

The multiple fuzzy origins of woodiness within Balsaminaceae using an integrated approach. Where do we draw the line?

Frederic Lens^{1,*}, Sharon Eeckhout^{2,3}, Rosa Zwartjes¹, Erik Smets^{1,2,4} and Steven B. Janssens²

¹Netherlands Centre for Biodiversity Naturalis-section NHN, PO Box 9514, NL-2300 RA Leiden, The Netherlands, ²Laboratory of Plant Systematics, Institute of Botany and Microbiology, K.U.Leuven, Kasteelpark Arenberg 31 box 2437, BE-3001 Leuven, Belgium, ³Pteridology, Department of Biology, Ghent University, KL Ledeganckstraat 35, B-9000 Ghent, Belgium and ⁴Hortus botanicus, Leiden University, PO Box 9516, 2300 RA Leiden, The Netherlands

* For correspondence. E-mail lens@nhn.leidenuniv.nl

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• **Background and Aims** The family Balsaminaceae is essentially herbaceous, except for some woodier species that can be described as ‘woody’ herbs or small shrubs. The family is nested within the so-called balsaminoid clade of Ericales, including the exclusively woody families Tetrameristaceae and Marcgraviaceae, which is sister to the remaining families of the predominantly woody order. A molecular phylogeny of Balsaminaceae is compared with wood anatomical observations to find out whether the woodier species are derived from herbaceous taxa (i.e. secondary woodiness), or whether woodiness in the family represents the ancestral state for the order (i.e. primary woodiness).

• **Methods** Wood anatomical observations of 68 *Impatiens* species and *Hydrocera triflora*, of which 47 are included in a multigene phylogeny, are carried out using light and scanning electron microscopy and compared with the molecular phylogenetic insights.

• **Key Results** There is much continuous variation in wood development between the *Impatiens* species studied, making the distinction between herbaceousness and woodiness difficult. However, the most woody species, unambiguously considered as truly woody shrubs, all display paedomorphic wood features pointing to secondary woodiness. This hypothesis is further supported by the molecular phylogeny, demonstrating that these most woody species are derived from herbaceous (or less woody) species in at least five independent clades. Wood formation in *H. triflora* is mostly confined to the ribs of the stems and shows paedomorphic wood features as well, suggesting that the common ancestor of Balsaminaceae was probably herbaceous.

• **Conclusions** The terms ‘herbaceousness’ and ‘woodiness’ are notoriously difficult to use in Balsaminaceae. However, anatomical observations and molecular sequence data show that the woodier species are derived from less woody or clearly herbaceous species, demonstrating that secondary woodiness has evolved in parallel.

Key words: Balsaminaceae, herbaceousness, *Hydrocera*, *Impatiens*, insular woodiness, light microscopy, primary woodiness, secondary woodiness, wood anatomy.

INTRODUCTION

Balsaminaceae are a family of horticulturally important herbs that are characterized by an enormous floral diversity (Yuan *et al.*, 2004; Janssens *et al.*, 2006, 2009). Although the family exceeds 1000 species, only two genera are recognized: *Impatiens* and *Hydrocera*. The species-rich genus *Impatiens* is primarily distributed in the highlands and mountains of the Paleotropics, yet a few species also occur in temperate Eurasia and North America (Yuan *et al.*, 2004; Janssens *et al.*, 2009). In contrast, the genus *Hydrocera* contains only one species, *Hydrocera triflora* (Fig. 1A), and is confined to the lowlands of Indo-Malaysia (Grey-Wilson, 1980b). Large differences in habitat can be observed between both genera: *Impatiens* is nearly always associated with an often humid environment as it often grows along small rivers (Fig. 1B) or in the spray zone of waterfalls – although some species grow on physiologically dry limestone outcrops (Fig. 1C) – while *Hydrocera* is a semi-aquatic plant, thriving in stagnant water of pools and ditches (Grey-Wilson, 1980a). The high species

diversity in *Impatiens* is reflected by the hypervariable floral morphology, of which the spurred sepal and the lateral petals show an extreme variability (Yuan *et al.*, 2004; Caris *et al.*, 2006; Janssens *et al.*, 2008). Despite the enormous floral variation, the vegetative morphology of *Impatiens* is well preserved, always having glandular toothed leaves and often a fleshy semi-succulent stem (Fig. 1B).

The majority of the (sub-)tropical balsams are considered to be annual, especially the species growing in wet microhabitats without dry periods (Grey-Wilson, 1980a). However, a considerable number of species are perennial and have specific root adaptations, such as, for example, tubers in *I. tuberosa* (Madagascar) and *I. mirabilis* (Thailand), which are needed to survive the (usually short) dry season (Perrier de la Bathie, 1948; Grey-Wilson, 1980a; Newman, 2008). Also epiphytic or semi-epiphytic species are considered to be perennials that are often adapted to short periods of water shortage due to the formation of tubers (Grey-Wilson, 1980a; Cheek and Fischer, 1999; Janssens *et al.*, 2010).



FIG. 1 Examples of the variation in growth forms of (A) *Hydrocera* and (B–D) *Impatiens*. (A) *Hydrocera triflora*, overview of the top part of the flowering stem, 4–5 ribbed (insert). (B) *Impatiens glandulifera*, relatively thick but herbaceous, semi-succulent stem with a limited amount of wood development, growing near the river Dijle, Leuven (Belgium). (C) *Impatiens mirabilis* (bottom left), very thick herbaceous, succulent stem consisting of abundant parenchyma tissue and a negligible amount of wood, growing on a limestone outcrop in Pulau Langgun (Malaysia); *I. mirabilis* is deciduous in the dry season (photo credit: Dr Max van Balgooy). (D) *Impatiens niammiamensis*, woody stems, growing in a tropical montane greenhouse of the Botanical Garden of Ulm (Germany).

A small number of species in Africa, South India and Madagascar have robust shoots that initially thicken and gradually become woody with age (Fig. 1D), sometimes almost becoming shrubby (Hooker and Thomson, 1859; Grey-Wilson, 1980a). Interestingly, Grey-Wilson (1980a) suggested that a woody habit is probably not related to any specific habitat type, but independently originated throughout the genus. We want to investigate this hypothesis, and assess (1) whether the woody species in *Impatiens* have originated from herbaceous relatives (secondary woodiness) or resemble the woody ancestral state for the Ericales order (primary woodiness); and investigate (2) whether these habit shifts have happened several times within the genus. Three independent strategies can be applied to investigate whether herbaceous lineages have evolved into secondarily woody species. A first strategy is to trace evolutionary shifts towards secondary woodiness using a robust, species-dense molecular phylogenetic framework. Most of these secondarily woody lineages are found on islands and are therefore also referred to as insular woody lineages (e.g. Böhle et al., 1996; Francisco-Ortega et al., 2002; Lee et al., 2005). A second option is to make woody mutants from herbaceous wild

types (Groover, 2005; Melzer et al., 2008; Lens et al., 2012). If molecular data are insufficient or even unavailable, which is still the case in many groups, a third source of evidence is to look for so-called pedomorphic features in the wood anatomy of the species under study (Carlquist, 1962, 1974, 1992, 2009; Koek-Noorman, 1976; Lens et al., 2005a, b, 2007, 2009; Dulin and Kirchoff, 2010). Pedomorphic or juvenile wood features resemble characters of the primary xylem that are protracted into the more mature secondary xylem (=wood) of secondarily woody species. Examples are the continuous decrease of vessel element length from the pith towards the cambium, the presence of wide gaping or gash-like intervessel pits resembling helical or reticulate tracheids in the primary xylem, and the absence of rays and/or the presence of rays with mainly square to upright ray cells. As stressed in Lens et al. (2009, 2012) and Dulin and Kirchoff (2010), scientists should make use of independent strategies to obtain sound conclusions on habit shifts towards secondary/insular woodiness, because studying merely wood anatomical observations or molecular data separately may lead to misinterpretation of the origin of woodiness within a particular group.

Stem anatomical observations in Balsaminaceae are extremely scarce. As far as we know, there are only two papers that describe the wood anatomy of *Impatiens*: Gerard (1917) includes a very brief description of only one species, and Lens et al. (2005b) provide a more detailed description of only two species. To rectify this lack of information, we have studied the stem anatomy of 68 *Impatiens* species from all major clades of the present molecular phylogeny (Yuan et al., 2004; Janssens et al., 2006, 2009) and *Hydrocera triflora*, and compared the anatomical observations with an improved phylogeny.

The objectives of this study are to present an overview of the stem anatomical variation in Balsaminaceae, and to investigate the origin of woodiness based on the anatomical observations in combination with an up-to-date molecular phylogeny. However, above all, this study wants to find a way to distinguish between herbaceousness and woodiness in a group that shows a continuous variation in wood development.

MATERIALS AND METHODS

Material

Stem samples from 69 Balsaminaceae species were collected from the living collection of the National Botanic Gardens of Belgium (BR), and the spirit and herbarium collection of the Netherlands Centre for Biodiversity Naturalis-section NHN (NCB Naturalis, L) (Appendix). Our sampling covers all major Balsaminaceae clades following the latest molecular phylogeny of Janssens et al. (2009). To increase the number of species for which molecular data are also available from earlier studies, we sequenced additional chloroplast *atpB-rbcL* and nuclear *ImpDEF1* and *ImpDEF2* sequences for the following species: *Impatiens eriosperma*, *I. grandis*, *I. kilimanjari* × *pseudoviola*, *I. stuhlmannii* and *I. repens* (GenBank accession nos HE617195–HE617200).

Since wood development – if present at all – is limited in many Balsaminaceae, we investigated only stem samples at the base of a mature plant during flowering. After sampling, we immediately stored the stems in 70 % alcohol to prevent the stems from drying out. This is important, because the majority of Balsaminaceae species have stems containing much parenchyma tissue that would otherwise completely shrink due to the drying process. Consequently, most of our samples had to be sampled from living collections grown in botanical gardens or from available spirit collections, and only a few samples of the woodiest species in the NCB Naturalis herbarium collection were added to our sampling (Appendix).

Wood anatomical descriptions and microtechnique

Stems of Balsaminaceae are typically soft because of the high ratio of parenchymatous vs. lignified tissues. Therefore, the standardized way of wood sectioning following Lens et al. (2005b) could only be applied for the most woody herbarium species. All the other species were embedded in LR White resin (hard grade, London Resin, UK) and sectioned with a rotary microscope according to the protocol described in Hamann et al. (2011). The LR White sections were

stained with either toluidine blue or Etzolds dye (a mixture of 10 mg of fuchsin, 40 of mg safranin and 150 of mg astra blue dissolved in 100 mL of water, added with 2 mL of acetic acid). Transverse sections and longitudinal sections were made for the most woody species, while the other species were represented by transverse sections only. For length-on-age curves, measurements for vessel elements were made using radial sections from the pith towards the cambium, and added with maceration slides taken from various distances between pith and cambium. The wood anatomical terminology follows the ‘IAWA list of microscopic features for hardwood identification’ (IAWA Committee, 1989).

Molecular analysis

DNA was extracted using a modified cetyltrimethylammonium bromide (CTAB) protocol (Janssens et al., 2006). Primers used for amplification and sequencing of the chloroplast *atpB-rbcL* spacer and the nuclear *ImpDEF1* and *ImpDEF2* are obtained from Janssens et al. (2006, 2007). Amplified DNA was sequenced by the MacroGen sequencing facility (MacroGen, Seoul, South Korea). Sequences obtained in this study were submitted to GenBank (see the Materials and methods). Alignment of the nuclear and chloroplast sequences was conducted with MUSCLE under default parameters (Edgar, 2004) as implemented in the software program Geneious v.4.7.5 (Biomatters Ltd, Auckland) and subsequently fine-tuned by hand. Chloroplast *atpB-rbcL* and nuclear *ImpDEF1/ImpDEF2* data matrices were analysed separately and combined using the probabilistic maximum likelihood (ML) method. Maximum likelihood analyses were performed using the RaxML search algorithm (Stamatakis et al., 2005) under the GTRGAMMA approximation of rate heterogeneity for each gene (Stamatakis, 2006). Five hundred bootstrap trees were inferred using the RaxML Rapid bootstrap algorithm (ML-BS) to provide support values for the best-scoring ML tree. A partition homogeneity test, as implemented in PAUP*4.0b10a (Swofford, 2002), was used to appraise whether the data sets provide different signals in the combined analyses.

