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What is *Nephrolepis* '*bostoniensis*'? Unravelling the origin of *Nephrolepis* hybrids and cultivars with molecular data

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ABSTRACT

Hybridization, polyploidization, and reticulate evolution are major mechanisms that shape the current fern diversity. Based on molecular phylogenetic analyses of chloroplast DNA (*psbA-trnH*, *trnG-trnR*) and low-copy nuclear DNA (*gapCp*) sequences and flow cytometric analysis, we attempt to clarify the origin of putative *Nephrolepis* hybrids and cultivars. Our results confirm the presence of several hybrids, among which *Nephrolepis 'bostoniensis'* and *Nephrolepis x hippocrepicis*. Two widespread species, *Nephrolepis biserrata* and *Nephrolepis cordifolia* are revealed as the central species in the formation of these hybrids, with *Nephrolepis brownii* probably occasionally contributing as well. The C-values range indicates that all analysed *Nephrolepis* taxa are diploid except for *Nephrolepis hirsutula*, which is probably a tetraploid. All hybrids and cultivars are found to be diploid or homoploid hybrids. Additionally, our results suggest that *Nephrolepis davallioides* might also be a hybrid, possibly of multiple origins.

1. Introduction

Hybridization and polyploidization are rampant in ferns (Barrington et al., 1989) and considered major mechanisms that shape the current diversity of fern species. At least 95% of all fern species are estimated to have been involved in polyploidization during their evolutionary history (e.g., (Grant, 1981; Haufler, 2002)), although Wood et al. (2009) estimated this figure to be much lower, 31%, based on data from cytogenetic and phylogenetic databases. More than seed plants, ferns are susceptible to interspecific hybridization as they lack pre-mating isolation mechanisms such as pollinator specificity, reproductive organ specificity, and phenological seasonality (Smith, 1971; Haufler, 2002). Eventually, the processes of hybridization and polyploidization have the potential to obscure species circumscriptions and phylogenetic relationships among fern species, especially in complexes of closely related species (e.g. *Asplenium*, (Shepherd et al., 2008); *Cheilanthes yavapensis* complex, (Grusz et al., 2009); *Pteris cadieri* complex, (Chao et al., 2012); *Dryopteris*, (Sessa et al., 2012); *Pteris cretica* group, (Jaruwattanaphan et al., 2013)) .

Nephrolepis is a pantropical genus of approximately 19 species (Hovenkamp and Miyamoto 2005), and is probably the most popular source of commercial cultivars in ferns. The genus is characterized by the presence of proliferous stolons promoting vegetative propagation, which has probably contributed substantially to its popularity in cultivation. *Nephrolepis* cultivars differ from the normally pinnate fronds found in this genus by frequently sporting bi- to quadripinnate fronds as well as forked and ruffled pinnae (Morton, 1958; Hoshizaki and Moran, 2001). The "Boston fern", *Nephrolepis exaltata* 'Bostoniensis' or *N.* '*bostoniensis*' is the acknowledged source of most of the cultivars (Benedict, 1916) and may well be the commonest of all indoor ferns. Its origin, however, has always been unclear (Benedict, 1915; Benedict, 1916), although it has been consistently associated with *N. exaltata* (Sw.) Schott. *N. exaltata* is a Neotropical species with a native range including Florida, the Caribbean, parts of Central America, northern South America, and Hawaii (Hovenkamp and Miyamoto, 2005). In *Nephrolepis*, hybrid origins have been suggested for *N. exaltata* (Hennequin et al., 2010), *N.* '*bostoniensis*' (Morton, 1958) and *N. x hippocrepicis* (Hovenkamp and Miyamoto 2005). That *N. exaltata*

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originated by hybridization between *N. cordifolia* (L.) C. Presl and another unknown species, was suggested by the fact that *N. exaltata* was nested within *N. cordifolia* in the molecular phylogenetic reconstructions of Hennequin et al. (2010). However, inferences about possible hybridization were limited as this study was entirely based on chloroplast DNA markers. Miyamoto in Hovenkamp and Miyamoto (2005) described *N.* x *hippocrepicis* Miyam., as a hybrid between *N. cordifolia* and *N. biserrata* (Sw.) Schott from Japan, morphologically similar to *N. exaltata*, but occurring outside the native range of the latter. The hybrid nature of this species has been confirmed by Kao et al. (2014). Other hybrids have been described (Nauman, 1979; Wagner et al., 1999), which all have abortive spores, in contrast to the normal spores formed by the species not suspected to be hybrids (Hovenkamp and Miyamoto 2005). Morton (1958) suggested that *N.* '*bostoniensis*', which also does not normally form viable spores (Benedict 1915; but see also Benedict, 1924, who reports the occurrence of a fertile form; Hoshizaki and Moran, 2001) arose as a hybrid between *N. exaltata* and another unknown parent, but no evidence has supported this hypothesis. Moreover, in contrast to other fern genera, available cytological data indicate that polyploidization, which is often associated with hybridogenous speciation, seems to be rare in *Nephrolepis* (e.g. (Manton and Sledge 1954; Löve et al., 1977; Obermayer et al., 2002; Tindale and Roy 2002; Lange et al., 2005)).

To resolve problems of hybridization, polyploidization, and reticulate evolution in ferns, phylogenetic analysis of single or low copy nuclear genes can be used (e.g. *LFY* intron, (Hoot et al., 2004); *pgiC* gene, (Juslen and Vare Wikstrom, 2011); *gapCp* gene, (Ebihara et al., 2005; Schuettpelz et al., 2008; Grusz et al., 2009; Jaruwattanaphan et al., 2013)). As nuclear genes are biparentally inherited, in contrast to cpDNA that is maternally inherited in ferns (Gastony and Yatskievych, 1992), a comparison of phylogenetic reconstructions based on markers from both genomes may identify hybrid species and with luck determine both of their parents (Small et al., 2004).

