

1 Resolving phylogeny and polyploid parentage using genus-wide
2 genome-wide sequence data from birch trees

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21 **Abstract**

22 Numerous plant genera have a history including frequent hybridisation and
23 polyploidisation, which often means that their phylogenies are not yet fully resolved.
24 The genus *Betula*, which contains many ecologically important allopolyploid tree
25 species, is a case in point. We generated genome-wide sequence data for 27 diploid
26 and 31 polyploid *Betula* species or subspecies using restriction site associated DNA
27 (RAD) sequences assembled into contigs with a mean length of 675 bp. We
28 reconstructed the evolutionary relationships among diploid *Betula* species using both
29 supermatrix and species tree methods. We identified progenitors of the polyploids
30 according to the relative rates at which their reads mapped to contigs from different
31 diploid species. We sorted the polyploid reads into different putative sub-genomes and
32 used the extracted contigs, along with the diploid sequences, to build new phylogenies
33 that included the polyploid sub-genomes. This approach yielded a highly evidenced
34 phylogenetic hypothesis for the genus *Betula*, including the complex reticulate origins
35 of the majority of its polyploid taxa. The genus was split into two well supported
36 clades, which differ in their seed-wing morphology. We propose a new taxonomy for
37 *Betula*, splitting it into two subgenera. We have resolved the parentage of many
38 widespread and economically important polyploid tree species, opening the way for
39 their population genomic study.

40

41 **Key words:** polyploidy, whole genome duplication, hybridization, phylogenomics,

42 *Betula*

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45 1. Introduction

46

47 The evolution of plant diversity cannot be fully understood unless we can reconstruct
48 evolutionary relationships for allopolyploids (hybrid species with duplicated
49 genomes). While phylogenomic approaches that use thousands of loci can resolve the
50 phylogenies of diploid taxa with a history of hybridization (Folk et al., 2017; Fontaine
51 et al., 2015; Li et al., 2016), allopolyploids, which are common in plants (Barker et al.,
52 2016), remain hard to place in phylogenetic trees (Oxelman et al., 2017). When
53 molecular markers are sequenced from polyploids, it is difficult to phase them into
54 their parental subgenomes (Eriksson et al., 2018), and it is easy to mistake
55 homoeologs (genes duplicated in allopolyploidisation events) for paralogs (genes
56 arising from duplication within a genome) and vice versa (Brysting et al., 2011;
57 Linder and Rieseberg, 2004). Approaches to resolving the phylogenetic origins of
58 tetraploids (but not higher ploidy levels) have determined parental genomes using
59 heuristic methods (Jones et al., 2013; Lott et al., 2009) or long-read sequences
60 (Rothfels et al., 2017). While phylogenomic approaches are sometimes used to detect
61 the presence of hybrid polyploids (McKain et al., 2016; Morales-Briones et al., 2018),
62 we are not aware of studies that have used phylogenomic data to resolve polyploid
63 origins.

64

65 Resolving parental origins of polyploid subgenomes unlocks progress in their
66 genomic characterisation. Knowledge of the origins of the allohexaploid genome of
67 *Triticum aestivum* (bread wheat) allowed further characterisation to be assisted by
68 sequencing of the close relatives of its parents: *Aegilops tauschii* and *Triticum*
69 *turgidum* ssp. *dicoccoides* (Avni et al., 2017; Luo et al., 2017). Similarly, the
70 allotetraploid genome of *Gossypium hirsutum* (Upland cotton) has been illuminated
71 by sequencing of two close diploid relatives of its progenitor genomes (Li et al., 2015).
72 However, for many polyploids of economic and ecological importance, we do not
73 know the identity of the closest living relatives of their progenitor genomes. We need
74 accessible phylogenomic approaches to make this possible.

75

76 A relatively inexpensive method of generating genome-wide marker data for
77 phylogenomics from large numbers of individuals is through sequencing of
78 restriction-site associated DNA (RAD) libraries with short reads of 50-150 bp. This is
79 widely used as a method of genome-wide SNP genotyping in non-model organisms
80 for population genomic analyses (Andrews et al., 2016; Barchi et al., 2011; Emerson
81 et al., 2010; Etter et al., 2011; Hohenlohe et al., 2010; Zohren et al., 2016). SNP data
82 from RAD-seq has also been used in phylogenetic reconstruction using supermatrix
83 approaches which assume that all loci have the same evolutionary history (Cariou et
84 al., 2013; Cruaud et al., 2014; Eaton and Ree, 2013; Eaton et al., 2016; Gonen et al.,
85 2015; Hipp et al., 2014; Massatti et al., 2016; Pante et al., 2015; Rubin et al., 2012;
86 Wagner et al., 2013). A few studies have used species tree approaches, which take into
87 account the possibility of different evolutionary histories for separate loci, for analysis
88 of short read RAD-seq data. For example, Eaton and Ree (2013) used RAD loci
89 inferred from single end reads to build a species tree in the genus *Pedicularis*
90 (lousewort) (Eaton and Ree, 2013). DaCosta and Sorensen (2016) used single end
91 reads to construct species trees in two avian genera and Hou et al. (2015) used
92 paired-end reads to build a species tree for the genus *Diapensia* (pincushion plant)
93 (DaCosta and Sorensen, 2016; Hou et al., 2015).

