sites (+I). We set JModelTest to calculate the best model according to the Bayesian information criterion (BIC) to limit overfitting (i.e., compared to AIC, see Hastie et al., 2005).

To reduce missing data in matrices for each gene, we trimmed the ends of sequences and applied indel coding. We trimmed the sequences so that neither the 5' or 3' ends of a matrix had more than 20% missing data. We applied indel coding to a concatenated matrix of all sequences only. Our indel coding comprised the simple, binary method of Simmons and Ochoterena(2000), implemented in SeqState (Müller, 2005).

We performed phylogenetic analyses using MrBayes version 3.2.2 (Ronquist et al., 2012) on the Cowboy supercomputer maintained by Oklahoma State University High Performance Computing Center (<https://hpcc.okstate.edu/>). These analyses consisted of two simultaneous runs for each gene independently, for concatenated matrices of chloroplast and nuclear genes independently, and for a concatenated matrix of all DNA data. We set the analyses to implement the model parameters resolved with JModelTest, and we used the best fitting model among those that MrBayes can implement (Table 2). The best models for all genes included a gamma rate distribution, which we approximated with ten rate categories. We ran our analyses in MrBayes with 11 hot chains and 1 cold chain for 15 million MCMC generations with sampling every 5000 generations (i.e., 3001 trees). For the analyses of the concatenated matrices, we partitioned the data by gene and unlinked the model parameters among partitions. We checked all runs visually for stationarity using Tracer version 1.6 (Rambaut and Drummond, 2007), and we determined that a 20% burnin was appropriate for all posterior distributions of trees. We implemented the burnin and combined the trees from the simultaneous runs using LogCombiner from the *BEAST package.

We performed coalescent analyses in *BEAST (Drummond and Rambaut, 2007) using three different datasets to generate species trees. Our datasets comprised (1) all seven genes partitioned individually, (2) all genes partitioned by organelle (chloroplast and nuclear), and (3) nuclear genes only partitioned individually. For all three datasets, we assigned each accession to its species, except that we removed *Billia* sp. from the matrix, because we could not determine with confidence whether it belonged to *B. hippocastanum* or *B. rosea* (see Table 1). Additionally, we treated the three outgroups as a single species represented by three sequences. In treating the outgroups in this way, we followed Heled and Drummond (2010); namely in that 'species' in the coalescent analysis refers to a lineage that ceased to interbreed with the rest of the group in the past, not a taxonomic rank. Moreover, using an outgroup represented by multiple sequences enabled a more rigorous implementation of the *BEAST tree building algorithm, because multiple sequences of a taxon are required to estimate its coalescent events and population size (Heled and Drummond, 2010). We implemented substitution models as shown in Table 2 with gamma categories set to ten and applied a random local molecular clock with no calibrations independently for each gene. We also set the birth-death model for speciation rates across the tree and modeled population size as piecewise and linear with a constant root. We started phylogenetic estimation in *BEAST with a UPGMA tree for each gene partition, and we ran *BEAST for ten million generations with sampling every 1000 generations for a total posterior distribution represented by ten thousand species trees. We checked the *BEAST results in Tracer, and manually removed 20% of trees as burnin. We performed the *BEAST analyses on the Cowboy supercomputer.

We generated maximum credibility trees for all post-burnin distributions of trees, including species trees, using TreeAnnotator, which is part of the *BEAST package. We set

TreeAnnotator to calculate the maximum credibility trees using only posterior probabilities of ≥ 0.5 and with the node heights of clades representing the median of the posterior distribution.

We visualized the maximum credibility trees using FigTree version 1.4.2

(<http://tree.bio.ed.ac.uk/software/figtree/>).

