

Phylogenetic analyses place the Australian monotypic *Revwattsia* in *Dryopteris* (Dryopteridaceae)

Meghan McKeown¹, Michael Sundue¹, David S. Barrington¹

¹ Pringle Herbarium, Department of Plant Biology, University of Vermont, 63 Carrigan Drive, Burlington Vermont, 05405 USA

Corresponding author: Meghan McKeown (mmckeown@uvm.edu)

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Abstract

Revwattsia fragilis (Watts) D.L. Jones (Dryopteridaceae), originally described as a *Polystichum* Roth by the pioneer Australian botanist Reverend W.W. Watts in 1914, is a rare epiphytic fern endemic to northeastern Queensland, Australia. Known from only a few populations, it is restricted to tropical rainforests in the Atherton Tablelands. We used the cpDNA markers *psbA-trnH*, *rbcL*, *rbcL-accD*, *rps4-trnS*, *trnG-trnR*, *trnL-trnF*, and *trnP-petG* to infer the relationships of *Revwattsia fragilis* within Dryopteridaceae. Based on our molecular analysis, we were able to reject Watts's 1914 hypothesis of a close relationship to *Polystichum*. Its closest allies are a suite of Asian *Dryopteris* Adans. species including *D. labordei*, *D. gymnosora*, *D. erythrosora* and *D. cystolepidota*; maintaining *Revwattsia* renders *Dryopteris* paraphyletic. The epiphytic habit and distinctive long-creeping rhizome of *Revwattsia* appear to be autapomorphies and do not warrant its generic status. In the course of our investigation we confirmed that polyphyly of *Dryopteris* is also sustained by the inclusion of *Acrorumohra* (H.Itô) H.Itô, *Acrophorus* C.Presl, *Arachniodes* Blume, *Diacalpe* Blume, *Dryopsis* Holttum & P.J.Edwards, and *Peranema* D.Don. The epithet *fragilis* is occupied in *Dryopteris*, therefore we provide the name *Dryopteris watsii* **nom. nov.** to accommodate *R. fragilis* in *Dryopteris*.

Keywords

Biogeography, Australia, Morphology, *Polystichum*, *Rumohra*

Introduction

The fern genera *Polystichum* Roth and *Dryopteris* Adans. are now understood to be closely allied members of the Dryopteridaceae. *Polystichum* and its allies *Cyrtomium* C.Presl and *Phanerophlebia* C.Presl are sister to *Arachniodes* Blume and *Dryopteris*

(Schuettpelez and Pryer 2007). The breadth of morphological diversity exhibited by *Polystichum* and *Dryopteris* has, in hindsight, had at least three impacts on the taxonomic history of these genera and their family. First, a large number of segregate genera have been removed from these two genera based on dramatic morphological transformations. Some of these segregates render *Dryopteris* and *Polystichum* paraphyletic; examples include *Sorolepidium* Christ, which belongs in *Polystichum* (Liu et al. 2007a), and *Lithostegia* Ching, which belongs in *Arachniodes* (Liu et al. 2007b). Second, these morphologically innovative lineages are sometimes superficially similar to (i.e. convergent with) remotely related ferns, leading to their circumscription as polyphyletic genera. For example, the morphologically anomalous *Polystichum speciosissimum* (Kunze) R.M.Tryon & A.F.Tryon was originally described in *Cheilanthes* Sw. (Pteridaceae). Third and central here, more remote members of the Dryopteridaceae superficially resemble species of *Polystichum* and *Dryopteris*. For instance, the epiphytic genus *Rumohra* Raddi was long included in *Polystichum*, presumably because of its pelate indusium (Diels 1902). However, Little and Barrington (2003) provided evidence for a close relationship of *Rumohra* to *Megalastrum* Holttum and *Lastreopsis* Ching, a conclusion confirmed in analyses with denser sampling more recently (Schuettpelez and Pryer 2007). This same relationship was implied by Tryon and Tryon (1982) who grouped *Rumohra*, *Megalastrum*, and *Lastreopsis* together in their key to dryopterid genera based on their shared central adaxial costal ridge.