Due to the horticultural importance of *Nephrolepis*, clarifying the evolutionary origin of hybrids and cultivars in this genus is of broad interest. In the present study, we aim to resolve the origins of the putative hybrids *N. exaltata*, *N.* '*bostoniensis*', and *N.* x *hippocrepicis*, based on sequences from two chloroplast regions, *psbA-trnH* and *trnG-trnR*, and a low-copy nuclear region, *gapCp*, and on nuclear weight-values for a number of *Nephrolepis* taxa.

2. Materials and methods

2.1. Taxon sampling

Taxon sampling comprised one specimen of *N. exaltata*, three of *N.* '*bostoniensis*', and one of *N.* x *hippocrepicis*, as well as specimens of *N. biserrata*, *N. brownii* (Desv.) Hovenkamp & Miyam., *N. cordifolia*, *N. davallioides* (Sw.) Kunze, *N. hirsutula* (G. Forst.) C. Presl, *N. lauterbachii* H. Christ, *N. pectinata* (Willd.) Schott, *N. rivularis* (Vahl) Mett. ex Krug, and *N. undulata* (Afzel.) J. Sm. The specimen of *N. exaltata* originated from Jamaica, the specimens of *N.* '*bostoniensis*' were obtained from commercial sources, and two DNA samples were taken from a plant of *N.* x *hippocrepicis* cultivated at the Hortus Botanicus in Leiden (donated by F. Miyamoto). Specimens from the other species were taken from fresh collections, silica dried material, or herbarium material from L and NY (Appendix A in Supplementary data), acronyms following Index Herbariorum (Thiers continuously updated). In case of the widespread species *N. biserrata*, *N. cordifolia*, and *N. undulata* at least one sample from all major parts of the geographical distribution areas of the species was sampled, where possible. As no material of the two other genera of Lomariopsidaceae included in earlier phylogenetic analyses (*Cyclopeltis* and *Lomariopsis*; Schuettpelz and Pryer, 2007; Hennequin et al., 2010) was available, a specimen of *Oleandra* sp. (Oleandraceae) was designated as outgroup representative. *Oleandra* has traditionally been treated as closely related to *Nephrolepis* (e.g. (Holttum, 1949; Schelpe, 1970; Verdcourt, 2001)), and belongs to the sister clade of Lomariopsidaceae within the Eupolypods I part 2 clade of Schuettpelz and Pryer (2007), similar to the outgroup representatives *Arthropteris* and *Tectaria* in Hennequin et al. (2010).

2.2. DNA extraction, amplification, cloning, and sequencing

Total DNA was extracted using the DNeasy Plant II Mini Kit (Qiagen, Hilden, Germany). PCR amplification of the chloroplast regions was performed in a volume of 50 μl containing 2.0 μl DNA template, 5.0 μl of 10× PCR buffer (Qiagen), 1.0 μl of 10 pmol of each primer, 2.0 μl of 2.5 mM dNTP's, 2.5 μl of 50 mM $MgCl_2$, 2.0 μl of 10 mg/ml bovine serum albumin (BSA; Promega, Madison, Wisconsin, USA) (if necessary, especially for the amplification of herbarium material), and 0.4 μl of 5 U/μl Taq DNA polymerase (Qiagen). The *psbA-trnH* region was amplified using primers psbAF and trnHR (Sang et al., 1997) and a PCR profile of an initial denaturation at 95 °C for 5 min, followed by 35 cycles of: denaturation at 95 °C for 30 s, annealing at 48 °C for 1 min, and elongation at 72 °C for 1 min and 40 s, and a final elongation step at 72 °C for 7 min. The *trnG-trnR* region was amplified using primers trnG1F and trnR22R (Nagalingum et al., 2007) and a PCR profile of an initial denaturation at 94 °C for 3 min, 35 cycles of: denaturation at 94 °C for 30 s, annealing at 50 °C for 30 s, and elongation at 72 °C for 1 min, and a final elongation step at 72 °C for 7 min.

For the *gapCp* region, PCR amplification was performed in a volume of 40 μl containing 2.0 μl DNA template, 4.0 μl of 10× PCR buffer (Qiagen), 2.0 μl of 10 pmol of each primer, 2.0 μl of 2.5 mM dNTP's, 2.0 μl of 50 mM $MgCl_2$, 0.4 μl of 10 mg/ml BSA, and 0.4 μl of 5 U/μl Taq DNA polymerase (Qiagen). Amplification primers were ESGAPCP8F1 and ESGAPCP11R1 (Schuettpelz et al., 2008), or, in the case of some recalcitrant specimens, gapCp_F2 (TCAACTGGTGCTGCYAAGGT) and gapCp_R2 (TCCTGCTTTTGCATCAAAGAT), which were newly designed using the program Primer3 (Rozen and Skaletsky, 2000). The amplification profile comprised an initial denaturation at 94 °C for 5 min, 35 cycles of: denaturation at 94 °C for 30 s, annealing at 59 °C for 30 s, and elongation at 72 °C for 1 min, and a final elongation step at 72 °C for 10 min.

Amplification of the *gapCp* region usually resulted in double bands. In case of double bands, the band of approximately 600 bp in size (cf. Schuettpelz et al., 2008; Grusz et al., 2009) was excised, purified with the Wizard® SV Gel and PCR Clean-Up kit (Promega, Madison, Wisconsin, USA) and cloned using the TOPO TA Cloning kit (Invitrogen, Carlsbad, California, USA) following the suppliers' protocols. After overnight incubation, 8–24 white colonies were selected for colony PCR amplification in a 30 μl reaction using primers M13Fi and M13Ri. The colony PCR amplification profile comprised an initial denaturation at 95 °C for 3 min, 35 cycles of: denaturation at 95 °C for 20 s, annealing at 50 °C for 20 s, and elongation at 72 °C for 90 s, and a final elongation step at 72 °C for 10 min.