We performed a NeighborNet network analysis in SplitsTree version 4.13.1 (Huson, 1998) for Hippocastaneae using DNA sequences from all genes. We performed the network analysis as a preliminary way to detect and visualize reticulate evolution, such as arises due to hybridization. There is no evidence for ongoing hybridization among *Aesculus*, *Billia*, and *Handeli dendron*, and they are probably too geographically separated for it to occur. Nevertheless, we cannot rule out ancient hybridization, especially because some species of *Aesculus* produce natural hybrids in areas of secondary contact or when cultivated in close proximity to one other (Hardin, 1957d). Within SplitsTree, we used the NeighborNet method because, it allows and graphs groupings that are incompatible in bifurcating phylogenies (Huson and Bryant, 2006). For performing the NeighborNet analysis, we calculated distances between sequences in SplitsTree as the proportion of different sites (Huson and Bryant, 2006), and we removed sequences of two accessions that resulted in reciprocally saturated distances (i.e., of 1 due to differentially missing data; *Billia hippocastanum* 2 and *Billia hippocastanum* 5 and additional information in Table 1). We determined the support for the splits in the network using 1000 bootstrap replicates. We drew the network in SplitsTree using the Equal Angle algorithm, which improves visual clarity by graphing parallel (i.e., non-crossing) edges, or branches (Klopper and Huson, 2008).

3. Results and Discussion

3.1 Phylogenetic Analyses

3.1.1 Single-Gene Phylogenies

Our phylogenetic analyses of individual genes yielded mixed results for the monophyly of *Billia* and *Aesculus* (Table 3). Five of the seven genes supported the monophyly of *Billia* (1.00pp), while *psbA-trnH* showed that *Billia* was paraphyletic and included *Handeliiodendron* (0.36pp), and *srd1* showed that *Billia* formed a grade into *Aesculus* (Table 3). The monophyly of *Aesculus* was supported by two chloroplast genes with pp = 1.00. However, *Aesculus* was paraphyletic and included *Billia* and *Handeliiodendron* according the other three chloroplast genes and both nuclear genes. The paraphyly in *Aesculus* had weak support (pp = 0.12-0.79). All single gene trees are presented in Supplementary file 1.

Among individual genes, phylogenetic positions were highly variable for *A. californica* (Spach.) Nutt, which is endemic to California and southern Oregon, *A. parviflora* Walt., which occurs narrowly in Alabama and Georgia, and members of sect. *Calothyrsus*, which have ranges within mainland eastern Asia. For example, *A. californica* was sister to sect. *Calothyrsus* in the *psbA-trnH* tree and sister to sect. *Aesculus*, comprising species disjunct in Japan and Europe, in the *petN-trnC* tree. The positions of *A. californica*, *A. parviflora*, and sect. *Calothyrsus* have also been inconsistent among prior studies (Forest et al., 2001; Hardin, 1957c; Harris et al., 2009; Xiang et al., 1998). In particular, *A. californica* was resolved within or as sister to sect. *Calothyrsus* according to cladistic analyses of cpDNA and morphology, but it is sister to *A. parviflora* according to published nuclear phylogenies (Forest et al., 2001; Hardin, 1957c; Harris et al., 2009; Xiang et al., 1998). The sister relationship of *A. californica* to *A. parviflora* may also be supported inductively by biogeography, because species pairs may be less likely to be disjunct among continents (i.e., Asia and North American) than more distantly related clades within genera (Wen, 1999). The inconsistent relationships among *A. californica*, *A. parviflora*, and sect.

Calothyrsus may be due to incomplete lineage sorting (Harris et al., 2009; Xiang et al., 1998) or ancient hybridization. In our present analyses, these three species behaved as wildcards (*sensu* Nixon and Wheeler, 1992) by occupying many weakly supported positions within and among the posterior distributions of gene trees, and they may have reduced support for or interfered with other nodes in our phylogeny (Nixon and Wheeler, 1992). Further work is needed to stabilize the phylogenetic positions of *A. californica*, *A. parviflora*, and sect. *Calothyrsus*; especially with greater taxonomic sampling depth within these three lineages to test hypotheses of incomplete lineage sorting and hybridization.