The rare Australian monotypic genus *Revwattsia* D.L.Jones presents a similarly intricate history (Fig. 1A). A high-canopy epiphyte, *Revwattsia fragilis* (Watts) D.L.Jones (Dryopteridaceae) is endemic to northeastern Queensland, where it is known from only a few small populations (Australia's Virtual Herbarium 2012). *Revwattsia fragilis* is confined to mid-elevation rainforest, where it grows inside rotting tree hollows and among other epiphytes (Jones 1998). The Reverend W.W. Watts originally described *R. fragilis* in 1915 ('1914') as a *Polystichum*, presumably because of its perceived similarity to *Rumohra adiantiformis* (G. Forst.) Ching, which was then included in *Polystichum*. In northern Queensland, *Rumohra adiantiformis* is a common species in the humid forests; the two share a few superficial similarities: a long-creeping dorsiventral rhizome and epiphytic habit (Watts 1914)(Fig. 1B). Watts accurately listed characters by which *R. fragilis* differed from *R. adiantiformis*, including its reniform indusia, its less coriaceous texture, and lamina axes lacking a central adaxial costal ridge (Fig. 1D). Andrews (1990) and later Jones (1998) both emphasized what they perceived to be unique characters of *R. fragilis*. Andrews (1990) suggested recognition as a separate genus for the taxon in his treatment of the ferns of Queensland. Jones (1998) followed this lead in establishing the genus *Revwattsia* in his treatment to the Dryopteridaceae of Australia.

Indeed, inclusion of *Revwattsia* in *Polystichum* is untenable morphologically. Long-creeping rhizomes, reniform indusia, and the epiphytic habit are not characteristic of *Polystichum*. The herbaceous dark-brown petiole scales of *Revwattsia* are unknown in *Polystichum*, which has pale scales or dark indurated petiole scales. The extensive glandular indument characteristic of *Revwattsia* (Andrews 1990) is unknown among the mature fronds of large *Polystichum* species. In addition, the symmetrical ultimate

