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2 **Paternally expressed imprinted genes under positive Darwinian**
3 **selection in *Arabidopsis thaliana***

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5 **Reetu Tuteja^{1†}, Peter C. McKeown^{1,*}, Pat Ryan¹, Claire C. Morgan²⁼, Mark T.A.**
6 **Donoghue^{1,+}, Tim Downing², Mary J. O'Connell^{3,4*}, Charles Spillane^{1,*}**

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8 ¹Genetics & Biotechnology Lab, Plant & AgriBiosciences Research Centre (PABC),
9 School of Natural Sciences, Ryan Institute, National University of Ireland Galway,
10 University Road, Galway H91 REW4, Ireland.

11 ²School of Biotechnology, Faculty of Biological Sciences, Dublin City University, Ireland.

12 ³Computational and Molecular Evolutionary Biology Research Group, School of
13 Biology, Faculty of Biological Sciences, The University of Leeds, LS2 9JT, UK.

14 ⁴Computational and Molecular Evolutionary Biology Group, School of Life Sciences,
15 University of Nottingham, NG7 2RD, UK

16 [†]current address: Center for Genomics and Systems Biology, New York University, New
17 York, NY, USA.

18 ⁼current address: Division of Diabetes, Endocrinology and Metabolism, Imperial
19 College London, UK.

20 ⁺current address: Memorial Sloan Kettering Cancer Center, New York, NY, USA.

21

22 *to whom correspondence should be addressed:

23

24 *Professor Charles Spillane, Genetics & Biotechnology Lab, Plant & AgriBiosciences
25 Research Centre (PABC), Ryan Institute, National University of Ireland Galway,
26 University Road, Galway H91 REW4, Ireland.

27 *Dr. Peter C. McKeown, Genetics & Biotechnology Lab, Plant & AgriBiosciences
28 Research Centre (PABC), Ryan Institute, National University of Ireland Galway,
29 University Road, Galway H91 REW4, Ireland.

30 *Dr. Mary J. O'Connell, Computational and Molecular Evolutionary Biology Group,
31 School of Life Sciences, University of Nottingham, NG7 2RD, UK.

32

33 charles.spillane@nuigalway.ie

34 peter.mckeown@nuigalway.ie

35 mary.o'connell@nottingham.ac.uk

36

37 **Keywords:** Genomic imprinting; genomic conflict; positive Darwinian selection;
38 endosperm; plant evolution

39

41 Abstract

42 Genomic imprinting is an epigenetic phenomenon where autosomal genes display
43 uniparental expression depending on whether they are maternally or paternally
44 inherited. Genomic imprinting can arise from parental conflicts over resource
45 allocation to the offspring, which could drive imprinted loci to evolve by positive
46 selection. We investigate whether positive selection is associated with genomic
47 imprinting in the inbreeding species *Arabidopsis thaliana*. Our analysis of 140 genes
48 regulated by genomic imprinting in the *A. thaliana* seed endosperm demonstrates
49 they are evolving more rapidly than expected. To investigate whether positive
50 selection drives this evolutionary acceleration, we identified orthologs of each
51 imprinted gene across 34 plant species and elucidated their evolutionary trajectories.
52 Increased positive selection was sought by comparing its incidence among imprinted
53 genes with non-imprinted controls. Strikingly, we find a statistically significant
54 enrichment of imprinted paternally expressed genes (iPEGs) evolving under positive
55 selection, 50.6% of the total, but no such enrichment for positive selection among
56 imprinted maternally expressed genes (iMEGs). This suggests that maternally- and
57 paternally-expressed imprinted genes are subject to different selective pressures.
58 Almost all positively selected amino acids were fixed across 80 sequenced *A. thaliana*
59 accessions, suggestive of selective sweeps in the *A. thaliana* lineage. The imprinted
60 genes under positive selection are involved in processes important for seed
61 development including auxin biosynthesis and epigenetic regulation. Our findings
62 support a genomic imprinting model for plants where positive selection can affect
63 paternally-expressed genes due to continued conflict with maternal sporophyte
64 tissues, even when parental conflict is reduced in predominantly inbreeding species.

66 **Introduction**

67 Rapid evolution under Positive Selection (PS) is a feature of many reproductive
68 proteins in both plants and animals, occurring either as a result of adaptive radiation
69 or of sexual conflict within and between genomes (Clark, et al. 2006). For example,
70 tests of selective pressure have shown that genes expressed in the highly reduced
71 male gametophyte of flowering plants (the pollen grain) display elevated PS
72 (Arunkumar, et al. 2013; Gossmann, et al. 2014). These increased levels of PS are
73 observed in genes expressed in the pollen tube but not the sperm cell, and are
74 interpreted to be a consequence of conflict driven by competition between pollen
75 grains for access to ovules (Bernasconi, et al. 2004). Conflict is also expected to occur
76 at loci regulated by genomic imprinting, in which genes are monoallelically expressed
77 under epigenetic regulation in a parent-of-origin specific manner, in violation of the
78 Mendelian rules of genetic inheritance (Haig 1997; Wilkins 2011). Indeed, genomic
79 imprinting is widely considered to have evolved due to conflict between parentally-
80 derived genomes over resource allocation to developing offspring which lead to genes
81 evolving different optimal expression levels depending upon whether they are
82 maternally- or paternally-derived (Willson and Burley 1983; Wilkins and Haig 2003b;
83 Haig 2004). Imprinting has been reported from both mammals and flowering plants, in
84 which it principally occurs in the endosperm (Gehring and Satyaki 2017), the second
85 product of double fertilization which provides maternally-derived resources to the
86 developing embryo in the seed (Walbot and Evans 2003). Imprinting leads to the
87 occurrence of imprinted maternally expressed genes (iMEGs) and imprinted paternally
88 expressed genes (iPEGs) (Haig and Westoby 1991; Garnier, et al. 2008; Köhler, et al.
89 2012). Kin conflict between iPEGs and iMEGs in plants is expected to arise from
90 differences in the optimal level of offspring resource allocation, and resulting offspring
91 size, between the maternal and paternal genomes as selection on the maternal
92 genome favours equal provision to all offspring (and iMEGs near-equal provision; see
93 (Trivers 1974)) while the paternal genome promotes growth of its own offspring alone
94 (Haig 2000; Costa, et al. 2012; Haig 2013; Willi 2013).

95 Such conflict can have different consequences at the molecular level, including conflict
96 relating to expression level and rapid evolution of nucleotide sequence (or epigenetic

97 signatures) associated with gene expression (Haig, et al. 2014). At the level of the
98 coding sequence, one prediction is that conflict can lead to positive selection on pairs
99 of reciprocally imprinted genes expressed from the maternally and paternally
100 inherited genomes, each having antagonistic effects on offspring growth (Wilkins and
101 Haig 2001; Mills and Moore 2004). We illustrate this occurring inside the endosperm
102 of the seed (yellow) in fig. 1A, within which iMEGs and iPEGs mutually interact. Some
103 support for this particular form of parental conflict has been found in mammals, for
104 example at the *Igf-2* and *callipyge* loci (Georges, et al. 2003; Reik, et al. 2003; Crespi
105 and Semeniuk 2004). Signatures of positive selection have also been detected at the
106 imprinted *MEDEA* locus in the flowering plant *Arabidopsis lyrata* (Spillane, et al. 2007;
107 Miyake, et al. 2009) which may support the hypothesis that imprinting can cause
108 positive selection on coding sequences of the loci concerned. On the other hand,
109 conflict can have other molecular effects, including selection for stable equilibria of
110 iMEG and iPEG expression levels (Haig 2014), and co-evolutionary scenarios between
111 iMEGs and cytoplasmic factors (Wolf and Hager 2006), as shown in fig. 1B. It has also
112 been suggested that conflict could occur between iPEGs and the tissues of the
113 maternal sporophyte (Willi 2013): the genes of the seed coat (SC) are also maternally-
114 derived and could therefore act in a manner antagonistic to iPEGs – this scenario of
115 ‘indirect conflict’ between the genes of the maternal seed coat (which we denote
116 scMEGs) and iPEGs in the endosperm is shown in fig. 1C. It has been alternatively
117 suggested that imprinting in plants could be related to the biology of gene expression
118 in triploid endosperm, for example as a dosage control mechanism, although a recent
119 study of gene expression in triploid embryos did not support this (Fort, et al. 2017).

120 Genomic imprinting also occurs in the model plant, *Arabidopsis thaliana* (L.) Heynh,
121 which is the sister species to *A. lyrata*, at *MEDEA* and several hundred other loci
122 (Gehring, et al. 2011; Hsieh, et al. 2011; McKeown, et al. 2011; Wolff, et al. 2011).
123 Furthermore, a subset of imprinted genes which are expressed early in *A. thaliana*
124 seed development (four days after pollination) display accelerated evolutionary rates
125 compared to non-imprinted genes (Wolff, et al. 2011) as measured by D_N/D_S . The rate
126 of nonsynonymous mutations per nonsynonymous site (D_N) and the rate of
127 synonymous mutations per synonymous site (D_S) is assumed to follow the neutral

128 evolutionary process and the ratio, such that D_N/D_S (also denoted ω), is therefore
129 approximate to the selective pressure on the protein product of a gene. A value of
130 $\omega > 1$ signifies positive selection (PS) at a site, $\omega \approx 1$ implies neutral evolution, while $\omega < 1$
131 indicates purifying selection. It should be noted that positive selection typically only
132 acts at a subset of amino acid sites while other sites are typically still under purifying
133 selection, so ω is still generally < 1 at the level of the whole gene even when PS has
134 occurred. Hence, comparisons between sets of candidate genes and relevant control
135 sets are needed to identify elevated levels of ω . Enrichment for sites with $\omega > 1$ in the
136 dataset of Wolff et al. (Wolff, et al. 2011) when compared with controls in this way
137 was therefore interpreted as a possible signature for conflict-driven selection within
138 plant imprinted genes.