3.1.2 Chloroplast and Nuclear Gene Phylogenies

We obtained different results from our analyses of the cp- and nuclear DNA. The concatenated cpDNA matrix resolved the monophyly of *Aesculus* and *Billia*: *Aesculus* comprised a clade with 1.00pp and *Billia* had 0.99pp. In contrast to the cpDNA, the concatenated nuclear DNA matrix yielded a topology in which *Aesculus* contained independent clades of *Billia* and *Handelidendron* (Table 3; trees not shown). Paraphyly within the nuclear gene tree may result from the wildcard behavior of *A. californica* and *A. parviflora*, which were sister, respectively, to *Billia*, and a clade of sect. *Aesculus*, *Billia*, and *A. parviflora*. In the coalescent analysis of nuclear genes only, we resolved *Billia* with 1.00pp but found only 0.09pp for *Aesculus* (Fig. S1A).

3.1.3 Total Evidence Analyses (Concatenated Matrix, Species Tree, Network)

We performed total evidence analyses of concatenated data matrices with and without indel coding. Both analyses yielded the same topology and highly similar branch lengths. Thus, we present and discuss the tree with indel, because it showed equal or slightly higher pp values at all nodes.

All total evidence analyses supported the monophyly of *Aesculus* and *Billia*. The analysis of concatenated data resulted in monophyletic clades of *Aesculus* and *Billia* each with 1.00pp. While this result must be regarded with caution, because concatenation can amplify either phylogenetic signal or noise (Gadagkar et al., 2005), it is corroborated by our two coalescent analyses of all genes (Fig. S1B-C). The coalescent analysis with partitioning by gene yielded species trees with 1.00pp for the monophyly both genera (Fig. S1B), and the analysis with partitioning by organelle resulted in 0.91pp for *Aesculus* and 1.00pp for *Billia* (Fig. S1C). The monophyly of both genera is corroborated by evidence from pollen, which is larger in *Billia* than in all of *Aesculus* according to prior studies (Harris et al., 2015; also see Pozhidaev, 1995). Additionally, our network analysis showed that *Aesculus* and *Billia* formed relatively well supported, distinct groups with bootstrap support of 81.6% and 100%, respectively (Fig. S2).

Among genera, we found support for a clade of *Aesculus* and *Billia* with *Handeliodendron* as its sister in all phylogenetic analyses except the coalescent analysis with nuclear genes only. Specifically, *Aesculus* and *Billia* were sisters in our analysis of the concatenated data matrix with 0.89pp (Fig. 1). The clade of *Aesculus* and *Billia* was also resolved in the coalescent analyses of all genes partitioned individually (0.67pp; Fig. S1B) and by organelle (0.41pp; Fig. S1C). The sister relationship between *Aesculus* and *Billia* may be corroborated by prior morphological studies, which unite the two genera and separate them from *Handeliodendron* on the basis of leaflets without glandular spots, anthers with apical and basal appendages (apical only in *Handeliodendron*), petals without appendages or appendages minute, and having a sessile ovary (Acevedo-Rodriguez et al., 2011; Judd et al., 1994; Xia et al., 2007). However, the coalescent analysis of nuclear genes only showed *Aesculus* as sister to *Handeliodendron* (pp = 0.50; Fig. A). *Aesculus* and *Handelidendron* also exhibit shared traits

including habitats that are within temperate latitudes and leaves with three to 11 leaflets in contrast to *Billia*, which occurs in the tropics and bears leaves with three leaflets (Hardin, 1957; Manchester, 2001; Xia et al., 2007). Overall, our support for the relationships among *Aesculus*, *Billia*, and *Handeliidendron* is relatively weak.

3.4 Summary

Aesculus and *Billia* are mutually monophyletic based on our analyses of all seven genes using traditional Bayesian and coalescent methods. However, phylogenies based on single genes or combined nuclear genes suggested weak support for the paraphyly of the two genera, especially *Aesculus*. We speculate that the paraphyly of *Aesculus* was an artifactual result of noise in our posterior distribution of trees caused by *A. californica*, *A. parviflora*, and members of sect. *Calothyrsus*, which seemed to behave as wildcards in phylogenetic analyses. Our results show low support for *Aesculus* as sister to *Billia* within Hippocastaneae. Nevertheless, the relationships among *Aesculus*, *Billia*, and *Handeliidendron* still need to be more completely resolved in future work with more robust data such as more plastid and nuclear genes.

