



# Phylogeny and biogeography of the pantropical genus *Zanthoxylum* and its closest relatives in the proto-Rutaceae group (Rutaceae)

Marc S. Appelhans<sup>a,b,\*</sup>, Niklas Reichelt<sup>a</sup>, Milton Groppo<sup>c</sup>, Claudia Paetzold<sup>a</sup>, Jun Wen<sup>b</sup>

<sup>a</sup> Department of Systematics, Biodiversity and Evolution of Plants, Albrecht-von-Haller Institute of Plant Sciences, University of Goettingen, Untere Karspuele 2, 37073 Goettingen, Germany

<sup>b</sup> Department of Botany, National Museum of Natural History, Smithsonian Institution, P.O. Box 37012, MRC 166, Washington, DC 20013-7012, USA

<sup>c</sup> Departamento de Biologia, Faculdade de Filosofia, Ciências e Letras de Ribeirão Preto, Universidade de São Paulo, Ribeirão Preto, São Paulo, Brazil

## ARTICLE INFO

### Keywords:

Bering Land Bridge

*Fagaria*

North Atlantic Land Bridge

*Toddalia*

Transatlantic Disjunction

## ABSTRACT

*Zanthoxylum* L. (prickly ash) is the only genus in the *Citrus* L. family (Rutaceae) with a pantropical distribution. We present the first detailed phylogenetic and biogeographic study of the genus and its close relatives in the proto-Rutaceae group. Our phylogenetic analyses based on two plastid and two nuclear markers show that the genus *Toddalia* Juss. is nested within *Zanthoxylum*, that earlier generic and intrageneric classifications need revision, and that the homochlamydeous flowers of the temperate species of *Zanthoxylum* are the result of a reduction from heterochlamydeous flowers. The biogeographic analyses reveal a Eurasian origin of *Zanthoxylum* in the Paleocene or Eocene with successive intercontinental or long-range migrations. *Zanthoxylum* likely crossed the North Atlantic Land Bridges to colonize the Americas in the Eocene, and migrated back to the Old World probably via the Bering Land Bridge in the Oligocene or Miocene. *Zanthoxylum* also colonized several Pacific Islands and the Hawaiian clade shows phylogenetic incongruence between the plastid and nuclear datasets, suggesting hybridization. The Hawaiian species are one of the rare examples of endemic Hawaiian lineages that are older than the current main islands.

## 1. Introduction

Rutaceae is a medium-sized family of flowering plants that is well known for the economically important genus *Citrus* L. While the family shows a worldwide distribution, *Zanthoxylum* L. (prickly ash) is the only genus that has been documented (extant or fossils) on all continents except Antarctica. The genus consists of about 225 species, which makes it the second largest genus of the family after *Melicope* J.R.Forst. & G.Forst. (about 300 species including several genera nested within it; Appelhans et al., 2014). It is distributed in tropical and subtropical areas in the Americas, Africa, Madagascar, Asia, Australia, and Pacific archipelagos, and a smaller number of species are adapted to temperate climates in eastern North America and Asia (Kubitzki et al., 2011). In the Americas, *Zanthoxylum* reaches the northern boundaries of its distribution in southern Ontario and south-western Quebec, Canada (Scoggan, 1978), while *Zanthoxylum mayu* Bertero is present in the southern limit of the genus (Juan Fernández Island, Chile, Reiche 1896); in Asia the northern boundaries are northern Japan (Hokkaido), Korea and northern and north-western China (Gansu, Hebei, and

Liaoning provinces) (Zhang et al., 2008). There are no extant species in Europe, but *Zanthoxylum* has a rich fossil record and the oldest fossils have been described from Early Eocene sediments in England (Chandler, 1961; Gregor, 1989). *Zanthoxylum* fossils have further been reported from the Late Oligocene onwards in the Americas (Graham and Jarzen, 1969; Tiffney, 1994), and from the Miocene onwards in Africa and Asia (Jacobs and Kabuye, 1987; Zhu et al., 2015). Because of the pantropical distribution and the rich fossil records, *Zanthoxylum* is an excellent model to study Cenozoic biogeography at the continental scale.

*Phellodendron* Rupr., *Tetradium* Lour. and *Toddalia* Juss. have been identified as close relatives of *Zanthoxylum* based on molecular phylogenetic data (Poon et al., 2007), a relationship that has long been hypothesized because of substantial phytochemical similarities (Fish and Waterman, 1973; Waterman, 2007). Several alkaloids have been identified that were regarded ancestral for Rutaceae and the group has therefore been called ‘proto-Rutaceae’ (Waterman, 1983). The four genera have been sampled in several phylogenetic studies at a broader scale. Poon et al. (2007) and Groppo et al. (2008, 2012) found a sister

\* Corresponding author at: Department of Systematics, Biodiversity and Evolution of Plants, Albrecht-von-Haller Institute of Plant Sciences, University of Goettingen, Untere Karspuele 2, 37073 Goettingen, Germany.

E-mail addresses: [mappelh@gwdg.de](mailto:mappelh@gwdg.de) (M.S. Appelhans), [niklas.reichelt@stud.uni-goettingen.de](mailto:niklas.reichelt@stud.uni-goettingen.de) (N. Reichelt), [groppo@fclrp.usp.br](mailto:groppo@fclrp.usp.br) (M. Groppo), [claudia.paetzold@uni-goettingen.de](mailto:claudia.paetzold@uni-goettingen.de) (C. Paetzold), [wenj@si.edu](mailto:wenj@si.edu) (J. Wen).

<https://doi.org/10.1016/j.ympev.2018.04.013>

Received 15 December 2017; Received in revised form 6 April 2018; Accepted 9 April 2018

Available online 10 April 2018

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group relationship of *Zanthoxylum* and *Toddalia*, while *Phellodendron* was nested within *Tetradium*, albeit with low support of several important nodes. Ling et al. (2009) and Bayly et al. (2013) showed that the African and Malagasy *Fagaropsis* Mildbr. ex Siebenlist belongs to the proto-Rutaceae as well. Appelhans et al. (2014) have so far sampled the highest number of *Zanthoxylum* species and provided the first evidence that *Toddalia* might be nested within *Zanthoxylum*.

*Zanthoxylum* species are easily recognizable by their usually pinnate leaves (rarely unifoliate, as in *Zanthoxylum unifoliolatum* Groppo & Pirani and *Z. schreberi* J.F.Gmelin, or bifoliate, as in *Z. bifoliolatum* Leonard; Groppo & Pirani 2017), and by their corky knobs with prickles (“knob thorns”) on the stems and main branches. In addition, prickles also occur on the leaf rachis and in pairs at a pseudo-stipular position in several species (Weberling, 1970; Zhang et al., 2008; Kubitzki et al., 2011). *Zanthoxylum* is further recognizable by its follicular fruits, in which the seeds remain attached to the open fruit by their funicle (Kubitzki et al., 2011). The seeds have a shiny, black pellicle and it is assumed that *Zanthoxylum* species are bird dispersed via endozoochory (Carlquist, 1966). The fruits of several species are used as a well-known and popular spice (“Sichuan pepper” or “hua jiao”) especially in Chinese cuisine (Zhu et al., 2015). The flowers show remarkable variation, which resulted in the recognition of two separate genera: *Zanthoxylum* s.str. with homochlamydeous flowers, and *Fagara* L. with heterochlamydeous flowers (Engler, 1931; Brizicky, 1962). In an influential publication, Engler (1931) further subdivided *Fagara* into the four sections *Blackburnia*, *Macqueria*, *Mayu* and *Tobinia*, but their monophyly has yet to be tested using molecular phylogenetic methods.

The main goals of this study are to (1) assess the monophyly of *Zanthoxylum* and its relationship with its closest relatives *Fagaropsis*, *Phellodendron*, *Tetradium* and *Toddalia* of the proto-Rutaceae group, (2) evaluate if *Zanthoxylum* s.str. and *Fagara* are distinct genera or if they should be merged, (3) test if Engler’s sectional classification is supported by phylogenetic analyses, and (4) estimate the geographic origin, age and major migration routes at the continental scale of the genus.

## 2. Material and methods

### 2.1. Taxon sampling

We sampled a total of 116 specimens (Table 1). Of these, 99 belonged to *Zanthoxylum* representing 54 out of the  $\pm$  225 species, as well as eight unidentified specimens. Specimens from all major areas of the genus’ distribution were included. The other four genera of the proto-Rutaceae were also sampled, including one of the four species of *Fagaropsis*, both species of *Phellodendron*, five of the nine species of *Tetradium*, as well as the monotypic *Toddalia* were included in the dataset.

One specimen of *Acronychia* J.R.Forst. & G.Forst. and *Melicope* each were chosen as outgroups based on previous phylogenetic studies (e.g., Appelhans et al., 2012; Bayly et al. 2013).

### 2.2. Molecular lab work

Total DNA was extracted from silica gel-dried leaf material or herbarium specimens using the DNeasy Plant Mini Kit (Qiagen, Hilden, Germany) with the following modifications: The cell lysis was extended to 30 min and only 75  $\mu$ L (instead of 100  $\mu$ L) of elution buffer was added at the final step.

Four marker regions were amplified via PCR; nuclear ETS (external transcribed spacer; primers Bur1 and 18S-IGS [Baldwin and Markos, 1998; Becerra, 2003]) and ITS (internal transcribed spacer; primers ITS2, ITS3, ITS4 and ITS5a [White et al., 1990; Stanford, et al., 2000]) as well as plastid *rps16* (primers *rpsF* and *rpsR2* [Oxelman et al. 1997]) and *trnL-trnF* (primers C, D, E and F [Taberlet et al., 1991]). PCR programs included an initial denaturation of 5 min at 95 °C, 35 cycles of 1 min at 95 °C, 1 min at 52 °C and 0:40–1:10 min at 72 °C (depending on

the size of the marker), and a final elongation of 7 min at 72 °C. PCR products were cleaned using ExoSAP-IT (affymetrix USB, Cleveland, Ohio, USA) and were sent to SeqLab (Goettingen, Germany) for sequencing on an ABI 3100 sequencer. All sequences have been deposited at Genbank (<https://www.ncbi.nlm.nih.gov>; Table 1).