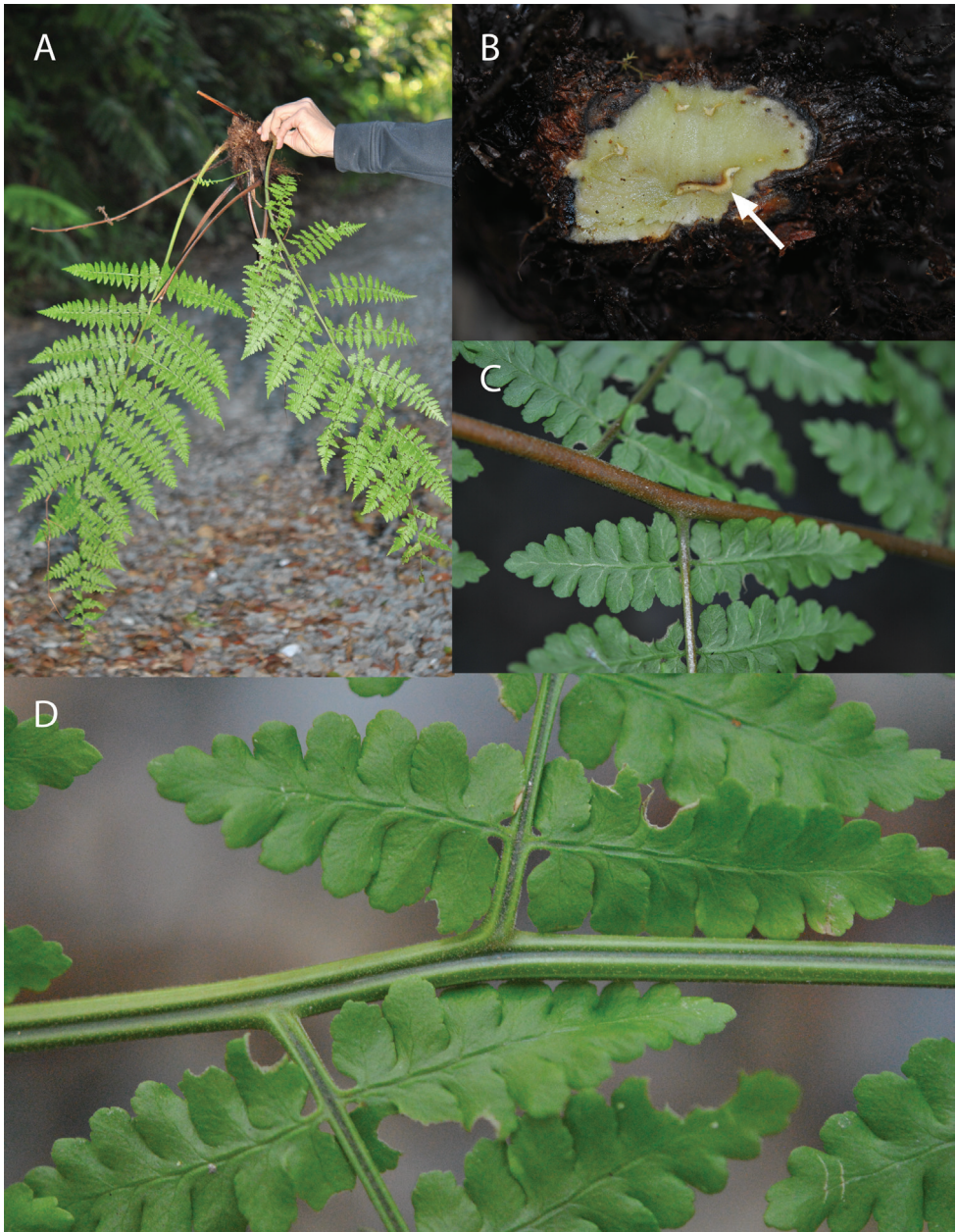


Figure 1. *Revwattsia fragilis*. **A** habit **B** Rhizome in cross section **C** Abaxial rachis and costa **D** Adaxial rachis and costa (M Kessler, M Sundue and M Lehnert 14293).

segments are unknown in *Polystichum* species with large laminae. *Revwattsia* does, however, present morphological features suggestive of a relationship to *Dryopteris*, including the reniform indusium and capitate-glandular indument; characters which are common in *Dryopteris*. On the contrary, the long-creeping rhizome of *Revwattsia* is

virtually unknown in *Dryopteris* (present in *D. amurensis* Christ and *D. angustifrons* (T. Moore) Kuntze), as is the epiphytic habit (known in the tropical American species *D. patula* (Sw.) Underw.). Furthermore, the dorsiventral rhizome is absent from the clade that includes *Polystichum* and *Dryopteris*.

Revwattisia presents a taxonomist's classic dilemma; taxonomic placement requires a considered set of decisions about which morphological characters are synapomorphies and which are not. To address this dilemma, we assembled a set of chloroplast DNA nucleotide data from seven markers to infer the phylogenetic relationships of *Revwattisia* and provide insight into its morphological evolution. Included in our inquiry was a test of Jones' 1998 assertion that *Revwattisia fragilis* requires a separate genus within the Dryopteridaceae. In order to understand implications of the taxonomic placement of *R. fragilis*, we also studied its critical morphological characters, namely those of the rhizome, indument, rachis and costa architecture, lamina segment shape, and indusium shape.

Methods

Material

Revwattisia fragilis was collected in the Cook District, Queensland, Australia, along the Mt. Lewis road, ca. 12 km before the shelter at the end of the rd. 16°36'S, 145°17'E, 900 m, M Kessler, M Sundue and M Lehnert 14293 (BRI, VT), 10 Aug 2011. Material for genetic analysis was stored in silica gel until DNA could be extracted. The permit used to collect this material was issued by Dept. of Environment and Resource Management Queensland (Michael Sundue, permit number WISP09438311).

Morphology

Characters for *Revwattisia fragilis* were scored from M Kessler, M Sundue and M Lehnert 14293 at The Pringle Herbarium (VT), and from previously published literature (Watts 1914, Andrews 1990, Jones 1998). We reviewed all salient features, but with particular attention to characters relevant to generic placement i.e. rhizome symmetry and morphology, rachis and costa architecture, lamina dissection, indument, and indusium shape.

Taxon sampling

One-hundred and ninety-eight taxa from 36 genera were used in the phylogenetic analyses including 32 from the Dryopteridaceae. Taxonomic sampling was informed by an initial blast search of the *Revwattisia fragilis rbcL* sequence against the NCBI