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Table 1: Taxonomic and molecular sampling of *Aesculus*, *Billia*, *Handeliidendron*, and outgroups (all other genera). Subscripted numerals to the right of taxon names differentiate between multiple accessions where necessary and correspond to numbered accessions appearing in trees. For voucher specimens, we give the collector and collection number. All voucher specimens are housed at US unless otherwise noted by a parenthetical herbarium acronym. Symbols: [‡] Vouchered by an herbarium specimen with accession number 797498 at RSA; ^{††} Vouchered by an additional herbarium specimen with accession number 4552 at the unregistered herbarium of the State Arboretum of Virginia. Numbers beneath marker names refer to Genbank accession numbers. Genbank does not accept sequences of length <200, so some sequence fragments can be obtained only via Treebase (submission 19364; <http://purl.org/phylo/treebase/phylows/study/TB2:S19364?x-access-code=e098844e0bb7703fdee93a378c80aed3&format=html> FOR REVIEWER ACCESS ONLY). These are marked as 'see Treebase.'

Taxon	Collector and number	Molecular markers						
		<i>at103</i>	<i>sqd1</i>	<i>psbA-trnH</i>	<i>trnC-petN</i>	<i>trnL-trnF</i>	<i>trnS-trnG</i>	<i>ycf1</i>
<i>Acer cappadocicum</i> Gled. var. <i>sinicum</i> Rehder	Wen 12548	-	KX277877	KX277794	KX277763	KX277731	KX277824	KX277906
<i>Acer negundo</i> L.	Wen 12532	KX277851	-	KX277800	-	KX277732	KX277829	KX277907
<i>Acer palmatum</i> Thunb.	Wen 12523	KX277852	KX277878	KX277812	KX277768	KX277733	KX277830	KX277908
<i>Acer saccharinum</i> L.	Wen 12540	KX277853	KX277879	KX277814	KX277767	KX277734	KX277828	KX277909
<i>Acer triflorum</i> Kom.	Wen 12544	KX277854	KX277880	KX277818	KX277765	KX277735	KX277826	KX277910
<i>Aesculus californica</i> (Spach.) Nutt.	Harris 2014-13	KX277855	KX277881	KX277795	KX277781	-	KX277841	KX277911
<i>Aesculus chinensis</i> ¹ Bunge	Wen 12550	KX277856	KX277882	-	KX277762	-	KX277823	KX277912
<i>Aesculus chinensis</i> ² Bunge	Harris 2014-14	KX277857	KX277883	KX277811	KX277780	KX277736	KX277840	KX277913
<i>Aesculus flava</i> ¹ Aiton	Wen 12543	KX277858	KX277884	KX277797	KX277766	KX277737	KX277827	KX277914
<i>Aesculus flava</i> ² Aiton	Harris 2014-07	-	KX277885	-	KX277786	-	KX277845	KX277915
<i>Aesculus glabra</i> Willd. var. <i>arguta</i> ¹ (Buckley) B.L. Rob.	Harris 2014-18	KX2778	KX277886	KX277817	KX277776	KX277738	KX277837	see Treebase