### 2.3. Sequence alignment and phylogenetic analyses

Alignments for all markers were constructed in CLC genomics workbench version 10 (Qiagen, Aarhus, Denmark) using the MUSCLE algorithm (Katoh et al., 2002; Katoh and Toh, 2008) and then edited manually in Mesquite 3.11.1 (Maddison and Maddison, 2015). Only specimens for which sequencing of at least three out of the four markers was successful were included in the alignments (116 specimens). A region of 20 bp in the *rps16* region could not be aligned with confidence and was excluded from the analyses. All other characters were included and the final alignment lengths were 507 bp (ETS), 761 bp (ITS), 1015 bp (*rps16*), and 1193 bp (*trnL-trnF*), respectively. We tested for congruence of datasets using a tree topology based hierarchical likelihood ratio test as implemented in Concatenator v 1.8a (Leigh et al., 2008). We tested for concatenation of plastid versus nuclear markers, both including and excluding the Hawaiian taxa. Phylogenetic analyses were carried out using Bayesian Inference (BI) and Maximum Likelihood (ML). The best fitting substitution models were determined in jModelTest 2.1.3 (Darriba et al., 2012) and the GTR +  $\Gamma$  model was employed for the *trnL-trnF* dataset, while GTR + I +  $\Gamma$  was used for the other markers. MrBayes 3.2.6 (Ronquist et al., 2012) was used for the Bayesian analyses and all tree searches consisted of two independent Markov Chain Monte Carlo (MCMC) runs with four chains each, that were observed for 10 million generations. A tree was saved every 100th generation, and the temperature parameter was set to 0.05. All runs reached stationarity (standard deviation of split frequencies < 0.01), and results were inspected in Tracer 1.6.0 (Rambaut et al., 2014) to check if effective sample sizes (ESS) were above 200 for all parameters. 50% majority-rule consensus trees were calculated after 10% of the trees were discarded as burn-in. Statistical support of branches was measured as posterior probability values, and only values of  $\geq$  0.95 pp were considered.

Garli 2.0 (Zwickl, 2006) was utilized for ML. The ML bootstrap analyses consisted of 100 pseudoreplicates, each based on five independent search replicates. 50% majority-rule consensus trees were calculated using SumTrees, which is part of the DendroPy 4.3.0 package (Sukumaran and Holder, 2010). Branches with bootstrap (bs) values of 50–69% were considered to be of low support, values of 70–89% bs to be of medium support, and values of 90% and higher to be of high support.

### 2.4. Biogeographic analyses

Molecular dating analyses were performed in BEAST 1.8.4 (Drummond et al. 2012). The analyses were based on both the 4-marker dataset that included the Hawaiian taxa and the 4-marker dataset in which the Hawaiian taxa were excluded in order to evaluate if the incongruent placement of the Hawaiian taxa has an influence on the dating. Two fossils were used as calibration points for the crown ages of *Zanthoxylum* and *Phellodendron/Tetradium*. *Zanthoxylum* has a rich fossil record (e.g. Chandler, 1969; Gregor, 1989; Tiffney, 1994), and the oldest known fossil has been assigned to the early Eocene (Chandler, 1961). We calibrated the crown of *Zanthoxylum* s.l. setting a prior with a lognormal distribution with an offset of 48.0 million years ago (MA), a mean of 1.0 MA and a standard deviation of 0.65 MA so that the prior distribution matched with the geologic epoch. The oldest fossil attributed to *Phellodendron* and *Tetradium* was dated to the Late Paleocene (Chandler, 1925, 1961). The fossil has been assigned to *Phellodendron* (*Phellodendron costatum* Chandler; Chandler, 1925, 1961) and to *Tetradium* (as *Euodia costata* (Chandler) Tiffney; Tiffney, 1981) and we

**Table 1**

Specimens used in the phylogenetic analyses including voucher information, geographic origin and Genbank accession numbers. A hyphen (-) stands for missing data. Sequences generated for this study are marked with an asterisk (\*). Abbreviations for genera are as follows: *A* = *Acronychia*, *F* = *Fagaropsis*, *M* = *Melicope*, *P* = *Phellodendron*, *Te* = *Tetradium*, *To* = *Toddalia*, *Z* = *Zanthoxylum*.

Species name	Collector, number and herbarium acronym	Geographical origin	ETS	ITS	<i>rps16</i>	<i>trnL-trnF</i>
<i>A. pedunculata</i>	de Wilde 6834 (L)	Thailand	HG002527	HG002398	MG975200*	HG002754
<i>F. spec</i>	Burge 3059 (US)	Ethiopia	MG975097*	MH016465*	MG975201*	MG975302*
<i>M. clusifolia</i>	Wood 12406 (PTBG)	Hawaii (Hawai'i)	HG002541	HG002411	MG975202*	HG002797 + HG002873
<i>P. amurense</i>	Miller 10646 (US)	Cultivated at Missouri Botanical Garden	HG971596	HG002503	MG975203*	HG002935
<i>P. chinense</i>	Gaoligong Shan Biodiversity Survey 33348 (GH)	China (Yunnan)	MG975098*	MH016466*	MG975204*	MG975303*
<i>Te. daniellii</i>	Wen 12118 (US)	China (Sichuan)	MG975099*	MH016467*	MG975205*	MG975304*
<i>Te. fraxinifolium</i>	Wen 2077 (US)	China (Yunnan)	HG971603	HG971450	MG975206*	HG971272
<i>Te. fraxinifolium</i>	Gaoligong Shan Biodiversity Survey 24087 (GH)	China (Yunnan)	MG975100*	MH016468*	MG975207*	MG975305*
<i>Te. fraxinifolium</i>	Gaoligong Shan Biodiversity Survey 26035 (GH)	China (Yunnan)	MG975101*	MH016469*	–	MG975394*
<i>Te. glabrifolium</i>	Wen 13348 (US)	China (Guangdong)	MG975104*	MH016472*	MG975208*	MG975306*
<i>Te. ruticarpum</i>	Wen 12154 (US)	China (Sichuan)	HG971604	HG002510	MG975209*	HG002941
<i>Te. ruticarpum</i>	Gaoligong Shan Biodiversity Survey 22387 (GH)	China (Yunnan)	MG975102*	MH016470*	MG975210*	MG975307*
<i>Te. ruticarpum</i>	–	Cultivated at US National Arboretum (Nr. NA-49204/H)	MG975103*	MH016471*	MG975211*	MG975308*
<i>Te. ruticarpum</i>	Wen 13365 (US)	China (Guangdong)	MG975105*	MH016473*	MG975212*	MG975309*
<i>Te. trichotomum</i>	Wen 2217 (US)	China (Yunnan)	MG975106*	MH016474*	MG975213*	MG975310*
<i>To. asiatica</i>	Wen 2104 (US)	China (Yunnan)	HG971605	HG002511	–	HG002942
<i>To. asiatica</i>	Wen 13271 (US)	China (Guangdong)	MG975107*	MH016475*	MG975214*	MG975311*
<i>Z. acuminatum</i>	Groppo 1120 (SPF)	Brazil	MG975108*	MH016476*	MG975215*	MG975312*
<i>Z. ailanthoides</i>	Konta 18323 (L)	Japan	MG975110*	MH016478*	–	MG975314*
<i>Z. ailanthoides</i>	Wen 12508 (US)	Japan	MG975109*	MH016477*	MG975216*	MG975313*
<i>Z. americanum</i>	Appelhans MA542 (GOET)	Cultivated at Goettingen Botanical Garden	MG975111*	MH016479*	MG975217*	MG975315*
<i>Z. armatum</i>	Wen 12410 (US)	Indonesia (Bali)	MG975112*	MH016480*	MG975218*	MG975316*
<i>Z. armatum</i>	Wen 12476 (US)	China (Wuhan)	MG975113*	MH016481*	MG975219*	MG975317*
<i>Z. armatum</i>	Wen 13308 (US)	China (Guangdong)	MG975114*	MH016482*	MG975220*	MG975318*
<i>Z. armatum</i>	Dickoré 13835 (GOET)	Pakistan	MG975115*	MH016483*	MG975221*	MG975319*
<i>Z. armatum</i>	Freitag 4769 (KAS)	Afghanistan	MG975116*	MH016484*	MG975222*	MG975320*
<i>Z. avicennae</i>	Wen 12808 (US)	China (Guangdong)	MG975117*	MH016485*	MG975223*	MG975321*
<i>Z. avicennae</i>	Wen 13311 (US)	China (Guangdong)	MG975118*	MH016486*	MG975224*	MG975322*
<i>Z. beecheyanum</i>	Wen 12812 (US)	Cultivated at Kunming Bot. Gard.	MG975119*	MH016487*	MG975225*	MG975323*
<i>Z. brachyacanthum</i>	Forster PIF28159 (L)	Australia	MG975121*	MH016489*	MG975226*	MG975324*
<i>Z. brachyacanthum</i>	Forster PIF25206 (L)	Australia	MG975120*	MH016488*	MG975227*	MG975325*
<i>Z. bungeanum</i> var. <i>punctatum</i>	Wen 13225 (US)	China	MG975122*	MH016490*	MG975228*	MG975326*
<i>Z. bungeanum</i>	Wen 13252 (US)	China (Shaanxi)	MG975123*	MH016491*	MG975229*	MG975327*
<i>Z. bungeanum</i>	Wen 1541 (US)	China (Yunnan)	MG975124*	HG002512	MG975230*	HG002943
<i>Z. caribaeum</i>	Bruniera 549 (SPFR)	Brazil	MG975125*	MH016493*	MG975231*	MG975329*
<i>Z. caribaeum</i>	Flores 5332 (MO)	Mexico	MG975126*	MH016492*	MG975232*	MG975328*
<i>Z. chalybeum</i>	Kindeketa 1090 (MO)	Tanzania	MG975127*	MH016494*	–	MG975330*
<i>Z. chalybeum</i>	Seegeler 2231 (MO)	Ethiopia	MG975128*	MH016495*	MG975233*	MG975331*
<i>Z. chalybeum</i>	Mhoro 6225 (US)	Tanzania	MG975129*	MH016496*	MG975234*	MG975332*
<i>Z. clava-herculis</i>	Wen 12771 (US)	USA (Florida)	MG975130*	MH016497*	MG975235*	MG975333*
<i>Z. clava-herculis</i>	Wen 12774 (US)	USA (Florida)	MG975131*	MH016498*	MG975236*	MG975334*
<i>Z. coco</i>	Kessler 3121 (GOET)	Bolivia	MG975132*	MH016499*	MG975237*	MG975335*
<i>Z. coco</i>	Ree & Wen 53858 (US)	Bolivia	MG975133*	MH016500*	MG975238*	MG975336*
<i>Z. davyi</i>	Schroeder 153 (GOET)	South Africa	MG975134*	MH016501*	MG975239*	MG975337*
<i>Z. deremense</i>	Mwangoka 7646 (MO)	Tanzania	MG975135*	MH016502*	MG975240*	MG975338*
<i>Z. dipetalum</i>	Trauernicht 750 (PTBG)	Hawaii (Kaua'i)	MG975137*	HG002513	MG975241*	HG002944
<i>Z. dipetalum</i>	Wood 13822 (PTBG)	Hawaii (Kaua'i)	MG975136*	HG002514	–	HG002945
<i>Z. dipetalum</i>	Wood 7664 (PTBG)	Hawaii (Kaua'i)	MG975138*	HG002515	MG975242*	HG002946
<i>Z. dissitum</i>	Wen 12840 (US)	China (Hubei)	MG975139*	MH016503*	MG975243*	MG975339*
<i>Z. echinocarpum</i>	Wen 13309 (US)	China	MG975140*	MH016504*	MG975244*	MG975340*
<i>Z. esquirolii</i>	Wen 12813 (US)	China	MG975141*	MH016505*	MG975245*	MG975341*
<i>Z. fagara</i>	Wurdack	Cultivated at Fairchild Botanical Garden (6611A)	MG975142*	MH016506*	MG975246*	MG975342*
<i>Z. foliolosum</i>	Zarate Marcos 124 (MO)	Mexico	MG975143*	MH016507*	MG975247*	MG975343*
<i>Z. gillettii</i>	Hamill 1079 (MO)	Uganda	MG975144*	MH016508*	–	MG975344*
<i>Z. gillettii</i>	Bourland s.n. [Bourland 1]	DR Congo	MG975145*	MH016509*	MG975248*	MG975345*
<i>Z. gillettii</i>	Bourland s.n. [Bourland 2]	DR Congo	MG975146*	MH016510*	MG975249*	MG975346*
<i>Z. gillettii</i>	Bourland s.n. [Bourland 3]	DR Congo	MG975147*	MH016511*	MG975250*	MG975347*
<i>Z. gillettii</i>	Bourland s.n. [Bourland 4]	DR Congo	MG975148*	MH016512*	MG975251*	MG975348*
<i>Z. gillettii</i>	Bourland s.n. [Bourland 5]	DR Congo	MG975149*	MH016513*	MG975252*	MG975349*
<i>Z. hawaiiense</i>	Wood 12463 (PTBG)	Hawaii (Kaua'i)	MG975150*	HG002516	MG975253*	HG002947
<i>Z. heterophyllum</i>	Loreno 2607 (MO)	Mauritius	MG975151*	–	MG975254*	MG975350*
<i>Z. holtzianum</i>	Rulangeranga 199 (US)	Tanzania	MG975152*	MH016514*	–	MG975351*
<i>Z. holtzianum</i>	Mwangoka & Mzubua 2162 (MO)	Tanzania	MG975153*	MH016515*	MG975255*	MG975352*

