

The investigation of pellicle peelability on Japanese chestnut cultivar of ‘Yakko’ (*Castanea crenata* Sieb. et Zucc.)

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1 **Title:** The investigation of pellicle peelability on Japanese chestnut cultivar of ‘Yakko’
2 (*Castanea crenata* Sieb. et Zucc.)

3

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17 **Abstract**

18 Japanese chestnuts (*Castanea crenata* Sieb. et Zucc.) generally have difficult-
19 peeling pellicles even after heating, making easy-peeling pellicle (EPP) an important
20 breeding target. Recently, EPP cultivars ‘Porotan’ and ‘Porosuke’ were released by a
21 government-funded breeding program. However, very few genotypes carry the major
22 recessive gene responsible for the EPP trait, resulting in inbreeding within a narrow
23 gene pool. To discover other genetic materials having the potential for EPP breeding,
24 we evaluated the pellicle peelability of 59 accessions (51 Japanese local cultivars and 8
25 wild individuals) by using the high-temperature oil peeling method. We discovered that
26 ‘Yakko’ had an exceptionally high pellicle peelability score (87%), close to that of
27 ‘Porotan’ (94%). The results of segregation ratio analysis of pellicle peelability and
28 genotype prediction by simple sequence repeat (SSR) markers among F₁ seedlings
29 suggested that the EPP alleles of ‘Porotan’ and ‘Yakko’ are at the same locus. However,
30 a haplotype structure analysis of the EPP genome region with SSR markers revealed
31 that both haplotypes of ‘Yakko’ differed from those of ‘Porotan’, suggesting that the
32 EPP gene of ‘Yakko’ had a different origin from that of ‘Porotan’ or was inherited from
33 a common ancestor many generations ago.

34

35 **Keywords:** Japanese chestnut, Breeding, Haplotype, Inbreeding, Pellicle peelability

36

37 **Highlights**

- 38 ● Pellicle peelability was evaluated in 59 Japanese chestnut accessions.
- 39 ● The goal was to detect accessions with easily peeled pellicles.
- 40 ● The pellicle peelability of ‘Yakko’ was exceptionally high.
- 41 ● Peelability of ‘Yakko’ and ‘Porotan’ appears to be controlled by the same locus.

42

43 **Abbreviations**

44 APR, average peeling rate; DPP, difficult-peeling pellicle; EPP, easy-peeling pellicle;

45 HOP, high-temperature oil peeling; MAS, marker-assisted selection; NARO, National

46 Agriculture and Food Research Organization; NIFTS, Institute of Fruit Tree and Tea

47 Science, NARO; QTLs, quantitative trait loci; SSR, simple sequence repeats

48

49 **1. Introduction**

50 There are four major chestnut species: Japanese chestnut (*Castanea crenata* Sieb.
51 et Zucc.), Chinese chestnut (*C. mollissima* Bl.), European chestnut (*C. sativa* Mill.), and
52 American chestnut (*C. dentata* Borkh.). Japanese chestnut is naturally distributed and is
53 grown in Japan and the Korean Peninsula, and many local cultivars have been
54 developed in Japan (Pereira-Lorenzo et al., 2012). Chinese chestnut is grown mainly in
55 China. European chestnut is commercially grown in Europe, Asia Minor, and North
56 Africa. American chestnut was a common species in eastern North America until the
57 early 20th century, when it was decimated by the accidental introduction of chestnut
58 blight (Woodroof, 1979). Japanese chestnut cultivars are believed to have been selected
59 from wild chestnuts of Japanese origin (Kotobuki, 1994). This hypothesis is supported
60 by the considerable genetic distance between local Japanese chestnut cultivars and
61 Chinese chestnut accessions, as determined using amplified fragment length
62 polymorphism markers (Yamamoto et al., 1998).

63 Many cultivars of Chinese chestnut and European chestnut have a pellicle that is
64 easy to peel (hereafter, an easy-peeling pellicle: EPP). In contrast, Japanese chestnut
65 cultivars generally have a pellicle that is difficult to peel (hereafter, a difficult-peeling
66 pellicle: DPP), even after heating (Kikuchi, 1948; Miller et al., 1996; Pereira-Lorenzo et
67 al., 2012; Tanaka et al., 1981). The pellicle of Japanese chestnut can be scraped away by
68 hand using a knife, but this is laborious and costly. Thus, releasing new Japanese
69 chestnut cultivars with EPP has been an important target for Japanese chestnut breeding,
70 in addition to large nut size, high eating quality, and high productivity. This program
71 started in 1947 at a national level and is currently managed by the Institute of Fruit Tree
72 and Tea Science, National Agriculture and Food Research Organization (NIFTS).

73 Recently, the breeding program released two Japanese chestnut cultivars with the EPP
74 trait: ‘Porotan’ in 2006 (Saito et al., 2009) and ‘Porosuke’ in 2016 (Saito et al., 2017).
75 The area planted to ‘Porotan’ has been increasing rapidly, reaching 212 ha in 2014. The
76 EPP trait of ‘Porotan’ is controlled by a single major recessive gene: the pellicle
77 peelability locus has been designated *P/p* (Takada et al., 2012), and a molecular marker
78 linked to this locus was developed (Nishio et al., 2013). Today, marker-assisted
79 selection (MAS) is available for the EPP trait in cross-derived populations, allowing
80 selection using large seedling populations and eliminating the need to raise the plants
81 until they are old enough to produce nuts, which is laborious and time-consuming.

82 So far, very few genotypes (offspring, selections, or cultivars) have been found to
83 carry the EPP gene. This is a concern because repeated crossing among specific genetic
84 resources within a narrow gene pool results in inbreeding depression, such as decreased
85 tree vigor and productivity, in woody fruit crops, including Japanese pear (Sato et al.,
86 2008) and persimmon (Yamada et al., 1994). This depression has not yet been observed
87 in Japanese chestnut, but based on the results for other tree species, seems likely to
88 develop as breeding progresses. Outcrossing can mitigate or eliminate inbreeding
89 depression by incorporating genes from accessions that are genetically distant from the
90 current cross parents in breeding, thus increasing genetic diversity.