Character mapping

It is known that most herbaceous species produce a limited amount of wood (Dulin and Kirchoff, 2010; Schweingruber et al., 2011; Lens et al., 2012), but how much wood does a species need to produce in order to be considered woody? All the species studied that do not form a wood cylinder are treated here as herbaceous (Table 1). In order to distinguish between ‘herbaceousness’ and ‘woodiness’ amongst the species that do form a wood cylinder at least at the base of their stems, we carefully measured the ratio of the double thickness of the wood cylinder over the entire stem diameter. Since the stems and wood cylinders are usually not perfectly cylindrical, we measured both stems and wood cylinders multiple times for each stem (eight measurements to calculate mean double wood cylinder thickness, four measurements to calculate mean stem diameter), and divided the mean values to obtain a habit quotient. The quotient for each species can

TABLE 1. Overview of selected stem anatomical characters within Balsaminaceae

Taxon	Outer layer, meristimatic activity	No. of cell layers collenchyma, meristimatic activity	No. of cell layers cortex, meristimatic activity	No. of cell layers wood InterF vs. intraF region	Wood distributed as cylinder or as individual islands	Thickness double wood cylinder (mm)	Average diameter entire stem (mm)	Ratio thickness double wood cylinder/ average stem diameter	Character state after gap weighting ($n = 26$) for species with wood cylinder	Four-character states	Two-character states
<i>Hydrocera triflora</i>	Epidermis, +	2–3, +	Aerenchyma 10, ±	0–7 vs. 18–45	Island	–	15.1	–	–	Herbaceous	Herbaceous
<i>Impatiens amplexicaulis</i>	Periderm, +	?	?	12–36	Cylinder	1.49	4.15	0.36	14	Slightly woody	Woody
<i>I. arguta</i>	Epidermis, –	3–4, –	2–4, –	10–15 vs. 12–20	Cylinder	0.56	3.85	0.15	5	Woody herb	Herbaceous
<i>I. aurea</i>	Initiating periderm, +	1–2, –	1–2, –	19–24 vs. 33–40	Cylinder	1.33	6.4	0.21	8	Woody herb	Herbaceous
<i>I. auricoma</i>	Initiating periderm, +	15–18, +	12–17, +	0 vs. 12–23	Island	–	18.1	–	–	Herbaceous	Herbaceous
<i>I. balfourii</i>	Periderm, +	?	?	50–100	Cylinder	2.73	5.28	0.52	21	Truly woody	Woody
<i>I. balsamina</i>	Epidermis, +	3–4, +	4–6, +	6–8 vs. 14–30	Cylinder	0.3	19	0.02	1	Woody herb	Herbaceous
<i>I. bicaudata</i>	Initiating periderm, +	20–25, +	12–15, +	0 vs. 40–45	Island	–	30	–	–	Herbaceous	Herbaceous
<i>I. biflora</i>	Periderm, +	1–3, –	8–12, –	24–67	Cylinder	1.56	5.16	0.3	12	Slightly woody	Woody
<i>I. burtonii</i> ssp. <i>burtonii</i>	Epidermis, ±	2–4, –	5–8, ±	0 vs. 2–3	Island	–	3	–	–	Herbaceous	Herbaceous
<i>I. campanulata</i>	Epidermis, –	5–7, +	5–7, +	18–20 vs. 25–35	Cylinder	1	7.9	0.13	5	Woody herb	Herbaceous
<i>I. capensis</i>	Periderm, +	1–2, –	2–3, –	20–25 vs. 25–30	Cylinder	1.34	6.3	0.21	8	Woody herb	Herbaceous
<i>I. catati</i>	Epidermis, –	2–7, –	2–4, +	0 vs. 2–3	Island	–	3.1	–	–	Herbaceous	Herbaceous
<i>I. cecili</i>	Epidermis, –	4–6, ±	4–7, +	40–45 vs. 40–55	Cylinder	2.01	5.8	0.35	14	Slightly woody	Woody
<i>I. clavigera</i>	Initiating periderm, +	6–8, –	6–10, –	0 vs. 6–8	Island	–	5.2	–	–	Herbaceous	Herbaceous
<i>I. curvipes</i>	Epidermis, ±	3–5, ±	3–5, ±	0 vs. 1–2	Island	–	2.8	–	–	Herbaceous	Herbaceous
<i>I. dewildeana</i>	Periderm, +	?	?	52–68 vs. 60–88	Cylinder	2.77	5.73	0.48	20	Truly woody	Woody
<i>I. edgeworthii</i>	Epidermis, –	3–5, –	2–4, –	0–3 vs. 12–20	Island	–	3.4	–	–	Herbaceous	Herbaceous
<i>I. eriosperma</i>	Epidermis, –	2–5, ±	3–5, +	0 vs. 2–3	Island	–	2.2	–	–	Herbaceous	Herbaceous
<i>I. eubotrya</i>	Periderm, +	?	?	37–58 vs. 56–69	Cylinder	1.74	5.1	0.34	14	Slightly woody	Woody
<i>I. flaccida</i>	Initiating periderm, +	5–8, +	3–5, +	60–80	Cylinder	2.89	6.78	0.43	17	Truly woody	Woody
<i>I. gesnerioides</i>	Periderm, +	?	?	47–68	Cylinder	3.45	6.14	0.56	23	Truly woody	Woody
<i>I. glandulifera</i>	Epidermis, +	6–11, ±	4–9, ±	17–20 vs. 30–35	Cylinder	0.5	27	0.018	1	Herbaceous	Herbaceous
<i>I. glandulosa</i>	Epidermis, +	8–10, –	5–12, +	0 vs. 1–2	Island	–	17	–	–	Herbaceous	Herbaceous

<i>I. grandis</i>	Epidermis, -	6-7, ±	4-7, ±	0 vs. 3-10	Island	-	10	-	-	Herbaceous	Herbaceous
<i>I. griffithii</i>	Periderm, +	1-2, -	2-3, -	10-14 vs. 15-29	Cylinder	0.62	3.35	0.19	7	Woody herb	Herbaceous
<i>I. havilandii</i>	Periderm, +	?	?	53-58 vs. 50-67	Cylinder	2.78	4.42	0.63	26	Truly woody	Woody
<i>I. hawkeri</i>	Epidermis, +	4-5, ±	3-5, ±	18-22	Cylinder	0.6	5	0.12	4	Woody herb	Herbaceous
<i>I. henslowiana</i>	Epidermis, -	4-6, +	3-7, ±	1-5 vs. 5-10	Cylinder	0.15	3.51	0.12	1	Woody herb	Herbaceous
<i>I. hians</i> var. <i>hians</i>	Epidermis, +	6-9, -	3-7, ±	12-20 vs. 25-35	Cylinder	0.6	8.2	0.07	3	Woody herb	Herbaceous
<i>I. hochstetteri</i> ssp. <i>hochstetteri</i>	Epidermis, -	9-11, -	10-13, +	0 vs. 8-18	Island	-	16	-	-	Herbaceous	Herbaceous
<i>I. inaperta</i>	Epidermis, -	2-3, -	4-5, -	0 vs. 1-2	Island	-	1.2	-	-	Herbaceous	Herbaceous
<i>I. irvingii</i>	Epidermis, -	4-8, +	6-11, +	18-23 vs. 30-40	Cylinder	1	7.5	0.13	5	Woody herb	Herbaceous
<i>I. jurpia</i>	Periderm, +	4-5, -	3-6, -	2-5 vs. 2-6	Cylinder	0.12	2.7	0.04	1	Woody herb	Herbaceous
<i>I. keillii</i> ssp. <i>keillii</i>	Epidermis, +	0-2, -	6-9, +	0 vs. 2-3	Island	-	5.4	-	-	Herbaceous	Herbaceous
<i>I. kilimanjari</i>	Epidermis, -	3-6, -	4-8, ±	0 vs. 1-2	Island	-	6.1	-	-	Herbaceous	Herbaceous
<i>I. kilimanjari</i> × <i>pseudoviola</i>	Initiating periderm, +	3-5, -	4-7, +	8-10 vs. 15-20	Cylinder	0.46	4.93	0.09	3	Woody herb	Herbaceous
<i>I. latifolia</i>	Periderm, +	?	?	13-18 vs. 25-28	Cylinder	0.78	3.28	0.24	9	Woody herb	Herbaceous
<i>I. leschenaultii</i>	Periderm, +	?	?	24-36 vs. 40-48	Cylinder	1.81	3.95	0.46	19	truly woody	Woody
<i>I. lyallii</i>	Periderm, +	?	?	15-20 vs. 22-36	Cylinder	0.94	4.5	0.21	8	Woody herb	Herbaceous
<i>I. mackeyana</i> ssp. <i>claei</i>	Periderm, +	5-10, -	8-13, ±	0 vs. 10-17	Island	-	15	-	-	Herbaceous	Herbaceous
<i>I. macrophylla</i>	Periderm, +	2-3, -	6-10, -	15-26	Cylinder	0.65	9	0.072	2	Herbaceous	Herbaceous
<i>I. masonii</i>	Epidermis, -	2-7, -	3-7, -	0 vs. 0-1	Island	-	2.1	-	-	Herbaceous	Herbaceous
<i>I. mengtzeana</i>	Epidermis, -	4-8, -	7-10, -	0 vs. 0-1	Island	-	1.8	-	-	Herbaceous	Herbaceous
<i>I. mirabilis</i>	Periderm, +	15-25, +	20-25, ±	0 vs. 1-2	Island	-	25	-	-	Herbaceous	Herbaceous
<i>I. namchabarwensis</i>	Epidermis, +	2-5, +	1-5, +	90-110	Cylinder	3.4	5.53	0.61	25	Truly woody	Woody
<i>I. niamniamensis</i>	Periderm, +	7-10, -	12-16, +	80-100	Cylinder	4.14	14	0.3	12	Slightly woody	Woody
<i>I. noli-tangere</i>	Epidermis, +	1-3, -	2-4, ±	7-15 vs. 16-25	Cylinder	1.4	10	0.14	5	Woody herb	Herbaceous
<i>I. nomenya</i>	Epidermis, -	2-3, -	7-9, +	0 vs. 2-5	Island	-	4.6	-	-	Herbaceous	Herbaceous
<i>I. omeiana</i>	Epidermis, -	3-5, -	6-8, -	0 vs. 1-6	Island	-	4	-	-	Herbaceous	Herbaceous
<i>I. opinata</i>	Epidermis, -	2-5, -	2-6, -	2-5 vs. 2-6	Cylinder	0.065	1.1	0.059	2	Herbaceous	Herbaceous
<i>I. parasitica</i>	Epidermis, -	11-14, -	5-6, ±	0 vs. 5-10	Island	-	10	-	-	Herbaceous	Herbaceous
<i>I. parviflora</i>	Epidermis, -	2-3, -	3-5, +	24-30 vs. 35-45	Cylinder	1.68	8.15	0.21	8	Woody herb	Herbaceous
<i>I. platypetala</i>	Epidermis, +	4-6, +	6-11, +	2-15 vs. 9-30	Cylinder	0.75	6.94	0.11	4	Woody herb	Herbaceous
<i>I. psittacina</i>	Epidermis, -	2-5, ±	3-6, -	0 vs. 1-4	Island	-	1.6	-	-	Herbaceous	Herbaceous
<i>I. pseudomacroptera</i>	Initiating periderm, +	4-6, -	4-6, -	12-20 vs. 18-30	Cylinder	1.09	6.16	0.18	7	Woody herb	Herbaceous