database (Altschul et al. 1990). The most similar *rbcL* sequences were *Dryopteris erythrosora* (D.C.Eaton) Kuntze, *Dryopteris cystolepidota* (Miq.) C.Chr., and *Dryopteris championii* (Benth.) C.Chr., with 98.6% pairwise identity. Accordingly, our sampling was heaviest in *Dryopteris*, but also included a diverse selection of Dryopteridaceae. We also included more distant outgroups from the Lomariopsidaceae and Pteridaceae. As several generic segregates of *Dryopteris* are suspected to be nested within the genus (Liu et al. 2007b), we included accessions of *Acrorumohra* (H.Itô) H.Itô, *Acrophorus* C.Presl, *Arachniodes* Blume, *Diacalpe* Blume, *Dryopsis* Holttum & P.J.Edwards, *Nothoperanema* (Tagawa) Ching, and *Peranema* D.Don in this study. Some of these taxa have combinations in *Dryopteris*, however recent authors (Liu et al. 2007b, Wu 1999) have treated them under these alternate genera. We use the alternate names to highlight their phylogenetic position. Sequences other than those for *R. fragilis* were downloaded from GenBank; they are primarily from the work reported in Sessa et al. 2012 and Liu et al. 2007a (accession number and herbarium voucher information, Appendix 1).

DNA extraction, amplification and sequencing

Total DNA extraction from silica-dried specimens was accomplished following the CTAB protocol of Doyle and Doyle (1987). Using the Techne TC3000 thermocycler (Techne, Duxford, UK) and the polymerase chain reaction (PCR), two intergenic spacers, *trnG-trnR* and *rps4-trnS*, were amplified for *Revwattisia fragilis*. The primers TRNG1F and TRNR22R (Nagalingum et al. 2007) were used to amplify *trnG-trnR*. Reactions were carried out in 25 mL volumes and included 2.5 mL of 10X PCR buffer, 0.5 mL of 10mM dNTPs, 0.5 mL of 100X BSA, 1.25 mL of the 10 mM forward primer, 1.25 mL of the 10mM reverse primer, 17.85 mL of ddH₂O, 0.15 mL of Ex Taq Polymerase, and 0.5 mL of extracted DNA from *Revwattisia fragilis*. The thermocycler conditions for amplifying *trnG-trnR* comprised an initial denaturation of 2 minutes at 95°C followed by a core sequence of 35 repetitions of 95°C for 30 seconds, 45°C for 30 seconds, and 71°C for 1 minute followed by a final extension of 5 minutes at 71°C. The primers *rps4-3er.f* (Skog et al. 2004) and *trnSr* (Souza-Chies et al. 1997) were used to amplify *rps4-trnS*. Reaction conditions for *rps4-trnS* were the same as for *trnG-trnR*. Thermocycler conditions for amplifying *rps4-trnS* comprised an initial denaturation of 3 minutes at 94°C followed by 35 repetitions of 94°C for 1 minute, 55°C for 1 minute, and 72°C for 2 minutes followed by a final extension of 8 minutes at 72°C. *Revwattisia rbcL* sequences were generated following Schuettpelz and Pryer (2007) using the primers ESRBCL1F and ESRBCL1361R. Resulting PCR products were electrophoresed on a 1% agarose gel in 1x Tris-borate-EDTA (TBE) buffer (pH 8.0) containing ethidium bromide to visualize bands. Automated sequencing took place on an ABI Prism 3130x1 sequencer at the Vermont Cancer Center, Burlington, Vermont, USA. Sequencing primers for *rps4-trnS* were the same primers used for the template amplification. For *trnG-trnR* analysis we used the following sequencing prim-

ers: TRNG1F, TRNR22R, TRNG43F, and TRNG63R (Nagalingum et al. 2007). For *rbcL* sequencing we used the amplification primers in addition to ESRBCL628F and ESRBCL654R (Schuettpelz and Pryer 2007).

Sequence alignment and coding

Sequences were edited and aligned using Geneious v5.4.2 (Drummond et al. 2011) and then manually checked for errors. Markers were analyzed separately using Modeltest v3.06 (Posada and Crandall 1998) to determine the model of evolution that each marker most closely fit (Table 1) using the Akaike information criterion (AIC). Indels were coded using the program SeqState 1.4.1 (Müller 2005) and treated in the matrix as standard data.

Phylogenetic analyses

Bayesian inference was conducted on the concatenated data set (*psbA-trnH*, *rbcL*, *rbcL-accD*, *rps4-trnS*, *trnG-trnR*, *trnL-trnF*, and *trnP-petG*) using MrBayes v3.2.0 (Ronquist et al. 2011) using the appropriate evolutionary models determined for each. Sampling of all seven loci was primarily within *Dryopteris*; the remaining taxa, including *Revwattsia fragilis*, had subsets of the seven loci. The Markov chain Monte Carlo permutation of tree parameters was conducted for 2 runs of 5,000,000 generations, sampling every 100th generation. A plot of generations versus log-likelihood was examined using Tracer v1.5 (Rambaut and Drummond 2009) to visually assess stationarity and verify that an appropriate burn-in was achieved. The burn-in was 500,000 generations. The 50% majority-rule tree was examined in FigTree v1.3.1 (Rambaut 2009).