91 Both ‘Porotan’ and ‘Porosuke’ are early-maturing cultivars, which results in early
92 cessation of EPP nut production in areas of cultivation and a concentration of harvest
93 dates within a brief period. Therefore, the development of a mid- or late-maturing
94 cultivar with EPP, which would extend the season when fresh nuts are available and
95 give farmers more time to harvest their crops, is a current chestnut breeding target at
96 NIFTS. Kotobuki et al. (1984) suggested that nut harvest time is controlled by

97 quantitative trait loci (QTLs), and Nishio et al. (2017) detected QTLs for nut harvesting
98 date. Thus, we wish to identify later-ripening Japanese chestnut accessions with some
99 level of EPP as cross parents for the breeding of mid- or late-maturing cultivars.

100 In books published about a century ago, Nakaoka (1913), Yagioka (1915), and
101 Tanaka (1933) described local Japanese chestnut cultivars having EPP on the basis of
102 their observations, but they did not report any test results. This suggests that some
103 unidentified EPP genotypes might exist among Japanese chestnut genetic resources,
104 including the local cultivars mentioned in those books. Our previous study suggested
105 the possibility of breeding novel EPP cultivars by crossing among DPP accessions with
106 relatively easily peeled pellicles (Takada et al., 2017). Thus, it is necessary to identify
107 accessions with relatively high pellicle peelability for breeding novel EPP cultivars. The
108 objective of this study was to discover Japanese chestnut accessions with the EPP trait
109 or with relatively high pellicle peelability by surveying 59 Japanese chestnut accessions
110 that were not included our previous study (Takada et al., 2017).

111

112 **2. Materials and methods**

113 *2.1. Pellicle peelability of 51 local cultivars and 8 wild individuals*

114 We tested a total of 59 Japanese chestnut accessions, consisting of 51 local
115 cultivars and 8 wild individuals, and used ‘Porotan’ as the standard for the EPP trait
116 (Table 1). We grew one tree per accession at NIFTS, in Tsukuba, Ibaraki (36°02′56″N,
117 140°05′56″E), Japan. The pellicle peelability of each accession was evaluated in either
118 2004 or 2007 (Table 1). All trees were grown following standard cultural techniques
119 used in commercial production in Japan.

120 The harvest day for each accession was the first day that ≥ 10 nuts could be
121 harvested. In 2004, it ranged from 25 August for ‘Yamaguchiwase’ to 6 October for
122 ‘Daihachi’, ‘Katayama’, and ‘Kinshiu’. In 2007, it ranged from 22 August for
123 ‘Hassaku’, ‘Tanabata’, and ‘Toyotamawase’ to 17 October for ‘Choubei’ and
124 ‘Shimokatsugi’. Among the 33 accessions harvested in 2004, 24 were harvested again
125 in 2007. The average harvest day of these 24 accessions was 18 September in 2004 and
126 27 September in 2007. Although there was a difference of about 10 days in mean
127 harvest day between the two years, the relative maturities of the accessions were similar
128 in each of the two years. Nuts were harvested after the bur opened and were then stored
129 at 5 °C for 1 month.

130 Ten nuts per accession were randomly used to evaluate pellicle peelability. For
131 accessions harvested in both 2004 and 2007, peelability was assessed only in 2004.
132 After the shells were removed, the nuts were fried in canola oil at 190 °C for 2 min (the
133 high-temperature oil peeling [HOP] method; Shoda et al., 2006). The pellicle peelability
134 of each nut was then determined by means of hand-peeling with a paring knife and was
135 scored by visual estimation of the percentage of the surface area that peeled away
136 without scraping (“peeling rate”), on a scale graded in 10% increments, where “0%”
137 represents 0%, “5%” represents $0% < \text{and } \leq 10\%$, “15%” represents $10% < \text{and } \leq 20\%$, ...
138 “85%” represents $80% < \text{and } \leq 90\%$, and “95%” represents $90% < \text{and } \leq 100\%$ (Takada et
139 al., 2017). Pellicle peelability was quantified as the average peeling rate of 10 nuts per
140 genotype evaluated (APR; %). The accessions with APR values $\geq 75\%$ were classified
141 as EPP; those with APR $< 75\%$ were considered DPP.

142

143 *2.2. Inheritance of pellicle peelability of ‘Yakko’*

144 As described in Results, ‘Yakko’ had an exceptionally high APR value relative to
145 the other accessions, suggesting that it has a major EPP gene. To test whether the mode
146 of inheritance of pellicle peelability of ‘Yakko’ was the same as that of ‘Porotan’, we
147 examined the segregation ratio of pellicle peelability among F₁ seedlings of crosses
148 made using ‘Yakko’ as a parent. We crossed ‘Porotan’ (*p/p*) × ‘Yakko’ in 2006 and
149 2010, and ‘Tanzawa’ (*P/p*) × ‘Yakko’ in 2005 and 2006. ‘Tanzawa’ was previously
150 shown to be heterozygous for the *p* allele found in ‘Porotan’ (Takada et al., 2012;
151 Nishio et al., 2013). Two-year-old offspring were planted in a space of 2 m × 5 m in the
152 NIFTS orchard. Nuts were harvested from each seedling of ‘Tanzawa’ × ‘Yakko’ in
153 2011 and of ‘Porotan’ × ‘Yakko’ in 2013 after the bur opened and stored at 5 °C for 1
154 month. Ten nuts from each seedling were randomly evaluated for pellicle peelability by
155 the HOP method as described in section 2.1. As above, seedlings having average APR
156 values of ≥75% were regarded as EPP. The segregation ratio of pellicle peelability for
157 the seedlings of ‘Tanzawa’ × ‘Yakko’ was tested by the chi-square goodness-of-fit test
158 for the hypotheses of a 1:1 segregation ratio.

159

160 *2.3. Association between pellicle peelability and genotype estimated by simple sequence* 161 *repeat markers*

162 Because ‘Yakko’ had an exceptionally high APR value, similar to that of
163 ‘Porotan’, we hypothesized that both cultivars had the same *p/p* genotype. Thus, we
164 estimated the pellicle peelability genotypes of F₁ seedlings derived from ‘Tanzawa’
165 (*P/p*) × ‘Yakko’ (described in section 2.2) by determining which allele from ‘Tanzawa’
166 was present in each seedling. Two simple sequence repeat (SSR) markers closely linked

167 to the *P/p* locus of ‘Tanzawa’ (PRB28 and PEB62; Nishio et al., 2013) were used to
168 genotype each seedling.