TABLE 1. *Continued*

Taxon	Outer layer, meristematic activity	No. of cell layers collenchyma, meristematic activity	No. of cell layers cortex, meristematic activity	No. of cell layers wood InterF vs. intraF region	Wood distributed as cylinder or as individual islands	Thickness double wood cylinder (mm)	Average diameter entire stem (mm)	Ratio thickness double wood cylinder/average stem diameter	Character state after gap weighting ($n = 26$) for species with wood cylinder	Four-character states	Two-character states
<i>I. pseudoviola</i>	Initiating periderm, +	4–9, +	6–8, +	0 vs. 2–4	Island	–	4.4	–	–	Herbaceous	Herbaceous
<i>I. purpureo-violacea</i>	Initiating periderm, +	2–5, –	3–5, ±	10–17 vs. 12–14	Cylinder	0.42	2.6	0.16	6	Woody herb	Herbaceous
<i>I. repens</i>	Epidermis, +	2–3, ±	5–10, +	0 vs. 2–10	Island	–	6.7	–	–	Herbaceous	Herbaceous
<i>I. shirensis</i>	Periderm, +	?	?	36–44 vs. 42–51	Cylinder	1.43	4.03	0.36	14	Slightly woody	Woody
<i>I. sodenii</i>	Epidermis, +	5–8, +	9–14, +	2–3 vs. 10–15	Cylinder	0.1	8.4	0.01	1	Woody herb	Herbaceous
<i>I. stenantha</i>	Periderm, +	?	?	17–25 vs. 32–52	Cylinder	1.6	3.55	0.45	18	Truly woody	Woody
<i>I. stuhlmannii</i>	Epidermis, –	4–7, –	4–7, ±	9–17 vs. 13–33	Cylinder	0.57	6.99	0.08	3	Woody herb	Herbaceous
<i>I. cf. stuhlmannii</i>	Epidermis, –	3–8, –	4–5, –	4–8 vs. 6–13	Cylinder	0.2	3.74	0.05	2	Woody herb	Herbaceous
<i>I. usambarensis</i>	Epidermis, –	4–9, +	4–8, +	1–6 vs. 5–12	Cylinder	0.32	6.68	0.05	1	Woody herb	Herbaceous
<i>I. vaughanii</i>	Epidermis, –	3–4, –	5–10, ±	0 vs. 1–2	Island	–	2.5	–	–	Herbaceous	Herbaceous
<i>I. violaeiflora</i>	Periderm, +	?	?	21–39	Cylinder	1.64	3.58	0.46	19	Truly woody	Woody
<i>I. viscida</i>	Epidermis, –	2–3, –	5–7, ±	0 vs. 5–15	Island	–	5	–	–	Herbaceous	Herbaceous
<i>I. vitellina</i>	Periderm, +	?	?	52–64 vs. 72–118	Cylinder	2.71	4.75	0.57	23	Truly woody	Woody

Species are arranged alphabetically. +, present; ±, sometimes present; –, absent; IntraF, intrafascicular; InterF, interfascicular.

be considered as a proxy for the continuously varying wood development we are interested in. Subsequently, this continuous character was treated as an ordered multistate character with 26 different character states using the gap weighting method of Thiele (1993), and implemented by the open-source software program MorphoCode (Schols *et al.*, 2004). Consequently, besides species without a wood cylinder which are considered herbaceous, this method allows three other classes amongst the species with a complete wood cylinder to be chosen: ‘woody’ herbs (character states 1–9), slightly woody species (character states 12–14) and truly woody species (character states 17–26; see Supplementary Data Fig. S1 for a distribution of the character states among the species). An alternative option is to have only two classes, i.e. herbaceous species (all the species without a wood cylinder and species having character states 1–9) and woody species (character states 12–26; Table 1; Supplementary Data Fig. S1). Nevertheless, plotting the two- or four-character state features onto our up-to-date phylogenetic tree leads to the same conclusions with respect the origin of woodiness (Fig. 7). Parsimony character optimization was carried out using MacClade 4.05 (Maddison and Maddison, 2002) under the ACCTRAN algorithm.

RESULTS

Anatomical descriptions

The stem anatomy of *Impatiens* is described separately from the stem anatomy of *Hydrocera* (Table 1). The wood anatomical description of *Impatiens* is based on the ten most woody species having character states 17–26: *I. balfourii*, *I. dewildeana*, *I. flaccida*, *I. gesneroidea*, *I. havilandii*, *I. leschenaultii*, *I. namchabarwensis*, *I. stanantha*, *I. violaeiflora* and *I. vitellina* (Figs 2F and 4; Table 2). For both genera examined, the numerator represents the number of species studied and the denominator includes the total number of species. Numbers without parentheses are ranges of means, while numbers between parentheses represent minimum or maximum values. A summary of selected wood features is shown in Table 2.

Impatiens (68/1000 + ; Figs 2–4; Tables 1 and 2; Supplementary Data Fig. S2). Continuous variation in wood development throughout the genus: ranging from islands of wood in the fascicular regions of some species (Fig. 2A), towards species with a small wood cylinder (Fig. 2B–D) over species with larger wood cylinders (Fig. 2E, F).

Stems hollow in *I. aurea*, *I. balfourii*, *I. biflora*, *I. capensis*, *I. dewildeana*, *I. eubotrya*, *I. gesneroidea*, *I. glandulifera*, *I. griffithii*, *I. havilandii*, *I. jerdoniae*, *I. latifolia*, *I. lyallii*, *I. macrophylla*, *I. noli-tangere*, *I. pseudomacroptera* and *I. usumbarensis*. Outer stem layer with (non-)meristematic epidermis attached in most species (Fig. 3A, B); initiating periderm observed in *I. aurea*, *I. auricoma*, *I. bicaudata*, *I. clavigera*, *I. flaccida*, *I. kilimanjari* × *pseudoviola*, *I. pseudoviola*, *I. pseudomacroptera* and *I. purpureo-violacea*, a more developed periderm present in most species that are considered to be slightly woody or truly woody except for *I. capensis*, *I. griffithii*, *I. jurpia*, *I. latifolia*, *I. lyallii*, *I. mackeyana*, *I. macrophylla* and *I. mirabilis*; enlarged cork cells in *I. niarniamensis* and *I. stanantha*. Collenchyma

3–6–(25) cell layers in width, meristematic activity mostly confined to species with large stem diameters (Fig. 3A, D). Cortex 3–7–(25) cell layers in width, meristematic activity mostly confined to species with large stem diameters (Fig. 3B). Cell groups radiating around remnants of primary xylem helical tracheids in pith observed in most species (Fig. 3D, E). Raphides born in mucilage sacs observed in collenchyma, cortex and pith parenchyma (Fig. 3F).

Growth ring boundaries in wood absent. Wood diffuse-porous (Fig. 4A–C). Vessels (3)–8–70–(84) mm⁻², usually solitary (Fig. 4A), sometimes in radial multiples of 2–3–(5) and/or occasionally in clusters of 3–5 (Fig. 4B, C), vessel outline angular. Vessel perforation plates simple. Lateral wall pitting typically wide gaping (pseudo-)scalariform (Fig. 4D) to sometimes reticulate, pits with minute borders, pit cavities 10–75 µm in horizontal size, in *I. balfourii* distinctly bordered alternate pitting, pit borders 6–12 µm in horizontal size, non-vestured. Tangential diameter of vessels (20)–27–61–(90) µm, vessel elements (100)–175–275–(400) µm long. Tracheids absent. Fibres septate in *I. balfourii*, *I. dewildeana* and *I. gesneroidea*, and also sometimes in *I. leschenaultii*, thin-walled, and relatively wide, (320)–420–640–(900) µm long, with mostly simple to occasionally minutely bordered pits equally distributed in radial and tangential walls, pits 2–3 µm in horizontal diameter. Axial parenchyma scanty paratracheal (Fig. 4C), 2–3 cells per strand. Rays absent in most species studied, tall and multiserial in *I. balfourii* (Fig. 4E) and *I. flaccida*, 3–15 cells wide and (1200)–2020 to >5000 µm high, 0–2 rays mm⁻¹, consisting of upright cells only, ray-like areas with fibre-like ray parenchyma cells observed in *I. gesneroidea*, *I. leschenaultii*, *I. namchabarwensis* and *I. vitellina* (Fig. 4F); sheath cells absent. Raphides observed in ray-like areas of *I. vitellina*.

Hydrocera (1/1; Fig. 5; Tables 1 and 2). Wood formation is mainly restricted to the ribs of the stem. In between rib regions, the thin ring-like wood cylinder is interrupted at some places (Fig. 5A).

Stems hollow. Outer stem layer with epidermis still attached. Collenchyma 2–3 cell layers in width, without meristematic activity. Cortex modified into aerenchyma with large intercellular spaces (Fig. 5A). Raphides observed in collenchyma, aerenchyma and pith parenchyma.

Wood mostly confined to the ribs of the stem. Growth ring boundaries absent. Wood diffuse-porous (Fig. 5B). Vessels (60)–70–(80) mm⁻², usually solitary and sometimes in radial multiples of two (Fig. 5B), vessel outline angular. Vessel perforation plates simple. Lateral wall pitting (pseudo-)scalariform near the primary xylem (Fig. 5D), 15–30 µm in horizontal size, and rapidly changing to an alternate pattern towards the cambium (Fig. 5E, F), pit border 5–6 µm in horizontal diameter, non-vestured. Tangential diameter of vessels (25)–56–(80) µm, vessel elements (200)–255–(400) µm long. Tracheids absent. Fibres non-septate, thin-walled, (400)–590–(800) µm long, with mostly simple to occasionally minutely bordered pits equally distributed in radial and tangential walls, pits 2–3 µm in diameter. Axial parenchyma scanty paratracheal, 2–3 cells per strand. Rays uniseriate and multiserial, 12–14 cells wide and >6200 µm high, 0–2 rays mm⁻¹, consisting of upright cells