Parsimony analyses using the same data set were conducted using TNT (Willi Hennig Society, Goloboff et al. 2008) implementing the parsimony ratchet (Nixon

Table 1. Characteristics of the cpDNA markers used in the phylogenetic analyses.

Marker	Model (AIC)	Aligned Length of Marker	% Parsimony Informative	Taxa sampled
rbcL	SYM+I+G (26503)	1506	19%	194
trnG-trnR	TIM+I+G (16303)	1290	40%	100
pbsA-trnH	TVM+G (4308)	584	30%	101
rbcL-accD	GTR+I+G (9942)	961	37%	99
trnL-trnF	GTR+G (4594)	297	57%	102
rps4-trnS	TVM+G (7704)	576	51%	101
trnP-petG	TIM+G (9370)	623	50%	99

1999), with the following search parameters: 1000 ratchets with 200 iterations per replicate, 10% weighting, holding 20 trees per ratchet, followed by tree-bisection-reconnection (TBR) branch swapping to completion. Clade support was assessed by implementing a bootstrap analysis of 1000 replicates with 10 ratchets per replicate and holding 20 trees per ratchet. The max RAM was set at 850 MB allowing for storage of 10,000 trees.

Results

Phylogenetic analyses

Of the 5425 total characters, 1717 characters (31.6%) were parsimony informative. In the maximum parsimony analysis (MP) 10,000 most parsimonious trees were retained before maximum storage capacity was reached. The shortest trees had a length of 5531 steps, a consistency index (CI) of 0.40, and retention index (RI) of 0.79. The topology of the Bayesian inference (BI) 50% majority rule tree was largely congruent with the topology of the MP tree but allowed greater resolution of the taxa allied to *Revwattisia fragilis*. Results of the BI and MP analyses place *R. fragilis* in a recently diverged clade within the genus *Dryopteris* (Figures 2 and 3).

In both analyses, there is strong support for placement of *Revwattisia fragilis* within a clade of *Dryopteris* comprising species from southern and eastern Asia. In the Bayesian analysis, *R. fragilis* is sister to the clade comprising *D. cystolepidota*, *D. erythrosora*, *D. gymnosora* (Makino) C.Chr., and *D. labordei* (Christ) C.Chr. (78% posterior probability). This clade in turn is sister to *D. championii* (92% posterior probability), followed by *D. triangularis* Herter (100% posterior probability). These same taxa form a clade in the MP analyses (93% bootstrap support), but relationships between these taxa collapse in the strict consensus of all most parsimonious trees.

Morphological assessment

Revwattisia fragilis exhibits a massive (3 cm diam.) long-creeping rhizome with dorsal leaves and ventral roots (Figure 1A). A rhizome cross-section revealed an elongate ventral meristele (Figure 1B arrow). The rhizome and basal petiole are densely provided with thin, dark brown attenuate scales. The rachis and costa are rounded abaxially (Figure 1C), and are shallowly grooved adaxially (Figure 1D). The grooves are shallowly continuous with the next-order axis (Figure 1D) and they lack a central ridge. These axes are densely provided with short capitate-glandular hairs (Figure 1D). Frond dissection is 2-pinnate-pinnatifid to 2-pinnate-pinnatisect with symmetrical (neither basiscopically nor acroscopically enlarged) pinnae and pinnules (Figure 1A). Fertile fronds have medial sori and light brown reniform indusia.