169 Genomic DNA was extracted from young leaves or young buds using a DNeasy
170 Plant Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer’s instructions.
171 Polymerase chain reaction products were separated and detected with a 3130xl Genetic
172 Analyzer (Life Technologies, Carlsbad, CA, USA). The size of each amplified band was
173 determined by comparison with a set of internal standard DNA fragments (400HD-ROX,
174 Life Technologies) in GeneMapper v. 5.0 software (Life Technologies).

175

176 *2.4 Haplotype structure around the P/p locus of ‘Yakko’ and ‘Porotan’*

177 To determine the haplotype structure around the *P/p* locus of ‘Yakko’ and
178 ‘Porotan’, we investigated an F₁ population derived from ‘Porotan’ × ‘Yakko’
179 (described in section 2.2). Genomic DNA was extracted as in section 2.3. The seedlings
180 were genotyped using 10 SSR markers associated with the *P* gene locus (PEA18,
181 PEA41, PEB62, PEB102, PRA51, PRB25, PRB28, PRD2, PRD52, PRD58; Nishio et
182 al., 2013). The size of each amplified band was determined as described in section 2.3.
183 The order and spacing of the markers were obtained from Nishio et al. (2013).

184 The fragment sizes of SSR markers PRA51 and PRB25 were 19 bp and 20 bp
185 larger, respectively, than those reported previously (Nishio et al., 2013). These size
186 differences are explained by a change in the forward primers from M13-tailed primers
187 (Schuelke, 2000) to fluorescently labeled primers. In addition, since the DNA sequencer
188 was changed from a PRISM 3100 DNA sequencer (Applied Biosystems, Carlsbad, CA,
189 USA) to a 3130xl Genetic Analyzer, a difference of 1 bp was found in some markers
190 (PRD2, PRB28, PEB62, PRD58) relative to the results of Nishio et al. (2013).

191

192 **3. Results**

193 *3.1. Pellicle peelability of 51 local cultivars and 8 wild individuals*

194 The APR of the 59 accessions and ‘Porotan’ ranged from 7.0% in ‘Katayama’ to
195 94.0% in ‘Porotan’ (Table 1). The frequency distribution of the APR values was
196 continuous in the 58 accessions with APR < 75% (Fig. 1). ‘Porotan’ and ‘Yakko’ had
197 exceptionally high APR values, which were discontinuous with those of the other
198 accessions (Fig. 1). The APR value of ‘Yakko’ was 87.0% and close to that of ‘Porotan’,
199 and only these two cultivars were classified as EPP. The mean APR value of the 58
200 DPP accessions was 43.3%. Among the DPP accessions, only ‘Otomune’ (72.0%),
201 ‘Fukunami’ (71.0%), and Shibaguri-166 (71.0%) had APR values of $\geq 70\%$ (Table 1).

202

203 *3.2. Inheritance of pellicle peelability of ‘Yakko’*

204 In a population of 16 F₁ offspring of the cross of ‘Porotan’ (*p/p*) \times ‘Yakko’,
205 pellicle peelability segregated in a ratio of 14 EPP to 2 DPP (Fig. 2). The two DPP
206 offspring had APR values of 59.0% and 61.0%. This segregation ratio was close to the
207 expected ratio of 1:0 for the progeny of parents homozygous for recessive alleles at the
208 same locus. In a population of 17 F₁ offspring from ‘Tanzawa’ (*P/p*) \times ‘Yakko’, pellicle
209 peelability segregated in a ratio of 6 EPP to 11 DPP (Fig. 2). The hypothesis of 1:1
210 segregation, as expected from a cross of a heterozygous parent by a homozygous
211 recessive parent, was not rejected at $P < 0.05$.

212

213 *3.3. Association between pellicle peelability and genotype estimated by SSR markers*

214 On the assumption that ‘Yakko’ has the same *p/p* genotype as ‘Porotan’, we
215 estimated the genotypes of 17 F₁ offspring from Tanzawa’ × ‘Yakko’ by using SSR
216 markers PRB28 and PEB62 and then compared the estimated genotypes with the APR
217 scores. The segregation was estimated as 7 offspring with the *p/p* genotype and 10 with
218 the *P/p* genotype, including one recombinant genotype that was judged to be *P/p* owing
219 to its very low APR value (3%). When these data were compared to the phenotypes, 6
220 of the 7 seedlings estimated as having the *p/p* genotype had APR ≥ 75% and were
221 classified as EPP (Fig. 3). The remaining seedling estimated as having the *p/p* genotype
222 had APR = 60.0% and was classified as DPP. All 10 seedlings estimated as having the
223 *P/p* genotype had APR < 29% and were classified as DPP (Fig. 3). The mean APR
224 values were 17.4% for the estimated *P/p* genotypes versus 83.7% for the estimated *p/p*
225 genotypes, indicating that the pellicle peelability of seedlings with the *p/p* genotype (as
226 estimated by SSR) was much higher than that of seedlings with the *P/p* genotype.

227

228 3.4. Haplotype structure around the *P/p* gene locus of ‘Yakko’ and ‘Porotan’

229 Overall, the two haplotypes of SSR markers around the *P/p* locus of ‘Yakko’
230 showed different structures from those of ‘Porotan’ (Fig. 4). In the region between
231 markers PEB62 and PEA41, all of which lie on the same side of the *P/p* locus, one
232 haplotype of ‘Yakko’ showed the same structure as both haplotypes of ‘Porotan’.
233 However, on the other side of the *P/p* locus, between markers PRD2 and PRD52, the
234 SSR marker haplotypes differed between ‘Porotan’ and ‘Yakko’, as well as within each
235 of the accessions.