PCR products were purified and sequenced at Macrogen Amsterdam (www.macrogen.com) using the amplification primers and, for *trnG-trnR*, the additional internal sequencing primers trnG43F1 and trnG63R (Nagalingum et al., 2007).

2.3. Sequence alignment and phylogenetic analysis

Sequences were edited and manually aligned in BioEdit (Hall, 1999). Two data sets, the combined chloroplast (*psbA-trnH*, *trnG-trnR*) and the nuclear (*gapCp*) sequences, were analysed separately

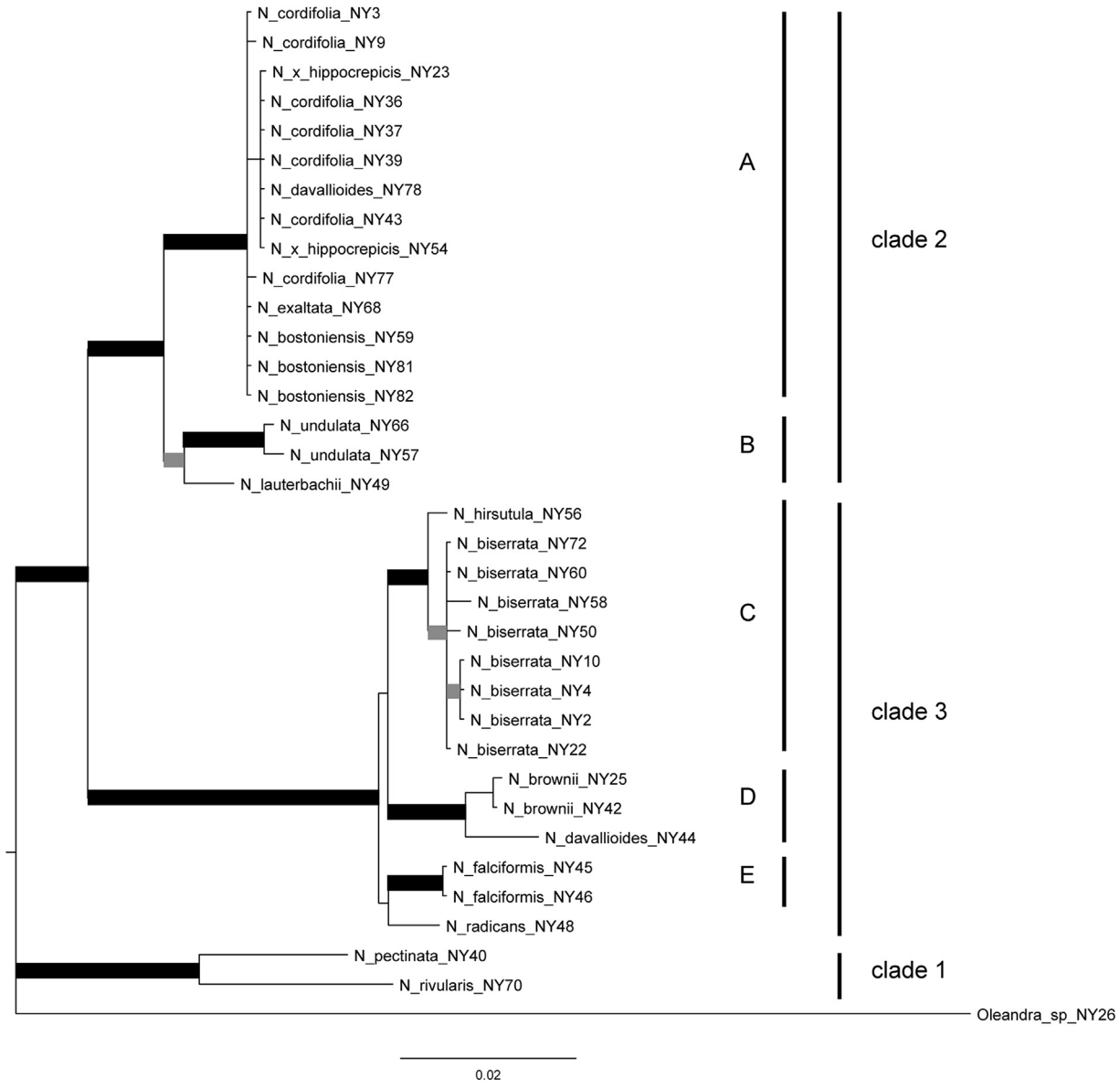

Fig. 1. Bayesian majority rule consensus tree for the combined chloroplast regions, *psbA-trnH* and *trnG-trnR*. Posterior probabilities (PP) and bootstrap support values from maximum parsimony analysis (BS) are indicated by branch formatting: Thick black branches indicate PP ≥ 0.95 and BS ≥ 95, thick grey branches PP ≥ 0.95 and BS ≥ 70, thin black branches indicate either low or no support. Branch lengths are proportional to mean node height. Numbers following species name are the accession numbers.

using maximum parsimony (MP) and Bayesian inference (BI). For *gapCp*, consensus allele sequences within the individual accessions were generated following the approach of Grusz et al. (2009). Consensus allele sequences were used in the phylogenetic analysis, together with sequence types represented by single clones. Gaps were treated as missing data or coded by the simple indel coding (SIC) approach of Simmons and Ochoterena (2000), as implemented in SeqState (Müller, 2005).

Parsimony analyses were performed in TNT 1.1 (Goloboff et al., 2003). All characters were treated as unordered and equally weighted. Tree searches were conducted using heuristic search methods with 100 replicates of random taxon addition combined with tree-bisection-reconnection (TBR) branch swapping. Bootstrap analyses (Felsenstein, 1985) were performed to determine the branch support using heuristic search with 1000 pseudoreplicates and TBR branch swapping. All analyses for both chloroplast and nuclear regions were run without and with gaps coded by SIC, in order to assess their effect on topology and clade support.