94

95 We reasoned that extra power for phylogenetic analysis may be gained by sequencing
96 RAD libraries with 300 bp paired-end reads and assembling these reads against a
97 reference genome to generate longer contigs spanning restriction enzyme and variable
98 sites. These contigs can be aligned to each other and individual phylogenies
99 reconstructed for each locus, for input into species tree methods, or the alignments
100 combined, for a supermatrix approach. So far, we are not aware of any studies which
101 have sequenced longer RAD loci in an attempt to gain greater power for species tree
102 methods.

103

104 The genus *Betula* (birches) includes about 65 species and subspecies with ranges

105 across the Northern Hemisphere (Ashburner and McAllister, 2016). Some act as
106 keystone species of forests across Eurasia and North America (Ashburner and
107 McAllister, 2016). Various birch species are planted for timber, paper, carbon
108 sequestration and ecological restoration, but some birch species are endangered with
109 narrow distributions and there is concern about the increasing threat posed by the
110 bronze birch borer (Muilenburg and Herms, 2012; Shaw et al., 2014). Previous
111 phylogenetic analyses of *Betula* using nuclear genes (ITS, NIA and ADH), chloroplast
112 genes (matK and rbcL) and AFLPs provided limited resolution of relationships among
113 species and partly contradicted each other (Järvinen et al., 2004; Li et al., 2005; Li et
114 al., 2007; Schenk et al., 2008). In addition, molecular phylogenies based on nuclear
115 genes contradict some species groupings proposed in a recent monograph based on
116 morphology, such as the placement of the ecologically and economically important *B.*
117 *maximowicziana* (monarch birch) (Ashburner and McAllister, 2016; Wang et al.,
118 2016).

119

120 Hybridisation is frequent and has been extensively documented in *Betula*
121 (Anamthawat-Jónsson and Tómasson, 1990; Anamthawat-Jónsson and Tómasson,
122 1999; Anamthawat-Jónsson and Thórsson, 2003; Anamthawat-Jónsson et al., 2010;
123 Barnes et al., 1974; Eidesen et al., 2015; Johnsson, 1945; Tsuda et al., 2017; Wang et
124 al., 2014). Polyploidy is also common within *Betula*, with nearly 60% of species
125 being polyploids (Wang et al., 2016) and ploidy ranging from diploid to dodecaploid
126 (Ashburner and McAllister, 2016). Some species contain different cytotypes, such as
127 *B. chinensis* (6x and 8x) (Ashburner and McAllister, 2016). The origins of most
128 polyploids in the genus are unresolved. One of the best studied is the tetraploid *B.*
129 *pubescens* (downy birch), with different lines of evidence suggesting as candidate
130 parents: *B. pendula* based on RAPD markers (Howland et al., 1995), *B. humilis* or *B.*
131 *nana* based on ADH (Järvinen et al., 2004), *B. humilis* based on morphology (Walters,
132 1968) or *B. lenta* based on SNPs (Salojarvi et al., 2017). This uncertainty hinders
133 genomic research on *B. pubescens*, the most widespread birch tree in Europe and
134 western Asia.

135

136 Here, in order to better resolve the phylogeny of *Betula* and elucidate the parental
137 origins of its polyploid species we use a RAD-seq approach with reads assembled
138 against the *B. pendula* reference genome (Salojarvi et al., 2017). We construct the
139 phylogeny of diploid species using supermatrix and species tree methods. As a
140 heuristic method for analyzing the origins of the polyploid species, we create a
141 reference using contigs from all diploid species and compare the genomic similarity
142 between each polyploid species and all diploids by mapping reads of each polyploid
143 species to the reference. Polyploid taxa should have a higher level of genetic
144 similarity to diploids closely related to their ancestors and hence a higher number of
145 mapped reads. These approaches together yielded a well-resolved history for the
146 genus *Betula*, including polyploid taxa.

147

148 **2. Materials and Methods**

149

150 **2.1. Sample collection**

151 Samples were obtained from living collections in Stone Lane Gardens (SL hereafter),
152 Ness Gardens (N hereafter), the Royal Botanic Garden Edinburgh (RBGE) or
153 collected from the wild by the research group (Table S1). The genome size of most of
154 these taxa has been obtained (Wang et al., 2016) and morphological characters were
155 used to confirm the identity of each taxon sampled. *Alnus inokumae* was chosen as the
156 outgroup as *Alnus* has been shown to be sister to *Betula* (Li et al., 2007). In addition,
157 *A. orientalis* and *Corylus avellana* were included for marker development. Herbarium
158 specimens of most of these samples have been deposited at the Natural History
159 Museum London and RBGE with accession numbers provided in Table S1.