236

237 4. Discussion

238 Japanese chestnut cultivars generally have DPP traits, but nearly a century ago,
239 pellicle peelability was described in some local cultivars as EPP or relatively EPP.
240 Cultivars ‘Shimokatsugi’ (Nakaoka, 1913), ‘Akaguri’, ‘Choubei’, ‘Imakita’, ‘Kenaga’,
241 ‘Mikado’, ‘Shimokatsugi’, ‘Shougatsu’, ‘Wasa’ (Yagioka, 1915), ‘Akaguri’, ‘Gora’,
242 ‘Gosha’, ‘Ideno’, ‘Terai’, ‘Teteuchi’, ‘Yakko’, and ‘Wasa’ (Tanaka, 1933) were
243 described as EPP or relatively EPP, but these descriptions provided no supporting data.
244 These cultivars were not grown widely, probably owing to low productivity, small nuts,
245 or other undesirable characteristics. Ten of these cultivars (all except ‘Akaguri’, ‘Ideno’,
246 ‘Mikado’, and ‘Wasa’) are conserved at NIFTS. At the time of the present study,
247 ‘Imakita’ and ‘Shougatsu’ had already been classified as DPP (APR = 41.0% and 35.0%,
248 respectively; Takada et al., 2017), so they were not retested here.

249 We evaluated the pellicle peelability of 7 local cultivars (‘Choubei’, ‘Gora’,
250 ‘Gosha’, ‘Kenaga’, ‘Shimokatsugi’, ‘Teteuchi’, and ‘Yakko’) from among the 14
251 cultivars previously described as EPP or relatively EPP. In two cases, cultivars
252 considered to be synonymous were used. In the first case, ‘Kenaga’, introduced by
253 Yagioka (1915), is considered synonymous with ‘Kenagaginyose’, which is conserved
254 at NIFTS. Similarly, ‘Teteuchi’, introduced by Tanaka (1933), seems to be synonymous
255 with cultivar ‘Ogawateteuchi’, which is conserved at NIFTS, because both cultivars
256 originated in Ogawa village in Hyogo prefecture. Among these cultivars, the APR
257 values were all less than 55% except for ‘Yakko’, which was 87.0% (Table 1). Thus,
258 only ‘Yakko’ was selected as having the EPP trait among this group of old cultivars.
259 According to the database of NARO Genebank, this cultivar has intermediate tree vigor,
260 intermediate nut size, and high eating quality. Since these 14 cultivars cited about a
261 century ago included 1 EPP cultivar, another might be hidden among the 5 cultivars that

262 were not evaluated in this study or by Takada et al. (2017). One of these, ‘Terai’, is
263 included in the NIFTS collection and should be evaluated for pellicle peelability. The
264 four cultivars that are not conserved at NIFTS will have to be acquired by exploration.

265 In our previous study (Takada et al., 2017), the high and continuous variation of
266 the APR values among the DPP accessions suggested the existence of quantitative gene
267 effects on pellicle peelability. This indicates the possibility of developing novel EPP
268 cultivars by accumulating QTLs for more easily peeled pellicle among the DPP
269 accessions with relatively high APR values. In a previous study, 70.0% (Shibaguri-37)
270 was the highest APR value among the DPP accessions (Takada et al., 2017). In the
271 present study, ‘Fukunami’, ‘Otomune’, and Shibaguri-166 had $APR \geq 70\%$, like
272 Shibaguri-37. These accessions can be considered as cross parents for attempts to breed
273 novel EPP cultivars. To develop such cultivars efficiently, it will be necessary to clarify
274 the mode of inheritance and to perform QTL analysis on the pellicle peelability of these
275 accessions.

276 The segregation of the APR values in the F_1 seedlings of ‘Porotan’ \times ‘Yakko’,
277 most of which were EPP, suggested that both ‘Porotan’ and ‘Yakko’ were homozygous
278 for recessive alleles at the same locus (i.e., both p/p). As noted, however, there were two
279 exceptional DPP offspring (Fig. 2). APR values have considerable environmental and
280 non-genetic variability (Takada et al., 2017); thus, the small sample size (10 nuts) may
281 explain the two-exceptional offspring. The distribution of the APR values in offspring
282 from ‘Tanzawa’ \times ‘Yakko’ suggests a bimodal distribution with peaks corresponding to
283 EPP and DPP but also showing environmental and non-genetic variability. ‘Tanzawa’
284 has genotype P/p (Takada et al., 2012), and the approximately 1:1 segregation in the F_1

285 progeny of ‘Tanzawa’ × ‘Yakko’ supports the hypothesis that ‘Yakko’ has a p/p
286 genotype at the same EPP locus as in ‘Porotan’ and ‘Tanzawa’ (Fig. 2).

287 Consistent with the assumption that the genotype of pellicle peelability in ‘Yakko’
288 is p/p , the seedlings from ‘Tanzawa’ × ‘Yakko’ that inherited the p allele of ‘Tanzawa’
289 were EPP, with one exception, and all of those that inherited the P allele of ‘Tanzawa’
290 were DPP (Fig. 3). The result also shows that EPP in ‘Yakko’ is controlled by the same
291 locus as in ‘Porotan’ (which is the same as that in ‘Tanzawa’). The SSR markers closely
292 linked to the p gene of ‘Porotan’ (Nishio et al., 2013) should also be able to predict the p
293 gene of ‘Yakko’ (Fig. 4), although the specific marker alleles would sometimes differ.
294 In practical use, these SSR markers would be highly effective in MAS of the pellicle
295 peelability trait in ‘Yakko’ in addition to that of ‘Porotan’ and its relatives. The APR
296 values were also influenced by quantitative minor effects responsible for genetic
297 variation in DPP cultivars. The low APR values in the F_1 seedlings of ‘Tanzawa’ ×
298 ‘Yakko’ with the P/p genotype (Fig. 3) may be partly due to quantitative gene effects
299 specific to ‘Tanzawa’ and ‘Yakko’.