Best-fit models of nucleotide substitution under Akaike's Information Criterion (AIC) were determined for each marker in MrModeltest 2.3 (Nylander, 2008). Bayesian analyses were performed employing the Markov Chain Monte Carlo (MCMC) approach as implemented in MrBayes v.3.2 (Huelsenbeck and Ronquist, 2001; Ronquist and Huelsenbeck, 2003). For the combined chloroplast data set, the analysis was run with two partitions, employing the best-fit model for each chloroplast regions; GTR + Γ (nst = 6, rates = gamma) for *psbA-trnH* and GTR + I (nst = 6, rates = propinv) for *trnG-trnR*. The best-fit model for *gapCp* was GTR + Γ (nst = 6, rates = gamma). For the analyses with gaps included, a binary model was applied to the respective indel matrices. Bayesian analyses were carried out with all other settings at default values using MrBayes on the CIPRES Science Gateway (Miller et al., 2010). The standard deviation of split frequencies (as calculated by MrBayes) as well as trace plots generated in Tracer v1.5 (Rambaut and Drummond, 2009) were used to confirm the convergence of the runs. Fifty-percent majority-rule consensus

trees and posterior probabilities were calculated by MrBayes after excluding the default burnin of 25%.

2.4. Flow cytometry

Measurements of DNA contents were obtained for *Nephrolepis* '*bostoniensis*', *N. brownii*, *N. cordifolia*, *N. davallioides*, *N. falcata*, *N. falciformis*, *N. lauterbachii*, *N. radicans*, and *N.* x *hippocrepicis*, to complement published C-values (Obermayer et al., 2002). For the isolation of nuclei, about 1 cm^2 of leaf or a few cm of root was chopped together with a piece of *Agave americana* L. 'Aureomarginata' as an internal standard. Chopping was done with a new razor blade in a Petri dish in 0.25 ml nuclei-isolation buffer (Galbraith et al., 1983) to which 0.25 mg RNase/ml was added (Zonneveld and Van Iren, 2001). After adding 1.75 ml propidium iodide solution (50 mg PI/l in isolation buffer) the suspension with nuclei was filtered through a 30 μm nylon filter. The fluorescence of the nuclei was measured half an hour and one hour after addition of propidium iodide, using a CA-II flow cytometer (Partec, Münster, Germany). The optical path contained a HBO mercury lamp, filters KG1, BG12, dichroic mirror TK500, filter OG570 and a Leitz 50 × 1 water immersion objective. Data were analysed by means of DPAC software (Partec). Fresh male human leucocytes (2C = 7.0 pg; 1 picogram = 10–12 g = 0.978 × 109 base pairs) (Dolezel et al., 2003) were chosen as primary standard (Tiersch et al., 1989). This yields 2C = 15.9 pg for nuclei of *A. americana*. The 2C DNA content of the sample was calculated as the sample peak mean, divided by the *Agave* peak mean, and multiplied with the amount of DNA of the *Agave* standard. Most samples were measured at least four times, each time with about 5000 nuclei. Most histograms revealed a coefficient of variation of around 5%. The standard deviation for nuclear DNA content, using all relevant measurements, was about 2%.

3. Results

3.1. Chloroplast DNA sequence variation and phylogeny

The length of combined chloroplast alignment was 1672 positions (*psbA-trnH* positions 1–460, *trnG-trnR* 461–1672), of which 200 were parsimony-informative (*psbA-trnH* 29, *trnG-trnR* 171). Simple indel coding added another 87 (*psbA-trnH* 22, *trnG-trnR* 65) parsimony-informative indel characters.

Phylogenetic reconstructions without and with indels included showed slightly different topologies, but no incongruence with respect to significantly supported clades. Therefore, in the following the reconstructions without indel coding are discussed.

Parsimony analysis yielded a single most parsimonious tree (MPT) of 526 steps. The topology of the majority-rule consensus tree was similar to the majority rule consensus tree obtained from Bayesian inference (BI) (Fig. 1). All trees recovered three well supported main clades that within the limits of our taxon sampling correspond to the results of Hennequin et al. (2010), and for ease of comparison we will adopt the same clade terminology. We recovered Clade 1 with posterior probability PP = 1, and parsimony bootstrap support BS = 100%, but in a different position than in Hennequin et al. (2010), as sister to the remaining ingroup. We recovered Clade 2 with PP = 0.99 and BS = 100%, and Clade 3 with PP = 1 and BS = 100%.

Our results confirmed the structure of Clade 2 with two well-supported subclades, the *N. cordifolia* clade (subclade A; PP = 1, BS = 100%) and the *N. lauterbachii-N. undulata* clade (subclade B; PP = 1, BS = 100%). Subclade A comprised, in addition to *N. cordifolia*, the sequenced specimens of *N.* '*bostoniensis*', *N. davallioides* p.p., *N. exaltata*, and *N.* x *hippocrepicis*. The latter formed an unsupported clade with one specimen of *N. davallioides* (NY78) and four of the *N. cordifolia* accessions originating mainly from Asia and South America. In subclade B, the two specimens of *N. undulata* from Africa and South America were supported as monophyletic (PP = 1, BS = 100). Clade 3 contained three supported subclades with maximum support, one containing *N. biserrata* and *N. hirsutula* (C), one with *N. brownii* and *N. davallioides* p.p. accession NY44 (D), and one containing the two accessions of *N. falciformis* (E).

3.2. Nuclear DNA sequence variation and phylogeny

The *gapCp* alignment consisted of 724 positions, of which 190 were parsimony-informative. Simple indel coding added another 45 parsimony informative indel characters. Analyses with or without indels included yielded highly similar trees, therefore only the reconstructions without indels included are discussed.