139 Evidence of elevated rates of adaptive substitution have also been reported for
140 imprinted genes of the outcrossing Brassicaceae species, *Capsella rubella*
141 (Hatorangan, et al. 2016). This suggests that increased PS could be a general
142 phenomenon for imprinted genes, supporting models of the parental conflict theory in
143 which conflict leads to rapid evolution of coding sequences. However, it is important
144 to note that elevated D_N/D_S values can be caused by other factors such as variable
145 effective population size, N_e (Kryazhimskiy and Plotkin 2008; Jensen and Bachtrog
146 2011) and selection on silent sites (Chamary, et al. 2006). It is also unclear whether
147 potential PS in *A. thaliana* or *C. rubella* is acting equally on iMEGs or iPEGs as would
148 be consistent with models of parental conflict involving direct interactions between
149 the proteins which they encode (fig. 1A): iMEGs and iPEGs both showed higher D_N/D_S
150 in the study of (Wolff, et al. 2011), although in *C. rubella* increased accumulation of
151 nearly neutral non-synonymous variants was restricted to iPEGs (Hatorangan, et al.
152 2016). Nor has it been shown whether past positive selection has led to fixation within
153 current plant populations, as would be expected if the selection acting on amino acids
154 is functionally significant for protein function.

155 To determine whether genomic imprinting in the seed endosperm is associated with
156 positive selection in plant genomes, we analyzed the selective pressures acting on a
157 comprehensive group of all confirmed imprinted genes of *A. thaliana* (Gehring, et al.
158 2011; Hsieh, et al. 2011; McKeown, et al. 2011; Wolff, et al. 2011). Specifically, we

159 addressed the following questions: 1) What selective pressures are imprinted genes
160 evolving under in *A. thaliana*? 2) If imprinted genes are evolving under positive
161 selection, does this lead to overall positive selection in iMEGs and/or iPEGs being
162 elevated compared to similar sets of biallelically expressed genes? And 3) Is there
163 evidence for fixation of positively selected sites in imprinted genes across sequenced
164 *A. thaliana* accessions? Our findings in relation to these questions extend our
165 understanding of the evolutionary drivers of genomic imprinting and the
166 consequences of parental conflict during reproduction.

167

168 **Results**

169 **Imprinted *Arabidopsis thaliana* genes are rapidly evolving**

170 Genomic imprinting has been predicted to evolve due to parental conflicts over
171 provision of maternal resources to offspring, which has been hypothesised to lead to
172 positive selection at loci involved in this conflict. The model eudicot *Arabidopsis*
173 *thaliana* has been reported to display genomic imprinting on at least 436 genes in its
174 seed endosperm (Gehring et al., 2011; Hsieh et al., 2011; McKeown et al., 2011; Wolff
175 et al., 2011), with growing consensus over a core set which appear to be stably
176 imprinted in many accessions (Gehring and Satyaki 2017; Schon and Nodine 2017;
177 Wyder, et al. 2017). The identification of genes subject to monoallelic expression in
178 the seed endosperm can be confounded by parent-of-origin specific expression
179 patterns that can also arise during early seed development from gametophytic
180 deposition of mRNA in the fertilised egg cell (zygote) or fertilised central cell
181 (endosperm), or from maternal-expression from genes expressed in the sporophytic
182 seed coat, which may be present as contaminants during RNA-seq analyses. To
183 determine the selective pressures acting on imprinted genes, while avoiding these
184 confounding scenarios, we focused our analyses on those genes with strong evidence
185 for uniparental expression in seeds due to imprinting. We classified these as genes
186 identified from RNA-seq-based studies (Gehring, et al. 2011; Hsieh, et al. 2011; Wolff,
187 et al. 2011) which are expressed from the paternal genome (iPEGs), and which
188 therefore cannot be due to contamination from maternal tissues; and those iMEGs for
189 which experimental validation of monoallelic expression and/or epigenetic regulation
190 in the endosperm has been performed *in planta* (Vielle-Calzada, et al. 1999; Kinoshita,
191 et al. 2004; Köhler, et al. 2005; Tiwari, et al. 2008; Gehring, et al. 2009; Hsieh, et al.
192 2011; McKeown, et al. 2011; Shirzadi, et al. 2011; Wolff, et al. 2011). This produced a
193 set of 140 high-confidence imprinted genes (supplementary table S1A,1B) of which 63
194 were iPEGs and 77 were iMEGs. By comparing the *A. thaliana* and *A. lyrata* orthologs,
195 we determined that both iPEGs and iMEGs within the 140 imprinted genes had mean
196 values of ω significantly higher than that of the background representing all other
197 remaining *A. thaliana* genes (table 1; U-test: iPEGs $p=9.9e-07$, iMEGs $p=1.9e-06$). This
198 provides large-scale empirical evidence that rapid evolution previously observed in

199 imprinted genes detected in seed offspring at 4 days after pollination from one set of
200 reciprocal crosses (Wolff, et al. 2011) applies more generally to the imprinted genes of
201 *A. thaliana*.

202

203 **Imprinted genes are evolving under positive selection in *A. thaliana***

204 PS can be detected at the population genomic level by assessing allele frequency and
205 coalescence time as variation subject to PS is expected to go to fixation (Nielsen 2005;
206 Sabeti, et al. 2006). Genes can display elevated ω for a range of reasons other than PS,
207 however, such as reduced functional constraint or pseudogenization. To test whether
208 the increase in ω observed across the imprinted iMEGs and iPEGs was due to positive
209 selection, we analyzed the evolutionary rates of iMEGs and iPEGs in the context of
210 clusters of orthologous genes from across the plant kingdom. This analysis was
211 conducted using an in-house plant database containing ortholog clusters from 34
212 sequenced plant species, either Embryophyte or Chlorophyte (supplementary fig. S1).
213 To further ensure the robustness of our analysis, we only considered clusters for
214 which orthologous genes could be identified from at least six species, in addition to *A.*
215 *thaliana* (see Methods), following recommended best practice for PAML analyses
216 derived from simulation studies (Anisimova, et al. 2001). Applying this filter, suitable
217 clusters for PAML (codeML) analyses were obtained for 64 of the 140 imprinted genes
218 (30 iMEGs and 34 iPEGs; fig. 2; supplementary table S1B). Sequence alignment quality
219 is also critical for correct sequence analysis (Markova-Raina and Petrov 2011) so all
220 alignments were also assessed using the norMD score as a proxy for alignment quality
221 (Thompson, et al. 2001) – see Methods for details. Two genes (iPEG AT4G11400, iMEG
222 AT5G53870) that had poor sequence alignment quality (norMD score <0.6) were
223 excluded from further analyses.

224 Applying standard codeML models to the remaining 62 imprinted genes, we identified
225 30 that are evolving under PS (table 2; fig. 3A; supplementary table S1). For 6 of the 30
226 positively selected imprinted genes, the PS was specific to the *A. thaliana* lineage (i.e.
227 lineage-specific PS; supplementary table S1A), while for 16 imprinted genes positive
228 selection was detected at individual codons in cross-lineage comparisons (i.e. site-
229 specific PS, supplementary table S1A). Eight imprinted genes displayed both lineage-

230 specific and site-specific PS (fig. 3A). To ensure that these results have not been biased
231 by any of the assumptions inherent in PAML, we also performed a HyPhy analysis
232 (Pond and Muse 2005) on these 62 genes, using a combination of FEL (Fixed Effects
233 Likelihood), SLAC (Single-Nucleotide Ancestor Counting), and MEME (Mixed Effects
234 Model of Evolution) packages, as described in the Methods. From these analyses, we
235 determined that PS is also predicted to be occurring on all 30 genes identified by
236 PAML (supplementary table S2). HyPhy and codeml-based models such as PAML differ
237 fundamentally in how they estimate site-specific rates: PAML models use random
238 effects likelihood while HyPhy models use fixed-effects likelihood, hence the
239 congruence between the results of the two approaches provides strong confirmation
240 of the robustness of the PS signature at the 30 imprinted loci.

241 Recently, a methodology has been published for directly estimating possible
242 confounding of imprinting gene analysis by contamination with maternal tissues
243 (Schon and Nodine 2017). Two of the datasets, of Gehring et al. (2011) and Hsieh et
244 al. (2011), were analysed by Schon and Nodine who suggested that 20 iMEGs from
245 these studies used in our analysis should be considered 'low-confidence' (although
246 variation in gene expression patterns under different growth conditions could itself
247 confound these conclusions). The RNA-seq dataset of Wolff et al. (2011) was not
248 analysed by the Schon and Nodine (2017), so we performed the tissue-enrichment
249 test of Schon and Nodine on the datasets used by Wolff et al. (2011) to determine
250 expression pattern (Belmonte et al., 2012). We conclude that these datasets do not
251 suffer from significant levels of cross-tissue contamination (supplementary fig. S2):
252 only the suspensor showing any potential contamination from non-suspensor specific
253 transcripts while none of the endosperm datasets used to identify imprinted genes
254 showed any enrichment for other tissues, including the maternal seed coat. We
255 conclude that the remaining 57/77 iMEGs used in our PAML and HyPhy analyses are
256 'high-confidence' imprinted genes, while a further 20 may be due to the presence of
257 maternally-derived transcripts (supplementary table S3). These include four genes
258 which are under positive selection according to both codeML and HyPhy, 10 others
259 which showed no evidence for PS and 6 which were not tested due to lack of sufficient
260 orthology clusters. We conclude that positive selection acts upon 19 iPEGs and 11

261 iMEGs, and that all of the iPEGs and at least 7 of the iMEGs are high-confidence
262 imprinted genes. Taken together, these results indicate that positive selection acts on
263 protein-coding genes regulated by genomic imprinting in the seeds of *A. thaliana*.

