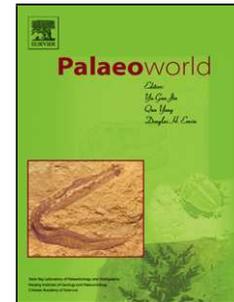# Accepted Manuscript

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Phylogeographic history of the woodwardioid ferns, including species from Himalaya

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Abstract

The woodwardioid ferns are well-represented in the Northern Hemisphere, where they are disjunctly distributed throughout the warm temperate and subtropical regions of North America, Europe, and Asia. To infer the biogeographic history of the woodwardioid ferns, the phylogeny of *Woodwardia* was estimated using *rbcL* and *rps4* sequences from divergent distribution regions including Himalaya. Phylogenetic results support *Woodwardia* as a monophyletic group with *Woodwardia areolatae* and *W. virginica* as basal, these two species from eastern North America diverged early, which are sister clades to the remaining species from America, Europe, and Asia. Based on analyses of the fossil records of these species for divergence times, *Woodwardia* species were estimated to have diverged initially in the Paleogene of North America. After its New World origin, a greater diversification and expansion of *Woodwardia* occurred in eastern Eurasia, with the Europen arrival of *Woodwardia radicans* during Middle Miocene. Compare to earlier reports, a migration back into North America via the Bering land bridge is consistent with these data.
Keywords: *Woodwardia*; *rbcL, rps4*; phylogeny; biogeography; divergence time.

The woodwardioid ferns, the genus *Woodwardia* Smith, as described in the most recent familial classification (Smith *et al*., 2006), includes *Anchistea* C. Presl, *Chieniopteris* Ching, and *Lorinseria* C. Presl. This group is characterized by anastomosing veins and indusiate sori borne on the outer arc of areolae. These three segregate genera have been interpreted to represent diversification within *Woodwardia* rather than major evolutionary divergence (Tryon and Tryon, 1982).

Woodwardia species are distributed disjunctly throughout the warm temperate and subtropical regions of Northern Hemisphere in a classic Arcto-Tertiary distribution pattern, a unique circumboreal distribution within the Family Blechnaceae, which otherwise exhibit a Gondwanan distribution in the temperate and montane tropical regions of Southern Hemisphere (Cranfill, 2001; Cranfill and Kato, 2003). At present the genus includes 14 species (Cranfill and Kato, 2003): *W. radicans* (L.) Sm. is endemic to Mediterranean Europe and Macronesia, six species are endemic to North and Central America and the remaining seven species are endemic to eastern and southeastern Asia with extension to Philippines, Indonesia and New Guinea (Fig.1).

The genus has been variously revised since its establishment in 1793 (see Cranfill and Kato, 2003 for a detailed review). Although the studies of Cranfill and Kato (2003) supported the monophyly of *Woodwardia* and three subunits of related species using both morphological and chloroplast nucleotide sequence data (*rps4* and *rbcL*), the phylogeny of these ferns received limited attention to date. For example, Himalayan species were not included in the analysis reported by Cranfill and Kato (2003). Also, the number of taxa sampled in that study was insufficient to determine the relationships of the three subunits of *Woodwardia.* The evolutionary history of *Woodwardia* has been reviewed by Collinson (2001) and Cranfill and Kato (2003), who suggested that this group first arose sometime in the Late Cretaceous in North America at high latitudes, with the diversification of its major lineages being completed by the Paleocene. Subsequently lineages spread southward into North America and westward into Asia, ultimately reaching Europe by the Miocene (see Fig 2). However, this interpretation has not been tested independently using genetic data. In addition, the origin of *Woodwardia* needs to be reconsidered carefully since little is known about the evolutionary and genetic relationship of these morphologically similar but inter-continentally isolated species. A well-corroborated, gene-based phylogenetic framework is necessary to assist in our inference of these complex biogeographic patterns.

The specific objectives of this study were to (1) determine molecular

phylogenetic relationships among species within the genus, (2) evaluate the placement of the Himalayan species specifically, and (3) use these results to test the Collinson-Cranfill/Kato interpretation of origin and formation of the present disjunct distribution pattern of *Woodwardia* in North America, Europe, and Asia.