(continued on next page)

Table 1 (continued)

Species name	Collector, number and herbarium acronym	Geographical origin	ETS	ITS	rps16	trnL-trnF
<i>Z. kauaense</i>	Wood 15034 (PTBG)	Hawaii (Kaua'i)	HG971606	HG002517	MG975256*	HG002948
<i>Z. kauaense</i>	Wood 14980 (PTBG)	Hawaii (Kaua'i)	MG975154*	HG002518	–	HG002949
<i>Z. kauaense</i>	Wood 15131 (PTBG)	Hawaii (Kaua'i)	MG975155*	HG002519	–	HG002950
<i>Z. kellermanii</i>	Torres-Diaz 1033 (MO)	Mexico	MG975156*	MH016516*	MG975257*	MG975353*
<i>Z. kellermanii</i>	Hammel 19397 (MO)	Costa Rica	MG975157*	MH016517*	MG975258*	MG975354*
<i>Z. kellermanii</i>	Bruniera 533 (SPFR)	Peru	MG975158*	MH016518*	MG975259*	MG975355*
<i>Z. lemairei</i>	Bourland s.n. [Bourland 1]	DR Congo	MG975159*	MH016519*	MG975260*	MG975356*
<i>Z. lemairei</i>	Bourland s.n. [Bourland 2]	DR Congo	MG975160*	MH016520*	MG975261*	MG975357*
<i>Z. limonella</i>	Wen 12411 (US)	Indonesia (Bali)	MG975161*	MH016521*	MG975262*	MG975358*
<i>Z. madagascariense</i>	Capuron 28595-SF (MO)	Madagascar	MG975162*	MH016522*	MG975263*	MG975359*
<i>Z. mayu</i>	Downloaded from Genbank	Downloaded from Genbank	–	HM851480	–	HM851516
<i>Z. megistophyllum</i>	Craven 107 (US)	Solomon Islands	MG975163*	MH016523*	–	MG975395*
<i>Z. mollissimum</i>	Reyes-Garcia 5972 (MO)	Mexico	MG975164*	MH016524*	MG975264*	MG975360*
<i>Z. nitidum</i>	Wen 13280 (US)	China (Guangdong)	MG975165*	MH016525*	MG975265*	MG975361*
<i>Z. ovalifolium</i>	Dockrill 1477 (L)	Australia	MG975166*	MH016526*	MG975266*	MG975362*
<i>Z. ovalifolium</i>	Gaoligong Shan Biodiversity Survey 29095 (GH)	China (Yunnan)	MG975167*	MH016527*	MG975267*	MG975363*
<i>Z. ovalifolium</i>	Schodde 2967 (US)	New Guinea	MG975168*	MH016528*	–	MG975364*
<i>Z. ovatifoliolatum</i>	SWA 3/76 (US)	Namibia	MG975169*	MH016529*	MG975268*	MG975365*
<i>Z. oxyphyllum</i>	Wen 2916 (US)	China (Tibet)	MG975170*	HG002522	MG975269*	HG002952
<i>Z. paniculatum</i>	Magdalena 001 (MO)	Mauritius	MG975171*	MH016530*	MG975270*	MG975366*
<i>Z. petiolare</i>	Groppo 1970 (SPFR)	Brazil	MG975172*	MH016531*	MG975271*	MG975367*
<i>Z. pinnatum</i>	Drake 282 (US)	Tonga	MG975173*	MH016532*	MG975272*	MG975368*
<i>Z. piperitum</i>	Breckle 13505 (GOET)	South Korea	MG975174*	MH016533*	MG975273*	MG975369*
<i>Z. poggei</i>	Bourland s.n. [Bourland 1]	DR Congo	MG975175*	MH016534*	MG975274*	MG975370*
<i>Z. retusum</i>	Groppo 2039 (SPFR)	Brazil	MG975176*	MH016535*	MG975275*	MG975371*
<i>Z. rhodoxylon</i>	Wen 11923 (US)	Jamaica	HG971607	HG002523	–	HG002953
<i>Z. rhodoxylon</i>	Wen 11907 (US)	Jamaica	MG975177*	HG002524	MG975276*	HG002954
<i>Z. rhoifolium</i>	Groppo 1057 (SPF)	Brazil	MG975178*	MH016536*	MG975277*	MG975372*
<i>Z. rhoifolium</i>	Stevens 33275 (MO)	Nicaragua	MG975179*	MH016537*	MG975278*	MG975373*
<i>Z. riedelianum</i>	Groppo 1342 (SPFR)	Brazil	MG975180*	MH016538*	MG975279*	MG975374*
<i>Z. riedelianum</i>	Groppo 1518 (SPFR)	Brazil	MG975181*	MH016539*	MG975280*	MG975375*
<i>Z. riedelianum</i>	Vargas 750 (MO)	Costa Rica	MG975182*	MH016540*	MG975281*	MG975376*
<i>Z. scandens</i>	Wen 13279 (US)	China (Guangdong)	MG975183*	MH016541*	MG975282*	MG975377*
<i>Z. scandens</i>	Wen 2064 (US)	China (Yunnan)	HG971608	HG002525	MG975283*	HG002955
<i>Z. scandens</i>	Gaoligong Shan Biodiversity Survey 30307 (GH)	China (Yunnan)	MG975184*	MH016542*	MG975284*	MG975378*
<i>Z. schinifolium</i>	Wen 12055 (US)	China (Jiangxi)	MG975185*	HG002526	MG975285*	HG002956
<i>Z. setulosum</i>	Kessler 182 (GOET)	Panama	MG975186*	MH016543*	–	MG975379*
<i>Z. simulans</i>	Appelhans MA706 (GOET)	Cultivated at Goettingen Botanical Garden	MG975187*	MH016544*	MG975286*	MG975380*
<i>Z. simulans</i>	Appelhans MA733 (GOET)	Cultivated by first author	MG975188*	MH016545*	MG975287*	MG975381*
<i>Z. spec</i>	Wen 12412 (US)	Indonesia (Bali)	MG975189*	MH016546*	MG975288*	MG975382*
<i>Z. spec</i>	Wen 12413 (US)	Indonesia (Bali)	MG975190*	MH016547*	MG975289*	MG975383*
<i>Z. spec</i>	Gaoligong Shan Biodiversity Survey 25121 (GH)	China (Yunnan)	MG975191*	MH016548*	MG975290*	MG975384*
<i>Z. spec</i>	Gaoligong Shan Biodiversity Survey 30354 (GH)	China (Yunnan)	MG975192*	MH016549*	MG975291*	MG975385*
<i>Z. spec</i>	Abeid 3356 (US)	Tanzania	MG975193*	MH016550*	MG975292*	MG975386*
<i>Z. spec</i>	Homeier 4494 (GOET)	Ecuador	MG975194*	MH016551*	MG975293*	MG975387*
<i>Z. spec</i>	Groppo s.n. (SPF)	Brazil	–	MG981028*	MG975294*	MG975388*
<i>Z. spec</i>	Munzinger 636 (MO)	New Caledonia	HG971609	HG971451	MG975295*	HG971273
<i>Z. tahitense</i>	Meyer 1038 (NY)	French Polynesia, Austral Islands, Rapa	MG975195*	HG002520	MG975296*	HG002951
<i>Z. tahitense</i>	Perlman 18007 (NY)	French Polynesia, Austral Islands, Rapa	–	MG981029*	MG975297*	MG975389*
<i>Z. thomsonianum</i>	Wurdack	Cultivated at Fairchild Botanical Garden (2002-0769A)	MG975196*	MH016552*	MG975298*	MG975390*
<i>Z. tingoassuba</i>	Groppo 1179 (SPF)	Brazil	MG975197*	MH016553*	MG975299*	MG975391*
<i>Z. viride</i>	Jongkind & Bilivegui 11383 (MO)	Guinea	MG975198*	MH016554*	MG975300*	MG975392*
<i>Z. zanthoxyloides</i>	Jongkind 1251 (US)	Ghana	MG975199*	MH016555*	MG975301*	MG975393*

used the fossil to calibrate the crown node of the clade of *Phellodendron* and *Tetradium*. We assigned a prior with a lognormal distribution, an offset of 55.4 MA, a mean of 0.5 MA and a standard deviation of 0.5 MA so that the prior distribution matched with the Late Paleocene. The prior for the root age was set to a normal distribution with a mean of 60.0 MA and a standard deviation of 3.0 MA based on the 95% HPD range of the split of the *Zanthoxylum* lineage from the clade that includes *Acronychia* and *Melicope* as estimated by Appelhans et al. (2012) in a study that sampled across the order Sapindales and was based on five fossil calibrations. We used the same substitution models as for BI and ML analyses, an uncorrelated lognormal relaxed clock, and a Birth-

Death Process tree prior together with a random starting tree. Two separate analyses were run for 50 million generations each, retaining trees every 5000th generation. Results were inspected in Tracer 1.6.0 (Rambaut et al., 2014) to confirm effective sample sizes (ESS) above 200 for all parameters. The trees of the two runs were combined in LogCombiner 1.8.4 (Drummond et al., 2012) and 10% of the trees were discarded as burn-in. A maximum clade credibility consensus tree was assembled with TreeAnnotator 1.8.4 (Drummond et al., 2012).