300 The crossing data indicate that it is highly possible that ‘Yakko’ has the same p
301 allele at the P/p locus as ‘Porotan’. However, the two SSR marker haplotypes around
302 the P/p locus of ‘Yakko’ showed somewhat different structure from those of ‘Porotan’
303 (Fig. 4). A previous study indicated that the recessive p allele in ‘Porotan’ was derived
304 from ‘Higan’, a local cultivar in Kyoto Prefecture, in central Japan (Nishio et al., 2014).
305 ‘Yakko’ is a local cultivar from the northern part of Osaka Prefecture, which is adjacent
306 to Kyoto Prefecture. Thus, some genetic relationship possibly exists between ‘Yakko’
307 and ‘Higan’, although no parent–offspring relationship was detected between them
308 (Nishio et al. 2014). If so, the p allele may have originated in a common ancestor many

309 generations ago, and the haplotypes may have changed owing to recombination over
310 time. Another possibility is that the *p* allele of ‘Yakko’ arose by mutation independently
311 from that of ‘Higan’ and ‘Porotan’. These questions may be answered by future DNA
312 sequencing of the EPP gene. So, it is suggested that the EPP alleles of ‘Porotan’ and
313 ‘Yakko’ are at the same locus but the EPP gene of ‘Yakko’ had a different origin from
314 that of ‘Porotan’ or was inherited from a common ancestor many generations ago.

315 Nishio et al. (2014) identified ‘Yakko’ and ‘Kanotsume’ as a parent–offspring
316 pair. The APR value of ‘Kanotsume’ was 57.0% in this study, which suggests that it has
317 the *P/p* genotype (assuming that ‘Yakko’ has the *p/p* genotype at this locus). In addition,
318 ‘Kanotsume’ and ‘Dengorou’ are a parent–offspring pair. ‘Dengorou’ had APR = 39.0%,
319 suggesting that it might have the *P/p* genotype. Thus, additional Japanese accessions
320 may carry the *p* allele as heterozygotes, and it would be impossible to identify these
321 genotypes only from the evaluation of pellicle peelability. Thus, it is important to
322 discover accessions with *p* alleles by genotyping linked SSR markers among a wider
323 range of genetic resources. For this purpose, developing markers tightly linked to the
324 *P/p* locus and identifying *p* alleles of different origins will be necessary.

325 Since the development of ‘Porotan’, only ‘Higan’ and its relatives have been used
326 at NIFTS for breeding of Japanese chestnut with the EPP trait. Repeated crossing
327 among these genetic resources will cause inbreeding, leading to depression of tree vigor
328 and productivity. Our haplotype structure analysis revealed that both EPP haplotypes
329 from ‘Yakko’ differ from those of ‘Porotan’, so their EPP alleles may have different
330 origins or an old common ancestor. Additionally, no parent–offspring relationships
331 were detected between ‘Yakko’ and either ‘Porotan’ or its ancestral cultivar ‘Higan’
332 (Nishio et al., 2014). Thus, ‘Yakko’ and its relatives will be effective as cross parents

333 for avoiding inbreeding depression risks arising from repeated use of ‘Higan’ and its
334 relatives in the Japanese chestnut breeding program at NIFTS.

335 Most of the accessions with the *p* gene derived from ‘Higan’ have early maturity
336 (e.g., usually around early September). For broadening the nut harvest period of the EPP
337 cultivars, making use of breeding materials that combine the EPP trait with later nut
338 harvest times would be desirable. The harvest day of ‘Yakko’ was 22 September 2004
339 and that of its offspring ‘Kanotsume’ was 15 September 2004, both later than those of
340 ‘Higan’ (11 September 2007) and ‘Porotan’ (4 September 2007). Also, the EPP gene
341 region of ‘Yakko’ can be predicted by the same SSR markers as those used to detect the
342 *p* gene of ‘Porotan’, which would enable efficient MAS of pellicle peelability in the
343 offspring of ‘Yakko’ and its relatives. In the breeding of the EPP cultivars, ‘Yakko’ and
344 ‘Kanotsume’ would therefore be useful as cross parents to lengthen the harvesting
345 period while also lowering the risk of inbreeding depression.

346

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352

353 **Declaration of Interest**

354 The authors declare no conflict of interest.

355

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424

425 **Fig. 1.** Frequency distribution of the average peeling rate (APR; %) of 10 nuts per
426 accession evaluated by the high-temperature oil peeling method among 59 accessions
427 and ‘Porotan’. Values falling at the edges of two adjacent bins were classified into the
428 lower bin (e.g., an APR of 15% would be classified into the “10–15” bin).

429

430 **Fig. 2.** Frequency distribution of the average peeling rate (APR; %) of 10 nuts per
431 offspring evaluated by the high-temperature oil peeling method among offspring of F₁
432 crosses of ‘Porotan’ (*p/p*) × ‘Yakko’ (left) and ‘Tanzawa’ (*P/p*) × ‘Yakko’ (right). EPP,
433 easy-peeling pellicle; DPP, difficult-peeling pellicle.

434

435 **Fig. 3.** Scatterplot of average peeling rate (APR; %) of the two genotypes estimated by
436 SSR analysis among 17 F₁ offspring of ‘Tanzawa’ (*P/p*) × ‘Yakko’. Estimation was
437 based on the assumption that ‘Yakko’ has the same *p/p* genotype as ‘Porotan’. APR was
438 assessed in 10 nuts per offspring by the high-temperature oil peeling method.

439

440 **Fig. 4.** Haplotype structure around the EPP genes in ‘Yakko’. That of ‘Porotan’ was
441 determined by Nishio et al. (2013). Numbers indicate allele size (bp). Genetic distances
442 from PRD2 were inferred from an integrated map of the 550-40 × ‘Tanzawa’ F₁
443 population (Nishio et al., 2013).

1 **Table 1.** Harvest day and average peeling rate (APR)^z of the 60 Japanese chestnut genotypes used in
 2 these experiments.