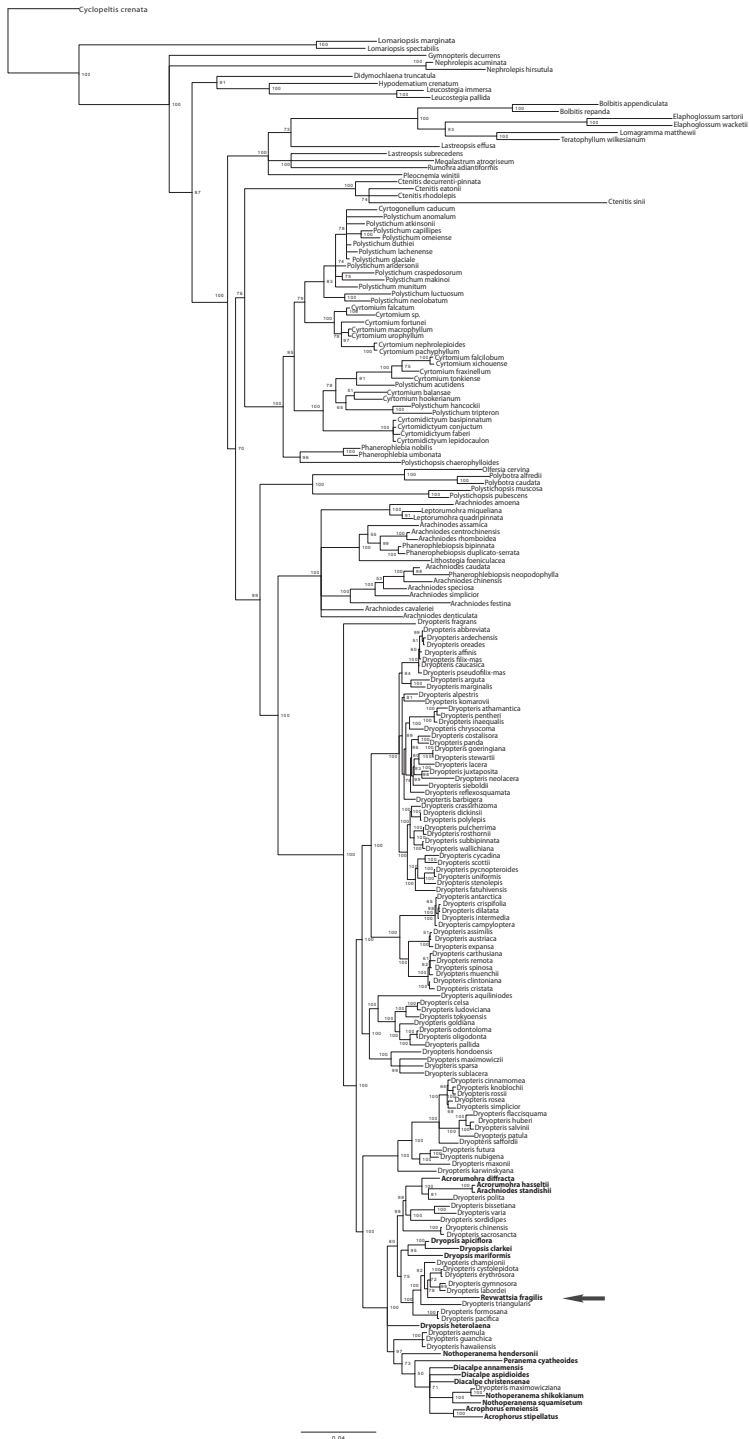


Figure 2. The 50% majority rule tree resulting from Bayesian analysis. Values indicate posterior probabilities, scale bar indicates 0.04 substitutions per site. Arrow indicates position of *Revwattisia fragilis*.

Discussion

Monophyly of *Dryopteris*

Results presented here demonstrate that the monotypic genus *Revwattsia* is nested within *Dryopteris* (Figures 2 and 3). Maintaining *Revwattsia* renders *Dryopteris* paraphyletic; we therefore recommend placing the monotypic *Revwattsia* in synonymy under *Dryopteris*.

Paraphyly of *Dryopteris* is further perpetuated by the inclusion of the sampled *Acrophorus* (two species), *Acrorumohra* (two species), *Arachniodes standishii* (T. Moore) Ohwi, *Diacalpe* (three species), *Dryopsis* (three species), *Nothoperanema* (three species), and *Peranema cyatheoides* D. Don. These results do not come as a surprise given the results of other recent phylogenetic studies (Liu et al. 2007b, Geiger and Ranker 2005). The paraphyly of *Dryopteris* presented here corroborates long-standing suspicion about the circumscription of *Dryopteris* segregate genera (Tryon and Tryon 1982) and underscores the need for rich taxon sampling, particularly from Asia, in studies of Dryopteridaceae.

Evolutionary implications

Our assessment of morphological characters largely corroborates those of Watts (1914), Andrews (1990), and Jones (1998). Most of the characters displayed by *Revwattsia fragilis* are known to occur within *Dryopteris*. The dark brown attenuate scales and capitate glandular hairs seen in *R. fragilis* occur frequently in *Dryopteris* (Kramer et al. 1990). The grooved rachis and costae are also typical of *Dryopteris* and many other dryopterid ferns (Holtum 1960). A reniform indusium is characteristic of most Dryopteridaceae and occurs throughout *Dryopteris* as it is currently circumscribed (other indusial shapes, which we take to be autapomorphies, are known from *Acrophorus*, *Diacalpe*, *Notheperanema*, and *Peranema*).