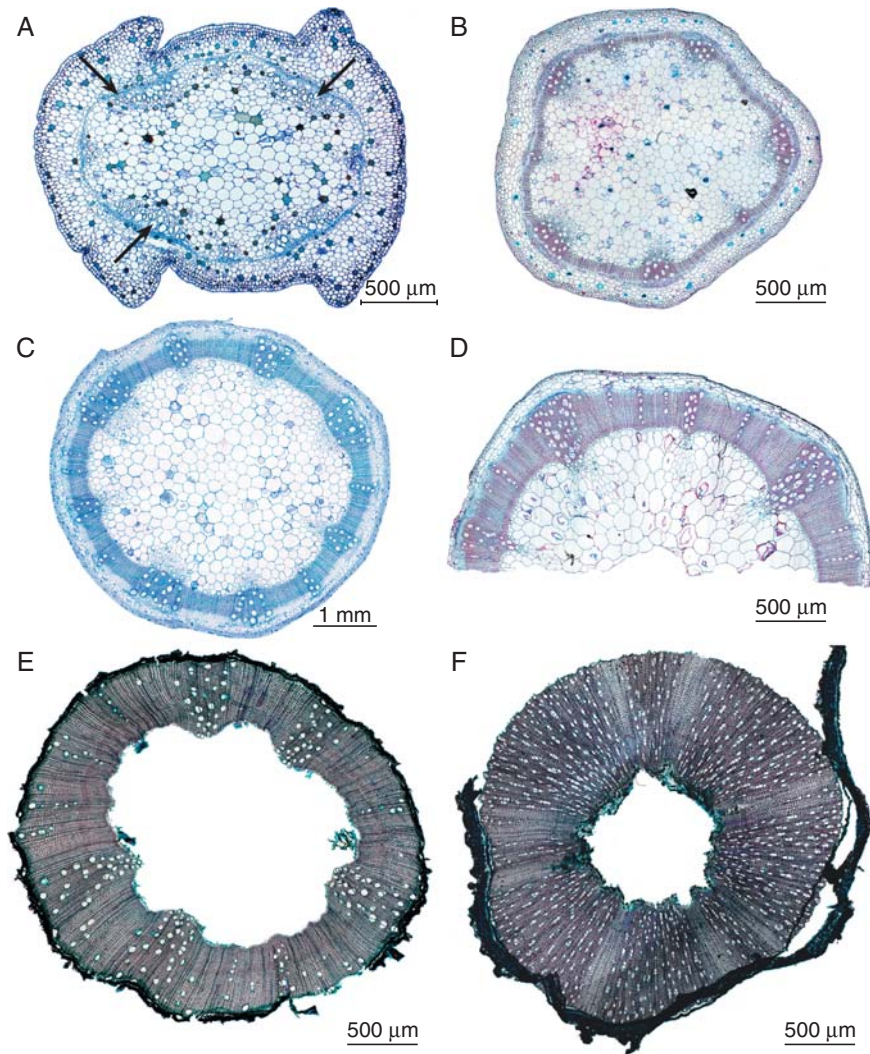


FIG. 2 Transverse light microscope sections of *Impatiens* showing variation in wood development at the base of the stem. (A) *Impatiens mansonii*, clearly herbaceous stem without wood cylinder, intrafascicular cambium produces few wood cells (arrows). (B) *Impatiens* cf. *stuhlmannii*, herbaceous stem with narrow wood cylinder; character state after gap weighting = 2. (C) *Impatiens hawkeri*, herbaceous stem with slightly larger wood cylinder, character state after gap weighting = 4. (D) *Impatiens latifolia*, herbaceous stem with the most developed wood cylinder observed, but still limited compared with the entire stem diameter; character state after gap weighting = 9. (E) *Impatiens eubotrya*, woody stem, ratio of double wood cylinder thickness over stem diameter is significantly larger than in (D); wood cylinder also extends to the upper parts of the stem; character state after gap weighting = 14. (F) *Impatiens gesnerioides*, clearly woody stem; character state after gap weighting = 23.

only (Fig. 5C); sheath cells absent. Raphides not observed in wood.

Phylogenetic analyses and character mapping

The combined data molecular matrix contains 3560 characters from which 961 (27%) are variable. The chloroplast *atpB-rbcL* data matrix consist of 879 characters (213 variable characters), whereas the nuclear *ImpDEF1* and *ImpDEF2* data matrices contain 710 (197 variable) and 1970 (551 variable) characters, respectively. Despite the inability to amplify some loci for a few species, the missing data had no notable impact in the combined matrix. Ambiguously aligned nucleotides of microsatellite regions were removed from both chloroplast and nuclear data matrices. Despite the fact that ML analysis of the *ImpDEF1/ImpDEF2* data sets resulted in a

much better resolved topology than the *atpB-rbcL* data set, no incongruent relationships were found between the two analyses. This congruence is confirmed by the partition homogeneity test, which found no significant difference between both partitions of the combined data set ($P > 0.05$). The combined data set yielded a well-resolved topology in which most of the lineages are resolved (Fig. 6). The obtained topology corroborates the major clades found by Janssens *et al.* (2006, 2007, 2008, 2009). In total, only three unresolved lineages are found: (1) the clade consisting of *I. arguta* and *I. namchabarwensis* forming a polytomy with the clade including *I. aurea*, *I. capensis* and *I. noli-tangere* and the additional *Impatiens* species; (2) *I. purpureo-violacea* forming a polytomy with the *I. burtonii*–*I. assurgens* clade and the *I. cecillii*–*I. shirensis* clade; and (3) the group represented by *I. repens*, *I. latifolia* and *I. leschenaultii*. Bootstrap analysis

TABLE 2. Overview of selected wood anatomical characters within the ten most woody *Impatiens* species observed and *Hydrocera triflora*

Taxon	Solitary vessels	Vessels in radial multiples	Vessels in clusters	Vessels confined to fascicular regions	Vessel diameter (µm)	Vessel density (per mm ²)	Vessel element length (µm)	Fibres septate	Ray-like areas present	Rays present	Ray width (no. of cells)	Multiseriate ray height (µm)
<i>Hydrocera triflora</i>	+	(2)	-	+	(25)-56-(80)	(60)-70-(80)	(150)-230-(400)	-	-	+	1-2, 12-14	>6200
<i>Impatiens balfourii</i>	+	(2)	-	-	(30)-48-(60)	(16)-25-(36)	(100)-190-(270)	+	-	+	(1-2), 3-10	(1200)-2020-(2600)
<i>I. dewildeana</i>	+	(2-3)	(3-5)	+	(30)-47-(70)	(5)-23-(42)	(225)-270-(350)	+	-	-	-	-
<i>I. flaccida</i>	+	(2-3)	-	-	(30)-60-(90)	(22)-36-(50)	(130)-220-(330)	-	-	+	7-15	>5000
<i>I. gesnerioides</i>	±	2-3-(5)	-	-	(25)-41-(60)	(53)-64-(75)	(170)-225-(300)	+	+	-	-	-
<i>I. havilandii</i>	+	(2)	(3)	+	(30)-61-(90)	(3)-8-(17)	(175)-275-(350)	-	-	-	-	-
<i>I. leschenaultii</i>	+	(2-4)	-	-	(30)-45-(60)	(45)-66-(84)	(160)-220-(310)	±	+	-	-	-
<i>I. namchabarwensis</i>	+	(2)	-	-	(30)-46-(60)	(8)-20-(38)	(150)-230-(350)	-	+	-	-	-
<i>I. stananiha</i>	+	(2)	-	+	(25)-39-(55)	(20)-27-(35)	(120)-175-(250)	-	-	-	-	-
<i>I. violaeiflora</i>	±	2-3	(3-5)	-	(30)-43-(70)	(48)-63-(80)	(190)-255-(320)	-	-	-	-	-
<i>I. vitellina</i>	+	(2-5)	-	+	(20)-27-(40)	(12)-29-(50)	(120)-175-(250)	-	+	-	-	-

Species are arranged alphabetically. Numbers between hyphens are mean values flanked by minimum and maximum values. +, present; ±, sometimes present; -, absent.

shows relatively high support for many of the lineages. Nevertheless, some of the nodes have relatively low bootstrap value below 75, and thus interpretation of these phylogenetic relationships should be treated with caution (for a discussion of bootstrap values in phylogenetic analyses, see Hillis and Bull, 1993; Soltis and Soltis, 2003), but this does not change the number of habit shifts in this study.

Maximum parsimony optimization of four states (0 = herbaceous species, 1 = ‘woody’ herbs, 2 = slightly woody species, 3 = woody species) or only two states [0 = herbaceous species (combining 0 and 1, and species without a wood cylinder), 1 = woody species (combining 2 and 3)], representing variation in habit in *Impatiens*, indicates a similar pattern in which secondary woodiness originated at least five times from a herbaceous Balsaminaceae ancestor (Fig. 7).

DISCUSSION

Stem anatomical diversity within *Impatiens*

Based on a careful screening of >100 *Impatiens* species in the herbarium of the Netherlands Centre for Biodiversity Naturalis-section NHN (L), taking into account multiple flowering specimens per species with roots attached, we observed that more than about 70 % of the species had very thin and completely flattened stems, indicating that the amount of wood formation is negligible to nearly absent. This can be confirmed by the *Impatiens* literature mentioning that the genus can be characterized by herbaceous, semi-succulent stems (Grey-Wilson, 1980a; Yuan et al., 2004). However, amongst the >1000 species present in *Impatiens*, several of them show a continuous variation in wood development at the genus level (Table 1; Fig. 2, Supplementary Data Fig. S1): truly herbaceous species only show a few wood cells in the fascicular regions (Fig. 2A), while the woodiest species have a significant wood cylinder of >50 cell layers in thickness at the base of their stems (Fig. 2F). It is known that most so-called herbaceous eudicot species develop a limited amount of wood at the base of their stems, either confined to the fascicular regions or somewhat more developed into a tiny wood cylinder (Krumbiegel and Kästner, 1993; Schweingruber, 2007; Schweingruber et al., 2011; Lens et al., 2012). Consequently, the continuous range in wood formation within the genus *Impatiens* emphasizes once more the fuzzy boundary between the terms ‘herbaceousness’ and ‘woodiness’ – in line with authors advocating the continuum morphology (e.g. Sattler, 1996) – which makes it extremely difficult to decide at which point a species can be considered woody. Some authors even propose to abandon both terms and use the Raunkiaer (1943) terminology instead, based on the presence/absence and the position of the surviving buds, to describe a plant’s life form (Dulin and Kirchoff, 2010).

We believe that the terms herbaceousness and woodiness remain valid as long as two criteria are fulfilled: (1) ‘herbaceousness’ should not be interpreted as ‘without wood formation’; and (2) a detailed description of the entire stem anatomy should be provided so that it is clear how much wood is developed in relation to the rest of the stem. Based on the carefully measured ratio of double wood cylinder thickness over total

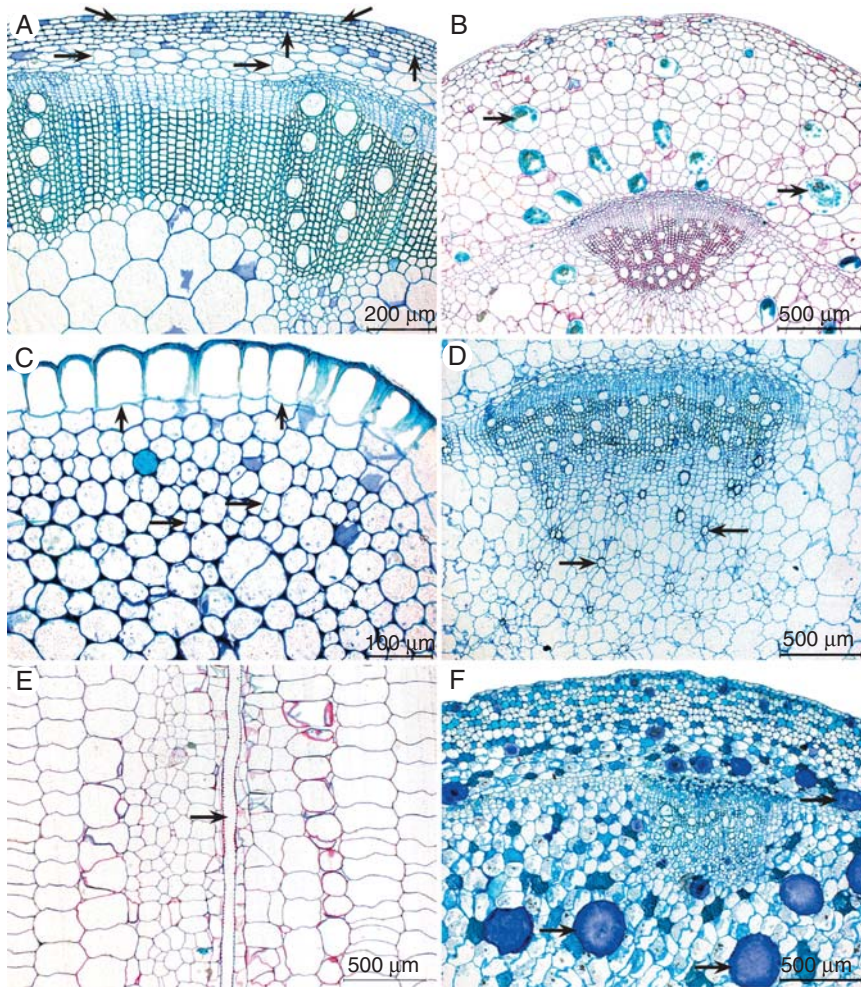


FIG. 3 Transverse (A–D, F) and longitudinal (E) light microscope stem sections of *Impatiens*. (A) *Impatiens hawkeri*, meristematic activity in epidermis (oblique arrows), collenchyma (vertical arrows) and cortex (horizontal arrows). (B) *Impatiens repens*, epidermis at the outside, collenchyma narrow, broad cortex with abundant meristematic activity and enlarged idioblasts containing raphide bundles (arrows). (C) *Impatiens pseudomacroptera*, detail of periderm with phellogen (vertical arrows) forming enlarged cork cells at the outside; horizontal arrows point to meristematic activity in collenchyma. (D) *Impatiens hochstetteri*, cell groups radiating around lost primary xylem helical tracheids that resemble flowers (arrows). (E) *Impatiens glandulifera*, longitudinal section through such a flower-like structure; the arrow points to a lost primary xylem helical element. (F) *Impatiens grandis*, unusually enlarged idioblasts (arrows) in pith and cortex containing raphides in mucilage sacs.