Ancestral Area Reconstruction (AAR) was carried out using the R package BioGeoBEARS 0.2.1. (Matzke, 2013; R version 3.3.3). The analyses were based on the dated phylogeny obtained from the BEAST

analysis that included the Hawaiian samples. The input tree was pruned in R using a BioGeoBEARS script (<http://phylo.wikidot.com/example-biogeobears-scripts>) so that species with more than one accession were only represented by a single accession in the analyses. For non-monophyletic species, one accession for each evolutionary lineage was kept and accessions that were not determined at the species level (*Z. spec*) were included. We defined seven geographic areas at the continental scale: A – North & Central America and the Caribbean; B – South America; C – mainland Asia; D – Africa, Madagascar and the Mascarene Islands; E – Malesia; F – Australia and nearby continental islands (New Zealand, New Caledonia); G – Pacific Islands. Up to three areas were assigned to each specimen. The area assignment was based on the distribution of the respective species, as determined using georeferenced occurrence data from the Global Biodiversity Information Facility ([www.gbif.org](http://www.gbif.org)). For specimens not identified at the species level, we used the collecting site to assign the geographic areas. For the outgroups, the geographic origin of the *Melicope-Acronychia* group (Appelhans et al., 2018) was chosen instead of the distribution of the two representatives of this group.

Several *Zanthoxylum* species as well as *Toddalia asiatica* occur in three areas (C-D-E or C-E-F). We thus set the maximum number of areas for ancestral nodes to three. Three models are implemented and compared in BioGeoBEARS: the DEC model, the DIVALIKE model, and the BAYAREA model. The DEC model corresponds to the dispersal-extinction-cladogenesis model, which is used in the program Lagrange (Ree and Smith, 2008). The DIVALIKE model is similar to the dispersal-variance model used in the program DIVA (Ronquist, 1997), and the BAYAREA model of Landis et al. (2013). An additional parameter for jump-dispersal (“J”; Matzke, 2013, 2014) can be added to the three models, and we used all models with and without jump-dispersal to evaluate which model fitted best to our data.

### 3. Results

#### 3.1. Phylogenetic analyses

The phylogenetic trees based on nuclear data alone (ETS, ITS, ETS & ITS combined [Fig. 1]) and those based on the plastid data alone (*rps16*, *trnL-trnF*, *rps16* & *trnL-trnF* combined [Fig. 2]) show strongly supported subclades, but have low resolutions at the backbone. The placement of the monophyletic Hawaiian group is not consistent between datasets. While the Hawaiian lineage is in all cases part of a larger Asian-Australian-Pacific clade, it is sister to Asian species in the nuclear datasets, or sister to the South Pacific species in the plastid datasets. However, the support in the plastid consensus trees is low (0.90 posterior probability in the Bayesian analysis, 57% bootstrap support in the ML analysis), so that the incongruence is not well supported. The topology-based congruence test rejected concatenation of nuclear and plastid markers in both tested scenarios, including ( $p = 0.000014$ ) and excluding ( $p = 0.002678$ ) the Hawaiian taxa. This test relies on each marker set resolving relationships nearly equally well. Considering the low resolution of the backbone in the plastid dataset (Fig. 2) as compared to the nuclear data set (Fig. 1), the inferred incongruence seems just as likely to originate from more different resolutions, as from different evolutionary histories. However, the significantly different *p*-values produced by including the Hawaiian taxa, seem to indicate an impact of these samples beyond methodological weaknesses. Since the topological incongruence had a low support and the results of the topology-based congruence test might be strongly influenced by the differences in resolution between plastid and nuclear datasets, we decided to also concatenate the nuclear and plastid datasets in order to show results from all perspectives. Tree searches of the concatenated datasets were done including and excluding the Hawaiian taxa.

The consensus trees of the concatenated 4-marker dataset show improved resolution and support regarding the backbone phylogeny compared to the nuclear or the plastid consensus trees. In the following,

phylogenetic relationships are described based on the 4-marker consensus trees including the Hawaiian taxa (Fig. 3). The results from the other analyses are mentioned only in cases of disagreement. Our results show that the proto-Rutaceae group consists of two major clades. The first clade (0.97 pp, 85% bs) unites the genera *Fagaropsis*, *Phellodendron* and *Tetradium*. These genera are each resolved as monophyletic and *Fagaropsis* is sister to the other genera. The second clade (1.00 pp, 100% bs) is composed of *Toddalia* and *Zanthoxylum*, with *Toddalia* nested within *Zanthoxylum*. The *Zanthoxylum* lineage is subdivided into four clades. Clade 1 (0.99 pp, 79% bs) is sister to the other clades of the *Zanthoxylum* lineage and contains all sampled African, Malagasy and Mascarene species of *Zanthoxylum* as well as the widespread Asian and African *Toddalia*. *Toddalia* is sister to all other taxa in this clade. Clade 2 (0.85 pp, lacking bootstrap support) consists of species from Asia, Malesia, Australasia and several Pacific Archipelagos. Within this clade, the Hawaiian group is resolved as monophyletic (1.00 pp, 100% bs) and is sister to a clade of the Asian taxa (1.00 pp, 95% bs). The clade of the Hawaiian group and the two successive sister lineages (highlighted in Fig. 3) is not supported in the Bayesian analysis (0.81 pp) and shows low support in the ML analysis (62% bs). This particular node is well supported (0.99 pp, 80% bs) when the Hawaiian samples were excluded.

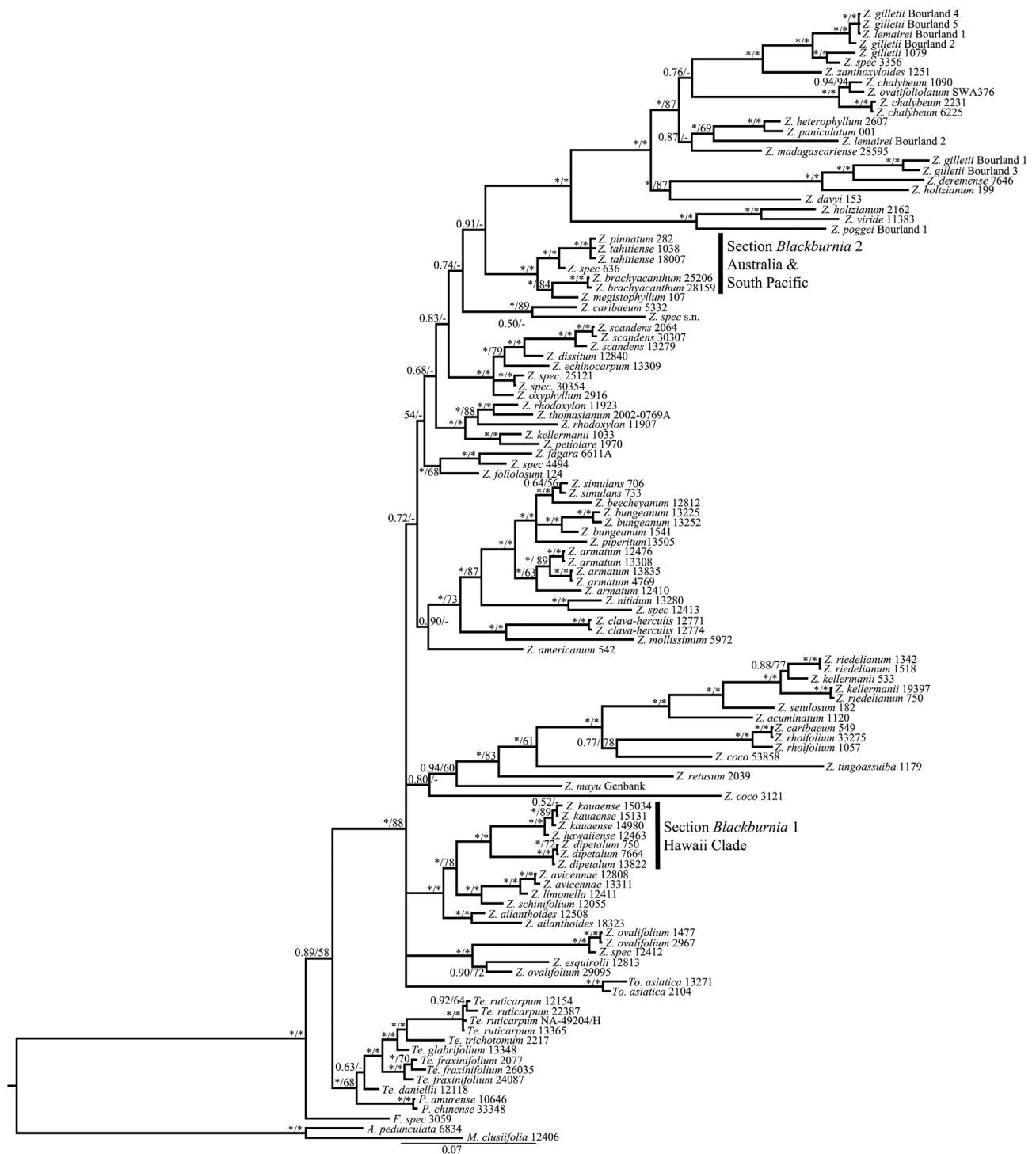
Clade 3 (1.00 pp, 79% bs) comprises taxa sampled from Central and South America and is sister to Clade 4 (1.00 pp, 83% bs), which unites American and Asian taxa. Within this clade, the Asian group (1.00 pp, 100% bs) is nested within a grade of American taxa. The distribution of the American taxa ranges from North to South America and the Caribbean, and the species that are closest relatives of the Asian taxa are from North and Central America.