Parsimony analysis yielded over 30000 most parsimonious trees (MPTs) of 349 steps. Both MP and BI reconstructions (Bayesian tree shown in Fig. 2) were congruent with respect to significantly supported clades and recovered a similar topology as in the chloroplast phylogeny, comprising clade 1 (PP = 1, BS = 96%), Clade 3 (PP = 1, BS = 99%), and Clade 2 without support.

In Clade 3 we distinguished six main subclades (A–F). Subclade A (PP = 1, BS = 91%) consisted of *N. biserrata* as well as *N. exaltata* and *N.* '*bostoniensis*' alleles. Subclade B (PP = 0.98, BS < 50%) only consisted of *N. falciformis* alleles. The unsupported subclade C comprised *N.* x *hippocrepicis* alleles together with allele 2 of *N. biserrata* NY22 and allele 1 of *N. biserrata* NY4. Subclade D (PP = 1, BS = 72%), contained alleles 2 and 3 of *N. exaltata*, allele 2 of *N. davallioides* NY78, allele 2 of *N. cordifolia* NY43, and both accessions of *N. brownii*. Subclade E (PP = 1, BS = 78%) only consisted of *N. biserrata* alleles. In subclade F (without support), all alleles of *N. radicans* and of *N. davallioides* formed monophyletic sister groups with maximum support, in contrast to the sistergroup relationship of *N. radicans* with *N. falciformis* in the chloroplast phylogeny (Fig. 1), together being sister to allele 1 of *N. hirsutula*. *Nephrolepis hirsutula*-alleles were reconstructed as non-monophyletic, although without significant support, as the other allele was recovered without support as sister to subclades A, B and C.

We distinguished three main subclades G–I in Clade 2. Subclade G (PP = 1, BS = 90%) included *N.* '*bostoniensis*', *N. cordifolia*, *N. davallioides* NY78, and *N.* x *hippocrepicis,* as sister to a clade containing allele 1 of *N. davallioides* NY44 and allele 1 of *N. exaltata*, and both together were sister to *N. cordifolia* NY77 from Hawaii. Subclade H (PP = 1, BS = 96%) consisted of only *N. lauterbachii* alleles and was sister to subclade G, in contrast to its sistergroup relationship with *N. undulata* in the chloroplast phylogeny. Subclade I was also well-supported (PP = 1, BS = 92%) and consisted of the alleles of the two accessions of *N. undulata,* which were reciprocally monophyletic.

3.3. DNA content

We measured the 2C DNA content of 26 accessions from 12 *Nephrolepis* species (Table 1), of which nine species are new records, namely *N.* '*bostoniensis*', *N. brownii*, *N. cordifolia*, *N. davallioides*, *N. falcata*, *N. falciformis*, *N. lauterbachii*, *N. radicans*, and *N.* x *hippocrepicis*.

The C-values obtained from our analysis range from 2C = 13.6 pg in *N. cordifolia* to 2C = 43.0 pg in *N. hirsutula* (Table 1). For taxa with multiple accessions, the C-value estimates may agree closely with each other (e.g. *N. brownii*, 2C = 20.6, 20.9, and 20.9 pg) while for other taxa, the range of estimates is wider (e.g. *N. cordifolia*, 2C = 13.6 to 20.6 pg) (Table 1). The C-value range for all the species in this study is more or less unequivocally within the published C-value range except for *N. hirsutula,* of which the value is two times higher than the estimates of other species and so far the highest estimate for *Nephrolepis*.