stem diameter, we calculated a habit character that continuously varied among the 42 species having a wood cylinder at the base of their stems. Following the gap weighting method of Thiele, we could define either two- or four-character states (Fig. 7, Supplementary Data Fig. S1). According to us, the two-character state solution [herbaceous (no wood cylinder and character states 1–9) vs. woody (character states 12–26)] is more appropriate from an anatomical point of view (Fig. 7A), because one of the species with character state 12, *I. niarniamensis*, has one of the largest wood cylinders studied in terms of number of cell layers (Table 1). Moreover, the wood cylinder at the base of the stems in both *I. niarniamensis* and *I. biflora* – the two species investigated with character state 12 – also extends to the upper stem parts (results based on hand sections), which is also true for the woodier species. On the other hand, the narrower wood cylinders in species with character states 1–9 are restricted to the

first few centimetres of the aboveground stem and are considered here as herbaceous.

In contrast to the large differences in amount of wood development within *Impatiens*, wood anatomical variation between the species observed is negligible. *Impatiens* species all have diffuse-porous wood with simple vessel perforation plates, very short vessel elements (frequently < 300 μm), flat or continuously decreasing length-on-age curves (Supplementary Data Fig. S2), wide gaping scalariform vessel wall pitting (Fig. 4D), thin-walled fibres with simple to minutely bordered pits, and scanty paratracheal parenchyma (Fig. 4C). Rays are usually absent, but, when present, they are clearly visible as tall, multiseriate structures with exclusively upright ray cells (Fig. 4E, Table 2). In some species, transverse sections show radial zones of slightly different cells suggesting rays (Fig. 4A), but tangential sections of the same wood samples demonstrate that the shape and size of the cells in these

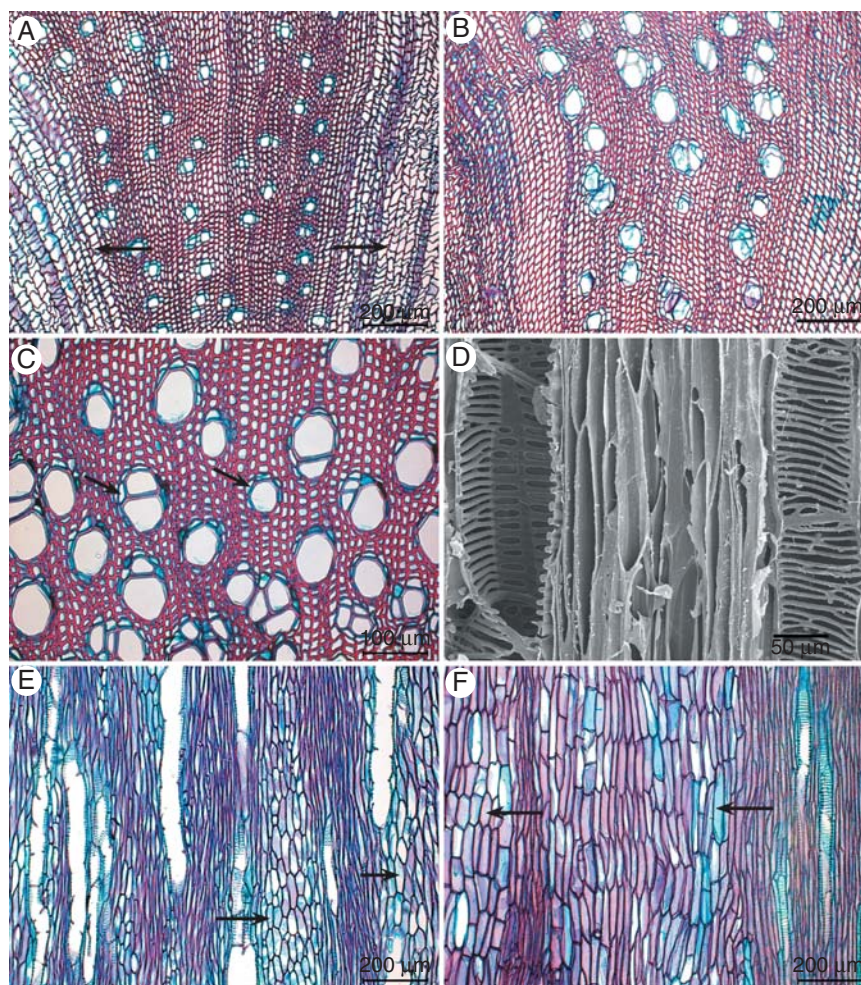


FIG. 4 Wood anatomical illustrations of *Impatiens* using light microscopy (A–C, E, F) and scanning electron microscopy (D). (A) *Impatiens vitellina*, transverse section showing mainly solitary vessels and ray-like areas (arrows). (B) *Impatiens dewildeana*, transverse section vessels arranged solitarily or in clusters, rayless wood. (C) *Impatiens shirensis*, detail of transverse section showing scanty paratracheal parenchyma (arrows). (D) *Impatiens flaccida*, tangential section showing wide gaping intervessel pitting. (E) *Impatiens balfourii*, tangential section showing true rays with exclusively upright ray cells (arrows). (F) *Impatiens vitellina*, tangential section showing broad ray-like areas with fibre-like ray cells (arrows).

so-called ray-like areas resemble those of libriform fibres (Fig. 4F).

When looking at the outer stem layers, many species with large stem diameters show meristematic activity in their cortex and collenchyma (Fig. 3A, B), explaining that secondary growth in *Impatiens* stems is not always triggered by a vascular cambium. For instance, the branch of *I. mirabilis* investigated – one of the species with the largest stems within the genus (Fig. 1C) – has a diameter of 25 mm, while the wood formation is negligible and only confined to the fascicular regions. In other words, stem diameter is not always a good proxy to distinguish between herbaceousness and woodiness. The presence of a periderm including a few layers of cork is better linked with woodiness, although some truly herbaceous species with large stem diameters have a periderm as well (*I. macrophylla*, *I. mackeyana* and *I. mirabilis*).

Two unusual features are found in the stems of *Impatiens*: ‘flower-like’ cells below the primary xylem regions observed

in the pith in most species (Fig. 3D, E), and unusually large idioblasts in the pith of *I. grandis* (Fig. 3F) and *I. parasitica*. The flower-like structures can best be interpreted as remnants of primary xylem helical tracheids that have been removed from the primary xylem by meristematic activity, and are surrounded by elongated parenchymatous cells giving the appearance of a flower (cf. Solereder, 1899; Fig. 3D). This interpretation is confirmed by longitudinal sections (Fig. 3E). Some of the extremely large idioblasts in the pith of *I. grandis* bear raphide bundles embedded in mucilage sacs, and resemble smaller raphide-containing idioblasts in the cortex, which are often observed in *Impatiens* stems.

Stem anatomy of *Hydrocera triflora*

The stem anatomy of *Hydrocera* strongly resembles that of *Impatiens*, except for the aerenchyma that is derived from the normal parenchymatous cortex (Fig. 5A). Since *H. triflora* often grows in shallow water (Grey-Wilson,

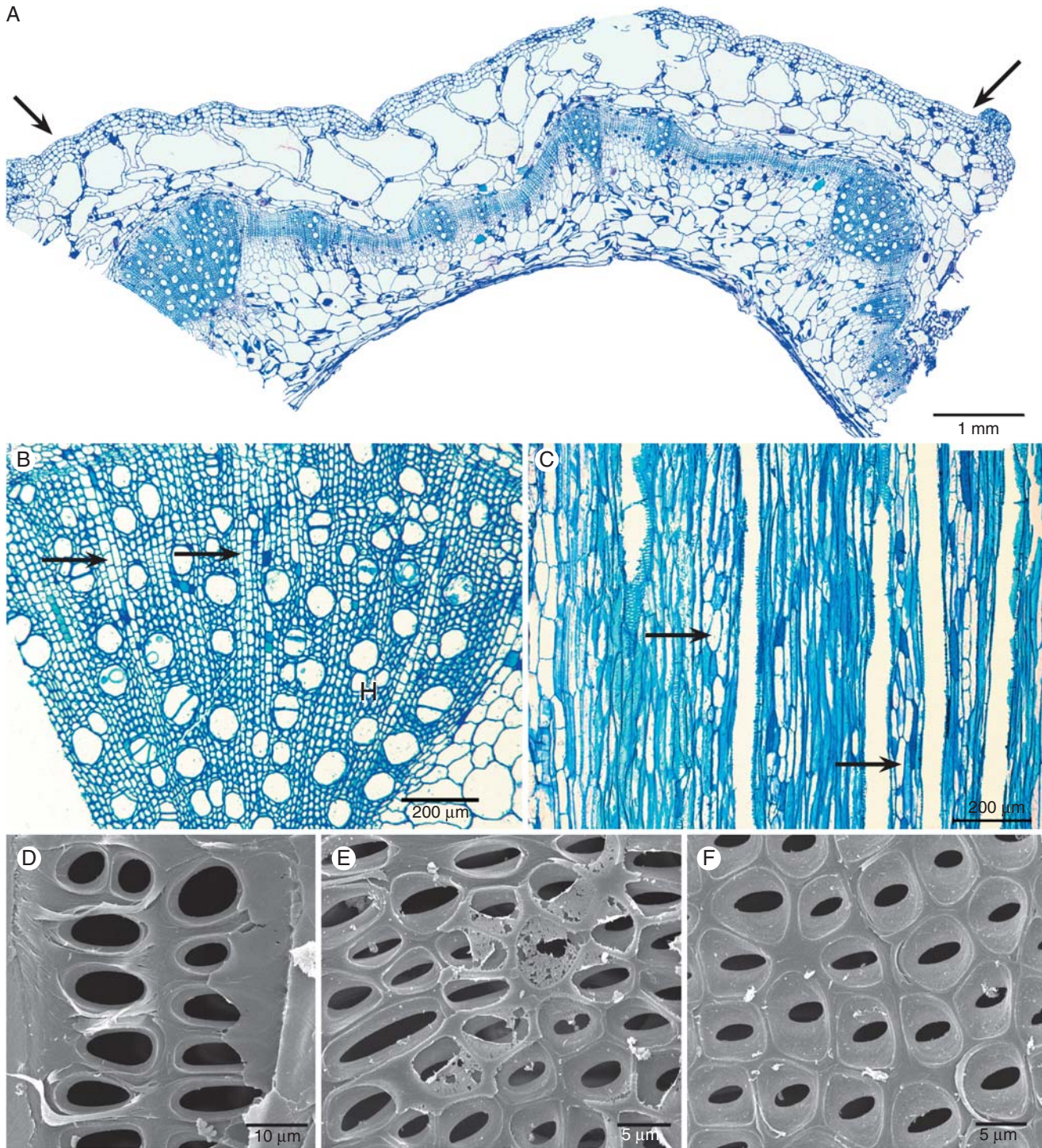


FIG. 5 Stem anatomical pictures of *Hydrocera triflora* using light microscopy (A–C) and scanning electron microscopy (D–F). (A) Transverse section showing two stem ribs (arrows) and an aerenchyma region in the outer layer with large intercellular spaces. (B) Detail of transverse section showing wood formation at the rib region, vessels solitary or in small radial multiples, rays present (arrows). (C) Tangential section showing true rays with exclusively upright cells (arrows). (D) Tangential section near the pith showing enlarged intervessel pits with reduced pit borders. (E) Tangential section closer to the cambium; smaller intervessel pits with more pronounced pit borders. (F) Tangential section near the cambium; distinctly bordered intervessel pits in an opposite to alternate arrangement.