264

265 **Imprinted genes are preferentially affected by positive selection**

266 The large number of imprinted genes subject to positive selection suggested that
267 genes epigenetically regulated by genomic imprinting could be under stronger positive
268 selection than biallelically-expressed genes. To test this hypothesis, we compared the
269 extent of positive selection in imprinted genes to that observed in randomly sampled
270 gene sets from across the whole genome (supplementary table S4A). Genomic
271 imprinting in plants mainly occurs in the seed endosperm, which can be subject to
272 different selective pressures related to its triploid genome dosage independent of
273 imprinting (Baroux, et al. 2002). Hence, we also conducted analysis of positive
274 selection for random samples of known endosperm-specific *A. thaliana* genes
275 (Belmonte, et al. 2013) (supplementary table S4B). For iPEGs, the odds ratio score for
276 lineage-specific positive selection indicated 3.3- and 2.6- fold enrichment in positive
277 selection in imprinted genes compared to whole-genome and endosperm controls,
278 respectively. These ratios equate to a significant enrichment of lineage-specific
279 positive selection in iPEGs when compared with either the genome-wide or
280 endosperm-specific controls (Fisher's test, $p=0.014$ and $p=0.041$ respectively; Fig. 3B).
281 Strikingly, no enrichment was found for iMEGs in either lineage-specific ($p=0.531$ vs.
282 genome-wide controls, $p=0.688$ vs. endosperm genes) or site-specific selective
283 pressure variation ($p=0.542$ vs. genome-wide controls, $p=0.764$ vs. endosperm genes)
284 (Fig. 3C), whether lower-confidence iMEGs were included or not. To determine if the
285 bias in enrichment of position selection in iPEGs as compared to iMEGs is due to
286 statistical threshold effect we identified an additional set of imprinted genes where
287 the significance level following LRT fell just below the cut off p-value of 0.05 (but
288 above 0.10): out of the set of six imprinted genes identified with this relaxed criteria,
289 only one imprinted gene is annotated as an iMEG, while the other five were iPEGs,
290 therefore we can discount any potential bias of this results due to thresholding. We
291 further tested the strength of the difference between the selective pressures acting on
292 iMEGs and iPEGs by performing a chi-squared test directly on the ω -values as

293 extracted from the branch site models (using likelihood ratio tests values from
294 (Morgan, et al. 2010)). We conclude that iPEGs, but not iMEGs, are subject to higher
295 levels of positive selective pressure, revealing a difference in the evolutionary
296 trajectory of imprinted genes depending on the parental genome from which they are
297 expressed.

298

299 **Most imprinted genes exhibit fixation of positively selected sites**

300 If the sites determined to be under positive selection in the *A. thaliana* lineage
301 improved plant fitness, then we could expect that these substitutions would be fixed
302 or exist at high frequency within *A. thaliana* populations due to full or partial selective
303 sweeps (Patwa and Wahl 2008). Hence, we tested the percentage conservation of *A.*
304 *thaliana*-specific amino acid sites under either lineage-specific PS or site-specific PS
305 (supplementary tables S5, S6). For almost all imprinted genes subject to lineage-
306 specific PS, the associated sites showed 100% conservation across the 80 *A. thaliana*
307 accessions for which full sequence data was available (posterior probability >0.95)
308 (supplementary table S7) (Cao, et al. 2011), with no difference observed between
309 iMEGs and iPEGs. Only two imprinted genes (AT1G48910 and AT1G55050) displayed
310 nonsynonymous mutations at the otherwise conserved positively selected position.
311 AT1G48910 encodes YUCCA 10, which is a flavin monooxygenase involved in auxin
312 biosynthesis predicted to have roles in morphogenetic development of pollen grains,
313 while AT1G55050 is a widely-conserved gene of unknown function. If variation at the
314 amino acids subject to positive selection confers phenotypic effects, this requires
315 distinct *A. thaliana* populations with known population histories to test for differing
316 intra-specific selection signatures driven by local environments (Huber, et al. 2014).
317 We consider that positive selective pressures at imprinted loci in the *A. thaliana*
318 lineage has been sufficiently strong, (i.e. with a selective advantage for these
319 alternative amino acids), to cause the fixation of these amino acid variants.

320

321 **Positive selection on the imprinted *NRPD1a* gene involved in sRNA regulation**

322 We noted that the imprinted genes subject to lineage-specific positive selection
323 included *NRPD1a*, which encodes a component of the RNA Pol IV complex response
324 for transcribing small RNA and, subsequently, transcriptional balance between

325 maternally and paternally inherited genomes in endosperm (supplementary table S8)
326 (Kanno, et al. 2005; Eamens, et al. 2008; Erdmann, et al. 2017). It has previously been
327 reported that nucleotide substitution rate of the Pol IV polymerase subunit encoded
328 by *NRPD1a* is 20 times higher than that observed in the equivalent subunit of Pol II
329 (Luo and Hall 2007), supporting a scenario whereby the *NRPD1a* gene is under positive
330 selection and suggesting a possible functional relationship between sRNA processing
331 and (imprinted) genes under positive selection. We assessed if positive selection at
332 *NRPD1a* might be due to selection occurring more generally on sRNA-processing
333 genes, perhaps because of their roles in controlling the balance of maternal and
334 paternal gene expression, and not due to the imprinting status of this gene
335 specifically. However, when we analysed the selective pressures acting on 23 non-
336 imprinted genes encoding components of the sRNA processing pathway, none
337 displayed any signature of positive selection (supplementary table S8). We consider
338 that the positive selection acting on *NRPD1a* is associated with its status as an
339 imprinted gene involved in small RNA production and, likely, with subsequent control
340 of gene expression in the endosperm.

341

342 **iMEGs and iPEGs have similar evolutionary ages**

343 One potential confounding factor in our analysis would be if iMEGs and iPEGs had
344 different evolutionary ages. To address this possibility, we determined the
345 evolutionary ages of the 140 imprinted genes using a phylostratigraphy approach
346 (Domazet-Lošo, et al. 2007) (fig. 4). Nine Age Classes (AC) were defined for available
347 plant genome sequences (<http://www.phytozome.net/>) where AC 0 includes the
348 youngest genes (i.e. those which have evolved since the divergence of *A. thaliana*) and
349 AC 9 the oldest, or most conserved. We then assigned imprinted genes to different
350 age classes using an e-value cutoff of $<10^{-3}$ (supplementary table S9). Notably, no
351 significant difference was observed between the age distributions of iMEGs and iPEGs
352 (Fisher's exact test, $p=0.7$), suggesting that differences in age are unlikely to explain
353 the differing levels of PS observed in these categories.

354 Interestingly, 11 of the imprinted *A. thaliana* genes have been shown to have
355 homologs regulated by imprinting in the sister species, *A. lyrata* (table S1A), according

356 to the analysis of (Klosinska, et al. 2016). These include three iMEGs and eight iPEGs,
357 including three iPEGs which we find to be under PS; these three all belonged to the
358 most conserved age classes (8 or 9; table S9) so may be good candidates for highly
359 conserved imprinting. In contrast, a total of seven imprinted genes did not show any
360 sequence similarity outside Brassicaceae (fig. 4), *i.e.* they were Brassicaceae-specific
361 orphans according to our previous definition (Donoghue, et al. 2011). Of these
362 Brassicaceae-specific imprinted orphan genes, one (AT4G31060) was found in *A.*
363 *thaliana* only and so represents the most recently-arisen imprinted gene known for
364 this species. The fact that some imprinted genes date from the evolution of the
365 angiosperms may indicate roles for these genes in the accompanying double
366 fertilization event by which the endosperm evolved (Gehring, et al. 2011), although
367 this remains to be tested.

368 We found that the imprinted gene set as a whole showed enrichment for participation
369 in the *At*- α whole genome duplication (WGD; 52 imprinted genes, Fisher's test,
370 $p=0.02$), whereas only 21 genes were found to have participated in either the *At*- β or
371 *At*- γ WGD events (Fisher's test, $p=0.14$) (fig. 4). The *At*- α WGD pre-dated the
372 diversification of core Brassicaceae from *Aethionema* (Franzke, et al. 2011), while *At*- β
373 and *At*- γ are older WGD events predating the emergence of Brassicaceae within the
374 Eurosids (Bowers et al., 2003). These findings are in agreement with the models of Qiu
375 et al., who suggested that many imprinted genes are descended from loci formed by
376 WGD during the evolution of Brassicales (Qiu, et al. 2014). However, there was again
377 no difference in this distribution between iMEGs and iPEGs across different WGD
378 events. In summary, we found no evidence for differing evolutionary histories or
379 recent iPEG diversification that could confound our molecular evolutionary
380 comparison between iPEGs and iMEGs.