*Zanthoxylum* s.str. as defined by Engler (1931) is only monophyletic if *Z. nitidum* (Roxb.) DC. is included, and consists of the Asian representatives of Clade 4 plus its two successive sister clades from North and Central America. *Fagara* sensu Engler (1931) is paraphyletic with respect to *Toddalia* and *Zanthoxylum*. Engler's (1931) *Fagara* sect. *Blackburnia* is resolved as monophyletic in the plastid consensus trees, but without support (Fig. 2). In the nuclear and 4-marker consensus trees (Figs. 1 and 3), the section is split into two lineages within Clade 2. The first lineage consists of all Hawaiian species, and the second lineage (1.00 pp, 100% bs) includes species from Australia, New Guinea and the South Pacific. Section *Mayu* consists solely of *Z. mayu*, an endemic of the Juan-Fernández Islands. *Zanthoxylum mayu* is part of Clade 3 and its closest relatives are from South America. Section *Tobinia* is confined to the Caribbean; and since only two out of about 13 species have been sampled in this study (part of Clade 4), we only tentatively treat this section as monophyletic. The species-rich section *Macqueria* is largely polyphyletic. All abovementioned sections, as well as *Toddalia* and *Zanthoxylum* s.str., are nested within section *Macqueria*, and representatives of the section are scattered throughout all four clades.

#### 3.2. Age and origin

The Maximum Clade Credibility tree from the BEAST analysis (Fig. 4) points towards an origin of the proto-Rutaceae in the Late Cretaceous (Maastrichtian) to Early Paleocene (mean estimate: 66.3 MA, 95% highest posterior density (HPD) interval: 62.6–70.4 MA). Shortly after that the split of the genera *Fagaropsis*, *Phellodendron* and *Tetradium* occurred. Compared to the old crown age of the proto-Rutaceae, the crown ages of the three genera are relatively recent. The lineages of the extant *Tetradium* species started to diversify between the Late Oligocene and Mid Miocene (19.7 MA; 12.9–29.4 MA), while the two extant species of *Phellodendron* split in Late Pliocene to Pleistocene (1.1 MA; 0.18–2.9 MA).

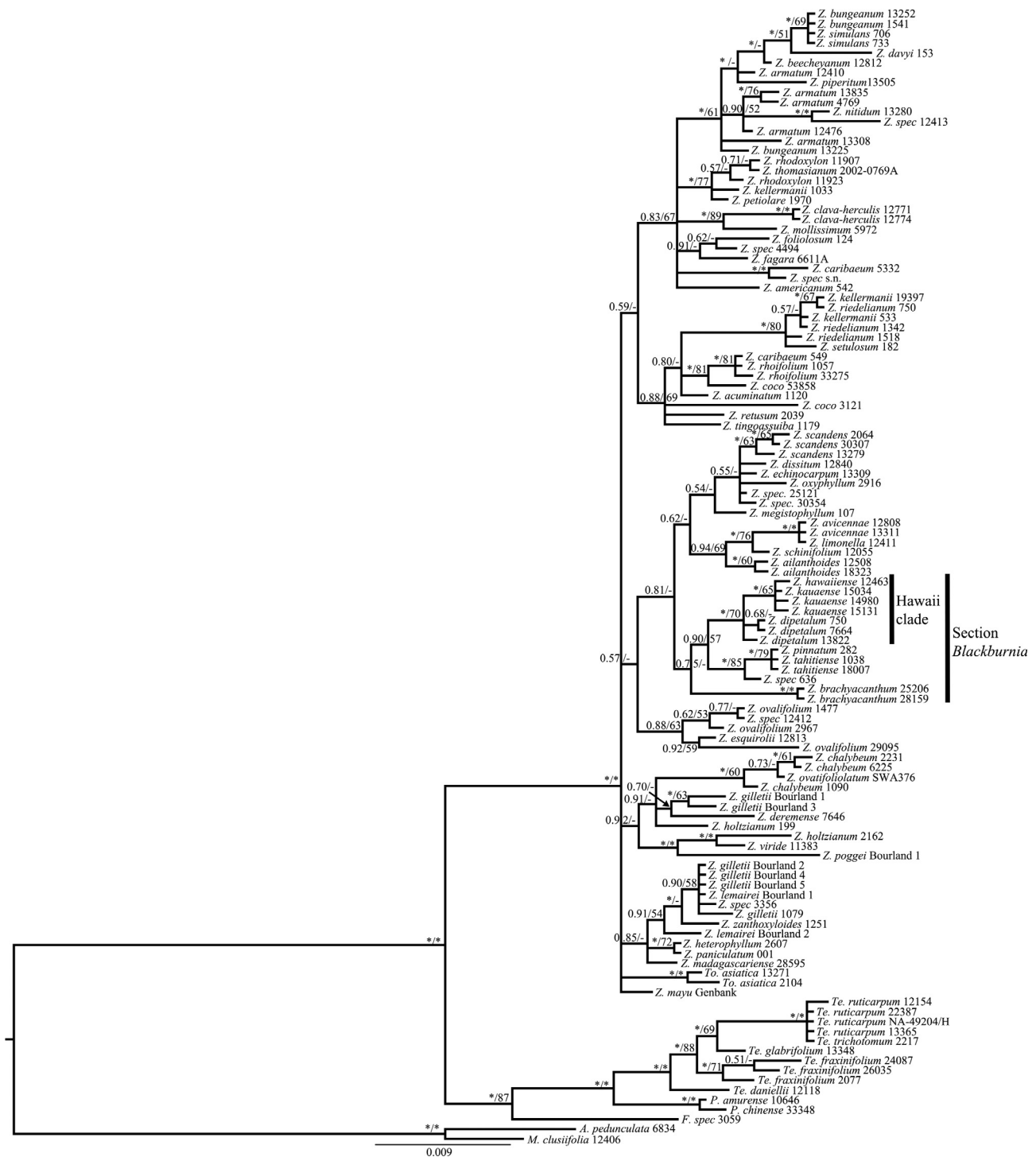
The *Zanthoxylum* crown group originated in the Early Eocene (50.5 MA; 48.5–53.9 MA) and the separation into the four main clades happened relatively shortly after the crown group formation. The African, Malagasy and Mascarene lineage (Clade 1) started to diversify in Early



**Fig. 1.** Phylogenetic reconstruction of *Zanthoxylum* and its closely related genera of the proto-Rutaceae based on the nuclear markers ETS and ITS. The Bayesian majority-rule consensus tree is shown with posterior probabilities as well as the bootstrap values from the GARLI analysis shown next to the branches (pp/bs). PP of 0.95 and higher and bs values of 90% and higher are marked with an asterisk (\*). Values below 0.50 pp or 50% bs are marked with a hyphen (-). The species of section *Blackburnia* are highlighted. Abbreviations: A = *Acronychia*, F = *Fagaropsis*, M = *Melicope*, P = *Phellodendron*, Te = *Tetradium*, To = *Toddalia*, Z = *Zanthoxylum*.

or Mid Eocene (47.3 MA; 43.0–51.6 MA). This estimated age also marks the split of *Toddalia* from the remainder of the group. The split of the two sampled *Toddalia* specimens is estimated to have occurred in the Late Miocene to Pleistocene (3.9 MA; 1.4–7.9 MA). The Mascarene and Malagasy species form a clade together with a specimen from DR Congo. The crown age estimate for this lineage is in the Late Oligocene to Early Miocene (20.9 MA; 14.9–27.0 MA). The two sampled species

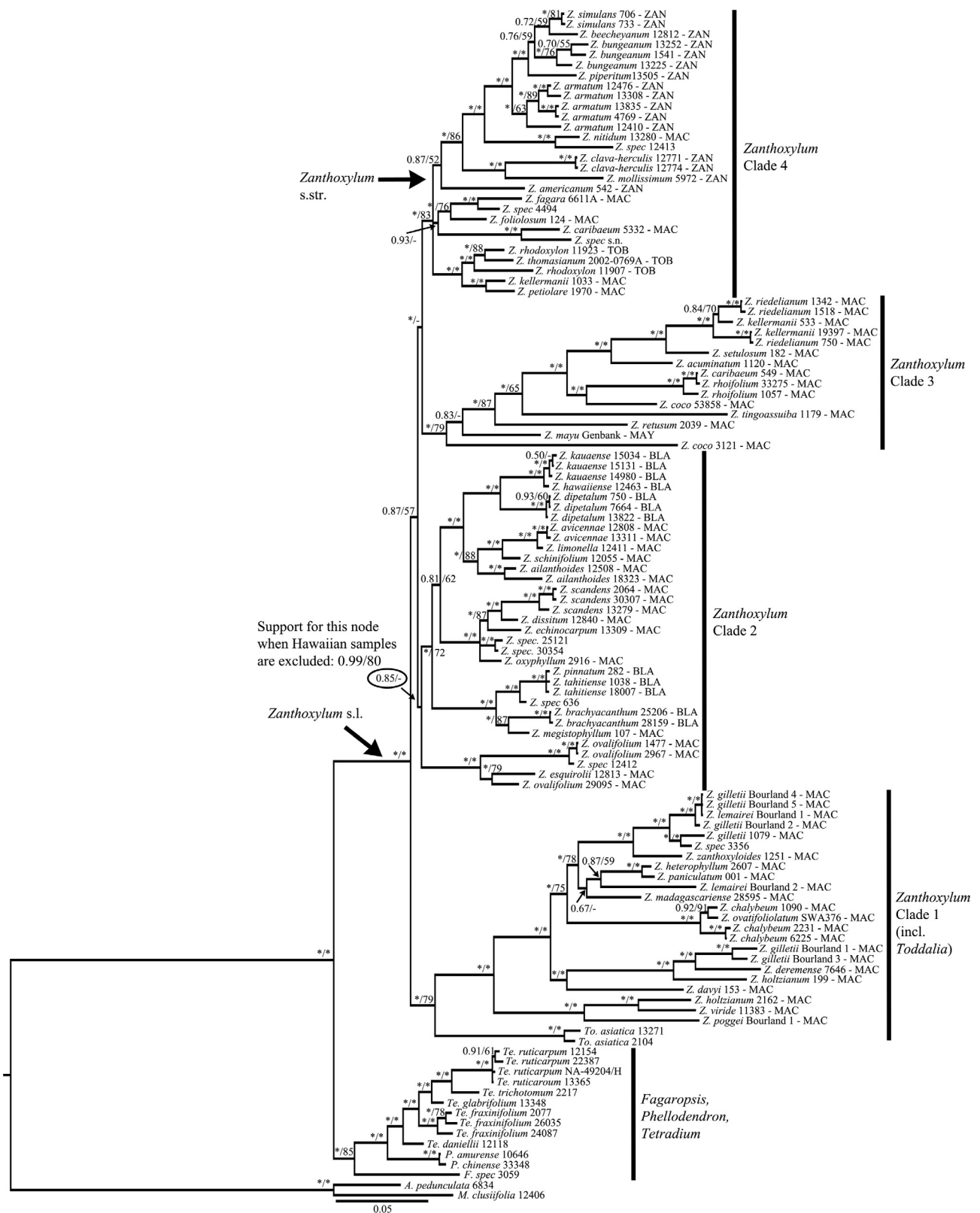
from Mauritius split from their relatives in the Miocene (16.7 MA; 10.7–23.1 MA) and diversified in the Pliocene or Pleistocene (2.4; 0.4–5.9 MA). The crown age of Clade 2 is in the Mid or Late Eocene (41.0 MA; 32.7–48.4 MA). This largely Asian, Malesian and Australian group also colonized the Pacific. One clade with species from New Caledonia, Tonga and Tahiti emerged in the Miocene (14.6; 9.3–21.5MA) and the New Caledonian species diverged from the



**Fig. 2.** Phylogenetic reconstruction of *Zanthoxylum* and its closely related genera of the proto-Rutaceae based on the plastid markers *rps16* and *trnL-trnF*. The Bayesian majority-rule consensus tree is shown with posterior probabilities as well as the bootstrap values from the GARLI analysis shown next to the branches (pp/bs). PP of 0.95 and higher and bs values of 90% and higher are marked with an asterisk (\*). Values below 0.50 pp or 50% bs are marked with a hyphen (-). The species of section *Blackburnia* are highlighted. Abbreviations: A = *Acronychia*, F = *Fagaropsis*, M = *Melicope*, P = *Phellodendron*, Te = *Tetradium*, To = *Toddalia*, Z = *Zanthoxylum*.