1980b), the adapted cortex into a zone with large air-filled cavities allows low resistance transport of oxygen and other gases in the plant between stem parts above water and submerged parts. Wood formation in *Hydrocera* is largely confined to

the ribs of the hollow stem (Fig. 5A) – interconnected by an extremely thin wood cylinder that is interrupted at some places – and is therefore interpreted as herbaceous in the present study. Although its wood anatomy is nearly identical

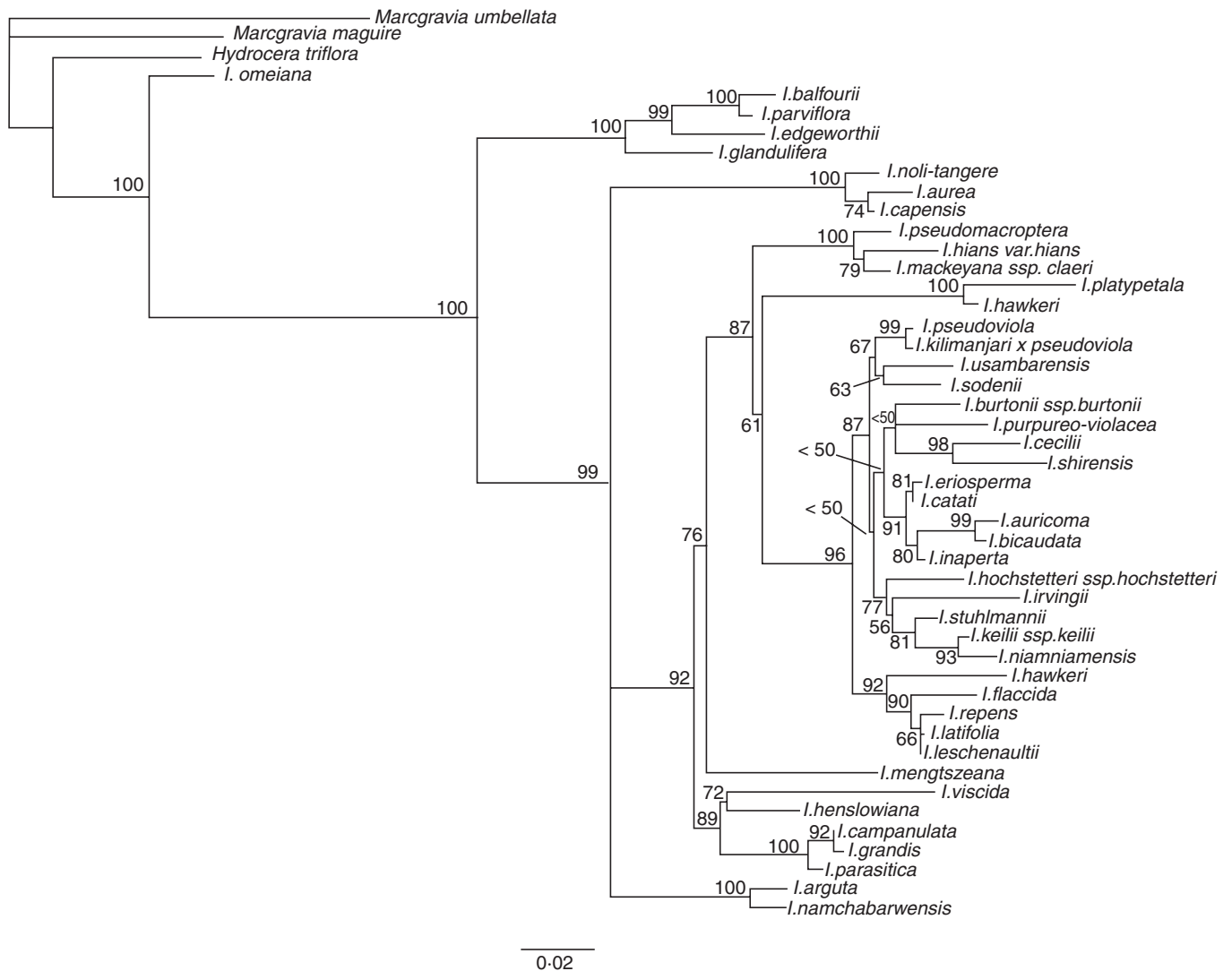


FIG. 6 Maximum likelihood analysis of the chloroplast marker *atpB-rbcL* and the nuclear markers *ImpDEF1* and *ImpDEF2*, representing only species from which anatomical observations are also made. Numbers above or below branches indicate bootstrap support values.

to the one of *Impatiens*, *Hydrocera* wood can be distinguished from that of *Impatiens* by the presence of a gradual transition of scalariform to alternate intervessel pitting from the pith region towards the cambium (Fig. 5D–F).

Secondary origin of wood formation within Balsaminaceae

As discussed before, the wood anatomy of Balsaminaceae is characterized by a number of pedomorphic wood features, including flat or decreasing length-on-age curves for vessel elements in all the woodiest *Impatiens* observed and in *Hydrocera* (Supplementary Data Fig. S2). The relatively small wood cylinder observed in these woodiest species do not allow reconstruction of length-on-age curves over a long distance, but we feel confident that the curves generated are informative to assess secondary woodiness. This is based on the ideas of Bailey (1920) who demonstrated that the vessel element length remains almost constant with age in species having short vessel elements (<300 μm) with simple perforations

(cf. Carlquist, 1962). However, from a hydraulic point of view, the length of entire vessels has proven to be more important in the water transport mechanism of plants, and greatly outweighs the importance of vessel element length (Sperry *et al.*, 2006, 2007; Lens *et al.*, 2011). A second pedomorphic wood feature that is often cited is the absence of rays or the presence of rays with exclusively upright ray cells (Fig. 4E; Carlquist, 1962, 1970, 1992, 2009), or the presence of ray-like areas containing fibre-like parenchyma cells (Fig. 4F). Whether or not the occurrence of wide gaping or gash-like intervessel pits in *Impatiens* (Fig. 4D) is a truly pedomorphic character and/or an adaptation to its parenchymatous semi-succulent stems is difficult to assess. The abundance of parenchyma cells in stems provides mechanical strength through cell turgor, which might compensate for the large intervessel pit apertures that weaken vessel walls (Dulin and Kirchoff, 2010). Alternatively, these wide gaping pits may be an adaptation to expansion and contraction of the slightly lignified wood during wet and drier periods,

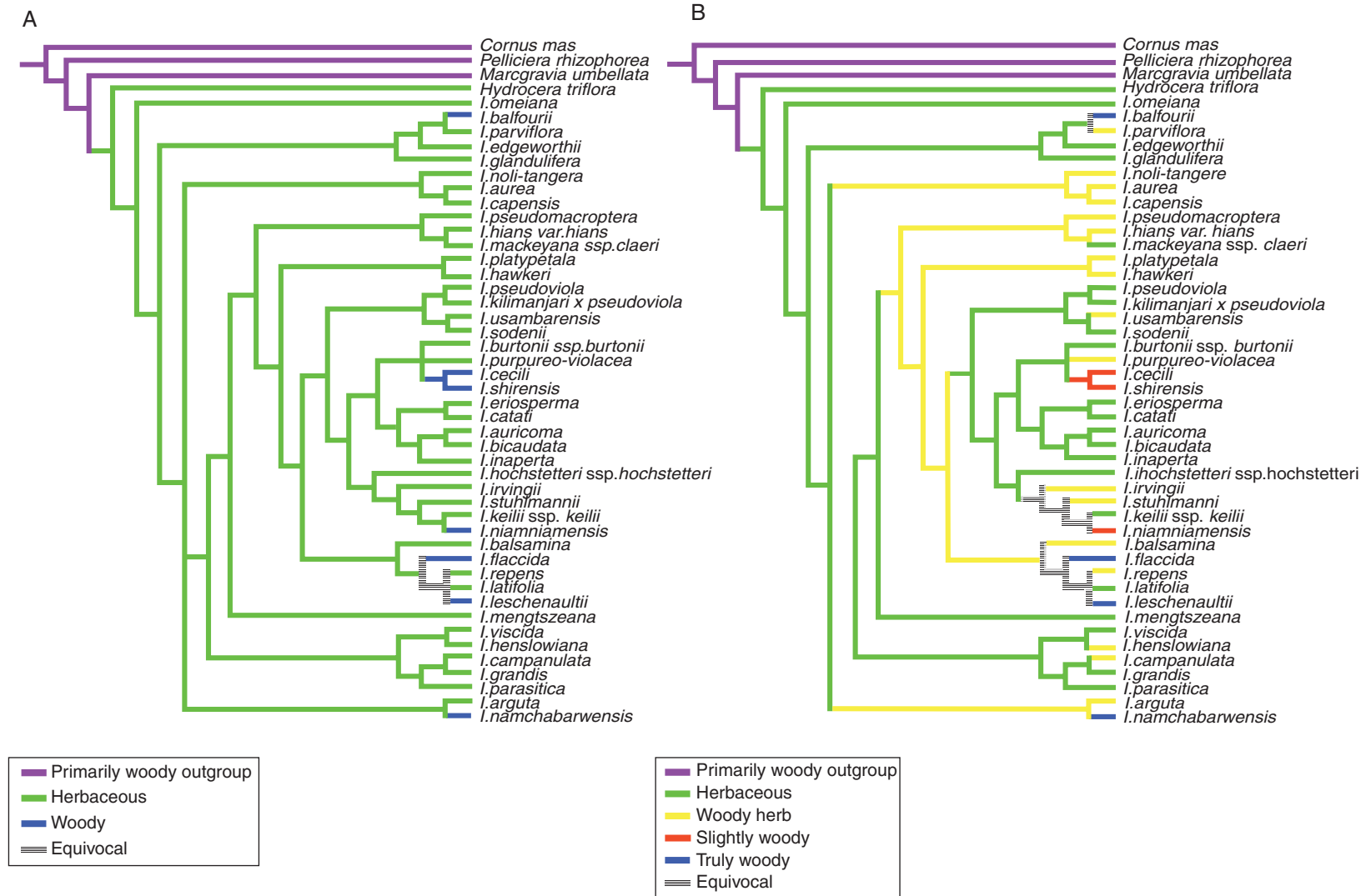


FIG. 7 Phylogenetic tree showing multiple habit shifts towards secondary woodiness within Balsaminaceae. The primarily woody *Cornus*, *Pelliciera* and *Marcgravia* are chosen as the outgroup. Character optimization of the habit character on the strict consensus tree from chloroplast *atpB-rbcL* and nuclear *ImpDEF1/ImpDEF2* sequences. (A) Two-character state analysis with two classes of species: herbaceous species (character states 1–9 plus all the species without a wood cylinder) and woody species (character states 12–26; Table 1). (B) Four-character state analysis with four classes of species: herbaceous species (species without a wood cylinder), ‘woody’ herbs (character states 1–9), slightly woody species (character states 12–14) and truly woody species (character states 17–26; Table 1).

respectively (Carlquist, 2009), although this has to be experimentally evaluated.