Fig. 1. Distribution of the genus *Woodwardia* and the hypothesized migration routes indicated by arrows based on *rbcL* sequence information from extant species. Line in black: migration route from NA to Eurasia; Line in green: a migration back to America from Asia; NA: North America, CA: Central America, EA: eastern Asia, SA: southeastern Asia.

1 Materials and methods

1.1 Taxon sampling

In order to facilitate comparison with the study of Cranfill and Kato (2003), we assembled an *rbcL* and *rps4* data matrix, which included both newly generated sequences and others obtained from GenBank. A total of 12 new *rbcL* and six *rps4* sequences were generated for this study. Our ingroup sample set comprised 43 taxa of *Woodwardia* which represent 15 species in the world, especially including two speceis endemic to Himalayan region (*Woodwardia himalaica* Ching et S. K. Wu and *Woodwardia magnifica* Ching et P. S. Chiu), however, two American species (*W. martinezii* and *W. semicordata*) were not included due to difficulties in acquiring material at present time.

Our sample includes all *rbcL* sequences currently available in GenBank, with multiple accessions for each species to quantify intraspecific and geographic variaton. We selected outgoup taxa from *Blechnum* L. following Cranfill and Kato (2003), who showed *Blechnum* to be one of sister groups to the woodwardioid ferns. The corresponding voucher specimens have been deposited in the Herbarium of the Yunnan University (PYU). Taxa, vouchers, and accession numbers are provided in Table 1.

1.2 DNA extraction, PCR amplification and DNA sequencing

Total genomic DNA was extracted from 2 g of fresh, or 1 g of silica gel dried, leaves using the CTAB procedure (Doyle and Doyle, 1987). The selected DNA regions were amplified with standard polymerase chain reaction (PCR). The protocols used to amplify *rbcL* and *rps4* genes were identical to those described by Li *et al.* (2004; 2008).

1.3 Sequence alignments

Alignments of all sequences (Table 1) were performed using Clustal X (Thompson *et al*., 1997) and subsequently edited manually in MacClade4 (Maddison and Maddison, 2005). There were no insertions or deletions (indels) in the sequence alignments. Phylogenetic analyses were performed by maximum parsimony (MP), maximum likelihood (ML), and Bayesian inference (BI) methods as implemented by PAUP* vers. 4.0b10 (Swofford, 2003), PHYML vers. 2.4.3 (Guindon and Gascuel, 2003), and MrBayes vers. 3.1.2 (Ronquist and Huelsenbeck, 2003). For MP analysis, unweighted analyses were performed by heuristic searches with tree-bisection-reconnection (TBR) branch swapping, the MulTrees in effect, steepest descent off using 1,000 random taxon-addition replicates, and one tree held at each step during stepwise addition. Bootstrap analyses (Felsenstein, 1981) were conducted to examine the relative level of support for individual clades on the cladograms of each search (MPBS), using 500 bootstrap replicates and the same tree search procedure as described above. For the ML and BI analyses, the best-fitting model of sequence evolution for each data was identified with the Akaike Information Criterion in Modeltest vers. 3.07 (Posada and Crandall, 1998). The SYM+I+G model was selected for the *rbcL* data set, and HKY+I+G model was selected for the *rps4* data set. Once the best sequence evolution model was determined, PHYML analysis was performed for each data set, the parameters such as base-composition, gamma-shape, and ratio of invariable sites were also estimated during each ML analysis. Nodal robustness on the ML tree was estimated by the nonparametric bootstrap technique (500 replicates, MLBS). Phylogenetic BI was conducted using the evolutionary models described above and default priors. Two concurrent analyses were performed, each with four chains of the Markov-Monte Carlo chain, sampling one tree every 100

generations of 2 x 1,000,000 generations and beginning with a random tree. The first 25 percent of the samples (5,000 trees) were discarded as ''burn-in'' (Ronquist and Huelsenbeck, 2003). At this point, the standard deviation of split frequencies was <0.01, indicating that convergence to a stationary distribution had been achieved. The posterior probability (PP) was used to estimate nodal robustness.