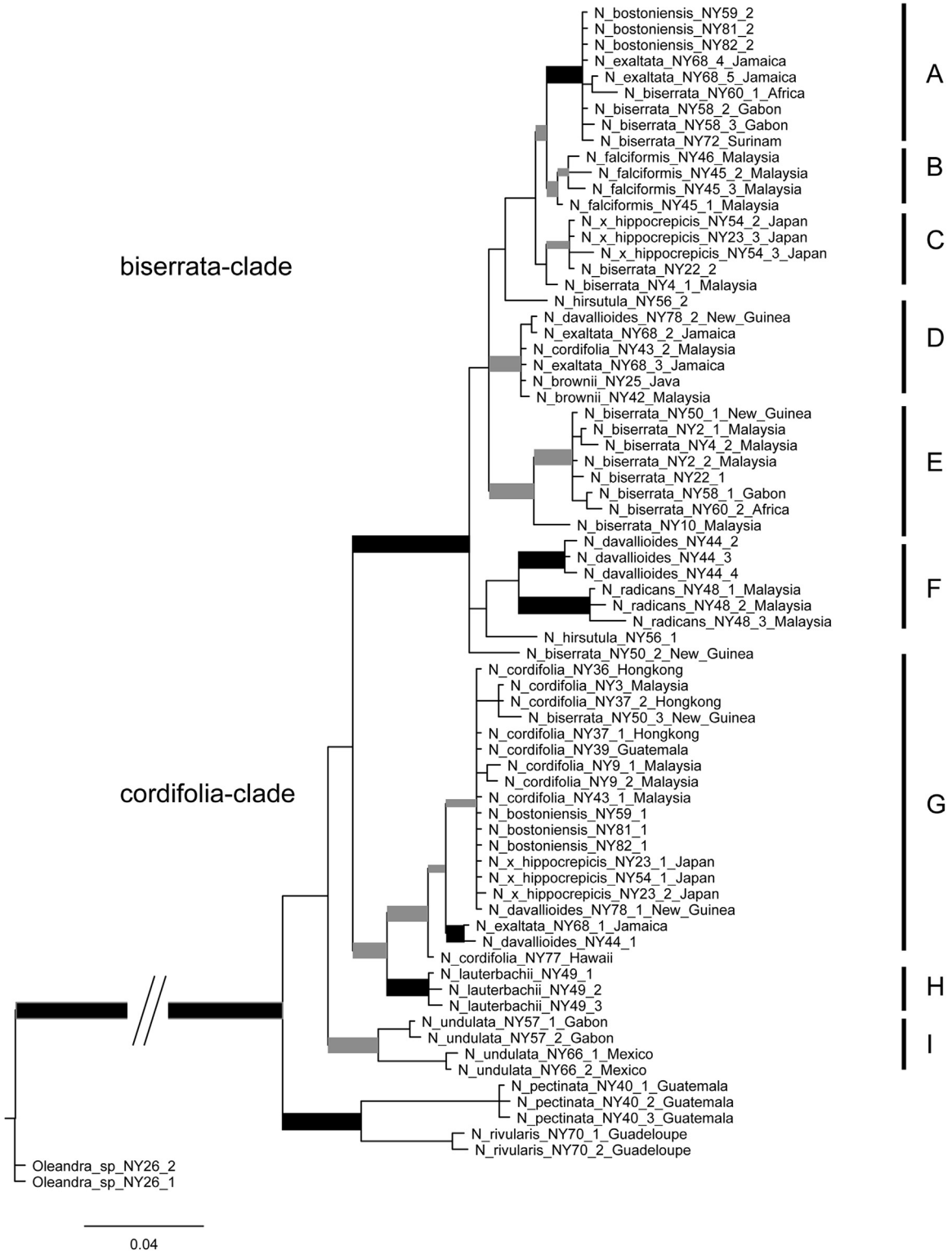

Fig. 2. Bayesian majority rule consensus tree of the nuclear region *gapCp*. Posterior probabilities (PP) and bootstrap support values from maximum parsimony analysis (BS) are indicated by branch formatting: thick black branches indicate PP ≥ 0.95 and BS ≥ 95, thick grey branches PP ≥ 0.95 and BS ≥ 70, semi thick grey branches PP ≥ 0.95 and BS < 70, and thin black branches indicate either lower or no support. Branch shortened (to c 1/2) for clarity is indicated by hash mark. Species names are followed by accession numbers, allele numbers and geographic origins of the specimens.

In order to determine roughly the ploidy level of our samples, we compared their C-values with the published *Nephrolepis* chromosome and C-value counts (Löve et al., 1977; Obermayer et al., 2002; Tindale and Roy 2002). All species with a published C-value in the lower range (*N. biserrata*, 2C = 19.0 pg; *N. cordifolia* 'duffii', 2C = 13.6 pg; and *N. exaltata*, 2C = 19.1 pg), of which chromosome numbers have been established, are considered diploids (Löve et al., 1977). This applies to all of our samples except for the analysed *N. hirsutula* (2C = 43.0 pg), which is accordingly inferred to represent a tetraploid.

TABLE 1
DNA C-values for 26 *Nephrolepis* studied together with chromosome numbers published in Manton and Sledge (1954), Löve et al. (1977), Obermayer et al. (2002), and Tindale and Roy (2002). HL: Hortus Botanicus Leiden registration number.

Samples	ID	Source or reference	Published 2*n*	DNA amount, 2C (pg)
N. biserrata (Sw.) Schott	NY2	HL20100060	82	15.2 ± 0.1
N. biserrata (Sw.) Schott	NY22	HL20010166	82	18.5 ± 0.3
N. biserrata (Sw.) Schott	NY4	HL20100061	82	18.6 ± 0.3
N. biserrata (Sw.) Schott	NY50	HL912019	82	19.4
N. '*bostoniensis*'		B. Zonneveld		17.8 ± 1.1
N. brownii (Desv.) Hovenkamp & Miyam.		HL23447		20.6 ± 1.1
N. brownii (Desv.) Hovenkamp & Miyam.	NY42	HL960317		20.9 ± 0.3
N. brownii (Desv.) Hovenkamp & Miyam.	NY25	HL950162		20.9 ± 1.0
N. cordifolia (L.) C. Presl	NY36	HL 20100213	82	13.6 ± 0.1
N. cordifolia (L.) C. Presl		HL 970956	82	13.7 ± 0.1
N. cordifolia (L.) C. Presl		HL 960313	82	14.0 ± 0.2
N. cordifolia (L.) C. Presl	NY37	HL 20100202	82	14.0 ± 0.4
N. cordifolia (L.) C. Presl		HL 20090086	82	14.2 ± 0.4
N. cordifolia (L.) C. Presl		HL 20031301	82	14.5 ± 0.3
N. cordifolia (L.) C. Presl	NY43	HL 960331	82	17.4 ± 0.2
N. cordifolia (L.) C. Presl	NY3	HL 20000576	82	20.6 ± 0.2
N. davallioides (Sw.) Kunze	NY78	HL 912022		17.5 ± 0.6
N. davallioides (Sw.) Kunze	NY44	HL 970975		21.4 ± 0.4
N. '*duffii*' T. Moore		HL 20090085	82	19.7 ± 0.4
N. falcata (Cav.) C. Chr		HL 912014		18.0 ± 1.9
N. falciformis J. Sm.	NY45	HL 960312		20.1 ± 0.6
N. falciformis J. Sm.	NY46	HL 960314		20.2
N. hirsutula (G. Forst.) C. Presl	NY56	HL 20000574	82, 164	43.0 ± 1.2
N. lauterbachii H. Christ	NY49	HL 930105	82	15.3 ± 0.1
N. radicans (Burm. f.) Kuhn	NY48	HL 933329		19.5 ± 0.4
N. x *hippocrepicis* Miyam.	NY23, NY 54	HL 20080064		15.8 ± 0.6