Molecular evolutionary trees support our wood anatomical hypothesis that the most woody *Impatiens* species are secondarily woody. Whether or not one chooses to divide the character growth form into four or two character states, the woodiest species remain scattered in the *Impatiens* topology in at least five different clades (Fig. 7).

As has been mentioned by Grey-Wilson (1980a), the question of why secondary woodiness occurs within *Impatiens* remains unanswered. Various hypotheses on the origin of secondary woodiness have been put forward, such as intraspecific competition (Darwin, 1859; Tilman 1988; Givnish, 1995), ability to produce more seeds (Wallace, 1878), counter-selection against inbreeding (Böhle et al., 1996), uniform climate (especially absence of frost; Carlquist, 1974) and the absence of large native herbivores (Carlquist, 1974). Most of these hypotheses are based on secondarily woody plants that are native to islands (insular woodiness) or island-like regions on continents, a distribution which is also characteristic of many *Impatiens* species (Grey-Wilson, 1980a; Janssens et al., 2009, 2010, 2011). However, when the habitat of the woodiest *Impatiens* species is compared with that of the majority of the herbaceous species, none of these hypotheses applies to *Impatiens*.

Surprisingly, despite the presence of paedomorphic wood features in Balsaminaceae and the huge range of habit differences within the balsaminoid Ericales clade (lianas, mangroves, trees, small shrubs and woody herbs), we can list two phylogenetically informative wood anatomical resemblances between Balsaminaceae on the one hand and the related families Tetrameristaceae *sensu lato* and Marcgraviaceae on the other: septate libriform fibres and raphides in ray cells (Lens et al., 2005a). Furthermore, the three families share vessels in radial multiples and simple perforation plates, alternate intervessel pitting (although mainly absent in *Impatiens*) and paratracheal parenchyma, although this combination of characters is common in other woody flowering plant families as well.

Genetic background of secondary woodiness

Recently, Melzer et al. (2008) revealed the first evidence for the genetic mechanism triggering secondary or insular woodiness in a mutant of *Arabidopsis thaliana*. In this species, which only produces a negligible amount of wood in the fascicular regions under normal growth conditions (Lens et al., 2012), two flowering time control genes were knocked out: *SUPPRESSOR OF OVEREXPRESSION OF CONSTANS 1* (*SOCI*) and *FRUITFULL* (*FUL*). Surprisingly, the resulting double mutant developed into a woody shrub after several months of growth, showing wood development throughout all stems and to a much larger extent than any *A. thaliana* mutant described so far. Moreover, the induced wood in this mutant showed the expected paedomorphic features, and strongly resembles other secondarily woody Brassicaceae native to islands, suggesting that knocking out *SOCI* and *FUL* triggers the normal pathway leading to secondary woodiness in *A. thaliana* (Lens et al., 2012). Besides the shrub-like habit, the double mutants showed a combination of perennial-

like features, such as a prolonged age up to 18 months, the co-occurrence of active vegetative and reproductive meristems, and recurrent flowering cycles (Melzer et al., 2008).

The fact that knocking out only two genes in a truly herbaceous species can result in a woody phenotype is not only amazing, but it may also offer an explanation as to how this relatively 'simple' genetic mechanism could perhaps trigger woodiness in many non-related flowering plant lineages on islands or island-like continental regions throughout the world (Carlquist, 1974; Böhle et al., 1996; Givnish, 1998; Lee et al., 2005; Lens et al., 2009). It also clearly demonstrates that herbaceous plants keep the genetic capability to develop woodiness, but the wood-forming genes need to be activated (Oh et al., 2003; Ko et al., 2004; Groover, 2005; Spicer and Groover, 2010). *SOCI* and *FUL* might not be the only key players: other upstream, downstream or in parallel-acting (positive or negative) regulatory genes could be more important. Evidently, more genetic insights into wood development are required to better understand one of the most appealing developmental aspects of plants (Chaffey et al., 2002; Nieminen et al., 2004; Schrader et al., 2004; Dharmawardhana et al., 2010; Agusti et al., 2011).

SUPPLEMENTARY DATA

Supplementary data are available online at www.aob.oxfordjournals.org and consist of the following. Figure S1: visual representation of the character states implemented by the gap weighting method of Thiele (1993) based on the 42 *Impatiens* species showing a wood cylinder at least at the base of their stems. Figure S2: continuously decreasing or flat length-on-age curves for vessel elements of the woody *Impatiens flaccida*, *I. namchabarwensis* and *I. niarniamensis* based on maceration slides taken at each millimetre between pith and cambium. The curves are representative of secondarily woody species.

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LITERATURE CITED

- Agusti J, Lichtenberger R, Schwarz M, Nehlin L, Greb T. 2011. Characterization of transcriptome remodeling during cambium formation identifies *MOL1* and *RUL1* as opposing regulators of secondary growth. *PLoS Genetics* 7: e1001312. <http://dx.doi.org/10.1371/journal.pgen.1001312>.
- Bailey IW. 1920. The cambium and its derivative tissues. II. Size variations of cambial initials in gymnosperms and angiosperms. *American Journal of Botany* 7: 355–367.
- Böhle UR, Hilger HH, Martin WF. 1996. Island colonization and evolution of the insular woody habit in *Echium* L. (Boraginaceae). *Proceedings of the National Academy of Sciences, USA* 93: 11740–11745.
- Caris PL, Geuten KP, Janssens SB, Smets EF. 2006. Floral development in three species of *Impatiens* (Balsaminaceae). *American Journal of Botany* 93: 1–14.
- Carlquist S. 1962. A theory of paedomorphosis in dicotyledonous woods. *Phytomorphology* 12: 30–45.

- Carlquist S. 1970. Wood anatomy of insular species of *Plantago* and the problem of raylessness. *Bulletin of the Torrey Botanical Club* **97**: 353–361.
- Carlquist S. 1974. Insular woodiness. In: *Island biology*. New York: Columbia University Press, 350–428.
- Carlquist S. 1992. Wood anatomy of sympetalous dicotyledon families: a summary, with comments on systematic relationships and evolution of the woody habit. *Annals of the Missouri Botanic Garden* **79**: 303–332.
- Carlquist S. 2009. Xylem heterochrony: an unappreciated key to angiosperm origin and diversifications. *Botanical Journal of the Linnean Society* **161**: 26–65.
- Chaffey N, Cholewa E, Regan S, Sundberg B. 2002. Secondary xylem development in *Arabidopsis*: a model for wood formation. *Physiologia Plantarum* **114**: 594–600.
- Cheek M, Fischer E. 1999. A tuberous and epiphytic new species of *Impatiens* (Balsaminaceae) from southwest Cameroon. *Kew Bulletin* **57**: 669–674.
- Darwin C. 1859. *On the origin of species by means of natural selection*. London: John Murray.
- Dharmawardhana P, Brunner AM, Strauss SH. 2010. Genome-wide transcriptome analysis of the transition from primary to secondary stem development in *Populus trichocarpa*. *BMC Genomics* **11**: 150. <http://dx.doi.org/10.1186/1471-2164-11-150>.
- Dulin MW, Kirchoff BK. 2010. Pedomorphosis, secondary woodiness, and insular woodiness in plants. *Botanical Review* **76**: 405–490.
- Edgar RC. 2004. MUSCLE: a multiple sequence alignment method with reduced time and space complexity. *BMC Bioinformatics* **5**: 113. <http://dx.doi.org/10.1186/1471-2105-5-113>.
- Francisco-Ortega F, Fuerte-Aguilar F, Kim SC, Santos-Guerra A, Crawford DJ, Jansen RK. 2002. Phylogeny of the Macaronesian endemic *Crambe* section *Dendrocrambe* (Brassicaceae) based on internal transcribed spacer sequences of nuclear ribosomal DNA. *American Journal of Botany* **89**: 1984–1990.
- Gerard AM. 1917. *Recherches sur la spécification histologique de différents bois de Madagascar, avec étude comparative des principaux bois industriels d'Europe*. Lons-Le-Saunier, France.
- Givnish TJ. 1995. Plant stems: biomechanical adaptation for energy capture and influence on species distributions. In: Gartner BL, ed. *Plant stems: physiology and functional morphology*. New York: Chapman and Hall, 3–49.
- Givnish TJ. 1998. Adaptive plant evolution on islands: classical patterns, molecular data, new insights. In: Grant PR, ed. *Evolution on islands*. Oxford: Oxford University Press, 281–304.
- Grey-Wilson C. 1980a. *Impatiens of Africa*. Rotterdam: Balkema.
- Grey-Wilson C. 1980b. *Hydrocera triflora*, its floral morphology and relationship with *Impatiens*. *Studies in Balsaminaceae*. *Kew Bulletin* **34**: 221–227.
- Groover AT. 2005. What genes make a tree a tree? *Trends in Plant Science* **10**: 210–214.
- Hamann TD, Smets E, Lens F. 2011. A comparison of paraffin and resin-based techniques used in bark anatomy. *Taxon* **60**: 841–851.
- Hillis DM, Bull JJ. 1993. An empirical test of bootstrapping as a method for assessing confidence in phylogenetic analysis. *Systematic Biology* **42**: 182–192.
- Hooker JD, Thomson T. 1859. Praecursores ad floram Indicam. Balsaminaceae. *Botanical Journal of the Linnean Society* **4**: 106–157.
- IAWA Committee. 1989. IAWA list of microscopic features for hardwood identification. *International Association of Wood Anatomists Bulletin, New Series* **10**: 219–332.
- Janssens SB, Geuten K, Yuan Y-M, Küpfer P, Smets EF. 2006. Phylogenetics of *Impatiens* and *Hydrocera* using chloroplast *atpB-rbcL* spacer sequences. *Systematic Botany* **31**: 171–180.
- Janssens SB, Geuten KP, Viaene T, Yuan Y-M, Song Y, Smets E. 2007. Phylogenetic utility of the AP3/DEF K-domain and its molecular evolution in *Impatiens* (Balsaminaceae). *Molecular Phylogenetics and Evolution* **43**: 225–239.
- Janssens SB, Viaene T, Huysmans S, Smets EF, Geuten KP. 2008. Selection on length mutations after frameshift can explain the origin and retention of the AP3/DEF-like paralogues in *Impatiens*. *Journal of Molecular Evolution* **66**: 424–435.
- Janssens SB, Knox EB, Huysmans S, Smets EF, Merckx VSFT. 2009. Rapid radiation of *Impatiens* (Balsaminaceae) during Pliocene and Pleistocene: result of a global climate change. *Molecular Phylogenetics and Evolution* **52**: 806–824.
- Janssens SB, Fischer E, Stévant T. 2010. New insights on the origin of epiphytic *Impatiens* (Balsaminaceae) in West Equatorial Africa based on molecular phylogenetic and biogeographical analysis. *Taxon* **59**: 1508–1518.
- Janssens SB, Dessein S, Smets E. 2011. Portrayal of *Impatiens nzabiana* (Balsaminaceae): a morphological, molecular and biogeographic study of a new Gabonese species. *Systematic Botany* **36**: 440–448.
- Ko JH, Han KH, Yang J. 2004. Plant body weight-induced secondary growth in *Arabidopsis* and its transcription phenotype revealed by whole-transcriptome profiling. *Plant Physiology* **135**: 1069–1083.
- Koek-Noorman J. 1976. Juvenile characters in the wood of certain Rubiaceae with special emphasis to *Rubia fruticosa* Ait. *International Association of Wood Anatomists Bulletin* **3**: 38–42.
- Krumbiegel A, Kästner A. 1993. Sekundäres Dickenwachstum von Sproß und Wurzel bei annuellen dicotylen. *Österreichische Akademie der Wissenschaften, Biosystematics and Ecology Series* **4**: 1–49.
- Lee C, Kim S-C, Lundy K, Santos-Guerra A. 2005. Chloroplast DNA phylogeny of the woody *Sonchus* alliance (Asteraceae: Sonchinae) in the Macaronesian islands. *American Journal of Botany* **92**: 2072–2085.
- Lens F, Caris P, Smets E, Serlet L, Jansen S. 2005a. Comparative wood anatomy of the primuloid clade (Ericales s.l.). *Systematic Botany* **30**: 162–182.
- Lens F, Dressler S, Jansen S, Van Evelghem L, Smets E. 2005b. Relationships within balsaminoid Ericales: a wood anatomical approach. *American Journal of Botany* **92**: 941–953.
- Lens F, Schönenberger J, Baas P, Jansen S, Smets E. 2007. The role of wood anatomy in phylogeny reconstruction of Ericales. *Cladistics* **23**: 229–254.
- Lens F, Groeninckx I, Smets E, Dessein S. 2009. Woodiness within the Spermaceae–Knoxieae alliance (Rubiaceae): retention of the basal woody condition in Rubiaceae or recent innovation? *Annals of Botany* **103**: 1049–1064.
- Lens F, Sperry JS, Christman MA, Choat B, Rabaey D, Jansen S. 2011. Testing hypotheses that link wood anatomy to cavitation resistance and hydraulic conductivity in the genus *Acer*. *New Phytologist* **190**: 709–723.
- Lens F, Smets E, Melzer S. 2012. Stem anatomy supports *Arabidopsis thaliana* as a model for insular woodiness. *New Phytologist* **193**: 12–17.
- Maddison DR, Maddison WP. 2002. *MacClade 4.04*. Sunderland, MS: Sinauer Associates Inc.
- Melzer S, Lens F, Gennen J, Vanneste S, Rhode A, Beekman T. 2008. Flowering time genes modulate meristem determinacy and growth form in *Arabidopsis*. *Nature Genetics* **40**: 1489–1492.
- Newman MF. 2008. *Impatiens pachycaulon* (Balsaminaceae) a new species from Laos. *Edinburgh Botanical Journal* **65**: 23–26.
- Nieminen KM, Kauppinen L, Helariutta Y. 2004. A weed for wood? *Arabidopsis* as a genetic model for xylem development. *Plant Physiology* **135**: 635–659.
- Oh S, Park S, Han KH. 2003. Transcriptional regulation of secondary growth in *Arabidopsis thaliana*. *Journal of Experimental Botany* **54**: 2709–2722.
- Perrier de la Bathie H. 1948. Révision des *Impatiens* de Madagascar et des Comores. *Mémoires de l'Académie des Sciences* **67**: 1–16.
- Raunkjær C. 1934. *Life forms of plants and statistical plant geography*. Oxford: Clarendon.
- Sattler R. 1996. Classical morphology and continuum morphology: opposition and continuum. *Annals of Botany* **78**: 577–581.
- Schols P, D'Hondt C, Geuten K, Merckx V, Janssens S, Smets E. 2004. MorphoCode: coding quantitative data for phylogenetic analyses. *Phyloinformatics* **4**: 1–4.
- Schrader J, Nilsson J, Mellerowicz E, et al. 2004. A high-resolution transcript profile across the wood-forming meristem of poplar identifies potential regulators of cambial stem identity. *The Plant Cell* **16**: 2278–2292.
- Schweingruber FH. 2007. *Wood structure and environment*. Berlin: Springer.
- Schweingruber FH, Börner A, Schulze E-D. 2011. *Atlas of stem anatomy in herbs, shrubs and trees*, Vol. 1. Heidelberg: Springer.
- Solereder H. 1899. *Systematische Anatomie der Dicotyledonen*. Stuttgart: Verlag von Ferdinand Enke.
- Soltis PS, Soltis DE. Applying the bootstrap in phylogeny reconstruction. *Statistical Science* **2**: 256–267.