1.4 Divergence time estimation

A likelihood-ratio test using baseml program implemented in PAML (Yang, 1997) rejected the presence of a molecular clock. Accordingly, the Bayesian relaxed-clock method (Thorne and Kishino, 2002) was used to transform the ML phylogram into a chronogram. This was calibrated with the earliest divergence of the woodwardioid ferns (55.8 Ma), because fossils assignable to *Woodwardia* are known from throughout the Tertiary, beginning in the Paleocene (Collinson, 2001), as used in Schuettpelz and Pryer (2009). To estimate divergence time, the Bayesian analyses were performed with the estbranches and multidivtime programs available at http://statgen.ncsu.edu/thorne/multidivtime.html, according to the protocol described by Rutschmann (2005). We also employed the likelihood method of Pyron (2010) to assess placement of the calibration point using PhyloBayes (Lartillot et al., 2007).

Table 1. Taxa examined and *rbcL* GenBank Accession Numbers*

Fig. 2. Paleogeographic maps, showing the distributions of *Woodwardia* fossils using references cited in Collinson (2001). The base maps with paleocoastlines of Paleocene (ca. 60 Ma), Eocene (ca. 50 Ma), and Miocene (ca.14Ma) were modified from the paleomaps of Scotese (1997).

2 Results

2.1 Phylogenetic analysis

Twelve *rbc*L sequences were newly obtained in this study. The alignment of the *rbcL* sequence in 45 accessions produced a data set of 1,323 characters, with 156 variable sites (11.8%), 139 of which were phylogenetically informative (10.51%). Parsimony analysis of the *rbcL* data set resulted in 157 most parsimonious trees (MPTs) with a total length of 203 steps, a consistency index (CI) of 0.798 and a retention index (RI) of 0.934.

All MP, ML and BI analyses from 45 *rbcL* sequences resulted in nearly identical topologies, only with differences at the statistical support values (Fig. 3). The 50 percent majority-rule consensus tree resulting from MP, ML and BI analyses is shown in Fig. 3. These analyses yielded a robust phylogenetic topology with the exception of a few nodes. Together, *Woodwardia areolata* and *W. virginica,* both from the eastern North America with the chromosome base number of *n*=35, are basal clades to the rest of *Woodwardia* species, the rest species analyzed here constitute more recent divergent groups, which in turn comprises four clades (labeled as Clade 1–4 in Fig. 3) including species from America, Europe, and Asia (Fig. 1). Strong support was obtained for three of these clades (clades 2–4, PP = 1.00 and MLBS, MPBS >= 95%). Only Clade 1 was associated with a moderate statistical support values (PP = 0.97 and MLBS, MPBS < 85%), though strong support was obtained for its two subclades. The four well-supported clades within *Woodwardia* are consistent in their geographic distributions. Clade 1 includes species from eastern Asia and southeastern Asia with the two subclades corresponding to Section *Chieniopteris* (Ching) Cranfill and Section *Japonica* (Ching) Cranfill respectively in Cranfill and Kato (2003); Clade 2 included species from eastern Asia along with the Himalayan endemic *Woodwardia magnifica*; Clade 3 included two species from western America and Central America; Clade 4 contained species from Himalaya and Europe.

MP, ML and BI analyses from 22 combined data of *rbcL* and *rps4* sequences resulted in nearly identical topologies (Fig. 4). This result appears to confirm the phylogenetic relationships based on the *rbcL* data alone, but with stronger statistical support values.

2.2 Divergence times.

Estimated divergence times for the nodes of the cladogram are summarized in Fig. 3. The basal divergence in the woodwardioid ferns is estimated to have occurred in the Paleocene (56.51 ± 2.89 Ma); the subsequent divergence occurred in the early Eocene (49.93 ± 4.18 Ma); the first two divergences of the Asian species (Clade 1, Clade2) were estimated at 45.04 ± 6.02 Ma and 32.78 ± 6.77 Ma; the split of American/European and Asian/species (Clade 4/Clade 3 and Clade 2) is estimated to have occurred in the middle Eocene (44.88 ± 5.43 Ma); the second divergence of American species (Clade 3) is estimated to have occurred at 27.45 ± 8.29 Ma during Oligocene; the split in *Woodwardia* between the European and the Asian species is estimated to have occurred at 29.99 ± 7.31 Ma; the split between the European

Woodwardia radicans and Himalayan *W. unigemmata* is estimated to have occurred at 13.78 ± 6.54 Ma during Middle Miocene.