Cultivar or accession name	JP acc. No. ^y	Origin (prefecture)	Harvest day ^x	APR (%)
Local cultivars				
‘Arima’	113832	Kanagawa	8 Sep. 2004	45.0
‘Bonguri’	113834	Japan ^w	28 Aug. 2007	38.0
‘Buzen’	113836	Oita	22 Sep. 2004	32.0
‘Choubei’	113838	Kyoto	17 Oct. 2007	25.5
‘Choukouji’	113839	Hyogo	30 Sep. 2004	29.0
‘Daihachi’	113841	Kyoto	6 Oct. 2004	30.0
‘Dengorou’	113842	Akita	22 Sep. 2004	39.0
‘Enanishiki’	113843	Gifu	28 Aug. 2007	62.0
‘Fukunami’	113844	Kyoto	25 Sep. 2007	71.0
‘Fukunishi’	113845	Osaka	15 Sep. 2004	30.0
‘Ginyose’	113849	Osaka	20 Sep. 2007	48.0
‘Gora’	113850	Hyogo	30 Sep. 2004	29.0
‘Gosha’	113851	Kanagawa	22 Sep. 2004	54.0
‘Hajikami’	113852	Japan	1 Sep. 2004	19.0
‘Hassaku’	113853	Japan	22 Aug. 2007	42.0
‘Hatayaoguri’	113854	Akita	30 Sep. 2004	24.0
‘Hayadama’	113855	Wakayama	19 Sep. 2007	50.0
‘Higan’	113856	Kyoto	11 Sep. 2007	60.0
‘Hokugin’	113857	Gifu	11 Sep. 2007	57.0
‘Ichikawawase’	176782	Kanagawa	19 Sep. 2007	38.0
‘Kantotsume’	113867	Kyoto	15 Sep. 2004	57.0
‘Kasaharawase’	113868	Gifu	11 Sep. 2007	55.0
‘Katayama’	113869	Gifu	6 Oct. 2004	7.0
‘Kenagaginyose’	113870	Osaka	8 Sep. 2004	26.0
‘Kinseki’	113872	Hyogo	22 Sep. 2004	52.0
‘Kinshiu’	113873	Tokushima	6 Oct. 2004	28.0
‘Matabei’	113876	Kyoto	30 Sep. 2004	14.0
‘Ninomiya’	176780	Chiba	30 Sep. 2004	50.0
‘Obiwase’	113878	Miyazaki	28 Aug. 2007	46.0
‘Obuse 2 gou’	113879	Nagano	2 Oct. 2007	65.0
‘Obuse 3 gou’	113880	Nagano	22 Sep. 2004	20.0
‘Ogawateteuchi’	113882	Hyogo	11 Sep. 2007	26.0
‘Ookoma’	116299	Japan	15 Sep. 2004	35.0

Cultivar or accession name	JP acc. No. ^y	Origin (prefecture)	Harvest day ^x	APR (%)
‘Osaya’	113884	Kanagawa	28 Aug. 2007	64.0
‘Otomune’	113885	Hyogo	19 Sep. 2007	72.0
‘Saimyouji 1 gou’	176786	Akita	2 Oct. 2007	24.5
‘Saimyouji 2 gou’	176787	Akita	19 Sep. 2007	18.0
‘Shimokatsugi’	113894	Osaka	17 Oct. 2007	54.0
‘Shuuhouwase’	113895	Yamaguchi	11 Sep. 2007	66.0
‘Taishouwase’	113897	Kanagawa	28 Aug. 2007	55.0
‘Tajiriginoyose’	113898	Osaka	15 Sep. 2004	30.0
‘Tamanishiki’	113900	Japan	11 Sep. 2007	51.0
‘Tanabata’	113901	Shizuoka	22 Aug. 2007	58.0
‘Toyotamawase’	113907	Tokyo	22 Aug. 2007	30.0
‘Tsuchidawase’	113908	Gifu	25 Sep. 2007	35.0
‘Tsunehisa’	113910	Kanagawa	4 Sep. 2007	67.0
‘Waseginzen’	113919	Japan	1 Sep. 2004	56.0
‘Yakko’	113913	Osaka	22 Sep. 2004	87.0
‘Yamaguchiwase’	113914	Hyogo	25 Aug. 2004	41.0
‘Yamaguchiwase 2 gou’	113915	Tokushima	1 Sep. 2004	16.5
‘Yourou’	113917	Gifu	9 Oct. 2007	33.0
Wild individuals				
Sandoguri Kouchi 2	113971	Kouchi	30 Sep. 2004	63.0
Shibaguri-67	113888	Hyogo	22 Sep. 2004	39.0
Shibaguri-82	176797	Hyogo	22 Sep. 2004	56.0
Shibaguri-91	113889	Hyogo	22 Sep. 2004	57.0
Shibaguri-166	113890	Hyogo	15 Sep. 2004	71.0
Shidareguri-Gifu	113892	Gifu	15 Sep. 2004	51.0
Shidareguri-Tatsuno 2	234092	Nagano	15 Sep. 2004	42.0
Shidareguri-Tochigi	113937	Tochigi	8 Sep. 2004	56.0
Cultivar				
‘Porotan’	230435	F ₁ of 550-40 × ‘Tanzawa’	4 Sep. 2007	94.0

3 ^z Average peeling rate (APR; %) of 10 nuts evaluated by the high-temperature-oil peeling method.

4 ^y Accession numbers in the National Agriculture and Food Research Organization (NARO)

5 Genebank (http://www.gene.affrc.go.jp/index_en.php).

6 ^x For accessions harvested in both 2004 and 2007, the 2004 harvest day is shown. For each accession

7 listed, APR was measured in the year indicated.

8 ^w Prefecture is unknown.