160

161 **2.2. DNA extraction, RAD library preparation and Illumina sequencing**

162 Genomic DNA was isolated from silica-dried cambial tissue or leaves following a
163 modified 2 X CTAB (cetyltrimethylammonium bromide) protocol (Wang et al., 2013).
164 The isolated DNA was assessed with a 1.0% agarose gel and measured with a Qubit

165 2.0 Fluorometer (Invitrogen, Life technologies) using Broad-range assay reagents.
166 RAD libraries were prepared following the protocol of Etter et al. (2011) with slight
167 modifications (Etter et al., 2011). Briefly, 0.5-1.0 µg of genomic DNA for each sample
168 was heated at 65°C for 2-3 hours prior to digestion with PstI (New England Biolabs,
169 UK). This enzyme has a 6 bp recognition site and leaves a 4 bp overhang. Digestion
170 was followed by ligation of barcoded P1 adapters. Ligated DNA was sheared using a
171 Bioruptor (KBiosciences, UK) instrument in 1.5 mL tubes (high intensity, duration 30
172 s followed by a 30 s pause which was repeated eight times). Sheared fragments were
173 evenly distributed between 100 bp and 1500 bp and fragments of ~600 bp were
174 selected using Agencourt AMPure XP Beads (New England Biolabs) following a
175 protocol of double-size selection. Briefly, a ratio of bead:DNA solution of 0.55 was
176 used to remove large fragments and then a second round of size selection was
177 conducted, using 5 µl of bead solution concentrated from a starting volume of 20 µl.
178 After end-repair and A-tailing, the size-selected DNA was ligated to P2 adapters (400
179 nm) and PCR amplified. PCR amplification was carried out in 25 µl reactions
180 consisting of 0.46 vol ddH₂O and template DNA (4-5 ng), 0.5 vol 2×Phusion Master
181 Mix (New England Biolabs), and 0.04 vol P1 and P2 amplification primers (10 nm),
182 using the following cycling parameters: 98°C for 30 s followed by 12 cycles of 98°C
183 for 10 s and 72°C for 60 s. Three or four independent PCR replicates were conducted
184 for each sample to achieve a sufficient amount of the library. The final library was
185 quantified using a Bioanalyzer and a Qubit 2.0 Fluorometer (Invitrogen, Life
186 Technologies) using high-sensitivity assay reagents and was normalized prior to
187 sequencing. The quantified library was sequenced on an Illumina MiSeq machine
188 using MiSeq Reagent Kit v3 (Illumina) at the Genome Centre of Queen Mary
189 University of London.

190

191 **2.3. RAD data trimming and demultiplexing**

192 The raw data were trimmed using Trimmomatic (Bolger et al., 2014) in paired-end
193 mode with the following steps. First, LEADING and TRAILING steps were used to
194 remove bases from the ends of a read if the quality is below 20. Then a

195 SLIDINGWINDOW step was performed with a window size of 1 and a required
196 quality of 20. Finally, a MINLENGTH step was used to discard reads shorter than
197 100bp. FastQC was used to check various parameters of sequence quality in both raw
198 and trimmed datasets (Andrews, 2014). The trimmed data were demultiplexed, using
199 the process_radtags module of Stacks (Catchen et al., 2013).

200

201 **2.4. Reads mapping, sequence alignment and trimming**

202 The whole genome assembly of *B. pendula* (Salojarvi et al., 2017) was used as a
203 reference for mapping our RAD data, to separate orthologous loci (i.e. mapped
204 segments of DNA) from paralogous loci (Wang et al., 2013), and to anchor reads with
205 adjacent restriction cutting sites. Mapping of trimmed reads for each sample was
206 conducted using the ‘Map Reads to Reference’ tool in the CLC Genomics Workbench
207 v. 8. A similarity value of 0.8 and the fraction value of 0.8 were applied as the
208 threshold. Reads with non-specific matches were discarded and any regions with
209 coverage of below three were removed. A consensus sequence with a minimum contig
210 length of 300bp was created for each sample. *Betula glandulosa* was excluded from
211 further analysis because only 216 loci were mapped at a sufficient read depth.
212 Multiple sequence alignments for individual loci were generated using mafft v.6.903
213 (Katoh et al., 2005) with default parameters. Aligned sequences were trimmed using
214 trimAl v1.2rev59 (Capella-Gutierrez et al., 2009); gaps present in 40% of taxa or
215 above were removed (-gt 0.6).