Fig. 3. Fifty-percent majority-rule consensus tree (BI, 45 *rbcL* sequence data) and simplified chronogram generated by applying Bayesian relaxed-clock to the ML tree. The ML and MP trees are identical in topology. Values above branches are posterior probability from BI; values below branches are bootstrap percentage ≥ 50% from MP and ML analyses. Four clades and geographic distributions of *Woodwardia* are marked in the phylogenetic tree.

Fig. 4. Fifty-percent majority-rule consensus tree from Bayesian inference (BI) of the 22-taxon phylogenetic tree based on *rbcL+rps4* sequence data. The ML and MP trees are identical in topology. Values above branches are posterior probability from BI; values below branches are bootstrap percentage ≥ 50% from MP and ML analyses. Four clades and geographic distributions of *Woodwardia* are marked in the phylogenetic tree.

3 Discussion
3.1 Phylogenetic relationships

In this study we have inferred divergence times using *rbcL* data alone because Himalayan *Woodwardia* species have not been analyzed with *rbcL* data previously. Other molecular data sets currently lack a sufficiently wide taxon sample on which to base a similar analysis.

In general, our results agree with those published in previous phylogenetic studies, especially Cranfill and Kato (2003). However, the increased taxonomic sampling—especially the unsampled species from Himalaya—do provide new insights into the evolutionary history of *Woodwardia*. Based on *rbcL* tree in this study, *Woodwardia* appears to have diverged first in North America. Later, a greater diversification occurred in eastern Eurasia with the four clades radiating into their current geographic distributions (Fig. 3). Species relationships within the genus, however, remain equivocal. Most *Woodwardia* species are polyphyletic in the *rbcL* tree; for example, both *Woodwardia unigemmata* and *W. himalaica* are not monophyletic, because *W. radicans* was nested within. *Woodwardia himalaica* was

considered as a new species by Ching (1983) and merged into *Woodwardia unigemmata* by Wu (1999). Similarly, *Woodwardia unigemmata* is usually considered as a geographical substitute species of *W. radicans* (Chiu, 1974). Whether these *Woodwardia* morphotypes should be treated as separate species or be considered conspecific requires more study. An increase of geographical coverage appears particularly important for delineating these putatively unnatural species. Another Himalayan species *Woodwardia magnifica* was placed in Clade 2 and unexpectedly clustered with *W. prolifera* and *W. orientalis*. Morphologically, *W. magnifica* is close to *W. japonica*. Obviously the phylogenetic position of *W. magifica* needs further study. This must await production of additional DNA sequence markers that are more informative and together with more morphological and developmental characteristics.

3. 2 Biogeography and evolution of *Woodwardia*

On the basis of fossil evidence, Kvacek (1994) hypothesized that woodwardioid ferns first arose in North America and later dispersed across Beringia to eastern Asia and eventually to Europe. Our present phylogenetic analyses and divergence time estimations agree with Kvacek's hypothesis (1994) based on fossil records; our results also offer support for a North American origin sometime in the Paleocene (56.51 ± 2.89 Ma). Fossils from Eastern Asia do not appear until the middle Eocene. Our molecular results suggest that the first diversification of Asian *Woodwardia* species (Clade 1) occurred at 45.04 ± 6.02 Ma (middle Eocene). *Woodwardia* fossils did not appear in Europe until the Miocene and appear to have arrived from Asia, not directly from North America. Our phylogenetic results provide support for the European *Woodwardia radicans* and Himalayan species (*W. unigemmata* and *W. Himalaica*) being part of the same lineage (Clade 4). Divergence estimates also provide support for the European arrival of *Woodwardia radicans* at c. 14.00 Ma, during Middle Miocene. Extant *Woodwardia* species from western America and Central America are included in one clade (Clade 3), which appears to have diverged from its closest Asian sister lineage during Oligocene (27.45 ± 8.29 Ma). Accordingly, our data are consistent with the hypothesis of, but do not prove, migration back into North America via the Bering Land Bridge (BLB, Fig. 1). Flora exchange between Asia and North America could have taken place via two divergent routes: either across the BLB or across the North Atlantic European–North American land bridge (NALB), but BLB has been considered to be more important (Wen, 1999), the distributions of *Woodwardia* fossils on paleogeographic maps (Fig. 2) and a current age estimates are

accommodated readily by prevailing ideas on the availability of the BLB pathway.