9

Figure 1
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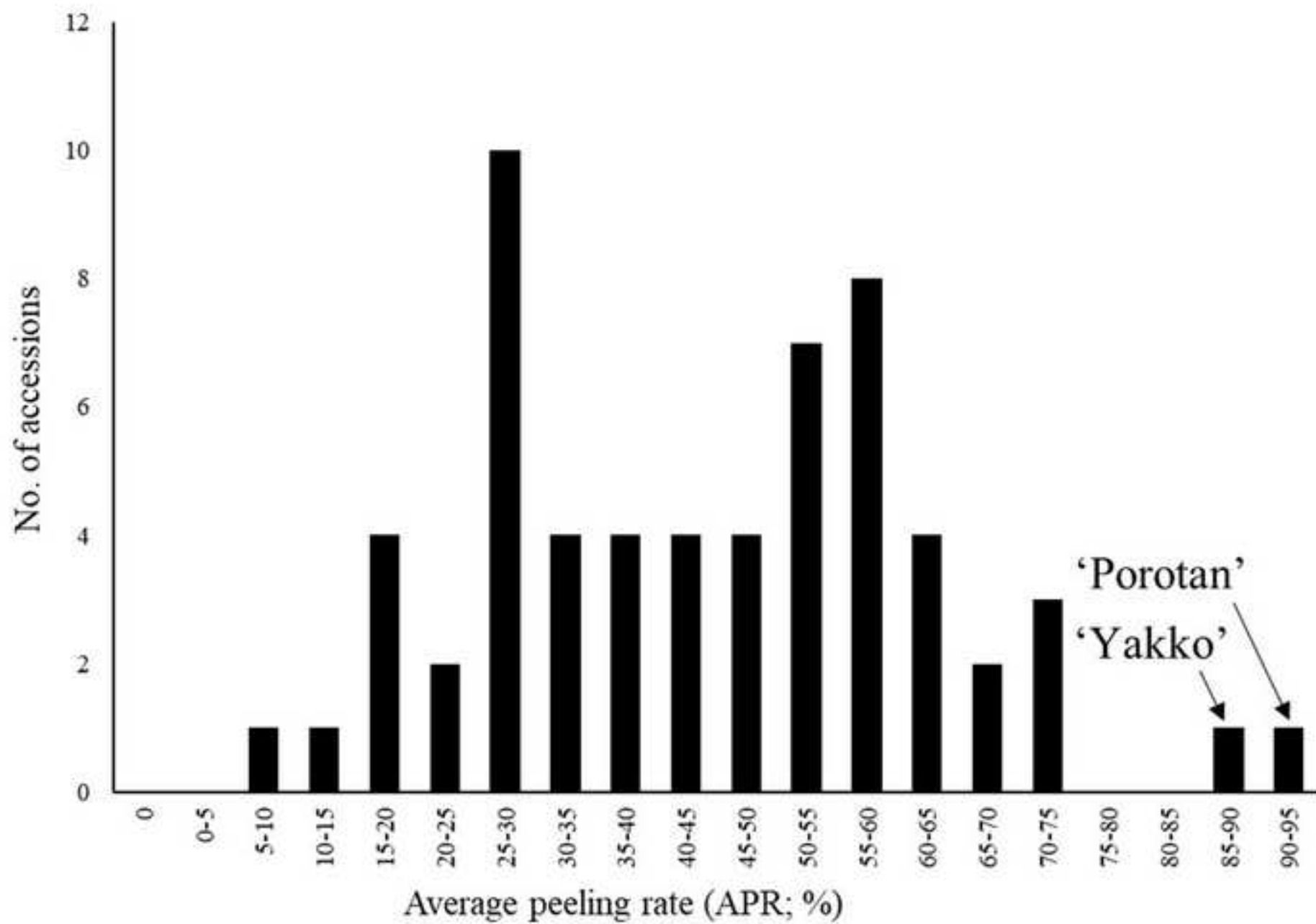


Figure 2
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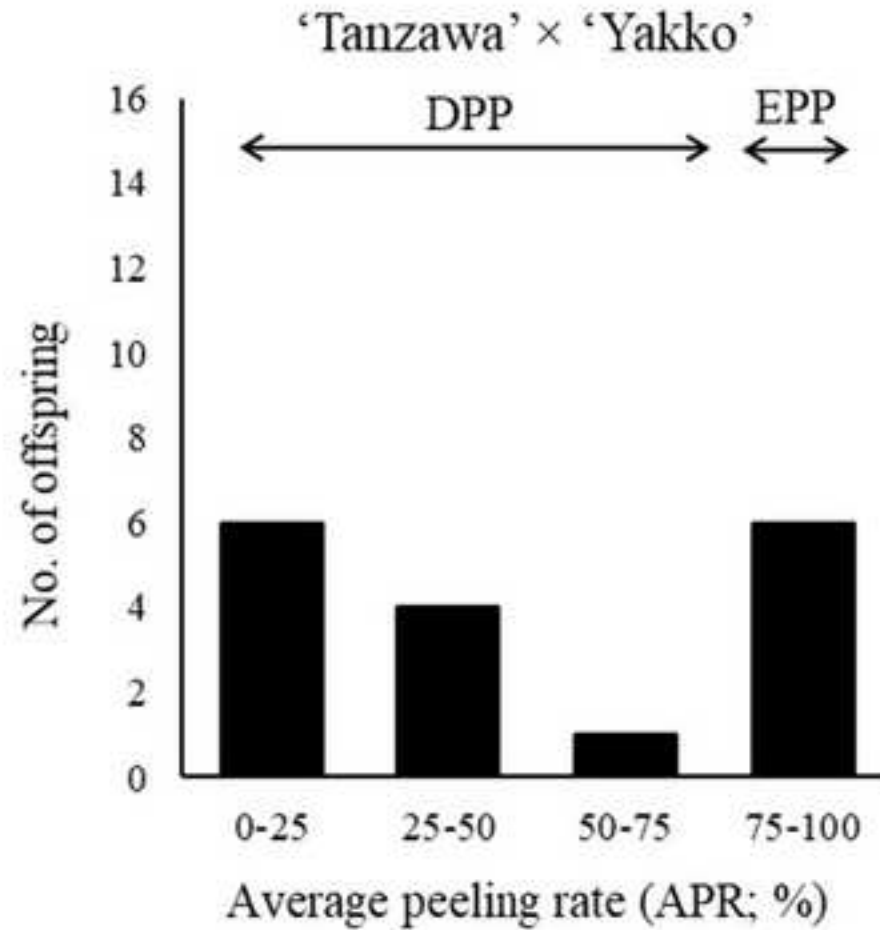
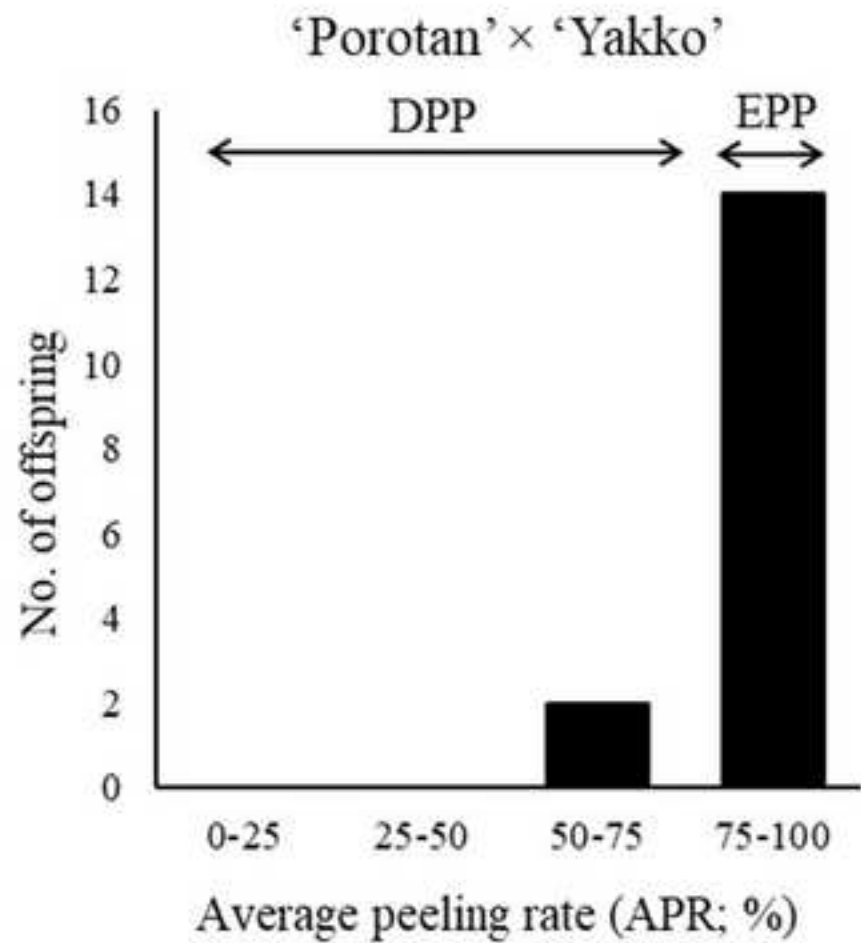