<i>Aesculus glabra</i> Willd. var. <i>arguta</i> ² (Buckley) B.L. Rob.	Harris 2014-12	-	KX277887	-	KX277782	-	KX277842	-
<i>Aesculus glabra</i> Willd. var. <i>glabra</i>	Wen 12617	KX2778	-	KX277810	KX277760	KX277739	KX277822	KX277917
<i>Aesculus hippocastanum</i> L.	Harris 2014-10	see Treebase	KX277888	see Treebase	KX277784	KX277740	KX277844	KX277918
<i>Aesculus indica</i> (Wall. ex Cambess.) Hook.	Harris 2014-11	KX277861	KX277889	-	KX277783	-	KX277843	KX277919
<i>Aesculus parryi</i> Gray	17320 (RSA living collection) [†]	KX277862	KX277890	KX277809	-	KX277741	KX277850	KX277920
<i>Aesculus parviflora</i> ¹ Walter	Wen 12546	KX277863	KX277891	KX277796	KX277764	KX277742	KX277825	KX277921
<i>Aesculus parviflora</i> ² Walter	Wen 12702	see Treebase	KX277892	KX277804	KX277759	KX277743	KX277821	KX277922
<i>Aesculus pavia</i> L. var. <i>flavescens</i> ¹ (Sarg.) Correll	Harris 2014-16	KX277864	KX277893	KX277806	KX277778	KX277744	KX277838	KX277923
<i>Aesculus pavia</i> L. var. <i>flavescens</i> ² (Sarg.) Correll	Harris 2014-17	KX277865	KX277894	KX277807	KX277777	KX277745	-	KX277924
<i>Aesculus pavia</i> L. var. <i>pavia</i> ¹	Harris 2014-01	KX277866	KX277895	KX277802	KX277788	-	KX277847	KX277925
<i>Aesculus pavia</i> L. var. <i>pavia</i> ²	Harris 2014-08	see Treebase	KX277896	KX277793	KX277785	KX277746	-	KX277926
<i>Aesculus sylvatica</i> ¹ Bart.	Harris 2014-04 ^{††}	KX277867	KX277897	see Treebase	KX277787	KX277747	KX277846	-
<i>Aesculus sylvatica</i> ² Bart.	Wen 12592	-	KX277898	-	KX277761	-	-	-
<i>Aesculus turbinata</i> Blume	Harris 2014-15	see Treebase	KX277899	-	KX277779	KX277748	KX277839	KX277927
<i>Aesculus wangii</i> Hu	Wen 12482	KX277868	KX277900	KX277799	KX277771	KX277749	KX277833	KX277928
<i>Aesculus wilsonii</i> ¹ Rehder	Harris 2012-01	KX277869	KX277901	KX277798	KX277789	-	KX277848	KX277929
<i>Aesculus wilsonii</i> ² Rehder	Wen 12470	see Treebase	KX277902	KX277801	KX277772	KX277750	KX277834	KX277930
<i>Billia hippocastanum</i> ² Peyr	D.E. Wilbur and R.L. Stone 8669	see Treebase	-	KX277816	-	-	-	-
<i>Billia hippocastanum</i> ³ Peyr	S.S. Tillett 636-40	KX277870	KX277903	KX277813	KX277773	-	KX277835	-
<i>Billia hippocastanum</i> ⁴ Peyr	Gorostiza s.n.	KX277871	see Treebase	KX277808	KX277790	KX277751	KX277849	KX277931
<i>Billia hippocastanum</i> ⁵ Peyr	Castillo 2055	see Treebase	-	KX277805	-	-	-	-
<i>Billia rosea</i> ¹ (Planch. & Linden) C. Ulloa & P. Jørg.	Jaramillo 7880	see Treebase	-	-	KX277774	-	-	-
<i>Billia</i> Peyr. sp.	Hammel 25983 (OKLA)	see Treebase	see Treebase	-	see Treebase	-	see Treebase	-
<i>Handeliendron bodinieri</i> ¹ Rehder	Xiang 1186 (NSC)	KX277872	see Treebase	see Treebase	KX277757	KX277752	KX277820	KX277932
<i>Handeliendron bodinieri</i> ² Rehder	Xiang 1193 (NSC)	KX277873	see Treebase	see Treebase	KX277758	KX277753	-	KX277933
<i>Koelreuteria bipinnata</i> Franch.	Wen 12495	KX277874	KX277904	KX277815	KX277769	KX277754	KX277831	KX277934
<i>Sapindus</i> L. sp.	Wen 12485	KX277875	KX277905	KX277803	KX277770	KX277755	KX277832	KX277935
<i>Ungnadia speciosa</i> Endl.	Harris s.n.	KX277876	-	KX277819	KX277775	KX277756	KX277836	-

Table 2: Information and statistics for our sequences of *at103*, *psbA-trnH*, *petN-trnC*, *sqd1*, *trnL-trnF*, *trnS-trnG*, and *ycf1*. 'All' refers to all sequences, whereas 'tribe' refers to the sequences of species of *Aesculus*, *Billia*, and *Handeliendron*. The 'best model' is the model with the highest likelihood according to the Bayesian information criterion, while the 'model used' has the highest likelihood among models available in MrBayes. In *BEAST, we used the HKY model in place of the simpler models, which are marked with asterisks (*).