381

382 **Most imprinted genes are functionally constrained**

383 Even if imprinted genes have been subject to positive selection in their evolutionary
384 histories, it is possible that their recent evolution has been more constrained, for
385 example by purifying selection. To estimate the relative roles of ancestral PS (*i.e.*
386 predating the most recent common ancestor of *A. thaliana* and *A. lyrata*) PS and

387 recent selective constraint, we performed McDonald-Kreitman tests (McDonald and
388 Kreitman 1991) on our entire set of 140 imprinted orthologs from *A. lyrata* and *A.*
389 *thaliana* (this included the imprinted genes for which orthologs were identified in
390 fewer than six other plant species, and which we had not been able to analysis by
391 PAML or HyPhy). Unambiguous *A. lyrata* orthologs were detected for 110 out of the
392 140 total imprinted *A. thaliana* genes (56 iPEGs and 54 iMEGs) on the basis of BLASTP
393 alignments (supplementary table S10A). This approach assumed that the number of
394 substitutions fixed between *A. thaliana* and *A. lyrata* was driven by ancestral positive
395 selection and neutral substitution at nonsynonymous sites (D_N), and by neutral
396 processes only at synonymous ones (D_S). As a result, a large D_N/D_S ratio may indicate
397 PS. We compared these D_N and D_S counts to the numbers of nonsynonymous (P_N) and
398 synonymous (P_S) polymorphisms within the population of 80 genome-sequenced *A.*
399 *thaliana* accessions to determine the fixation index (FI) such that $FI=(D_N/D_S)/(P_N/P_S)$.
400 Both P_N and P_S reflect a combination of neutral and deleterious alleles and thus
401 represent an expected value for a neutral D_N/D_S if no ancestral PS has occurred. If $FI>1$,
402 then ancestral adaptation through beneficial non-synonymous changes in the most
403 recent common ancestor of *A. thaliana* and *A. lyrata* can be concluded to have
404 occurred; alternately, if $FI<1$, then it implies that purifying selection on the ancestral
405 lineage was the predominant selective force. For the 110 imprinted genes, we found
406 that D_N/D_S (1.139) approximated P_N/P_S (1.196) with $FI=0.952$ (table 3) and conclude
407 that there is no evidence of relaxed selective constraints. (We note that neither D_N/D_S
408 and P_N/P_S ratios of these imprinted gene sets were biased by outliers (Daub, et al.
409 2014)). To further examine the recent selective pressures acting on *A. thaliana*
410 imprinted genes, we also performed Direction of Selection (DoS) analysis which can
411 produce more accurate estimates of selection, especially for highly conserved genes.
412 In agreement with the results of the McDonald-Kreitman test, DoS analysis did not
413 indicate any evidence of relaxed selective constraints (supplementary table S10B)
414 according to the Tarone and Greenland Neutrality Index ($NI_{TG}=1.237$; table 3). Here, NI
415 >1 indicates that negative selection is preventing fixation of harmful mutations.

416 We also compared these values to those of the *A. thaliana* genome as a whole and
417 found no evidence for imprinted genes differing from the genome-wide pattern (fig.

418 5). This suggests that the imprinted genes have been subject to similar selective
419 processes as other genes since the divergence of *thaliana-lyrata* (supplementary fig.
420 S3): the same relative proportions showed patterns of PS ($D_N/D_S \gg P_N/P_S$), ancestral
421 purifying selection (low D_N/D_S), neutrality ($D_N/D_S \sim P_N/P_S$), or potential
422 pseudogenization evidenced by relaxed selective constraint (high P_N/P_S and high
423 D_N/D_S) (Yang, et al. 2011; Wang, et al. 2012). In contrast to the PAML and HyPhy
424 analysis of selection from before the *thaliana-lyrata* divergence, no difference was
425 apparent between iMEGs and iPEGS (supplementary fig. S3). Both McDonald-Kreitman
426 and DoS analysis identified signatures of purifying selection on the same group of 13
427 genes (12% of the total, supplementary table S10A and B) while six putative
428 pseudogenes were discovered (5% of the total, supplementary table S11): as
429 expected, none of these showed any evidence of PS. As imprinted pseudogenes could
430 potentially bias the overall analysis, their effect was assessed by comparing the
431 baseline FI (0.952) to the expected fixation index (eFI , 1.205) determined from the
432 expected contingency table values of D_N , D_S , P_N , P_S for each of the 110 imprinted genes
433 (Axelsson and Ellegren 2009). This higher eFI suggested population-level mutations
434 were negatively correlated with purifying selection, presumably due to deleterious
435 alleles segregating within the 80 accessions and supporting previous reports of high P_N
436 values in *A. thaliana* (Huber et al. 2014). This is also important as relaxed selective
437 constraints (evident from a high level of within-*A. thaliana* nonsynonymous changes)
438 would have confounded our interspecies tests for positive selection, and because
439 previous work has shown that the average effect of nonsynonymous changes in *A.*
440 *thaliana* is slightly deleterious (Bustamante, et al. 2002).

441 Comparison of the results of PAML and HyPhy analysis, McDonald-Kreitman tests and
442 DoS demonstrates that the imprinted genes subject to positive selection in
443 interspecies analysis using at least six genomes do not show any strong evidence of
444 positive selection since the divergence of *A. thaliana* and *A. lyrata*. We conclude that
445 genes with different evolutionary trajectories are regulated by genomic imprinting in
446 *A. thaliana*, including some subject to pseudogenization while non-pseudogenized
447 genes show signatures of ancestral PS with stronger signatures of PS predating the
448 *thaliana-lyrata* split. Estimating the timing of these events with greater accuracy, and

449 determining their effects in extant populations, will provide a basis for future
450 determination of the selective pressures involved in the evolution of imprinted genes
451 in plants.

452

453 **Discussion**

454 Evolutionary trajectories of genes in mammals and angiosperms can be influenced by
455 their association with tissues involved in maternal provisioning, creating the possibility
456 for conflict over resource allocation and positive selection (PS) on the loci involved,
457 among other molecular signatures (fig. 1). In this study we have concentrated on the
458 molecular signatures of conflict acting on coding sequences of imprinted genes in
459 which alleles are expressed at different levels depending on whether they are
460 maternally- or paternally-derived (denoted iMEGs and iPEGs respectively; (Köhler, et
461 al. 2012)). The phenotypes associated with certain imprinted genes under PS in
462 animals (*Igfr*) and plants (*AIMEDEA*) supports the possibility of conflict-driven PS
463 (Spillane, et al. 2007; Miyake, et al. 2009; Wawrzik, et al. 2010; McCole, et al. 2011).
464 However, we have previously demonstrated that there is no strict concordance
465 between evidence of positive selection and imprinting status in mammals (O'Connell,
466 et al. 2010), and how conflict affects imprinted plant genes in general remains
467 unknown.

468 In this study, we have performed a comprehensive ortholog-based analysis of
469 selective pressures on genes subject to genomic imprinting in the seed endosperm of
470 *A. thaliana* and have demonstrated signatures of elevated PS (tables 1 and 2; figs. 2
471 and 3; fig. S1). To ensure these conclusions are robust, we have considered and
472 accounted for the effects of possible endosperm-specific effects and of differences in
473 gene age (fig. 4) and have accounted for potential confounding by genes expressed
474 uniparentally from maternal tissues (fig. S2). As approaches for inferring selection
475 pressures may be limited by their own inherent assumptions, we took a multiple-
476 methodology approach. For example, PAML makes the assumption that selective
477 pressures do not change on the branches where it is inferred, while HyPhy allows
478 branch-specific selection to change across all branches. We used two methodologies
479 for our ortholog-based analyses (PAML and HyPhy) and for our analysis of extant *A.*

480 *thaliana* populations (McDonald-Kreitman and Direction of Selection tests). In fact, the
481 30 imprinted genes found to be under PS by PAML analysis were confirmed in every
482 case confirmed as such by at least two HyPhy methods (tables S1, S2), while similar
483 conclusions were derived from both McDonald-Kreitman and DoS approaches (table
484 3). We also note that it is not currently feasible to assess such changes at gene
485 regulatory sequences across lineages, so our estimates for selection levels across loci,
486 based as they are on coding-sequences alone, may in fact be underestimates.

487 It should be noted that some assumptions still remain within our analyses. For
488 example, all Dn/Ds based methods for estimating selective pressure variation from
489 sequence data assume that Ds is a proxy for neutral evolution, *i.e.* silent sites are not
490 under selective pressure, even though we know for example that exon splice sites can
491 be subject to selection to function the spliceosomal machinery (albeit mostly in intron-
492 rich genomes (Warnecke, et al. 2008)). To control for this, we made use of non-
493 imprinted controls, both from genome-wide data and from genes specifically
494 expressed in the endosperm in which genomic imprinting occurs in flowering plants
495 (supplementary table S4). The robustness of the results from these analyses is
496 furthermore supported by the robustness of the phylogeny used, which is
497 uncontroversial (fig. 4; <https://phytozome.jgi.doe.gov/pz/portal.html>), and on the
498 number of species used in each alignment, which was set at a minimum of six,
499 following experimentally-determined best practice (Anisimova et al., (2001)).

500 Combining together these analyses, and their comparison with relevant controls, we
501 conclude that accelerated evolution and preferential tendency to PS are general
502 features of imprinted genes in *A. thaliana*.

503

504 **Fixation of selected sites and significance of mating system**

505 Extant plant lineages have undergone multiple transitions between self-fertilising and
506 out-crossing reproduction. It is expected that parental conflict will be minimized by
507 increased levels of self-fertilization, which reduces or eliminates the genetic
508 divergence between maternally- and paternally-derived genomes (Haig 1997, 2013;
509 Gehring and Satyaki 2017), as well as slightly reducing the efficacy of purifying
510 selection across the genome (Payne and Alvarez-Ponce 2018). Consistent with this,

511 previous investigations of the imprinted maternally expressed gene (iMEG) *MEDEA*
512 found that *MEDEA* was under positive selection in the outcrossing Brassicaceae
513 species, *Arabidopsis lyrata*, while its non-imprinted paralog *SWINGER* was not; but
514 that neither gene was under positive selection in the largely inbreeding congener, *A.*
515 *thaliana* (Spillane, et al. 2007; Miyake, et al. 2009). This was interpreted as a
516 consequence of reduced genomic conflict due to inbreeding (Garnier, et al. 2008;
517 McKeown, et al. 2013). The findings of our present study indicate that almost all of the
518 positively selected sites are now fixed across populations in extant *A. thaliana* which
519 may indicate that conflict has been reduced in this largely self-pollinated species:
520 while the levels of outcrossing in *A. thaliana* can reach 18% in natural populations in
521 exceptional cases, it is generally much lower (Bomblies, et al. 2010).