216

217 **2.5. Species tree inference**

218 Two datasets were used for phylogenetic analysis: dataset 1 (D1 hereafter) includes 20
219 diploid *Betula* samples and dataset 2 (D2 hereafter) 27 diploid samples. In D2, some
220 species were represented by more than one sample (Table S2). RAD loci ≥ 300 bp in
221 length that occurred in a minimum of four *Betula* samples were used for gene tree
222 inference. The gene tree for each locus was estimated using the maximum-likelihood
223 method (ML) in RAxML v. 8.1.16 (Stamatakis, 2006). A rapid bootstrap analysis with
224 100 bootstraps and 10 searches was performed under a GTR+GAMMA nucleotide

225 substitution model. The species tree was estimated from the gene trees with
226 ASTRAL-II v5.5.7 (Mirarab and Warnow, 2015) and ASTRID (Vachaspati and
227 Warnow, 2015). Branch support in the ASTRAL and ASTRID trees was assessed via
228 calculation of local posterior probabilities based on the gene tree quartet frequencies
229 (Sayyari and Mirarab, 2016) and bootstrapped gene trees (Vachaspati and Warnow,
230 2015), respectively. All loci used for building gene trees and inferring the species trees
231 were concatenated into a supermatrix, using custom shell scripts, which was analysed
232 in RAxML v. 8.1.16 using the same settings as above. The consensus tree generated
233 above was visualised in FigTree v.1.3.1 (<http://tree.bio.ed.ac.uk/software/figtree>).

234

235 **2.6. Phylogenetic Networks**

236 We used the Species Networks applying Quartets (SNaQ) method (Solís-Lemus and
237 Ané, 2016) implemented in the software PhyloNetworks 0.5.0 (Solís-Lemus et al.,
238 2017) to investigate whether the species tree without hybridisation events or a
239 phylogenetic network with one or more hybridisation events better describes the
240 diploid species relationships within *Betula*. Phylogenetic trees generated in RAxML
241 were used to estimate quartet concordance factors (CFs), which represent the
242 proportion of genes supporting each possible relationship between each set of four
243 species. These CFs were then used to reconstruct phylogenetic networks under
244 incomplete lineage sorting (ILS) and differing numbers of hybridisation events, and to
245 calculate their respective pseudolikelihoods. To determine whether a tree accounting
246 for ILS or a network better fits the observed data, we estimated the best phylogenetic
247 network with hybridisation events (h) ranging from 0 to 5 using the phylogeny
248 obtained with ASTRID as a starting tree. Using a value of $h=0$ will yield a tree
249 without reticulation, $h=1$ will yield a network with a maximum of one reticulation and
250 so forth. The fit of trees and networks to the data was evaluated based on
251 pseudo-deviance values, and estimated inheritance probabilities (i.e. the proportion of
252 genes contributed by each parental population to a hybrid taxon) were visualised. This
253 test compares the score of each network based on the negative log-pL, where the
254 network with the lowest value has the best fit.

255

256 **2.7. Identification of putative diploid progenitors of polyploid species**

257 We sought to infer the putative origins of polyploid species of *Betula* by
258 read-mapping. First, we used RAD loci present in at least 15 out of 20 diploid *Betula*
259 taxa to create a reference; the reference comprised 88,488 sequences in total,
260 representing 5,045 loci. All the sequences were concatenated and separated with
261 lengths of 500 Ns. Trimmed reads of polyploid taxa were mapped individually to this
262 single reference containing loci from all diploid taxa using strict parameters in CLC
263 Genomic Workbench: a fraction value of 0.9 and a similarity value of at least 0.995.
264 Reads with non-specific matches were discarded. Any region with a coverage of
265 below three was removed and the consensus sequences for each polyploid with a
266 minimum length of 300 bp were extracted. Variable sites were represented by
267 ambiguity codes in the consensus. Given the fact that the number of loci available for
268 each diploid species was variable (Table S2), we plotted the proportion of loci in the
269 reference for each diploid species to which reads from each polyploid species mapped.
270 We expected a higher proportion of consensus loci to be mapped in those diploids that
271 were progenitor species of each polyploid species. We therefore sought to identify
272 diploid progenitors for each polyploid based on the number of mapped consensus loci
273 assuming that the number of progenitors could not be more than half the ploidy level
274 of each polyploid. In addition, we mapped reads from diploid species to the reference
275 containing loci from all diploid taxa using the same parameters as described above.
276 We found a small number of loci of each diploid were mapped to by reads from other
277 diploid species, with the exception of *B. calcicola* and *B. potaninii*, for which a
278 relatively high number of loci (>1000) were mapped in each species by reads from the
279 other (Fig. S1).

280

281 **2.8. Phylogeny incorporating polyploid species**

282 For those polyploids for which we could identify putative diploid parental species, we
283 separated their homoeologues for each RAD-locus using another concatenated single
284 reference, similar to the one described in the paragraph above, but this time containing

285 all 50,870 loci present in a minimum of four *Betula* taxa of D1. For each of these
286 polyploids, we extracted a RAD consensus sequence from each mapped diploid locus,
287 with a minimum length of 200 bp; we excluded any sequence where the polyploid had
288 not mapped to their putative parents for that locus. We then excluded loci where reads
289 from one diploid had mapped to another diploid species (see above - Identification of
290 putative diploid progenitors of polyploid species). We constructed phylogenies
291 including the phased polyploid RAD loci with the diploid RAD loci (Fig. S2).
292 Individual gene trees were constructed in RAxML v. 8.1.16 using the same parameters
293 as described above (see - Species tree inference) and the species tree was inferred
294 using ASTRID. The putative diploid progenitors which we included for phylogenetic
295 analysis are provided in Table S2.