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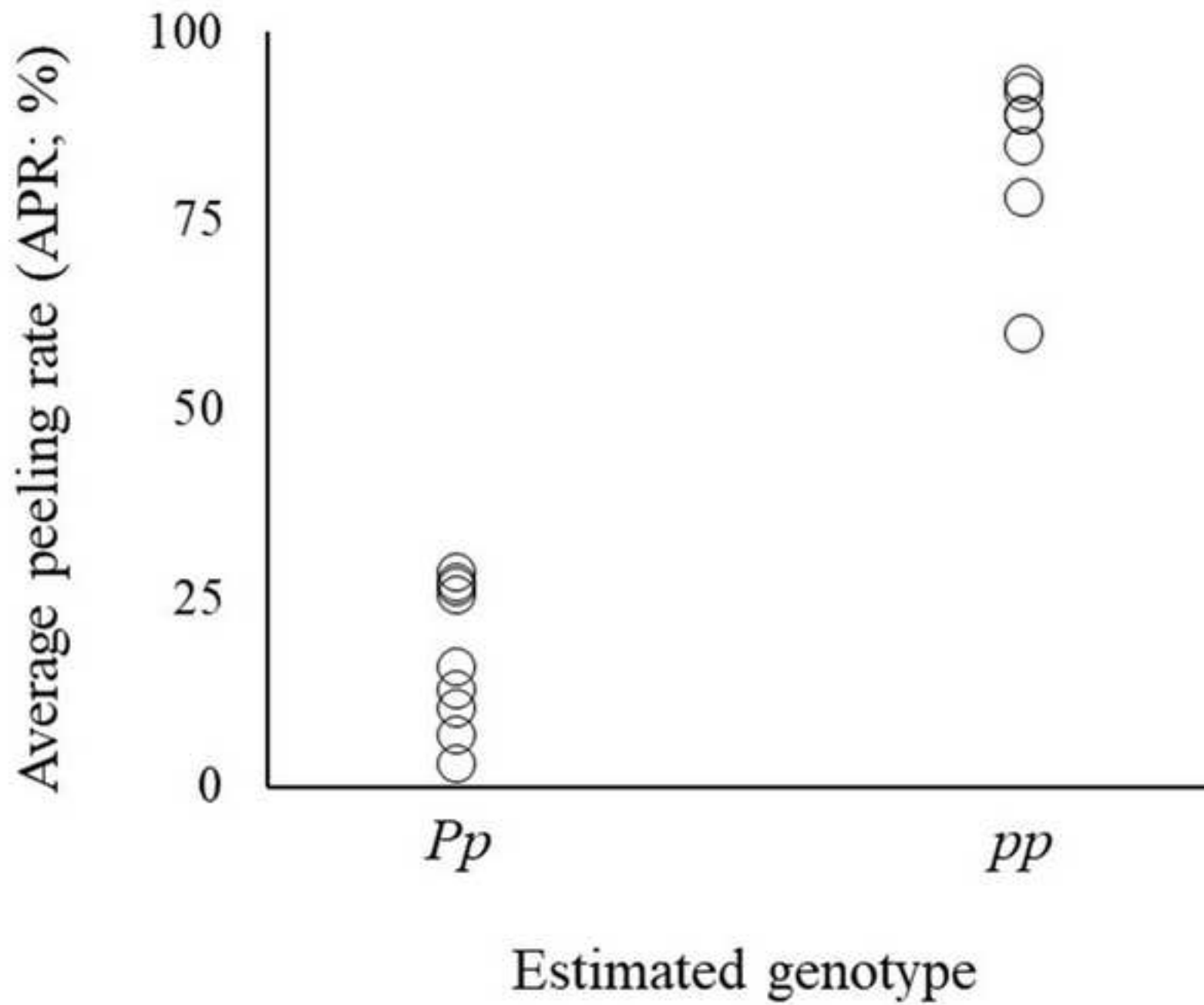


Figure 4
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	'Yakko'		'Porotan'		Genetic distance (cM)
PRD2	133	133	143	147	—
PRA51	270	270	273	268	9.6
PEA18	120	120	120	114	11.8
PEB102	171	171	176	171	11.8
PRB28	125	135	135	133	13.2
PRD52	144	144	150	150	16.6
peeling	<i>p</i>	<i>p</i>	<i>p</i>	<i>p</i>	
PEB62	169	159	169	169	19.8
PRD58	196	210	196	196	20.9
PRB25	157	157	157	157	22.3
PEA41	104	102	104	104	59.6