Polynesian lineage in the Late Miocene or Pliocene (5.6 MA; 2.5–9.2 MA). The Polynesian lineage diversified in the Late Pliocene to Pleistocene (1.0 MA; 0.17–2.3 MA). The second Pacific lineage consists of the Hawaiian species, which split from their sister group in the Late Oligocene to Early Miocene (22.4MA; 15.8–29.4MA). The crown group of the Hawaiian lineage, which marks the split of *Z. dipetalum* H. Mann from the other Hawaiian species, is dated to the Mid to Late Miocene

(11.8 MA; 6.9–17.5 MA). The further diversification on the Hawaiian Islands occurred from the Late Pliocene to Pleistocene. Clades 3 and 4 mainly consist of American species with an Asian subclade nested within it. Both lineages have estimated crown ages in the Mid Eocene to Early Oligocene (Clade 3: 39.7 MA; 33.4–46.1 MA; Clade 4: 35.5 MA; 28.8–43.8 MA). The crown age of the *Zanthoxylum* s.str. lineage within Clade 4 is dated to the Late Eocene to Late Oligocene (33.0 MA;



**Fig. 3.** Phylogenetic reconstruction of *Zanthoxylum* and its closely related genera of the proto-Rutaceae. The Bayesian majority-rule consensus tree of the 4-marker dataset is shown with posterior probabilities as well as the bootstrap values from the GARLI analysis shown next to the branches (pp/bs). PP of 0.95 and higher and bs values of 90% and higher are marked with an asterisk (\*). Values below 0.50 pp or 50% bs are marked with a hyphen (-). The suffixes after the taxon names and the collection numbers indicate whether a taxon has been assigned to *Zanthoxylum* s.str. (ZAN) or to *Fagara* sections *Blackburnia* (BLA), *Macqueria* (MAC), *Mayu* (MAY) or *Tobinia* (TOB) by Engler (1931). Abbreviations: A = *Acronychia*, F = *Fagaropsis*, M = *Melicope*, P = *Phellodendron*, Te = *Tetradium*, To = *Toddalia*, Z = *Zanthoxylum*.



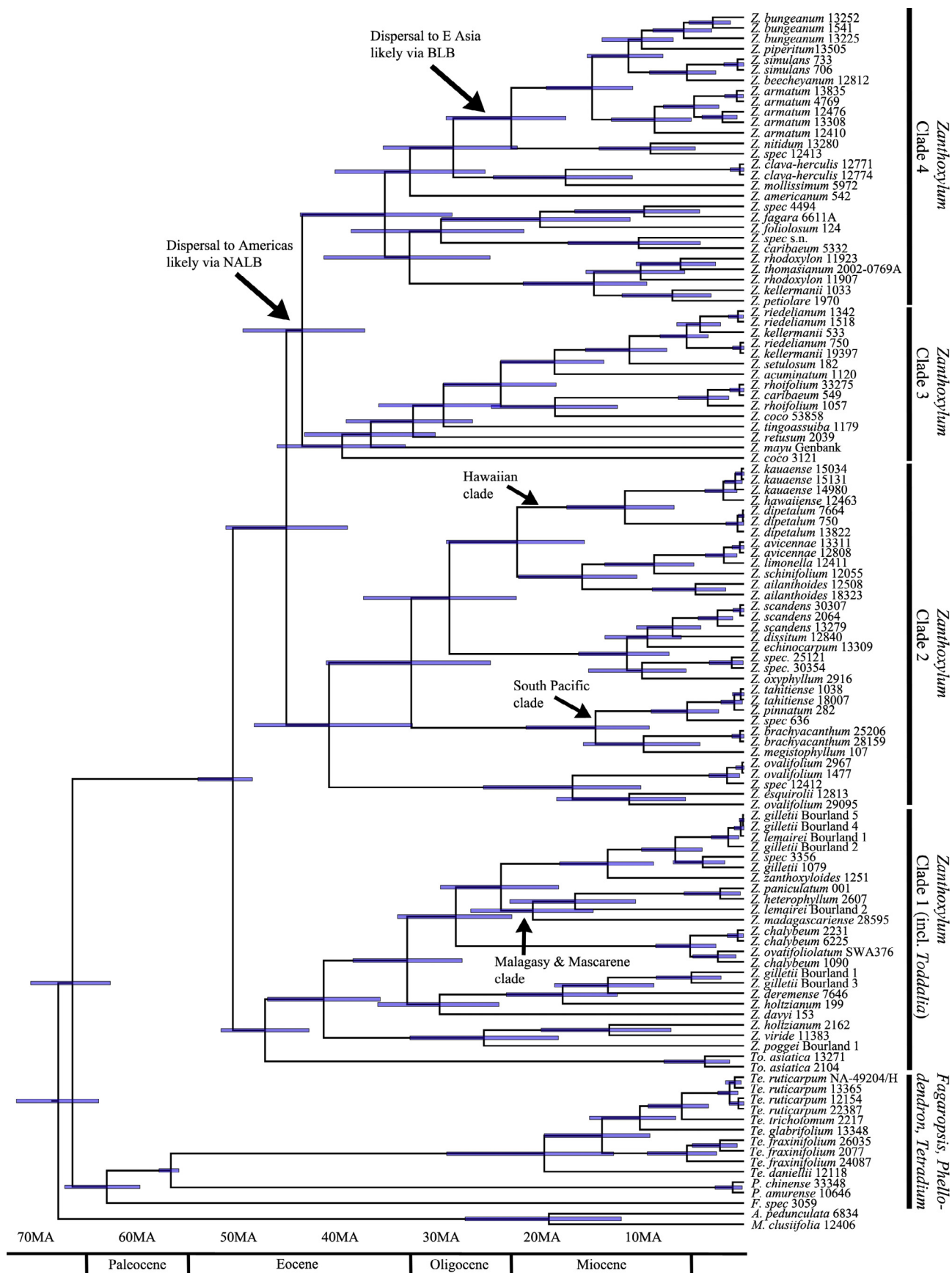


Fig. 4. Maximum clade credibility tree of the BEAST analysis based on the 4-marker dataset of *Zanathoxylum* and its closely related genera. The 95% credible intervals are shown for each branch. Clades 1–4 are identical to those in Fig. 3. Abbreviations: A = *Acronychia*, BLB = Bering Land Bridge, F = *Fagaropsis*, M = *Melicope*, NALB = North Atlantic Land Bridges, P = *Phellodendron*, Te = *Tetradium*, To = *Toddalia*, Z = *Zanathoxylum*.

**Table 2**

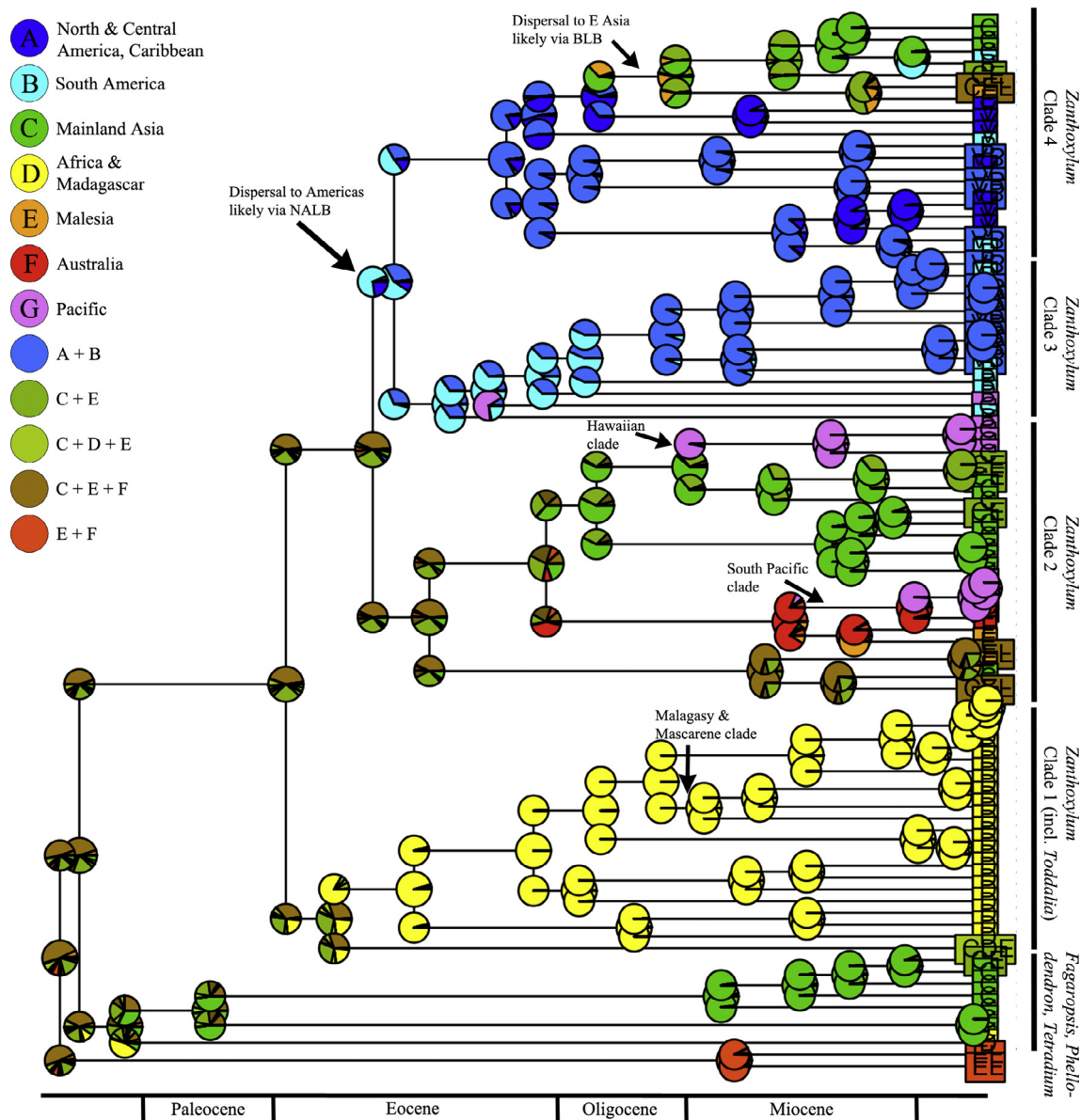
Results of the BioGeoBEARS analysis. The table shows likelihood (LnL) values for the three models with or without parameter “J” for jump dispersal and AIC (Akaike Information Criterion) values for the comparison of the models with or without the parameter “J”.