Marker	# taxa		best model	model used	average unaligned length	aligned length with gaps	# gaps coded and removed	final number of characters	% non-identical nucleotide sites		% missing nucleotides	
	all	tribe							all	tribe	all	tribe
<i>at103</i>	35	28	TVM+I+G	GTR+I+G	373.1	584	27	345	94.0	84.8	10.5	13.4
<i>psbA-trnH</i>	31	23	F81+I+G	F81+I+G*	441.2	740	94	200	87.6	82.8	8.2	10.6
<i>petN-trnC</i>	35	28	TVM+I+G	GTR+I+G	626.6	772	36	525	53.2	27.9	1.7	1.8
<i>sqd1</i>	34	28	JC+I+G	JC+I+G*	254.3	258	7	258	25.6	17.3	5.4	6.5
<i>trnL-trnF</i>	26	18	JC+I+G	JC+I+G*	887.3	1047	58	310	39.6	17.0	< 0.1	0.0
<i>trnS-trnG</i>	33	25	HKY+I+G	HKY+I+G	752.1	1025	98	400	80.0	19.6	5.8	3.8
<i>ycf1</i>	30	23	TrN+I+G	HKY+I+G	756.4	816	34	742	44.1	24.5	2.9	3.7

Table 3: Summary of independent Bayesian phylogenetic analyses of seven genes and combined nuclear and chloroplast genes, respectively, showing posterior probability (pp) support values for the monophyly of *Aesculus* and *Billia*. We indicate monophyly by showing the pp support. In cases where the genera were not monophyletic, we present notes and the pp for the most highly supported node that rejects monophyly.

Marker	<i>Aesculus</i>	<i>Billia</i>
<i>at103</i>	pp = 0.55; includes <i>Billia</i> and <i>Handeliodendron</i>	1.00
<i>psbA-trnH</i>	pp = 0.56; includes <i>Billia</i> and <i>Handeliodendron</i>	pp=0.36;includes <i>Handeliodendron</i>
<i>petN-trnC</i>	1.00	0.99
<i>sqd1</i>	pp = 0.14; includes <i>Handeliodendron</i>	pp=0.20; <i>Billia</i> grades into <i>Aesculus</i>
<i>trnL-trnF</i>	pp = 0.06; includes <i>Billia</i> and <i>Handeliodendron</i>	NA - 1 sample
<i>trnS-trnG</i>	pp = 0.79; includes <i>Billia</i> and <i>Handeliodendron</i>	1.00
<i>ycf1</i>	1.00	NA - 1 sample
<i>combined cpDNA</i>	1.00	0.99
<i>combined nDNA</i>	pp= 0.12; includes <i>Billia</i> and <i>Handeliodendron</i>	1.00

Figure Captions

FIG. 1. Total evidence, maximum credibility phylograms resulting from Bayesian analysis of seven molecular markers **A** - with and **B** - without use of indel coding. Clades of *Aesculus*, *Billia*, and *Handeliodendron* are delimited with gray boxes. The tree is rooted with *Ungnadia speciosa*, *Sapindus sp.*, and *Koelreuteria bipinnata*, and these outgroups are indicated by their genus names only. Other genus names are abbreviated, and *Ae.* represents *Aesculus*, while *Ac.* represents *Acer*. We show pp support on nodes. Nodes marked with asterisks (*) have pp values of 1.00, and unmarked nodes have pp values of 1.00 or less.

Figure 1

*Graphical Abstract