296

297 **2.9. Simple sequence repeat analysis**

298 To develop markers for future use in all *Betula* species for population genetic analyses,
299 mapped consensus sequences with length equal to or greater than 300 bp were mined
300 for simple sequence repeats (SSR) using the QDD pipeline version 3.1.2 (Meglécz et
301 al., 2014). Consensus sequences with a repeat motif of 2–5 bp, and repeated a
302 minimum of five times, were screened using the Downstream QDD pipeline version
303 3.1.2. Primer pairs were designed within 200 bp flanking regions using PRIMER3
304 software (Untergasser et al., 2012). The primer table output by the QDD version 3.1.2
305 pipeline allows selection of the best primer pair design for each SSR locus. We
306 filtered primer pairs according to parameters provided by QDD version 3.1.2. The
307 selected SSR loci had: a minimum number of 7 motif repeats within the SSR
308 sequence; a maximum primer alignment score of 5; a minimum of 20 bp forward and
309 reverse flanking region between SSR and primer sequences; and a high-quality primer
310 design without homopolymer, nanosatellite and microsatellite sequence in the primer
311 or flanking sequences. For polyploid species of *Betula*, *A. inokumae*, *A. orientalis* and
312 *C. avellana*, we selected SSR loci with a minimum number of 5 motif repeats as a
313 majority of loci had 5 or 6 motif repeats within the SSR sequence.

314

315 **3. Results**

316

317 **3.1. RAD data description**

318 The number of trimmed reads per diploid taxon ranges from 1,065,196 to 2,560,486
319 (average of 1,508,904) with between 881,333 and 2,252,171 (80.60% - 90.75%)
320 mapped to the *B. pendula* genome for each of the 27 diploid *Betula* and 707,914
321 (51.64%) for the outgroup *A. inokumae* (Table S1). In D1, 162 loci are present in all
322 20 *Betula* diploid taxa and 7,002 present in only four of these (Fig. 1A), whereas for
323 D2 99 loci are present in all 27 *Betula* diploid individuals and 6,078 present in only
324 four (Fig. 1B). Contigs of ≥ 300 bp, with an average length of 580.8bp - 755.8bp,
325 varied in number between 13,597 in *A. inokumae* and 30,717 in *B. pendula* (Fig. 1C,
326 D).

327

328 **3.2. Phylogenetic inference**

329 The concatenated D1 (50,870 loci) and D2 (58,442 loci) datasets include 31,815,738
330 and 35,859,769 nucleotides with 60.25% and 63.12% missing data (gaps and
331 undetermined characters), respectively. The three approaches used for phylogenetic
332 analysis of D1 (ASTRAL, ASTRID and supermatrix) all produced well resolved trees
333 that split the genus into two major clades. The ASTRAL species tree (Fig. 2A) and
334 concatenation tree (Fig. 2B) have identical topologies, whereas the ASTRID tree for
335 this dataset differs in the placement of *B. cordifolia* (Fig. S3). Phylogenetic trees for
336 D2 inferred with the species tree methods also separate the genus into two major
337 clades, similar to the D1 trees, but with some differences in the placement of a small
338 number of taxa within the largest clade (Fig. S4-S5). The concatenation tree of D2
339 does not recover the same major clades as the other analyses, although these
340 differences are not well supported (Fig. S6).

341

342 **3.3. Phylogenetic Networks**

343 The pseudolikelihood values of hybrid nodes decreased sharply from $h = 0$ to $h = 2$,
344 with only marginal improvements when further increasing the number of

345 hybridisation events (Fig. S7), suggesting the best-fitting phylogenetic model is one
346 involving two main hybridisation events. The D1 phylogenetic network when $h = 2$ is
347 similar to the phylogenetic trees for this dataset (Fig. 3), but with evidence for
348 hybridisation events involving four separate lineages within the largest of the major
349 clades.

350

351 **3.4. Read-mapping of polyploid species**

352 For 28 of the polyploid species or varieties (80%) the mapping analysis identified two
353 or more parental lineages (Fig. 4), represented by 17 diploid species (Table 1). Five
354 polyploids, all with the ploidy level $\geq 8x$, have putative progenitors from both of the
355 two major diploid clades. *Betula nigra* seems not to be the putative progenitor of any
356 polyploid species whereas *B. humilis* represents the putative parental lineage of up to
357 nine polyploids (Table 1).

358

359 **3.5. Phylogeny incorporating polyploid species**

360 When we included phased homoeologues from polyploids for which we could identify
361 putative parents in a phylogenetic analysis, for 22 of the 26 the polyploids their
362 homoeologues form clades with each of the putative parental diploid species (Table 1;
363 Fig. 5). For example, subgenomes of *B. pubescens* and its varieties formed
364 monophyletic clades which were sister to *B. pendula* and *B. platyphylla*, respectively.