522 The fixation of sites under positive selection in imprinted genes of *A. thaliana* is
523 consistent with hypotheses that imprinting may in some cases be a relic of its
524 outbreeding past (Brandvain and Haig 2005), perhaps because loss of imprinting to
525 protect against deleterious recessive mutations only occurs very slowly (Wilkins and
526 Haig 2003b). In other words, the signatures of selection detected by non-synonymous
527 changes to coding sequences retain evidence of past conflict even after any such
528 equilibrium has been reached: our PAML analysis is in fact identifying sites which have
529 changed under positive selection but are now at a stable equilibrium, and which no
530 longer show signatures of such pressures in current populations (whether measured
531 by McDonald-Kreitman tests or by Direction of Selection tests; tables S10 and S11).
532 Whether amino acid changes at these sites have also become fixed across other plant
533 lineages with different levels of inbreeding would be an interesting test of this
534 hypothesis, and will be possible to test empirically when once genomic data from
535 multiple accessions of sufficient numbers of outcrossing and inbreeding plant species
536 becomes available. It should also be noted that clonal interference arising from
537 inbreeding is expected to marginally reduce the efficiency of selection across the
538 genome (Neher, et al. 2013) and potentially mask signatures of positive selection,
539 although rates of neutral evolution at silent sites should not be affected (Good, et al.
540 2014), provided that the beneficial alleles co-occur in the same period of selection.

541 Therefore, clonal interference would mean tests for positive selection would be more
542 prone to false negatives rather than false positives.

543 In addition, we have compared our rates of positive selection in imprinted loci to the
544 genome-wide pattern for *A. thaliana*, which also adjusts for any potential confounding
545 effects of inbreeding. Whether fixed or not, imprinted genes which have been under
546 PS are likely to have been important for plant fitness and represent strong candidates
547 for future functional investigations.

548

549 **Imbalance between selective pressures acting on iMEGs and iPEGs**

550 Imprinted genes in mammals can undergo different evolutionary trajectories
551 (O'Connell, et al. 2010; McCole, et al. 2011). Our results from this study in plants
552 demonstrate that differential selective pressures act on imprinted genes that are
553 expressed from either the maternal or the paternal genomes. Specifically, iPEGs
554 display higher D_N/D_S values, and are significantly more likely to be subject to PS. This
555 finding of asymmetric selection pressures on iPEGs vs iMEGs does not fit neatly with
556 expectations of kin conflict which predict that any PS driven by intra-genomic conflict
557 should likely act on both genomes due to the mutual antagonism between the parents
558 over resource allocation to the offspring, possibly on pairs of reciprocally imprinted
559 genes encoding physically interacting offspring growth regulators (Moore and Haig
560 1991; Mills and Moore 2004).

561 Our identification of PS in iPEGs also lacks concordance with theories that propose
562 that imprinting results from maternal-offspring co-adaptation or cytonuclear co-
563 evolution as illustrated in fig. 1B (Wolf and Hager 2006), in line with the lack of
564 experimental support for this model (Haig 2013; Haig 2014). Although co-evolutionary
565 scenarios can lead to rapid evolution of genes (Wolf and Brandvain 2014), both of
566 these scenarios would be expected to preferentially affect iMEGs (assuming maternal
567 cytonuclear inheritance). Nor is PS in iPEGs due to genome dosage effects in the
568 endosperm, as the levels of positive selection for iPEGs are significantly higher than
569 biallelically-expressed endosperm genes (fig. 3). We can also rule out the possibility
570 that PS in iPEGs could be an artifact of these genes being younger than iMEGs,
571 because (1) there is no significant age difference between iPEGs and iMEGs, and (2) PS

572 does not affect the more recently evolved iPEGs (figs. 2 and 5). We do note that levels
573 of PS in the endosperm-expressed control set are slightly greater than the background
574 control set (fig. 3B), which could indicate the existence of unreported iPEGs within this
575 dataset, or other causes related to the role of the endosperm in seeds. Finally, our
576 results do not support an evolutionary scenario where imprinted genes arise as a
577 result of pseudogenization following gene duplication (Wolff et al., 2011), as we could
578 only identify six possible examples of this (fig. 2).

579 The finding that *A. thaliana* iPEGs are preferentially affected by PS compared with
580 iMEGs provides an interesting parallel with the evolutionary flexibility of iPEGs
581 observed in comparisons to *A. thaliana*'s sister species, *Arabidopsis lyrata*. Analysis of
582 *A. lyrata* endosperm found that iPEGs were more highly expressed in *A. lyrata* than *A.*
583 *thaliana*, while expression levels of iMEGs were more highly conserved (Klosinska, et
584 al. 2016). These changes were also associated with greater variation in CHG
585 methylation and histone modification marks between at least some conserved iPEGs
586 in the two species (Klosinska, et al. 2016). Furthermore, a study in *Capsella rubella*
587 showed that iPEGs display higher levels of non-synonymous substitution, a possible
588 indicator of PS (Hatorangan, et al. 2016), suggesting that this pattern may not be
589 restricted to the *Arabidopsis* genus either but may be a common feature of imprinting
590 in, at least, the Brassicaceae. One possible explanation for the differences between
591 selective pressures acting on iMEGs and iPEGs is that kin conflict more commonly
592 involves interactions between iPEGs and genes expressed in maternal tissues such as
593 the sporophytic seed coat (which are also involved in maternal provisioning (Orozco-
594 Arroyo, et al. 2015)), rather than with iMEGs in the endosperm. This would lead to
595 conflict that was indirect in nature, rather than involving physical interactions
596 between antagonistic pairs of iMEGs and iPEGs (McVean and Hurst 1997). Intriguingly,
597 an analysis of parental conflict in *A. lyrata* populations with different levels of
598 outbreeding suggested that conflict involving indirect interactions between paternal
599 factors and the female sporophyte ('the kinship model') was favoured in more self-
600 fertile populations, while direct interactions between proteins encoded by imprinted
601 genes in the endosperm tended to be lost as outcrossing reduced (Willi 2013). This
602 would also fit with the discovery that genes which are strongly expressed in the seed
603 coat of *A. thaliana* can also evolve under positive selection (Schon and Nodine 2017).

604 We also note that antagonism between the developing endosperm and another
605 maternal tissue, the nucellus, has been proposed as a key characteristic of seed
606 development in *A. thaliana* (Xu, et al. 2016). Analysis of the genetic interactions
607 between maternal seed coat or nucellus with iPEGs which regulate seed size (such as
608 *ADMENTOS*; (Kradolfer, et al. 2013)) will therefore be required to clarify whether
609 parental conflict occurs in *A. thaliana* and related species, and if so by what
610 mechanism.

611 Further possible explanations for the differences in selective pressures acting on
612 iMEGs and iPEGs could include differential breadth of expression patterns (including in
613 somatic tissues) or wider interaction networks which could theoretically place iMEGs
614 under greater constraints due to risk of pleiotropic interactions. Alternatively PS could
615 also be due to so-called 'arms races' between siblings that do not share the same
616 paternal parent (Sadras and Denison 2009), which is more likely among paternally-
617 derived 'patrigenes' than maternally-derived 'matrigenes' (Haig 2013). It has been
618 shown that PS in flowering plants can be driven by pre-fertilization sexual conflict
619 between male genomes during pollen tube competition (Gossmann, et al. 2014), in a
620 manner analogous to competition between animal sperm (Torgerson, et al. 2002),
621 such that positive selection at iPEGs could be triggered by conflict between the
622 paternal genomes of endosperm tissues within seeds developing on the same plant
623 (or in the same fruit). Paternal genetic variation is known to influence resource
624 allocation in embryos by up to 10% in *A. thaliana* (House, et al. 2010), which could be
625 sufficient to drive conflict between paternal alleles. Finally, if this pattern was also
626 conserved in monocots, it could explain reports that paternally-derived expression-
627 QTLs (eQTLs) have major roles in determining transcription levels in hybridized maize
628 seed (Swanson-Wagner, et al. 2009). Finally, the most active evolutionary signatures
629 acting at iPEGs in different species of Brassicaceae (this study; (Hatorangan, et al.
630 2016; Klosinska, et al. 2016)), in which multiple shifts of mating system have occurred,
631 could suggest that shifting patterns of paternal relatedness, and hence, [patrigenic](#)
632 [phenotypic optima for seed size](#), could lead to continual evolutionary pressure
633 manifested in different ways, such as changes to transcription level, epigenetic marks,
634 and changes to the nucleotide and amino sequence. More generally, models of
635 imprinting and conflict suggest that matrigenes typically favour phenotypes

636 intermediate to those favoured by patrigenes and maternal alleles (Burt and Trivers
637 1998; Wilkins and Haig 2002, 2003a; Haig 2013), in which case, positive selection for
638 conflict with maternal tissues would be stronger on paternally expressed imprinted
639 genes than on maternally expressed ones. If so, the same trend might be expected to
640 be common across seed plants: analysis of selective pressures acting on imprinted
641 genes in a more distantly related group such as the cereals could be instructive in
642 testing this hypothesis.