Acknowledgements

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Table 1. Taxa examined and *rbcL* GenBank Accession Numbers*

Species	Locality	Voucher[a]/ Reference	GenBank accession no.	
			rbcL	*rps4*
Blechnum occidentale L.	Cultivated	Cranfill and Kato	AY137670	AF533868
Blechnum serrulatum Rich.	Cultivated	Cranfill and Kato	EF423158	AF533869
Woodwardia areolata (L.) T. Moore	USA.	Cranfill and Kato	**AF425102**	**AF425155**
Woodwardia areolata (L.) T. Moore [b]	-	-	AB040596	-
Woodwardia auriculata Blume	Malaysia	Cranfill and Kato	AY137661	AF533858
Woodwardia fimbriata Sm.	California	Wolf et al., (1994)	U05950	-
Woodwardia fimbriata Sm.[b]	-	-	AB040597	-
Woodwardia fimbriata Sm.	Cultivated	Cranfill and Kato	AY137662	AF533859
Woodwardia harlandii Hook.	-	-	AB040598	-
Woodwardia harlandii Hook.[b]	-	-	AB040599	-
Woodwardia harlandii Hook.	Taiwan	Cranfill and Kato	AY137663	AF533860
Woodwardia harlandii Hook.	Japan	Ebihara et al., (2010)	AB575056	-
Woodwardia himalaica Ching et S. K. Wu	China, Xizang	Lu S-G/XZ9	**This study**	-
Woodwardia himalaica Ching et S. K. Wu	China, Xizang	Lu S-G/XZ10	**This study**	**This study**
Woodwardia himalaica Ching et S. K. Wu	China, Xizang	Lu S-G/XZ11	**This study**	-
Woodwardia himalaica Ching et S. K. Wu	China, Xizang	Lu S-G/XZ12	**This study**	-
Woodwardia japonica (L. f.) Sm.	China, Hunan	Lu S-G/FH3	**This study**	**This study**
Woodwardia japonica (L. f.) Sm.	China,	Lu S-G/FJS66	**This study**	-
Woodwardia japonica (L. f.) Sm.	China,	Lu S-G/GY51	**This study**	-
Woodwardia japonica (L. f.) Sm.	Japan	Ebihara et al., (2010)	AB575057	-
Woodwardia japonica (L. f.) Sm.[b]	-	-	AB040600	-
Woodwardia japonica (L. f.) Sm.	Japan	Cranfill and Kato	AY137664	AF533861
Woodwardia kempii Copel.	China, Hainan	Lu S-G/V36	**This study**	**This study**
Woodwardia kempii Copel.	Taiwan	Cranfill and Kato	AY137665	AF533861
Woodwardia kempii Copel.	Japan	Ebihara et al., (2010)	AB575058	-
Woodwardia kempii Copel.[b]	-	-	AB040601	-
Woodwardia magnifica Ching et P. S. Chiu	China, Yunnan	Lu S-G/LJS9	**This study**	**This study**
Woodwardia orientalis Sw.[b]	-	-	AB040602	-
Woodwardia orientalis Sw.	Japan	Sano et a., (2000)	AB021727	-
Woodwardia orientalis var. *formosana*	Japan	Ebihara et al., (2010)	AB575688	-
Woodwardia orientalis Sw. var. *orientalis*	Japan	Ebihara et al., (2010)	AB575059	-
Woodwardia prolifera Hook. & Arn.	China, Fujian	Lu S-G/WY18	**This study**	**This study**
Woodwardia prolifera Hook. & Arn.	Cultivated	Cranfill and Kato	AY137666	AF533864
Woodwardia prolifera Hook. & Arn.[b]	-	-	AB040603	-
Woodwardia radicans (L.) Sm.	Canary Islands	Cranfill and Kato	AY137667	AF533865
Woodwardia spinulosa M. Martens &	Mexico	Cranfill and Kato	AY137668	AF533866
Woodwardia spinulosa M. Martens &	-	-	AB040604	-
Woodwardia unigemmata (Makino) Nakai	China, Hunan	Lu S-G/FH2	**This study**	**This study**
Woodwardia unigemmata (Makino) Nakai	China,	Lu S-G/FJS65	**This study**	-
Woodwardia unigemmata (Makino) Nakai	China,	Lu S-G/GY48	**This study**	-
Woodwardia unigemmata (Makino) Nakai	Taiwan	Cranfill and Kato	AY139669	AF533867
Woodwardia unigemmata (Makino) Nakai	Japan	Ebihara et al., (2010)	AB575061	-
Woodwardia unigemmata (Makino) Nakai[b]	-	-	AB040605	-
Woodwardia virginica (L.) Sm.	USA, Carolina	Cranfill and Kato	AY137660	AF533857
Woodwardia virginica (L.) Sm.[b]	-	-	AB040606	-