Model	LnL	AIC
DEC	−185.7823	375.6
DEC + J	−183.2180	372.4
DIVALIKE	−197.7682	399.5
DIVALIKE + J	−197.4597	400.9
BAYAREA	−176.5355	357.1
BAYAREA + J	−161.5579	329.1

25.6–40.4 MA). The Asian clade of *Zanthoxylum* s.str. split from the American relatives in the Late Eocene to Late Oligocene (28.7 MA; 22.4–35.6 MA) and started to diversify in the Late Oligocene (23.0 MA; 17.6–29.4 MA).

The BAYAREA+J model fit our dataset best (Table 2), and the

results of the AAR using this model are shown in Fig. 5. The AAR revealed an origin of the proto-Rutaceae in the Old World. The areas C (mainland Asia) and E (Malesia) and a combination of these areas with either area D (Africa, Madagascar, Mascarenes) or area F (Australia) are the most likely origins of the group. Within the *Fagaropsis-Phellodendron-Tetradium* lineage, the ancestor of *Fagaropsis* migrated to Africa, while the other lineages are largely confined to area C, and one sub-clade within *Tetradium* extended its range to area E. The African and Malagasy lineage of *Zanthoxylum* (Clade 1) has only one representative – *Toddalia asiatica* – that occurs outside of area D. With the data at hand, the origin of *Toddalia* remains obscure. Clade 2 contains mainly tropical Asian and Malesian species, of which some extend into Australia. The AAR estimated an origin of this clade in these three areas. Two lineages within Clade 2 colonized Pacific archipelagos. The most likely origin of the Hawaiian group is either area C or areas C and E, while the origin of the South Pacific lineage is in Australasia (area F or areas E and F). Clades 3 and 4 split from the Old World to the Americas and two migrations out of the Americas can be observed. *Zanthoxylum*



**Fig. 5.** Ancestral areas of *Zanthoxylum* and its closely related genera from the BioGeoBEARS analysis using the BAYAREA+J model. The areas are plotted on the maximum clade credibility tree from the BEAST analysis (Fig. 4) after multiple accessions of the same species were pruned. Clades 1–4 are identical to those in Figs. 3 and 4. Abbreviations: BLB = Bering Land Bridge, NALB = North Atlantic Land Bridges.

*mayu* from the Juan-Fernández Islands is derived from a South American ancestor, while the Asian subclade has a higher probability of a North and Central American origin.

## 4. Discussion

### 4.1. Relationships at the genus level within the proto-Rutaceae

The five genera (*Fagaropsis*, *Phellodendron*, *Tetradium*, *Toddalia*, and *Zanthoxylum*) of the proto-Rutaceae are characterized by the presence of certain alkaloids (1-benzyltetrahydroisoquinoline alkaloids), which are otherwise only found in Ranunculales (Fish and Waterman, 1973; Waterman, 1975, 1983, 2007), a phylogenetically distant group from Rutaceae. The surprising resemblance with Ranunculales has led Waterman (1983, 2007) to the hypotheses that Riales might be closely related to basal dicots, and that the proto-Rutaceae represents an early branching clade within Rutaceae. Both hypotheses are not supported by molecular phylogenetic studies (Chase et al., 1999; Magallón and Castillo, 2009; Groppo et al., 2008; APG, 2016). The presence of these alkaloids in the proto-Rutaceae is a strong indication (synapomorphy) for the close relationships among these genera, while the presence of the alkaloids in Rutaceae and Ranunculales must be interpreted as a convergence.

The close relationship of *Fagaropsis*, *Phellodendron*, *Tetradium*, *Toddalia* and *Zanthoxylum* largely disagrees with the traditional taxonomy of Rutaceae, which mainly emphasized fruit characters (Engler, 1931). While *Fagaropsis*, *Phellodendron* and *Toddalia* have drupaceous fruits and have been placed in the subfamily Toddalioidae, *Tetradium* and *Zanthoxylum* have follicular or rarely capsular fruits, and have been placed in Rutoideae (Engler, 1931). Molecular phylogenetic studies revealed that the traditional subfamily classification does not reflect the evolutionary history of the family (Chase et al., 1999; Poon et al., 2007; Groppo et al., 2008; Morton and Telmer, 2014), and that fruit characteristics are not suited to define large groups in Rutaceae (see Fig. 6B in Groppo et al., 2008). A similar example of closely related taxa with fleshy and dry fruits in a major clade within Rutaceae is the *Melicope* complex. In this group, several genera with drupaceous fruits are nested within *Euodia* J.R. Forst. & G. Forst. and *Melicope*, respectively, both of which have follicular/capsular fruits (Appelhans et al., 2014; Holzmeyer et al., 2015; Appelhans et al., 2018). Several intermediate fruits types (e.g., subfleshy capsules, tardily dehiscent capsules, and drupes that easily open in a plant press) exist in the *Melicope* complex (Hartley, 2001, 2013; Appelhans et al., 2017) suggesting that the difference between a dehiscent and a non-dehiscent fruit in Rutaceae is rather gradual, and that it might simply be determined by the thickness of the pericarp.

Instead of fruit types, the phyllotaxis, habit and aculeate protuberances on branches are of taxonomic value in the proto-Rutaceae. While *Fagaropsis*, *Phellodendron* and *Tetradium* have opposite leaves, grow as trees or shrubs, and do not have any protuberances on branches, *Toddalia* and *Zanthoxylum* have alternate leaves, are either trees or scandent shrubs to woody climbers, and are characterized by aculeate branches (Kubitzki et al., 2011). The number of ovules per carpel also has taxonomic value. While *Toddalia* and *Zanthoxylum* have two ovules per carpel, *Fagaropsis* and *Phellodendron* have one ovule per carpel (Kubitzki et al., 2011). *Tetradium* is intermediate, having flowers with one or two ovules. The two early branching clades (*T. daniellii* (Benn.) T.G. Hartley, *T. fraxinifolium* (Hook.) T. G. Hartley) have two ovules per carpel which both develop into seeds, while the other clades have either one ovule per carpel, or two ovules per carpel of which one develops to a seed while the other one is aborted (Hartley, 1981).

### 4.2. *Fagara* vs. *Zanthoxylum* and Engler's sections

Our results clearly show that *Zanthoxylum* s.str. is deeply nested within *Fagara*. *Zanthoxylum* s.str. differs markedly from *Fagara* by

having a homochlamydeous perianth (Engler, 1931; Waterman, 1975) and several hypotheses about the origin of the homochlamydeous flowers of *Zanthoxylum* have been developed. Engler (1931) considered the flowers of *Zanthoxylum* as an ancestral type, which is otherwise unknown in the family. Engler (1931) further noted that the flowers of *Zanthoxylum* should not be regarded as derived from a *Fagara*-like ancestor. Other hypotheses were formulated by Linnaeus, who regarded the flowers of *Zanthoxylum* as apetalous (according to Brizicky, 1962), and Eichler (1878), who classified the flowers as aseptalous. The latter two hypotheses imply a reduction from a heterochlamydeous ancestor (Brizicky, 1962), and thus better reflect the phylogenetic position of *Zanthoxylum* s.str. deeply nested within *Fagara* than Engler's (1931) hypothesis. Several species, nevertheless, show intermediate morphologies regarding the perianth. The Southeast Asian *Z. dimorphophyllum* Hemsl. has a variable perianth morphology, and specimens can either have clearly heterochlamydeous flowers, or flowers with an undifferentiated perianth (Hartley, 1966; Zhang et al., 2008). At least four species from Central and South America also show a similar pattern of intermediate flower morphologies (Brizicky, 1962). Unfortunately, we did not have material of either *Z. dimorphophyllum* or the above-mentioned American species at hand. Inclusion of these species in future studies will help enhance our understanding of the evolution of flower morphology in this group.

No worldwide monograph has been published about *Zanthoxylum* in the past decades, so that Engler's (1931) account still remains an influential publication. Regarding Engler's (1931) sections, beyond the monotypic section *Mayu*, section *Tobinia* is possibly monophyletic, although our taxon sampling of this section is very limited. Species of section *Tobinia* are characterized by trimerous flowers and basally connate sepals and are endemic to the Caribbean (Engler, 1931). These characters clearly separate section *Tobinia* from the Caribbean species in section *Macqueria*, although it should be noted that some species in section *Tobinia* may develop tetramerous flowers (Beurton, 2008). A better taxon sampling of this section is needed to evaluate its monophyly. Our results show conflicting results concerning the monophyly of section *Blackburnia*. The Hawaiian species were either resolved as sister to the remaining species of section *Blackburnia* (plastid dataset), or nested within an Asian clade of section *Macqueria* (nuclear, and the 4-marker datasets). This conflict might be the result of a hybridization event prior to the colonization of the Hawaiian archipelago. The sister clade of the Hawaiian species in the nuclear and the 4-marker analyses consists of species from Asia. Two species – *Z. avicennae* (Lam.) DC. and *Z. limonella* (Dennst.) Alston – in this lineage have a large distribution area that ranges from Southern China to the Moluccas (*Z. avicennae*) and from India to New Guinea (*Z. limonella*), respectively (Hartley, 1966; Zhang et al., 2008). Putatively close relatives of the Australian *Z. brachyacanthum* F. Muell., a member of the non-Hawaiian clade of section *Blackburnia*, are endemic to New Guinea (Hartley, 2013) and *Zanthoxylum megistophyllum* (Burr) T.G. Hartley, another member of, this lineage, is also endemic to New Guinea (Hartley, 1966). The overlapping distribution of these two clades provides a contact zone for possible hybridization events. Interestingly, the four Hawaiian species have been subdivided into two groups, and independent colonization events have been assumed for them (Wagner et al., 1999). While *Z. dipetalum* shows morphological affinities to Asian taxa, the remaining Hawaiian species are similar to South-Western Pacific taxa (Wagner et al., 1999, mentioning pers. comm. with Hartley). Our results do not corroborate two independent colonization events. A possible explanation for the different morphotypes on Hawaii may be that one group is morphologically more similar to the Asian parent, while the other rather resembles the parent that colonized the South Pacific. A hybridization event prior to the colonization of the Hawaiian Islands is also supported by chromosome numbers. *Zanthoxylum hawaiiense* Hillebr. has a chromosome number of  $2n = 136\text{--}144$  (Kiehn and Lorence, 1996). This number is the highest count in the genus, together with  $2n = 136$  in *Z. armatum* DC. (as *Z. alatum* Roxb.) and  $2n = \text{ca. } 132$  in *Z.*

*simulans* Hance, which are not closely related to the Hawaiian species (Guerra, 1984; Kubitzki et al., 2011). In general, chromosome numbers range from  $n = 16$  to  $n = 68(-72)$  in *Zanthoxylum* (Kiehn and Lorence, 1996; Kubitzki et al., 2011), and the fact that higher numbers occur in unrelated species groups points toward several polyploidization events in the genus. Whether these events are linked to hybridization or just autopolyploidization needs to be tested in future studies based on next-generation sequence data.