365

366 **3.6. Simple sequence repeat analysis**

367 We developed between 58 and 565 microsatellite primer pairs for the diploid *Betula*
368 taxa and between 40 and 633 for polyploid *Betula* taxa. In addition, 100, 84 and 41
369 microsatellite primers pairs were developed for *A. inokumae*, *A. orientalis* and *C.*
370 *avellana*, respectively (Table S3).

371

372 **4. Discussion**

373

374 **4.1. A well resolved diploid phylogeny for *Betula***

375 We used both supermatrix, and more unusually, species tree approaches to construct
376 phylogenies based on RADseq data. We used longer contigs than is usual with
377 RADseq (Tripp et al., 2017), with an average length of 675 bp; these contigs were
378 generated from paired MiSeq reads, mapped to a reference genome (Salojarvi et al.,
379 2017). The use of multiple gene trees also enabled us to detect evidence for
380 hybridisation events among diploid species.

381

382 Two major clades were found in all analyses, which were not found by previous
383 molecular or morphological analyses (Ashburner and McAllister, 2016; Bina et al.,
384 2016; Järvinen et al., 2004; Li et al., 2005; Li et al., 2007; Nagamitsu et al., 2006;
385 Schenk et al., 2008; Wang et al., 2016). Interestingly, species of Clade 1 exclusively
386 have no or very narrow seed wings and species of Clade 2 exclusively have obvious
387 seed wings. The fact that some species of Clade 2 have very wide geographic
388 distributions is likely due to their strong dispersal ability. Ashburner and McAllister's
389 taxonomic sections *Asperae*, *Lentae* and *Nipponobetula* were grouped exclusively
390 into Clade 1 and sections *Acuminatae*, *Dahuricae*, *Betula*, *Costatae*, were grouped
391 exclusively into Clade 2, but species of section *Apterocaryon*, which are dwarf in
392 form, are split between both clades. Within *Apterocaryon*, *B. michauxii*, with a
393 geographic distribution in North America, is nested into Clade 1 whereas *B. humilis*
394 and *B. nana*, with a geographic distribution across Eurasia, are nested within Clade 2.
395 Thus the shrubby dwarf forms are likely due to independent evolution. Independent
396 evolution of dwarf forms has been occasionally observed in other genera, such as in
397 *Artemisia* (Tkach et al., 2007) and *Eucalyptus* (Foster et al., 2007). Another trait that
398 may have evolved independently in the genus is resistance to the bronze birch borer:
399 species reported to be largely resistant to the bronze birch borer (Muilenburg and
400 Herms, 2012) are split between the two clades.

401

402 On the basis of the above, we suggest that section *Apterocaryon* should be dissolved,
403 and *B. michauxii* placed in section *Lentae*, and *B. humilis* and *B. nana* in section

404 *Betula*. In the case of section *Acuminatae*, our analysis shows *B. luminifera* and *B.*
405 *haninanensis* form a clade, but *B. maximowicziana* is sister to section *Costatae*. The
406 incongruence between morphology and molecular evidence for these three species is
407 likely explained by hybridisation as indicated by our phylogenetic network analysis.
408 We suggest that *B. maximowicziana* should be moved to section *Costatae*. These
409 changes, taking into account the effects of hybridisation and convergent evolution,
410 mean that the seven remaining sections of the genus *Betula*, and the three remaining
411 subgenera proposed by Ashburner and McAllister, are all monophyletic in our diploid
412 trees.

413

414 We also note that in our analyses, *B. corylifolia*, the single species of section
415 *Nipponobetula*, was in a monophyletic group with species of section *Asperae*. Such a
416 relationship has previously been indicated based on ITS (Nagamitsu et al., 2006;
417 Wang et al., 2016). We therefore suggest that this species is placed in section *Asperae*,
418 reducing the genus to two sub-genera that correspond to the two major clades of our
419 diploid phylogenies. These are subgenus *Betula* containing sections *Acuminatae*,
420 *Costatae*, *Dahuricae* and *Betula*, and subgenus *Aspera* containing sections *Asperae*
421 and *Lentae*.

422

423 **4.2. Inferring polyploid parentage of *Betula* species**

424 Our results provide novel insights into parental species for a majority of *Betula*
425 polyploids (Table 1). For example, tetraploids of section *Costatae* (excluding *B. utilis*
426 ssp. *occidentalis*) have a high proportion of loci mapped to in *B. ashburneri* and *B.*
427 *costata*, indicating their likely parentage. This is consistent with morphological
428 characters, based on which Ashburner and McAllister placed these species within
429 section *Costatae* (Ashburner and McAllister, 2016). The parentage of *B. pubescens*,
430 which is widely planted, has been controversial for decades and has been suggested as
431 *B. pendula* (Howland et al., 1995; Walters, 1968), *B. humilis*, *B. nana* (Howland et al.,
432 1995; Järvinen et al., 2004; Walters, 1968) and *B. lenta* c.f. (Salojärvi et al., 2017).
433 Here we find *B. pendula* and its sister species *B. platyphylla* to be the most likely