*Newly generated sequences are in the submission process to GenBank. Note. Circumscription of species follows Ching and Wu (1983) and Wu (1999). Accession numbers in boldface type for newly generated sequences.

[a] Voucher information for newly generated sequences.
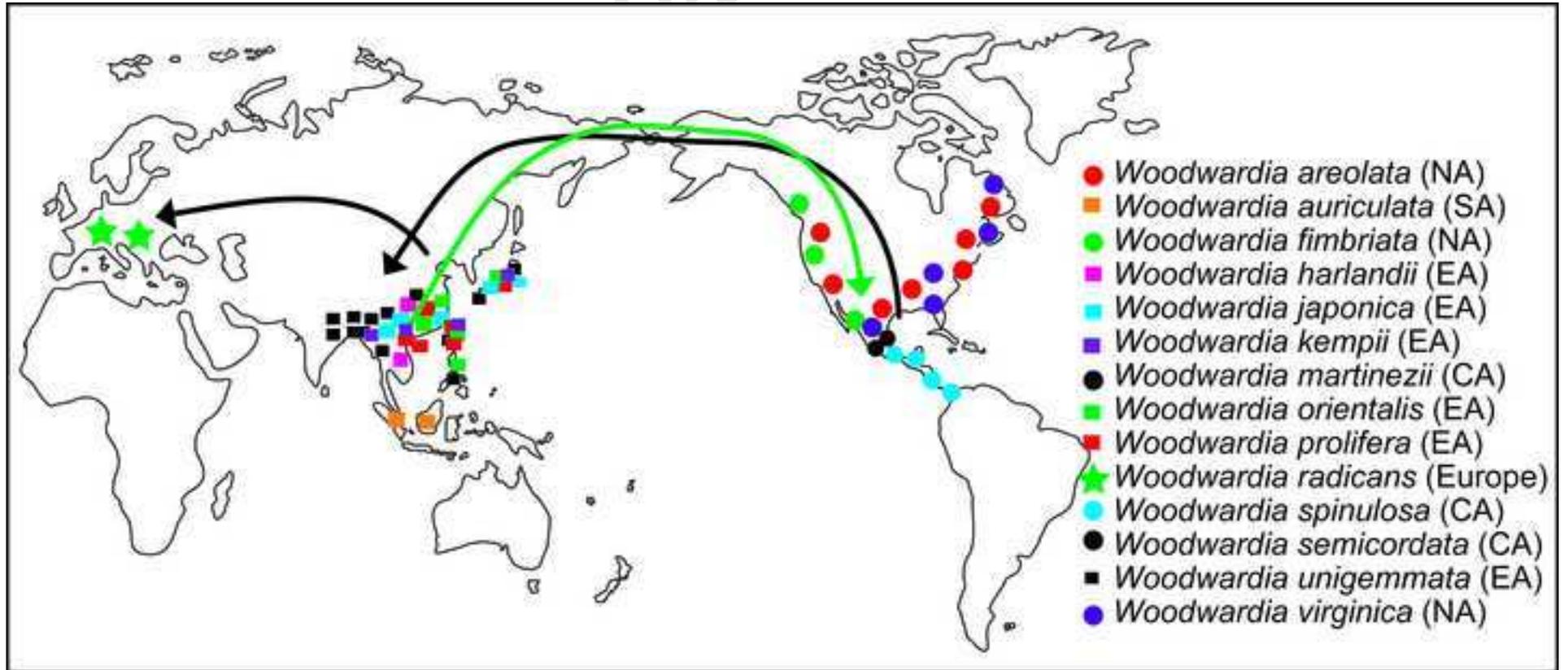[b] Published only in GenBank database.