By far the most species-rich section of *Zanthoxylum/Fagara* is section *Macqueria*. Our results show this section to be largely polyphyletic. The other sections are of more restricted distribution and have narrower morphological circumscription (Engler, 1931). In contrast, section *Macqueria* occurs throughout nearly the entire distribution of the genus and is morphologically very diverse. The polyphyly of section *Macqueria* is therefore not surprising.

#### 4.3. Biogeographic patterns

Our analyses suggest an origin of the proto-Rutaceae in the Old World in the Late Cretaceous to Early Paleocene. The AAR shows an increasing uncertainty towards the root of the phylogeny, but still revealed an important role of mainland Asia and Malesia, but also of Africa and Australia. The importance of Australia early in the evolution of the proto-Rutaceae seems implausible given the long distance between the Australian tectonic plate and Eurasia in the Cretaceous/Paleocene (Hall, 2011), and our outgroup selection might have led to an overestimation of the importance of Australia. The *Melicope-Acronychia* group probably has an Australasian origin (Appelhans et al., 2018), but the group represents only one out of several clades related to the proto-Rutaceae. While the immediate relatives of the proto-Rutaceae are not known, the more distantly related lineages also include taxa from mainland Asia and Africa (Poon et al., 2007; Groppo et al., 2008; Morton and Telmer, 2014). Taking into account that the oldest fossils of proto-Rutaceae are from Europe (Chandler, 1961; Gregor, 1989), a Eurasian origin of the group is conceivable (Europe in addition to areas C and E). The proto-Rutaceae group is among the oldest lineages within Rutaceae and likely established soon after the split of Rutaceae into its main lineages (Appelhans et al., 2012; Muellner-Riehl et al., 2016). Kubitzki et al. (2011) hypothesized that a north-Tethyan origin might be conceivable for the family, which is in agreement with an Eurasian origin of the proto-Rutaceae. Shortly after the establishment of the main clades within the proto-Rutaceae and *Zanthoxylum*, migration to Africa and to the Americas might have taken place. The African lineage also led to the colonization of Madagascar and the Mascarene Islands in the Oligocene or Miocene. Within the African clade, *Toddalia* is also found throughout tropical mainland Asia and Malesia (Kubitzki et al., 2011). The two *Toddalia* samples included in this study are from China. Thus, it remains unclear if *Toddalia* has an African origin or if it has an Asian origin and dispersed to Africa and Madagascar later. Sampling *T. asiatica* throughout its distributional range in the future is necessary to identify the geographic origin and dispersal patterns as well as to assess if this extremely widespread taxon indeed represents one species or not.

The migration of *Zanthoxylum* from the Old World to the Americas probably took place in the Eocene, which is in agreement with the oldest New World *Zanthoxylum* fossils from the Late Oligocene sediments (Graham and Jarzen, 1969; Tiffney, 1994). A vicariance event connected to the breakup of Gondwana is unlikely for *Zanthoxylum* because the divergence of the lineages occurred too recently (McLoughlin, 2001). Instead, there are two main dispersal pathways for taxa moving across Eurasia and North America: the North Atlantic Land Bridges (NALB; Tiffney, 1985) and the Bering Land Bridge (BLB; Wen et al., 2016). The NALB is well known for plant and animal migrations especially in the Eocene (Tiffney, 1985; Wen, 1999, 2001; Sanmartín et al., 2001; Wen et al., 2010). Considering the Early Eocene fossils from Europe (Chandler, 1961), dispersal via the NALB is a likely scenario for *Zanthoxylum*. A subsequent colonization event was inferred

from the Americas back to Asia in *Zanthoxylum*. This event probably occurred in the Late Eocene to Late Oligocene. The Asian species in this lineage are mostly found in eastern Asia, including Japan and Korea, and the many eastern Asian species as well as their closest relatives in North and Central America are adapted to temperate or subtropical climates (Engler, 1931). Because of these two factors, dispersal across the Bering Strait via BLB is the more likely explanation. The adaptation to temperate climates might have been a prerequisite for the migration along this northerly route in the colder climate of the Oligocene (Liu et al., 2009). *Zanthoxylum* is one of many examples of a plant genus with a Eurasian-American disjunct distribution (Donoghue et al., 2001; Qian and Ricklefs, 2004; Wen et al., 2010, 2016; Harris et al., 2017), but it represents a rare example of a genus for which migrations between Eurasia and the Americas via both the NALB and the BLB have been inferred. Other examples with similar inferences include *Ampelopsis* Michx. (Vitaceae; Nie et al., 2012), *Cornus* L. (Cornaceae; Xiang and Thomas, 2008), *Liquidambar* L. (Altingiaceae; Ickert-Bond and Wen, 2006), and *Toxicodendron* Mill. (Anacardiaceae; Nie et al., 2009).

*Zanthoxylum* colonized Pacific archipelagos at least two or three times. The Juan-Fernández endemic *Z. mayu* diverged from its South American relatives in the Eocene. This split clearly exceeds the age of the archipelago, whose oldest islands and nearby seamounts are about 5.8–8.5 myr old (Reyes et al., 2017). A likely explanation for the old divergence time of *Z. mayu* is an artefact due to the limited taxon sampling of South American taxa in this study. Future studies might identify closer relatives of *Z. mayu*, so that its separation from mainland South America may be dated more precisely. The topological conflicts concerning the placement of the Hawaiian species and the monophyly of section *Blackburnia* have been discussed above, and only the geographic origin and age are discussed in this paragraph. Since *Zanthoxylum* occurs both East and West of the Pacific, several possibilities exist for the origin of the Pacific species. The sister group relationships of the Hawaiian and South Pacific taxa with Asian and Australian species reflect morphological similarities (Wagner et al., 1999) as well as the fact that Asian and Australian lineages account for the largest number of colonizers of the Hawaiian flora. The age estimate for the Hawaiian group is surprisingly high. A more complete taxon sampling might identify closer relatives of the Hawaiian group, thereby reducing the stem age of the group. However, also the crown age of the Hawaiian group (age estimate: 6.9–17.5 MA) is much older than the oldest current islands, which are about 5 myr old (Price and Clague, 2002; Neall and Trewick, 2008). It is possible that *Zanthoxylum* colonized older islands of the Hawaiian-Emperor seamount chain, which are now either seamounts or low islands without forests (Price and Clague, 2002). The initial split into the two main Hawaiian lineages might have happened on these islands followed by two independent dispersal events to the current main islands. A bottleneck for dispersal to Hawaii has been proposed between 8 and 5 MA, since larger and densely spaced islands were lacking in the Hawaiian-Emperor seamount chain during this period (Price and Clague, 2002). Molecular dating studies of Hawaiian plant lineages support this bottleneck, since most lineages are younger than the formation of the oldest current islands (Keeley and Funk, 2011). However, there are lineages that exhibit a similar pattern as *Zanthoxylum*, e.g., Hawaiian lobeliads (Campanulaceae; Givnish et al., 2009), the largest radiation of Hawaiian plants, and *Melicope*, also within Rutaceae (Appelhans et al., 2018).

#### 5. Taxonomic implications

*Toddalia* is clearly nested within *Zanthoxylum*, and has been resolved as the sister to the African and Malagasy species of the genus. *Zanthoxylum* is a Linnean genus (Linnaeus, 1753), while *Toddalia* has been described by Jussieu (1789). *Toddalia* is a conserved name, but Rickett and Stafleu (1959) regarded the conservation as superfluous. Also, article 14.5 of the International Code of Nomenclature for algae, fungi, and plants (Melbourne Code; McNeill et al., 2012) specifies that

if a conserved name competes with an earlier name against which it has not explicitly been conserved, that early name is adopted. Therefore, *Zanthoxylum* has the priority over *Toddalia* and also over *Fagara* (see also Fosberg, 1959). Mziray (1992) transferred a number of taxa originally described as *Toddalia* species to *Vepris* Comm ex. A. Juss., which has been confirmed in a recent phylogenetic study (Morton, 2017). We think it would be redundant to mention these as names excluded from *Toddalia* since Mziray (1992) shows a complete list.

**We propose the following taxonomic change for *Toddalia asiatica*:**

*Zanthoxylum* L., Sp. Pl. 1: 270. 1753. Type species: *Zanthoxylum americanum* Mill. (designated by Fosberg, 1959).

*Toddalia* Juss., Gen. Pl. 371. 1789, nom. cons. – Type species: *Toddalia asiatica* (L.) Lam. (= *Zanthoxylum asiaticum* (L.) Appelhans, Groppo & J.Wen)

*Fagara* L., Syst. Nat., ed. 10. 897. 1759, nom. cons. – Type species: *Fagara pterota* L. (= *Zanthoxylum fagara* (L.) Sarg.)

*Zanthoxylum asiaticum* (L.) Appelhans, Groppo & J.Wen, comb. nov.

*Paullinia asiatica* L., Sp. Pl. 1: 365. 1753. *Toddalia asiatica* (L.) Lam., Tabl. Encycl. 2: 116. 1797. Type: SRI LANKA, s.d., Herb. Hermann 3: 45, sub No. 143 (conserved type, designated by Barrie and Nair (1991), Taxon 40, 646. 1991: BM! [BM000621969]).

## Acknowledgements

We are grateful to Nils Bourland (Royal Museum for Central Africa, Tervuren, Belgium), Ken Wurdack (US) and the curators of BM, GH, KAS, L, MO and NY for providing leaf samples or photographs of type specimens. We thank two anonymous reviewers for their constructive and helpful comments.

## Acknowledgements

MG acknowledges Fapesp (grant 2016/06260-2) as well as the Conselho Nacional de Pesquisa (CNPq) for financial support.

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