434 parents of *B. pubescens*. We found a relatively low but still a considerable proportion
435 of mapped loci from *B. pubescens* to *B. nana* and *B. humilis*, but several studies have
436 found evidence for introgressive hybridisation among these species since the
437 formation of *B. pubescens* (Bona et al., 2018; Jadwiszczak et al., 2012; Thórsson et al.,
438 2001) which could account for sequences from *B. nana* and *B. humilis* in *B. pubescens*
439 genomes. Previous hypotheses for *B. lenta* c.f. as a parental species of *B. papyrifera*
440 and *B. humilis* cf. as a parental species of *B. ermanii* (Järvinen et al., 2004) were not
441 supported by our results. Our results also show evidence of complex layering of
442 hybridisation and polyploidisation events in the history of some taxa. For example, *B.*
443 *luminifera* and *B. hainanensis* were identified as the extant representatives for the
444 progenitors of two tetraploids, but they themselves have a possible hybrid origin (or
445 have been subject to significant introgression) on the basis of the network analysis;
446 the two tetraploids with *B. luminifera*/*B. hainanensis* identified as progenitors both
447 have the next highest proportion of loci mapped to in *B. maximowicziana*, which
448 might suggest the evidence for hybridisation between *B. luminifera*/*B. hainanensis*
449 and *B. maximowicziana* identified from the network analysis occurred before the
450 origin of the polyploids. Given such complex evolutionary histories, it is perhaps
451 unsurprising that for eight of the 35 polyploids, we could not clearly identify all
452 putative parents. This may also be because these eight polyploids are older than the
453 polyploids for which we have identified putative parents, and thus more divergent
454 from the diploid species. Another possibility is that they are derived from diploid
455 species which are now extinct, have not yet been discovered or were not included in
456 our phylogenetic analyses (i.e. *B. glandulosa*).

457

458 **5. Conclusion**

459 Here, by generating a new phylogenetic hypothesis for *Betula*, and providing new
460 evidence for the progenitors of many of its polyploid taxa, we have provided a
461 framework within which the evolution and systematics of the genus can be understood.
462 Knowledge of the parentage of the allopolyploids, some of which are widespread and
463 economically important, opens the way for their genomic analysis. The approach we

464 have used is relatively cheap and straightforward and could be applied to many other
465 plant groups where allopolyploidy has impeded evolutionary analyses.

466

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471

472 **Author Contributions**

473 NW and RB conceived the project. NW, RB and HM collected samples. HM
474 identified the samples based on morphology. NW carried out lab work. NW, JZ and
475 LK analysed data. NW, RB and LK wrote the manuscript. All the authors contributed
476 to editing the manuscript.

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739 **Figure legends**

740 **Figure 1** Detailed information of number of shared loci and number of contigs (A)
741 number of shared loci only in between four and 20 of the diploid *Betula* species of D1;
742 (B) number of shared loci only in between four and 27 of the diploid *Betula* species of
743 D2; (C) number of contigs with length above 300bp for *A. inokumae* and each of the
744 27 diploid *Betula* species of D2; (D) length of contigs for *A. inokumae* and each of the
745 27 diploid *Betula* species of D2. The whiskers of the boxplot from the bottom to the
746 top indicate the minimum, the first quartile, the median, the third quartile and the
747 maximum value of contig length excluding outliers.

748 **Figure 2** Species tree from the maximum likelihood analysis of the 20 *Betula* diploids
749 of D1 using ASTRAL (A) and the supermatrix (B) approach based on data from
750 50,870 loci. Asterisks on the branches of (A) indicate local posterior probabilities of 1
751 and numbers on the branches of (B) are bootstrap support values. The scale bar below
752 (B) indicates the mean number of nucleotide substitutions per site. Species were
753 classified according to Ashburner and McAllister (2016).

754 **Figure 3** Best network inferred from SNaQ analysis of the 20 *Betula* diploids of D1
755 with the number of hybridization events $h=2$. Blue lines indicate hybrid edges and
756 values beside the blue line indicate estimated inheritance probabilities.

757 **Figure 4** Mapping patterns of polyploids to the diploid reference. Numbers on the x
758 axis indicate number of mapped loci.

759 **Figure 5** Species tree incorporating polyploids from the maximum likelihood analysis
760 using ASTRID. Species were classified according to Ashburner and McAllister
761 (2013).

762 **Figure S1** Mapping patterns of the 20 diploid *Betula* species of D1. Numbers on the x
763 axis indicate number of mapped loci.

764 **Figure S2** The schematic illustration of methods used for analyzing polyploids.

765 **Figure S3** Species tree from the maximum likelihood analysis of the 20 *Betula*
766 diploids using the ASTRID approach based on 50,870 gene trees. Species were
767 classified according to Ashburner and McAllister (2016).

768 **Figure S4** Species tree from the maximum likelihood analysis of the 27 *Betula*

769 diploids using the ASTRAL approach based on 58,442 gene trees. Asterisks on the
770 branches indicate 1.00 local posterior probabilities. Species were classified according
771 to Ashburner and McAllister (2016).