643 Given these different, and non-mutually exclusive possibilities, careful analysis of the
644 functions of the genes and codons subject to PS will be needed to clarify the
645 underlying impacts of the patterns we observe on the biology of the plant. Although
646 experimental characterization for many genes has yet to be fully performed, we note
647 that one of the iPEGs we have identified to be under PS is *NRPD1a*, which encodes a
648 subunit of RNA Pol IV, while other sRNA genes are not subject to PS (table S8). RNA Pol
649 IV is involved in control of transposable elements via RNA directed DNA methylation
650 (RdDM) and has recently also been identified as a regulator of allelic dosage in the
651 endosperm (Erdmann, et al. 2017). Interestingly, the largest subunits of PolV (NRPE1),
652 which is also implicated in the activity of 24-nt sRNAs in RNA-directed DNA
653 methylation (RdDM), has also been reported to evolve rapidly through restructuring of
654 intrinsically disordered repeats within its Argonaute-binding platform (Trujillo, et al.
655 2016). In the case of *NRPD1a*, this subunit is involved in physically binding
656 transposable elements including those expressed in maternal tissues in seeds
657 (Mosher, et al. 2009). Hence, it is possible that PS could be driven by conflict between
658 paternally-expressed proteins and maternally-controlled transposable elements, or to
659 interactions with the maternally-derived genomes of the endosperm in the case of
660 dosage control (Erdmann, et al. 2017). Interestingly, *NRPD1a* does not appear to be an
661 iPEG in *A. lyrata*, although two other genes encoding subunits of complexes involved
662 in the RdDM pathway are (Klosinska, et al. 2016). Further functional characterization
663 of the positively selected subunits will be needed to distinguish these possibilities.

664 We note that positive selection has been reported from the iMEG *MEDEA* in the
665 predominantly outcrossing *A. lyrata*, but that this selective pressure has been lost in
666 the inbreeding *A. thaliana* lineage (Spillane, et al. 2007). This lends further support to
667 the hypothesis that positive selection persists between iPEGs and the maternal

668 sporophyte but not between iPEGs and iMEGs during the transition to self-fertilization
669 (Willi 2013). Analysis of signatures of selective pressure on the components of the FIS
670 complex across multiple plant species will be essential for clarifying the effects of
671 parental conflict in imprinting, endosperm development and speciation.

672

673 **Conclusions**

674 The study of imprinted genes in both plants and mammals has identified examples of
675 positive Darwinian selection (Spillane, et al. 2007; O'Connell, et al. 2010; Wawrzik, et
676 al. 2010). Our study demonstrates that while imprinted genes expressed in the
677 endosperm of *Arabidopsis thaliana* are rapidly evolving due to positive selection, such
678 positive selection is preferentially associated with imprinted paternally expressed
679 genes (iPEGs). This raises the possibility that ongoing intra-genomic conflicts between
680 paternally-expressed imprinted genes (iPEGs), or between iPEGs and genes
681 functioning in the maternal sporophyte, could be evolutionary drivers and maintainers
682 of imprinting in plants. The iPEG and iMEG genes we have identified under positive
683 selection are involved in processes such as auxin biosynthesis (e.g. *YUCCA10*, *TAR1*)
684 and epigenetic regulation involving small RNAs and chromatin remodelling (*NRPD1a*).
685 Overall, our results identify the subset of imprinted genes, both iPEGs and iMEGs,
686 which are strong candidates for having functional effects that are antagonistic with
687 other molecular factors, in a manner that results in their evolution under positive
688 selection.

689

691 **Methods**

692 **Identification of imprinted genes and orthologs**

693 An *A. thaliana* imprinted gene set was compiled from a number of high-throughput
694 expression screens (Gehring, et al. 2011; Hsieh, et al. 2011; McKeown, et al. 2011;
695 Wolff, et al. 2011), supplemented by other studies (Vielle-Calzada, et al. 1999;
696 Kinoshita, et al. 2004; Köhler, et al. 2005; Jullien, et al. 2006; Tiwari, et al. 2008;
697 Gehring, et al. 2009; Gerald, et al. 2009) to yield 140 high-confidence imprinted genes
698 (supplementary table S1). Orthologs were identified across 34 plant species for which
699 assembled whole genome sequences were publically available (fig. 4). Peptide and
700 CDS sequences for 32 species were downloaded from Phytozome v8.0 (Goodstein, et
701 al. 2012); *Cajanus cajan* sequences were accessed from (Varshney, et al. 2012) and
702 *Lotus japonicus* from the PlantGDB database (Dong et al., 2004). In all cases, the
703 longest transcript was used as the representative transcript for each gene. To
704 minimize the number of false positives and ensure tight clustering of genes families,
705 we detected orthologous relationships between sequences using OrthoMCL (Li, et al.
706 2003; Chen, et al. 2007). We also chose to use maximum likelihood methods based on
707 codon models of sequence evolution as these are considered to be more robust than
708 alternative methods such as sliding window approaches (Schmid and Yang 2008). As
709 the power of maximum likelihood methods increases with greater taxonomic
710 representation and breadth (Anisimova, et al. 2001), we considered only the 62
711 imprinted genes for which orthologous genes could be identified from at least six
712 other species (in addition to *A. thaliana* itself). As controls, random sets of 100 genes
713 were generated representing the entire *A. thaliana* genome, and a subset of
714 endosperm-specific genes derived from (Belmonte, et al. 2013) (Supplementary table
715 S4). To ensure a valid comparison with the imprinted dataset, only genes belonging to
716 orthology clusters present in at least six other species (Anisimova, et al. 2001) were
717 included in these control sets.

718

719 **Multiple sequence alignments**

720 Multiple sequence alignments for each gene family were constructed using MUSCLE
721 (Edgar 2004) and MAFFT (Kato and Toh 2008) and were compared in AQUA (Muller,

722 et al. 2010). RASCAL (Thompson, et al. 2003) was used to refine the alignments and
723 norMD (Thompson, et al. 2001) was used to assess their quality. Alignments with a
724 norMD score <0.6 were considered as low quality. Poorly aligned sequences were
725 removed from alignments with norMD <0.6 and norMD was recalculated: if the
726 norMD score subsequently increased to >0.6 , the alignment was retained for further
727 analysis. Nucleotide sequence alignments were generated for each family using the
728 amino acid alignment and original nucleotide sequence files, using in-house software.
729 Recombinant sequences were also removed identified using RDP3 (Martin, et al. 2010)
730 with two substitution-based methods – GENECONV (Sawyer 1989) and MaxChi (Smith
731 1992) – and two phylogenetic-based methods – BOOTSCAN (Martin, et al. 2005) and
732 SiScan (Gibbs, et al. 2000). Sequences were considered as recombinant if a
733 recombination event was significantly predicted by at least one substitution-based
734 method *and* at least one phylogenetic-based method. The percentage of gaps in the
735 alignments were calculated using TrimAL (Capella-Gutierrez, et al. 2009) (-sgc option)
736 and predicted sites of positive selection which overlapped with regions of poor
737 alignment (gaps $> 40\%$) were discarded.

738

739 **Tree building**

740 Models for protein sequence evolution were generated using modelgenerator (Keane,
741 et al. 2006). Phylogenetic trees were inferred using RAxML (Randomized Axelerated
742 Maximum Likelihood) version 7.2.6 (Stamatakis 2006) with 1000 bootstrap replicates
743 and the rapid bootstrapping algorithm. The codeML analysis was run on all clades of
744 interest for genes with >80 sequences in their orthology clusters (supplementary table
745 S12A) and on control genes from genome-wide and endosperm-expressed datasets
746 (supplementary table S12B).

747

748 **Selective pressure analysis**

749 Selective pressure analysis was conducted using PAML version 4.4e (Yang 2007). Both
750 lineage-specific models (Yang 1998; Yang and Nielsen 2002) and site-specific models
751 (Yang and Swanson, 2002) were evaluated using likelihood ratio test (LRT). Sequences
752 were considered to exhibit lineage-specific selective pressure if the likelihood ratio
753 test for ModelA was significant in comparison to both ModelA null and M1Neutral,

754 where M1Neutral is a neutral model that allows two site classes: $\omega_0=0$ and $\omega_1=1$.
755 Model A assumes the two site classes are the same in both foreground and
756 background lineages ($\omega_0=0$ and $\omega_1=1$) and ω_1 was calculated from the data. Model A
757 null is the null hypothesis for this model and allows sites to be evolving under either
758 purifying selection, or to be neutrally evolving in the background lineages. For site-
759 specific analyses, LRTs were conducted to compare models M7 and M8a with model
760 M8. The test compared the neutral model M7, which assumes a β distribution for ω
761 over sites and the alternative model M8 (β and ω), which adds an extra site class of
762 positive selection. M8a is the null hypothesis of M8 where the additional category is
763 neutral, i.e. $\omega=1$. An automated CodeML wrapper (VESPA, (Webb, et al. 2017)) was
764 used to prepare all the codeML files, to parse the PAML output and perform the
765 likelihood ratio test. After ML estimates of model parameters were obtained, we used
766 two bayesian approaches to infer the posterior probability of the positively selected
767 sites: Bayes Empirical Bayes (BEB) and Naïve Empirical Bayes (NEB). BEB reduces the
768 rate of false positives when analyzing small datasets and retains the power of NEB
769 when analyzing large datasets (Yang and Nielsen 2002). Therefore if NEB and BEB
770 were both predicted the results from BEB were preferred.