Figure 1

Figure 2

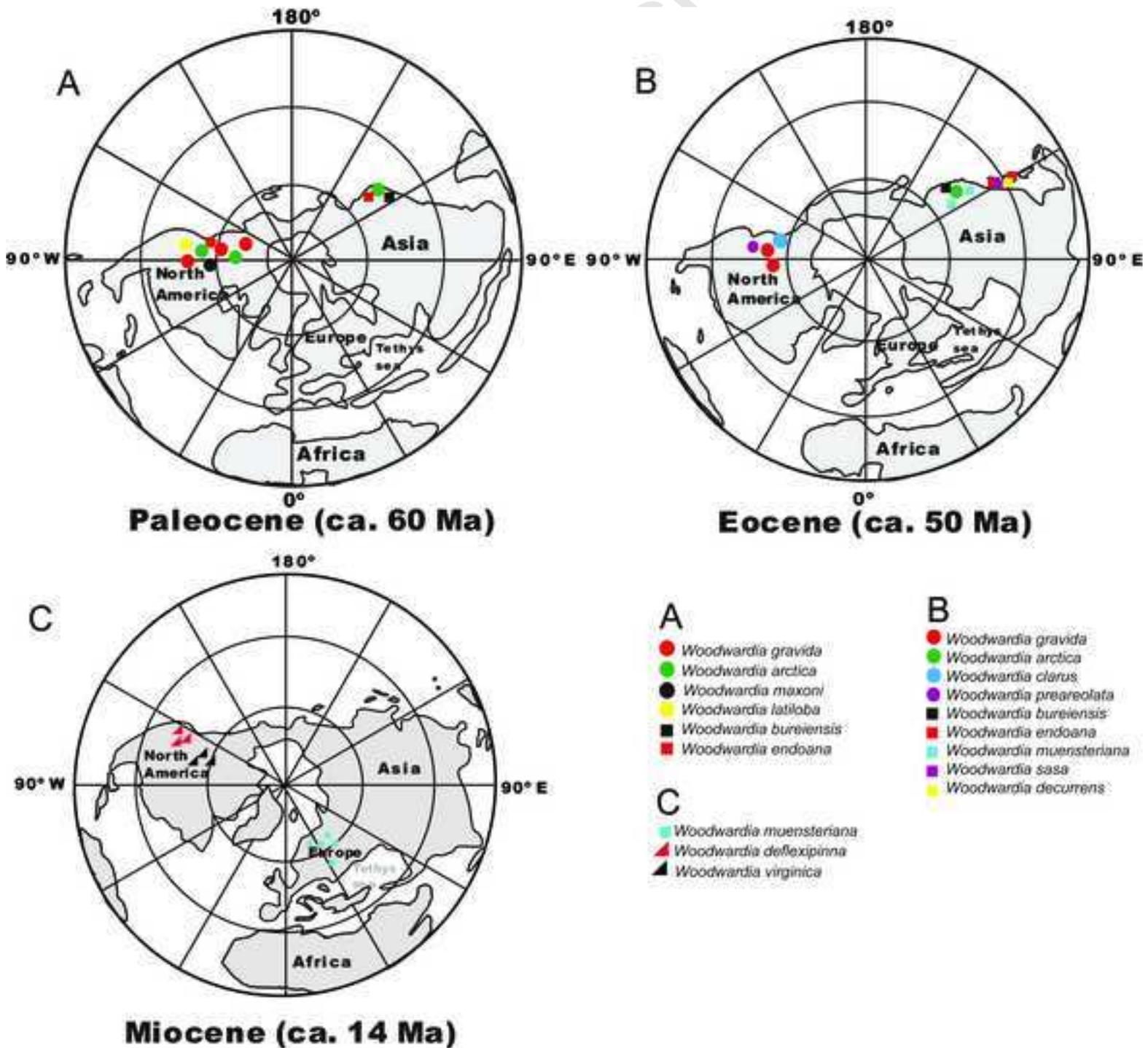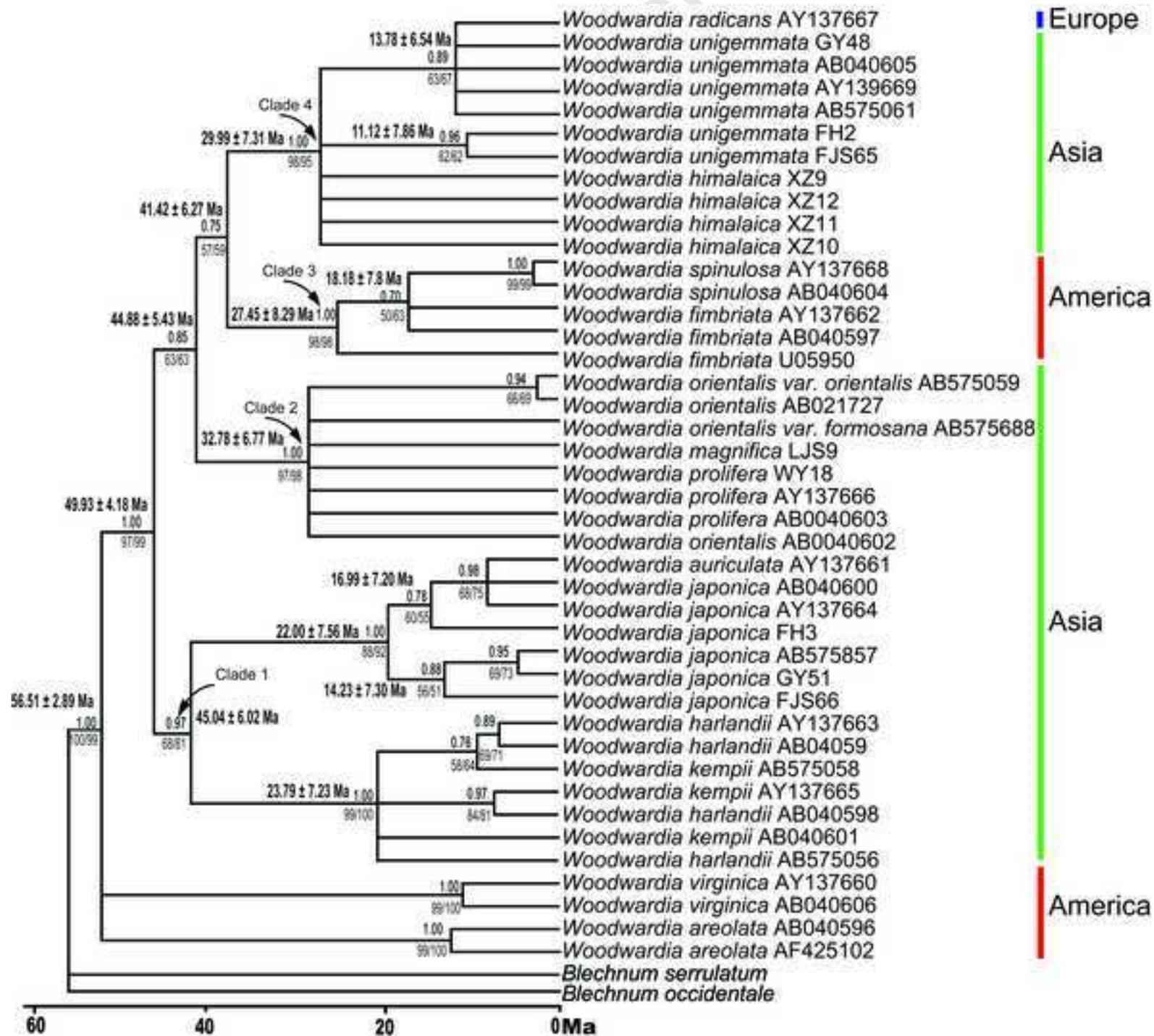Paleocene (ca. 60 Ma)

Eocene (ca. 50 Ma)

Miocene (ca. 14 Ma)

A
● Woodwardia gravida
● Woodwardia arctica
● Woodwardia maxoni
● Woodwardia latiloba
■ Woodwardia bureiensis
■ Woodwardia endoana

B
● Woodwardia gravida
● Woodwardia arctica
● Woodwardia clarus
● Woodwardia preareolata
■ Woodwardia bureiensis
■ Woodwardia endoana
■ Woodwardia muensteriana
■ Woodwardia sasa
● Woodwardia decurrens

C
□ Woodwardia muensteriana
◢ Woodwardia deflexipinna
◢ Woodwardia virginica

Figure 3

Figure 4