772 **Figure S5** Species tree from the maximum likelihood analysis of the 27 *Betula*
773 diploids using the ASTRID approach based on 58,442 gene trees. Numbers above
774 branches are support values. Marked with star indicates branches with low support
775 values. Species were classified according to Ashburner and McAllister (2016).

776 **Figure S6** Species tree from the maximum likelihood analysis of the 27 *Betula*
777 diploids using the supermatrix approach based on 58,442 gene trees. Numbers above
778 or below branches are support values. The scale bar indicates the mean number of
779 nucleotide substitutions per site. Species were classified according to Ashburner and
780 McAllister (2016).

781 **Figure S7** The pseudolikelihood values for the number of hybridization events from 1
782 to 5.

783

784 **Table legend**

785 **Table 1** Putative diploid progenitors suggested for polyploids of *Betula* and included
786 for phylogenetic analysis.

787

788 **Supplementary data**

789 **Table S1** Detailed information about *Betula* species used in this study and mapping
790 results of RADseq.

791 **Table S2** Number of loci for each diploid species represented in the reference.

792 **Table S3** Detailed information about microsatellite markers mined from assembled
793 contigs of species of *Betula*, *Alnus* and *Corylus*.

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800 **Table**

801 **Table 1.** Putative diploid progenitors suggested for polyploids of *Betula* and included
802 for phylogenetic analysis

Species ¹	Ploidy level	Putative diploid progenitors
<i>B. pubescens</i> var. <i>pubescens</i>	4	<i>B. pendula</i> / <i>B. platyphylla</i>
<i>B. pubescens</i> var. <i>litwinowii</i>	4	<i>B. pendula</i> / <i>B. platyphylla</i>
<i>B. pubescens</i> var. <i>celtibérica</i>	4	<i>B. pendula</i> / <i>B. platyphylla</i>
<i>B. pubescens</i> var. <i>pumila</i>	4	<i>B. pendula</i> / <i>B. platyphylla</i>
<i>B. pubescens</i> var. <i>fragrans</i>	4	<i>B. pendula</i> / <i>B. platyphylla</i>
<i>B. papyrifera</i>	6	<i>B. cordifolia</i> / <i>B. populifolia</i> / <i>B. occidentalis</i>
<i>B. papyrifera</i> var. <i>commutata</i>	6	<i>B. cordifolia</i> / <i>B. populifolia</i> / <i>B. occidentalis</i>
<i>B. pumila</i>	4	<i>B. populifolia</i> / <i>B. occidentalis</i>
<i>B. albosinensis</i>	4	<i>B. ashburneri</i> / <i>B. costata</i>
<i>B. albosinensis</i> var. <i>septentrionalis</i>	4	<i>B. ashburneri</i> / <i>B. costata</i>
<i>B. utilis</i> ssp. <i>pratii</i>	4	<i>B. ashburneri</i> / <i>B. costata</i>
<i>B. utilis</i>	4	<i>B. ashburneri</i> / <i>B. costata</i>
<i>B. ermaninii</i>	4	<i>B. ashburneri</i> / <i>B. costata</i>
<i>B. ermaninii</i> var. <i>lanata</i>	4	<i>B. ashburneri</i> / <i>B. costata</i>
<i>B. cylindrostachya</i>	4	<i>B. luminifera</i> / <i>B. hainanensis</i>
<i>B. alnoides</i>	4	<i>B. luminifera</i> / <i>B. hainanensis</i>
<i>B. alleghaniensis</i>	6	<i>B. lenta</i>
<i>B. murrayana</i>	8	<i>B. lenta</i> / <i>B. occidentalis</i> / <i>B. populifolia</i>
<i>B. medwediewii</i>	10	<i>B. humilis</i> / <i>B. lenta</i> / <i>B.</i>

		<i>maximowicziana/B. michauxii/B. luminifera</i>
<i>B. megrelica</i>	12	<i>B. humilis/B. lenta/B. maximowicziana/B. michauxii/B. luminifera</i>
<i>B. chinensis</i>	6	<i>B. calcicola/B. potaninii/B. chichibuensis</i>
<i>B. chinensis</i>	8	<i>B. calcicola/B. potaninii/B. chichibuensis</i>
<i>B. fargesii</i>	10	<i>B. calcicola/B. potaninii/B. chichibuensis</i>
<i>B. delavayi</i>	6	<i>B. calcicola/B. potaninii</i>
<i>B. bomiensis</i>	4	<i>B. calcicola/B. potaninii</i>
<i>B. globispica</i>	10	<i>B. corylifolia/B. chichibuensis/B. lenta/B. michauxii/B. costata</i>
<i>B. insignis</i>	10	<i>B. corylifolia/B. chichibuensis/B. lenta/B. michauxii/B. luminifera/B. maximowicziana</i>

803 ¹*B. insignis* (marked in blue) was provided with six putative progenitors due to the
 804 possible ploidy level either 10 or 12.

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