771

772 **Use of HyPhy to estimate rates of Darwinian selection**

773 A second positive selection pressure analysis of genes which were predicted to be
774 under positive selective pressure by PAML was conducted using HyPhy version 2.2.4
775 (Pond and Muse 2005). We employed the following three approaches from the HyPhy
776 package: FEL (Fixed effects Likelihood), SLAC (Single-Nucleotide Ancestor Counting),
777 and MEME (Mixed Effects Model of Evolution). FEL tests for both positive and negative
778 selection per individual site, and can identify individual sites that have undergone
779 pervasive diversifying selection while SLAC is an approximate method similar to FEL
780 (Kosakovsky Pond and Frost 2005). We also applied the MEME model from the HyPhy
781 package which tests for episodic selection at individual sites and on specific branches:
782 MEME does not assume that the strength and direction of selection is constant across
783 all lineages (Murrell, et al. 2012). Only sites resolved as being under PS by at least two
784 methods were considered confirmed by HyPhy.

785

786 **Tests including population-level variation**

787 *A. lyrata* orthologs of 140 imprinted *A. thaliana* genes were identified using reciprocal
788 best hits (RBH) of which 110 were also derived as the best hits of the *A. thaliana* genes
789 in reciprocal BLAST. *A. thaliana* and *A. lyrata* CDS were aligned as described above. 80
790 accession SNP data for *A. thaliana* was downloaded from the 1001 genome project
791 (http://1001genomes.org/data/MPI/MPICao2010/releases/current/genome_matrix)
792 and SNPs mapped to the reference genome using a custom-made python script.
793 McDonald-Kreitman tests were performed on each imprinted gene using a python
794 script that uses egglib library to calculate D_N , D_S , P_N and P_S values and calculated the
795 ratio using Fisher's exact test. Fixation indices (FI) were determined as $FI =$
796 $(D_N/D_S)/(P_N/P_S)$ with expected fixation index (eFI) calculated as reported previously
797 (Axelsson and Ellegren 2009). Genes with zero D_N/D_S and P_N/P_S were not considered
798 for FI calculations. Direction of selection (DoS) ((Stoletzki and Eyre-Walker 2011) was
799 calculated using $D_N/(D_N+D_S)-P_N/(P_N+P_S)$; the Tarone and Greenland Neutrality Index
800 (NI_{TG}) was calculated using the Distribution of Fitness Effect (DoFE) package.

801

802 **Acknowledgements**

803 This work was supported by grant funding from Science Foundation Ireland (SFI) to CS
804 (Principal Investigator Grants 08/IN.1/B193 and 13/IA/1820) and MJO'C (Research
805 Frontiers Programme Grant EOB2673). We would like to thank the DJEI/DES/SFI/HEA
806 funded Irish Centre for High-End Computing (ICHEC) for the provision of
807 computational facilities and support. The support of the NUI Galway Thomas Crawford
808 Hayes Trust is also acknowledged.

810 **Tables**

811

812 **Table 1**

813

Gene Class	Mean ω (D_N/D_S)	Median ω (D_N/D_S)
iPEGs	0.4265±0.053	0.3339
iMEGs	0.5045±0.061	0.3314
whole genome	0.2436±0.002	0.1814

814

815 **Table 1.** D_N/D_S ratios (ω) of iPEGs and iMEGs compared to whole genome.

816

818 **Table 2**

819

	iMEGs	iPEGs	Total
Total number of genes tested	30	32	62
Genes subject to lineage-specific selection only	2 (6.7%)	4 (12.5%)	6 (0.9%)
Genes subject to site-specific selection only	7 (23.3%)	9 (28.1%)	16 (25.8%)
Genes subject to both lineage- and site-specific selection	2 (6.7%)	6 (18.8%)	8 (12.9%)
TOTAL	11 (36.7%)	19 (59.4%)	30 (48.4%)

820

821 **Table 2:** Numbers of iMEGs and iPEGs determined to be under positive selection.

823

824 **Table 3.**

825

826	Parameter	Polymorphism	Divergence
827	Non-synonymous substitutions (D_N)	1988	4740
828	Synonymous substitutions (D_S)	1662	4161
829	Ratio of Non-synonymous		
830	/Synonymous (D_N/D_S) substitutions	1.196	1.139
831	Fixation Index (FI) ¹		0.952
832	Expected Fixation Index (eFI) ²		1.205
833	Neutrality Index (NI_{TG}) ³		1.237
834	α ⁴		-0.210

835

836 **Table 3.** Calculations derived from McDonald-Kreitman analyses of genes regulated by
837 genomic imprinting in the *A. thaliana* endosperm; values were derived from
838 comparisons between 80 sequenced *A. thaliana* accessions, using *A. lyrata* as
839 outgroup. Full gene-by-gene results from which these figures were derived are
840 presented in Suppl. table S7. ¹Observed fixation index, calculated according to $FI =$
841 $(D_N/D_S)/(P_N/P_S)$. ²Expected fixation index (eFI). ³The Tarone and Greenland Neutrality
842 Index (NI_{TG}). ⁴Proportion of fixed nonsynonymous mutations driven by fixed positive
843 selection fixed in *A. thaliana*, $\alpha = (FI - eFI)/eFI$.

844

846 **Figure legends**

847

848 **Fig. 1. Summary of scenarios for selection on imprinted plant genes.** Schematic of *A.*
849 *thaliana* seed summarising the impacts of genomic imprinting on genetic selection as
850 predicted by major hypotheses for genomic imprinting. In each case, the diploid F1
851 embryo is shown in dark green, surrounded by the triploid F1 endosperm, shown in
852 yellow) in which imprinting occurs, and the diploid seed coat (SC) which is part of the
853 maternal sporophyte, shown in light green. (A) Intra-Genomic conflict in which
854 antagonism between matrigenes and patrigenes over resource allocation results in
855 physical interactions between iMEGs and iPEGs (Spillane, et al. 2007). (B) Co-
856 adaptation models predict that any selective pressure should be concentrated on
857 iMEGs which are co-inherited with cytoplasmic genomes in *A. thaliana* (Wolf and
858 Brandvain 2014). (C) Indirect conflict or "Kinship Model" predicts that conflict
859 between iPEGs and genes expressed in maternal tissues (e.g. seed coat, scMEG, or
860 other sporophyte tissues) leads to positive selection on iPEGs (Willi 2013).

861

862 **Fig. 2. Size of orthology clusters to which imprinted *A. thaliana* genes belong.**
863 Orphans are defined according to (Donoghue, et al. 2011); genes present in orthology
864 clusters >6 were considered for further selective pressure variation analysis.

865

866 **Fig. 3. Summary of the number of genes under positive selection in the dataset.** (A)
867 Numbers of imprinted *A. thaliana* genes under site and/or lineage specific PS; (B, C)
868 the percentages of *A. thaliana* iMEGs and iPEGs subject to lineage-specific (B) or site-
869 specific (C) PS compared to the percentages in control sets of endosperm-expressed
870 ('Endosperm') or genome-wide ('Genome') biallelic genes; control gene-sets are listed
871 in supplementary table S4.

872

873 **Fig. 4: Phylogeny of the 34 species included in our analyses and the age distribution**
874 **of iMEGs and iPEGs.** (A) shows the frequency of age class (AC) for the iMEGs and
875 iPEGs tested. AC0, *A. thaliana* specific; AC1, *A. lyrata*; AC2, Brassicaceae; AC3,
876 Brassicales-Malvales; AC4, Rosid; AC5, Eudicot; AC6, Angiosperm; AC7, Tracheophyte;

877 AC8, Embryophyte; AC9, Viridiplantae. (B) Consensus phylogenetic relationships of all
878 34 species; the phylogenetic position of the age classes and the known whole genome
879 duplication events for the species included in the study are also highlighted (Vanneste,
880 et al. 2014).

881

882 **Fig. 5. Distribution of D_N/D_S and P_N/P_S ratios for imprinted genes compared to all**
883 **protein-coding genes in *A. thaliana*.** X-axis depicts P_N/P_S ratios, Y-axis represents
884 D_N/D_S ratios. Green dots = genes under purifying selection; red dots = genes under
885 positive selection; yellow dots = genes under neutral evolution; black triangles = *A.*
886 *thaliana* imprinted genes; blue triangles = pseudogenes with high D_N/D_S and high
887 P_N/P_S . No clustering was observed.

888

890 **Supplementary material legends**

891 **Suppl. fig. S1. Schematic of workflow for identifying positive Darwinian selection.**

892 **Suppl. fig. S2. Tissue specific transcript enrichment of previously published datasets.**

893 The test for tissue specific transcript contamination (Schon and Nodine 2017) was
894 applied to the datasets generated by (Wolff, et al. 2011), indicating that only the
895 suspensor datasets exhibited an enrichment for non-tissue specific transcripts.
896 Endosperm datasets exhibited no significant enrichments for other tissues.