4. Discussion

4.1. Species circumscriptions and patterns of sequence variation

The cpDNA markers analysed here resolve the same three clades that were found by Hennequin et al. (2010), and we will follow their numbering (clades 1–3) in our discussion. The hybrid origin of all three putative hybrid taxa, namely *N. exaltata*, *N.* '*bostoniensis*', and *N.* x *hippocrepicis*, is confirmed, as they have sequences that fall into clade 2, in particular within the sequence variation of *N. cordifolia* (Fig. 1) as well as sequences that fall in clade 3, but more widely distributed there (see detailed discussion below). The *gapCp* sequences resolve seven of the putative non-hybrid species (*N. davallioides*, *N. falciformis*, *N. lauterbachii*, *N. pectinata*, *N. radicans*, *N. rivularis*, *N. undulata*, and possibly *N. brownii*) as monophyletic, whereas relationships of the remaining taxa are more complicated based on their allelic diversity (Fig. 2). This is not surprising as far as the three taxa or cultivars that were already suspected to be of hybrid origin are concerned. However, we also found a greater than expected allelic diversity in *N. cordifolia, N. biserrata, N. davallioides and N. hirsutula*, which have not been recognized as putative hybrids so far. Coupled with the diversity in nuclear weights observed for these taxa, this suggests that hybridization may have occurred much more frequently in *Nephrolepis* than previously thought, with introgression by backcrossing as a possible explanation for the lack of hybrid morphology.

4.1.1. *Origin of* N. exaltata

The placement of the *N. exaltata* sequences in our phylogenetic reconstructions supports the hypothesis by Hennequin et al. (2010) that *N. exaltata* is of hybrid origin. Its position in the cpDNA phylogeny (Fig. 1) is in agreement with Hennequin et al. (2010) and adds support to *N. cordifolia* as its maternal parent. The sequenced specimen of *N. exaltata* exhibits five types of *gapCp* sequences, of which three are substantially different, and two differ only in a single position at the start of the alignment. The alleles fall into three well supported subclades of mainly different species and different geographic origins, namely subclades A (African and South American *N. biserrata* plus *N.* '*bostoniensis*'), D (Asian *N. brownii, N. cordifolia,* and *N. davallioides*), and G (mostly Asian and Central American *N. cordifolia* plus *N.* '*bostoniensis*' and *N.* x *hippocrepicis*) (Fig. 2), suggesting that our accession is a trigenomic hybrid. In addition to supporting the conjecture of Hennequin et al. (2010) on *N. biserrata* as paternal parent, the present data suggest that an extra parent (most likely *N. brownii)* contributed towards formation of our accession. *Nephrolepis brownii* is widespread in tropical Asia, but less widely distributed compared to the other two potential parent species, *N. biserrata*, and *N. cordifolia,* which are both pantropical. However, *N. brownii* is has very recently expanded its distribution invasively (Hovenkamp and Miyamoto, 2005). In Hawaii, *N. brownii*, *N. cordifolia*, and *N. exaltata* play major roles in the formation of the recently described *Nephrolepis* hybrids *N.* x *copelandii* and *N.* x *medlerae* (Wagner et al., 1999).

For our accession, we did not have a sample for flow cytometric analysis. The source material for our sequences is a plant that at first sight appears to be normally fertile, but produces few, if any, well-formed spores. Published C-values (Obermayer et al., 2002) and chromosome counts (Walker, 1966), however, indicate that *N. exaltata* is a fertile diploid. Our data thus strongly suggest that our source (which is the same accession that was used in Hennequin et al., 2010) does not correspond to what is usually considered *N. exaltata*, but is a plant of complex hybrid origin, that, similar to *N. hippocrepicis,* shows an "exaltata-like" morphology as a result of the combination of characters from different parents.

4.1.2. *Origin of* N. '*bostoniensis*'

The origin of *N.* '*bostoniensis*' is of broad interest to pteridologists and horticulturalists. This cultivar is conventionally thought to be derived from *N. exaltata* and hence it is often referred to as *N. exaltata* 'Bostoniensis'. The present phylogenetic reconstructions finally provide insights into its origin. In the cpDNA phylogeny, all three accessions of *N.* '*bostoniensis*' cluster together with *N. cordifolia* (Fig. 1), revealing that it shares chloroplast sequences with the latter species (in fact, sequences are identical to those of *N cordifolia* accession NY3).

Our *N.* '*bostoniensis*' accessions have two types of nuclear *gapCp* alleles, which are separated into two subclades (Fig. 2). Allele 1 groups with *N. cordifolia*, an allele of *N. exaltata*, and one of *N.* x *hippocrepicis* (Clade 2, subclade G), whereas allele 2 groups with African, Caribbean and South American *N. biserrata* and another allele of *N. exaltata* (Clade 3 subclade A). We can therefore confirm Morton's (1958) hypothesis that *N.* '*bostoniensis*' is a hybrid, while we can identify allele 1 with the maternal parent, and allele 2 with the paternal one, which may be derived from *N. biserrata* or possibly another, not sampled parent in Clade 3. Flow cytometry indicates that *N.* '*bostoniensis*' is a homoploid hybrid without chromosome doubling (Table 1), which might explain its sterility.

4.1.3. *Origin of* N. *x* hippocrepicis

Kao et al. (2014) recently resolved *N.* x *hippocrepicis* as a hybrid between *N. cordifolia* and *N. biserrata*. Our reconstructions, based on different plants and using partly different markers, support their conclusion. Both sequenced accessions of *N.* x *hippocrepicis* are nested within the clade with mainly Asian *N. cordifolia* in the cpDNA phylogeny (Fig. 1), confirming that *N. cordifolia* is the maternal parent of this hybrid.

In the *gapCp* phylogeny (Fig. 2), accessions NY23 and NY54 contain three alleles which clearly cluster in two different clades: alleles 1 and 2 of NY23 and allele 1 of NY54 are nested within the *N. cordifolia* (plus *N.* '*bostoniensis*', *N. davallioides,* and one allele of *N. exaltata*) clade (subclade G), corresponding to the cpDNA data, whereas the other alleles are grouped together with *N. biserrata* (subclade C), confirming *N. biserrata* as the paternal parent. Based on our flow cytometric analysis our specimen is inferred to be a homoploid hybrid. We consider that the presence three different alleles in each of the accessions is most likely due to errors during cloning or PCR: alleles NY23_1 and NY23_2 differ only in a few ambiguities, most of which near the end of the sequence, while alleles NY54_2 and NY54_3 differ in just 2 ambiguity codes. Apparently, *N.* x *hippocrepicis* is generated by ongoing, bidirectional hybridization when the two parents occur together (Kao et al., 2014), and may be more common than the currently known specimens indicate.