- Sperry JS, Hacke UG, Pittermann J. 2006. Size and function in conifer tracheids and angiosperm vessels. *American Journal of Botany* **93**: 1390–1500.
- Sperry JS, Hacke UG, Field TS, Sano Y, Sikkema EH. 2007. Hydraulic consequences of vessel evolution in angiosperms. *International Journal of Plant Sciences* **168**: 1127–1139.
- Spicer R, Groover A. 2010. Evolution of development of vascular cambia and secondary growth. *New Phytologist* **186**: 577–592.
- Stamatakis A. 2006. RAxML-VI-HPC: maximum likelihood-based phylogenetic analyses with thousands of taxa and mixed models. *Bioinformatics* **22**: 2688–2690.
- Stamatakis A, Ludwig T, Meier H. 2005. RAxML-III: a fast program for maximum likelihood-based inference of large phylogenetic trees. *Bioinformatics* **21**: 456–463.
- Swofford DL. 2002. *PAUP**. *Phylogenetic Analysis Using Parsimony (*and Other Methods)*. Version 4.0b. Sunderland, MA: Sinauer Associates.
- Thiele K. 1993. The holy grail of the perfect character: the cladistic treatment of morphometric data. *Cladistics* **9**: 275–304.
- Tilman D. 1988. *Resource competition and community structure*. Princeton, NJ: Princeton University Press.
- Wallace AR. 1878. *Tropical nature and other essays*. London: Macmillan.
- Yuan Y-M, Song Y, Geuten K, et al. 2004. Phylogeny and biogeography of Balsaminaceae inferred from ITS sequence data. *Taxon* **53**: 391–404.

APPENDIX

List of taxa investigated in this study with reference to their locality and vouchers.

- Hydrocera triflora* (L.) Wight & Arn., grown at BR, 20060140-55; *Impatiens amplexicaulis* Edgew., India, Maas & Geesteranus s.n., NCB Naturalis, L0388626; *I. arguta* Hook.f. & Thomson, grown at BR, 20060001-13; *I. aurea* Muhl., USA, Meeuse 13, NCB Naturalis, L0388634; *I. auricoma* Baill., grown at BR, 20060034-46; *I. balfourii* Hk.f., France, Wright senior s.n., NCB Naturalis, L0388666; *I. balsamina* L., grown at BR, 20091329-10; *I. bicaudata* H.Perrier, grown at BR, 20020131-10; *I. biflora* Walter, France, student's expedition 210, NCB Naturalis (U 74807); *I. burtonii* Hook.f. ssp. *burtonii*, Uganda, collector unknown, grown at BR, 20090336-70; *I. campanulata* Wight, grown at BR, 20020132-11; *I. capensis* Meerb., France, Mennema s.n., NCB Naturalis, L0388795; *I. catati* Baill., grown at BR; *I. cecili* N.E.Br., grown at BR, 20060002-14; *I. clavigera* Hook.f., Thailand, Maxwell 1278, NCB Naturalis, L0201398; *I. curvipes* Hook.f., Thailand (Chiang Mai), Maxwell 654, NCB Naturalis, L0383007; *I. dewildeana* Grey-Wilson, Sumatra, de Wilde & de Wilde-Duyfjes 14030, NCB Naturalis, L001382; *I. edgeworthii* Hook.f., Germany, Janssens 003-2008 (LV); *I. eriosperma* H.Perrier, grown at BR, 20090397-55; *I. eubotrya* Miq., Sumatra, van Borssum Waalkes 2164, NCB Naturalis, L0388893; *I. flaccida* Arn., grown at BR, 19680576; *I. gesneroidea* Gilg., Laarman s.n., DR Congo, NCB Naturalis, L0388905; *I. glandulifera* Royle, Belgium, Lens & Janssens s.n, LV; *I. glandulosa* Tardieu, origin unknown, collector unknown, NCB Naturalis, L0383043; *I. grandis* B.Heyne, grown at BR, 20090002-41; *I. griffithii* Hook.f. & Thomson, Malaysia, Samsuri Amad. 299, NCB Naturalis, L0388920; *I. havilandii* Grey-Wilson, Borneo, Chew, Corner & Staiton 1050, NCB Naturalis, L0388925; *I. hawkeri* W.Bull, unknown origin, Janssens 006 (LV); *I. henslowiana* Arn., Sri-Lanka, collector unknown, grown at BR, 20090338-86; *I. hians* Hook.f. ssp. *hians*, grown at BR, 20060003-15; *I. hochstetteri* Warb. ssp. *hochstetteri*, grown at BR, 20091294-72; *I. inaperta* H.Perrier, Madagascar, grown at BR, 20090340-88; *I. irvingii* Hook.f. ex Oliv., grown at BR, 20081389-61; *I. jurpia* Buch.-Ham. ex Hook.f. & Thomson, Thailand (Chiang Mai), Maxwell 490, NCB Naturalis, L0383002; *I. keilii* Gilg ssp. *keilii*, Tanzania, grown at BR, 20090341-89; *I. kilimanjari* Oliv., grown at BR, 2006004-16; *I. kilimanjari* Oliv. × *pseudoviola* Gilg, grown at BR, JMG 94613; *I. latifolia* L. ssp. *bipartita* Grey-Wilson, Sri Lanka, Comanor 92, NCB Naturalis, L0389078; *I. leschenaultii* Wall., India, Hohenacker 1138, NCB Naturalis, L038990; *I. lyallii* Baker var. *trichogyna* H.Perrier, Madagascar, Bai & Vohinar s.n., NCB Naturalis, L0389107; *I. mackeyana* Hook.f. ssp. *clari* (N.Hallé) Grey-Wilson, grown at BR, 20090003-42; *I. macrophylla* Gardner ex Hook.f., Sri Lanka, Kostermans 24608, NCB Naturalis, L0389876; *I. masonii* Hook.f., Thailand (Chiang Mai), Maxwell s.n., NCB Naturalis, L0799401; *I. mengtzeana* Hook.f., Thailand, Maxwell 1426, NCB Naturalis, L0201700; *I. mirabilis* Hook.f., Malaysia (Pulau Langkawi), van Balgooy 2361, NCB Naturalis, L0383038; *I. namchabarwensis* Morgan R.J., Yuan YM & Ge XJ, grown at LV; *I. nianniamensis* Gilg emend. G.M.Schulze, grown at BR, 19770093; *I. noli-tangere* L., Germany, Janssens 005-2008 (LV); *I. nomenya* Eb.Fish. & Raheliv., grown at BR, 20090349-00; *I. omeiana* Hook.f., grown at Botanic Garden in Ghent, 2002-1323; *I. opinata* Craib, Thailand (Kanchanaburi), Maxwell 1160, NCB Naturalis, L0382997; *I. parasitica* Bedd., grown at BR, 20020134-13; *I. parviflora* DC., Belgium, Lens & Janssens s.n., LV; *I. platypetala* Lindl., grown at BR, 20090005-44; *I. pseudomacroptera* Grey-Wilson, Gabon, Dessein 2023 (BR); *I. pseudoviola* Gilg, grown at BR; *I. psittacina* Hook.f., Thailand (Chiang Mai), Maxwell 1072, NCB Naturalis, L0382984; *I. purpureo-violacea* Gilg, Rwanda, grown at BR, 20090353-04; *I. repens* Moon, grown at BR; *I. shirensis* Baker F., Malawi, Brass 16423, NCB Naturalis, L0389737; *I. sodenii* Engl. & Warb. ex Engl., grown at BR, 20090006-45; *I. stenantha* Hook.f., India, Hooker 1239, NCB Naturalis, L0389743; *I. stuhlmannii* Warb., E. Africa, grown at BR, 16583; *I. cf. stuhlmannii* Warb., grown at BR, 20090356-07; *I. usambarensis* Grey-Wilson, grown at BR, 20090359-10; *I. vaughanii* Hook.f., Thailand (Nan), Maxwell 765, NCB Naturalis, L0201341; *I. violaeiflora* Hook.f., Thailand, Maxwell s.n., NCB Naturalis, L0389801; *I. viscida* Wight, grown at BR, 20020133-12; *I. vitellina* Grey-Wilson, Sumatra, de Wilde & de Wilde-Duyfjes 13812, NCB Naturalis, L0389805.