The long-creeping rhizome and elongate ventral meristele of *Revwattsia* (the latter first demonstrated here, Fig. 1B) are distinctive autapomorphies. Although a long-creeping rhizome is known to occur in *Dryopteris amurensis* and *D. angustifrons*, neither is closely allied to *R. fragilis*. These two characters occur in combination sporadically in Eupolypods I (e.g., in *Lomariopsis* Fée (Holtum 1978), the Bolbitidoid clade (Moran et al. 2010), and *Rumohra* (Kato 1974)) and appear to have evolved multiple times. In our experience this combination of characters appears to be correlated with strong dorsoventrality of the rhizome. We take this convergence between our subject species and *Rumohra adiantiformis*, the plant to which Watts presumably thought it most closely related, to be coincidental; Watts never cited these characters in his protolog.

Biogeographic implications

Biogeographic patterns in *Dryopteris* were recently examined by Sessa et al. (2012)—however patterns among Australian taxa were not explicitly addressed. In addition to

Revwattsia fragilis, Australia is home to three species of *Dryopteris*—*D. atrata* (Wall.) Ching, *D. cycadina* (Franch. & Sav.) C. Chr., *D. sparsa* (D. Don) Kuntze (Jones 2012)—and *Acrorumohra hasseltii* (Blume) Ching. All but *D. atrata* are included in our analysis. Unlike *R. fragilis*, these species have relatively broad ranges including India and Sri Lanka, southern China and Japan, and Malesia. In each of these cases, the closest relatives are distributed in southern and eastern Asia, suggesting this region as ancestral for each of the Australian taxa. These species are resolved in clades distinct from each other and from *R. fragilis*, indicating that at least four separate migration events are necessary to explain the current distribution of *Dryopteris* (including *R. fragilis* and *A. hasseltii*) in Australia. The inclusion of the unsampled *D. atrata* in future studies may increase the inferred number of migrations. Our results are comparable to those of Li et al. (2007), who revealed similar migration events from Southern Asia to Australia in the closely related genus *Polystichum*. Although the Sunda and Sahul shelves are currently divided by a deep oceanic trench, these regions were in close proximity 23 mya during the time of the divergence of *Dryopteris* (Sessa et al. 2012, Lohman et al. 2011). It remains unclear whether the migration of *Dryopteris* can be attributed to long distance dispersal or incremental range expansion.

Circumscription of *Dryopteris*

The phylogenetic position of species treated as *Acrophorus*, *Acrorumohra*, *Arachniodes standishii*, *Dryopsis*, *Nothoperanema*, *Peranema*, and *Revwattsia fragilis* demonstrate that the circumscription of *Dryopteris* needs to be expanded. Several of these genera include unique character states that do not occur in *Dryopteris* as currently defined. In addition to the morphological redefinition, expansion of *Dryopteris* to include these segregate genera necessitates numerous nomenclatural innovations. We provide here a name for *Revwattsia fragilis* in *Dryopteris*. The name *Dryopteris fragilis* is previously occupied; therefore a new name is provided.

Taxonomy and nomenclature

Dryopteris watsii, M. McKeown, Sundue, & Barrington nom. nov. \equiv *Polystichum fragile* Watts, Proc. Linn. Soc. New South Wales 39: 775. 1914 (1915). \equiv *Revwattsia fragilis* (Watts) D.L. Jones [as “*Revwattsia fragile*”], Flora of Australia 48:711. 1998. non *Dryopteris fragilis* C. Chr. TYPE: Australia, Queensland, Majors Homestead, near Ravenshoe, W. W. Watts s.n., Aug 1913 (Syntypes: BRI n.v., MEL n.v., NSW n.v.).

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Appendix

GenBank accession numbers for McKeown et al. 2012 (doi: 10.3897/phytokeys.14.3446.app) File format: Adobe PDF file (PDF).

Explanation note: Genbank Accession numbers are listed in the following order: psbA-trnH, rbcL, rbcL-accD, rps4-trnS, trnG-trnR, trnL-trnF, trnP-petG. The “—” indicates markers that were not available for the taxon.

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