The intermediate morphology of *N. x hippocrepicis* with an auriculate acroscopic base as in *N. cordifolia* and reniform indusia as in *N. biserrata* (Hovenkamp and Miyamoto, 2005; Kao et al., 2014) makes this hybrid difficult to distinguish from *N. exaltata.*

4.1.4. *Status of* N. davallioides

The polyphyly of *N. davallioides* in the cpDNA phylogeny (Fig. 1) could indicate the presence of a cryptic species with *N. davallioides* morphology. The *gapCp* phylogeny (Fig. 2), shows that both accessions have alleles in Clade 2 as well as Clade 3, with different closest relatives in the latter (*N. radicans* or possibly *N. brownii*, respectively; Fig. 2). These results imply that *N. davallioides* could be of multiple hybrid origins, with *N. cordifolia* involved in the hybridization in both cases.

4.1.5. N. hirsutula—*allotetraploid or autotetraploid?*

Nephrolepis hirsutula in our study shows a tetraploid cytotype (Table 1). It is significantly supported as sister species to *N. biserrata* in the cpDNA phylogeny (Fig. 1). However, in the *gapCp* phylogeny (Fig. 2), allele 1 is sister to *N. radicans* and *N. davallioides* (subclade F, but without support) and allele 2 is sister to subclades A, B and C, (also without support). This suggests an allopolyploid origin for *N. hirsutula*. However, due to the lack of support the possibility that it originated by autopolyploidization in *N. biserrata* cannot be excluded, but its divergent morphology strongly argues for the involvement of another parent. Previously published ploidy levels of *N. hirsutula* show two cytotypes, diploid with $2n = 82$ (Manton and Sledge, 1954; Abraham et al., 1962) and tetraploid with $2n = 164$ (cf. Löve et al., 1977), but considering the confusion that has always existed between *N. brownii* and *N. hirsutula*, these reports most likely refer to *N. brownii*, which is by far the commoner species in gardens, and weedy in many areas (Hovenkamp and Miyamoto, 2005)

4.2. *Timing and hybridization*

Of all the taxa for which we have recovered a hybrid origin, five have an origin which bridges the gap between Clades 2 and 3. Hennequin et al. (2010) estimated that the divergence between these clades is one of the very earliest divergences in *Nephrolepis*, with an estimated divergence date of 49.45 (28.48–72.31 interval at 95% posterior probability) Myr.

The dates for the hybridization events can be inferred to be much more recent (all dates are taken from Hennequin et al., 2010) for all events that include *N. cordifolia* as one of the parents, as *N. cordifolia* is the result of one of the most recent radiations (c. 1 Myr or less), and thus the hybridization event cannot be older. The origin of *N.* '*bostoniensis*' can be more precisely dated to the 19th Century (Benedict, 1915; Benedict, 1916), although direct eye-witness evidence unfortunately is lacking. *Nephrolepis* x *hippocrepicis* is most likely still being formed, but some clones likely have been in existence long enough to spread by vegetative means over the Ryukyu Islands (Okinawa and Miyako, as reported in Hovenkamp and Miyamoto, 2005).

It is also noteworthy that four of the hybrids display a morphology very similar to *N. exaltata*. *N. exaltata* is a species that shows a strong geographic discontinuity, which is coupled partly with some morphological variability (Wagner et al., 1999; Hovenkamp and Miyamoto, 2005). The frequent re-occurrence of *N. exaltata*-like morphology in hybrids suggests that these disparities may be explained by multiple independent origins of *N. exaltata*, thus increasing the number of hybridization events to more than those we have been able to diagnose unambiguously. If *N. exaltata* is indeed of hybridogenous origin, it must have acquired normal fertility in some way, without chromosome doubling, as must have also occurred in *N. davallioides*.

The recent ages of these hybrids should be viewed in the light of the old age of the divergence they bridge. Rothfels et al. (2015) argue that the lack of reproductive barriers allows ferns to bridge deeper splits than most other organisms. Their point is strongly confirmed by our results. The depth of the divergence bridged by these *Nephrolepis* hybrids is comparable to the one crossed by the hybrid x*Cystocarpium roskamianum* Fraser-Jenk.: the 95% posterior probability ranges almost fully overlap (x*Cystocarpium*: 40.2–76.2 mya, *Nephrolepis*: 28.48–72.31). In addition, it should be noted that *Nephrolepis* has a very effective way of vegetative reproduction by regrowth from creeping stolons. This allows hybrids to persist for long periods, which may have contributed to the acquisition of fertility. Thus, our results show that hybridization across a deep split need not result only in the formation of sterile hybrids, but may substantially contribute to the species diversity in a genus.

5. Implications for *Nephrolepis* horticulture

Nephrolepis '*bostoniensis*' is one of the most frequently cultivated ferns. However, the genetic basis for this business is extremely narrow—it is likely that all popular cultivars trace back to a single plant found in a shipment of 200 plants from Philadelphia (Benedict, 1915; Benedict, 1916). The recurrence of *N. exaltata*-like morphology in hybrids and the involvement of *N. cordifolia* and *N. biserrata* in all of these, and possibly *N. brownii* in one, suggests that it may be possible to broaden the currently very narrow genetic basis for *Nephrolepis*-cultivation by actively pursuing a hybridization program involving *N. cordifolia* and a number of com-

mon *Nephrolepis* species, with *N. biserrata* being the most obvious candidate.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.scienta.2016.04.001.

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