897 **Suppl. fig. S3. Distribution of D_N/D_S and P_N/P_S ratios for imprinted genes compared
898 to all protein-coding genes in *A. thaliana* disaggregated between iPEGs and iMEGs.**

899 **Suppl. table S1. 140 experimentally-validated genes subject to genomic imprinting in
900 the *A. thaliana* endosperm and original references.**

901 **Suppl. table S2. Confirmation of positive selection by HyPhy.**

902 **Suppl. table S3. Comparison of imprinted genes with methodology of Schon and
903 Nodine (2017) for identifying potential maternal contaminants.**

904 **Suppl. table S4. Results of selection analysis for control datasets.**

905 **Suppl. table S5. Details of all sites subject to positive selection in imprinted genes of
906 *A. thaliana*. Genes whose imprinted status is predicted to be 'low-confidence'
907 according to the methodology of Schon and Nodine (2017) are indicated.**

908 **Suppl. table S6. Amino acids encoded by sites subject positive selection in imprinted
909 genes of *A. thaliana*.**

910 **Suppl. tables S7. Percentage of fixation of lineage-specific positively selected sites in
911 *Ath* 80 population data identified by codeml. Data for all sites with posterior
912 probability >0.5 is displayed.**

913 **Suppl. table S8. Results of tests for positive selection on genes of the sRNA
914 processing pathway.**

915 **Suppl. table S9. Age classes of iMEGs and iPEGs (as shown in fig. 3).**

916 **Suppl. table S10. Results of all McDonald-Kreitman (10A) and Direction of Selection
917 (DoS, 10B) tests.**

918 **Suppl. table S11. High Dn/Ds and High Pn/Ps of *A. thaliana* imprinted genes**
919 **identified as pseudogenes.**

920 **Suppl. table S12. Gene trees for (A) imprinted genes and (B) non-imprinted control**
921 **genes tested for positive selection.**

922

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925

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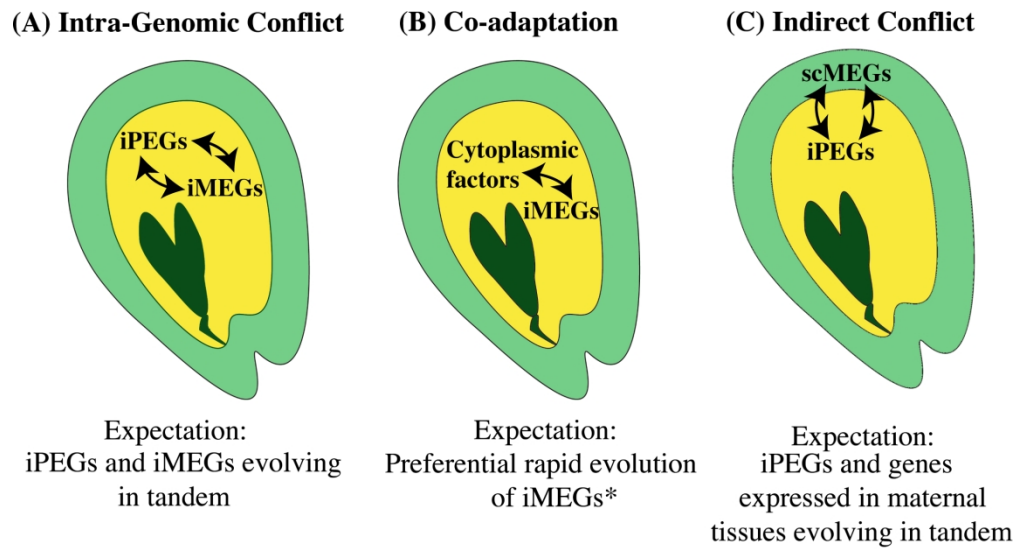
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*assumes maternal cytonuclear inheritance

Figure 1

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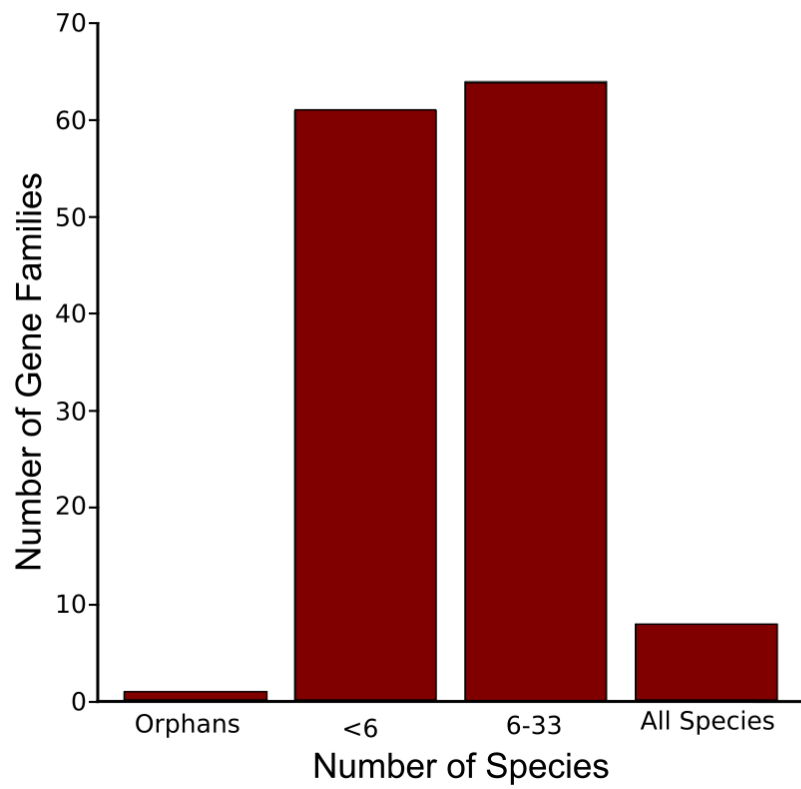


Figure 2

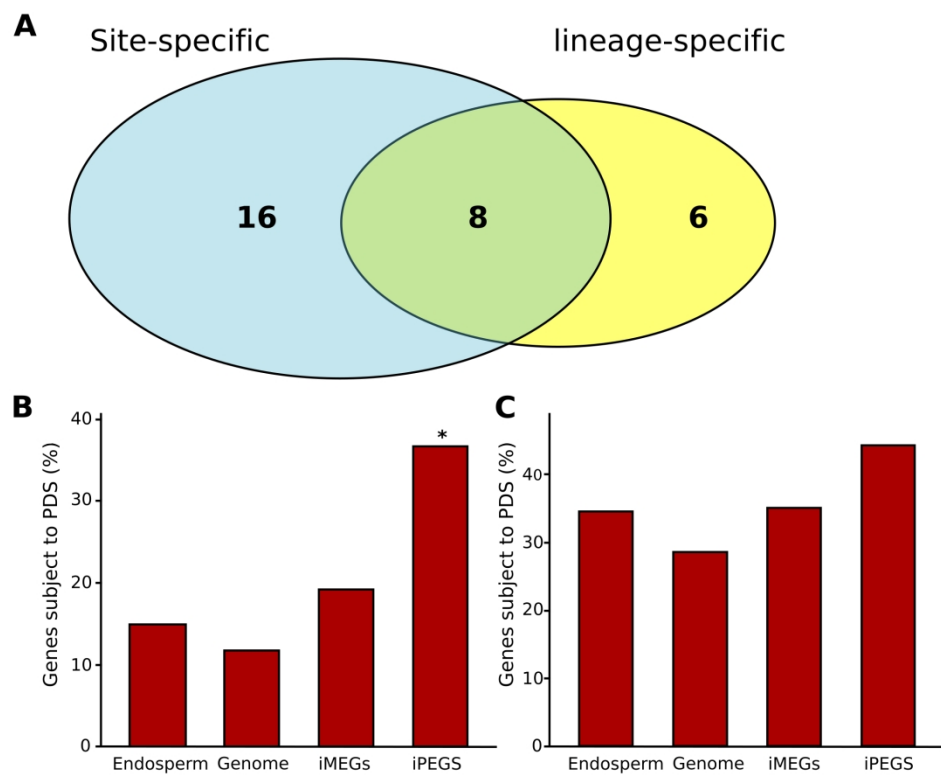


Figure 3

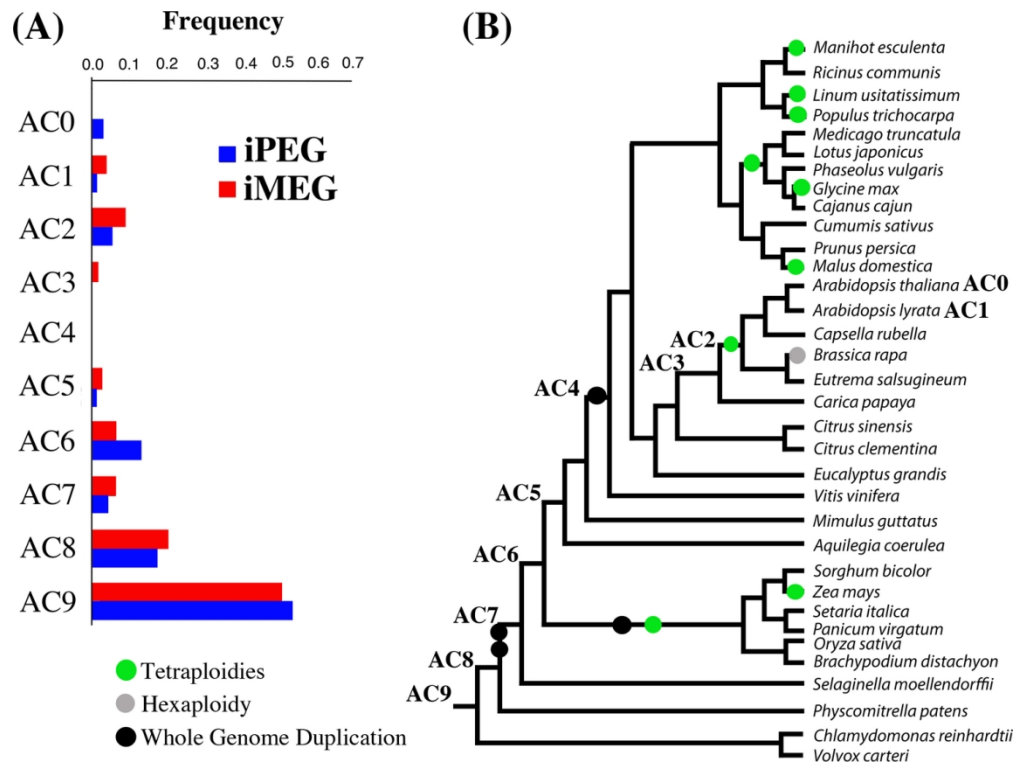


Figure 4

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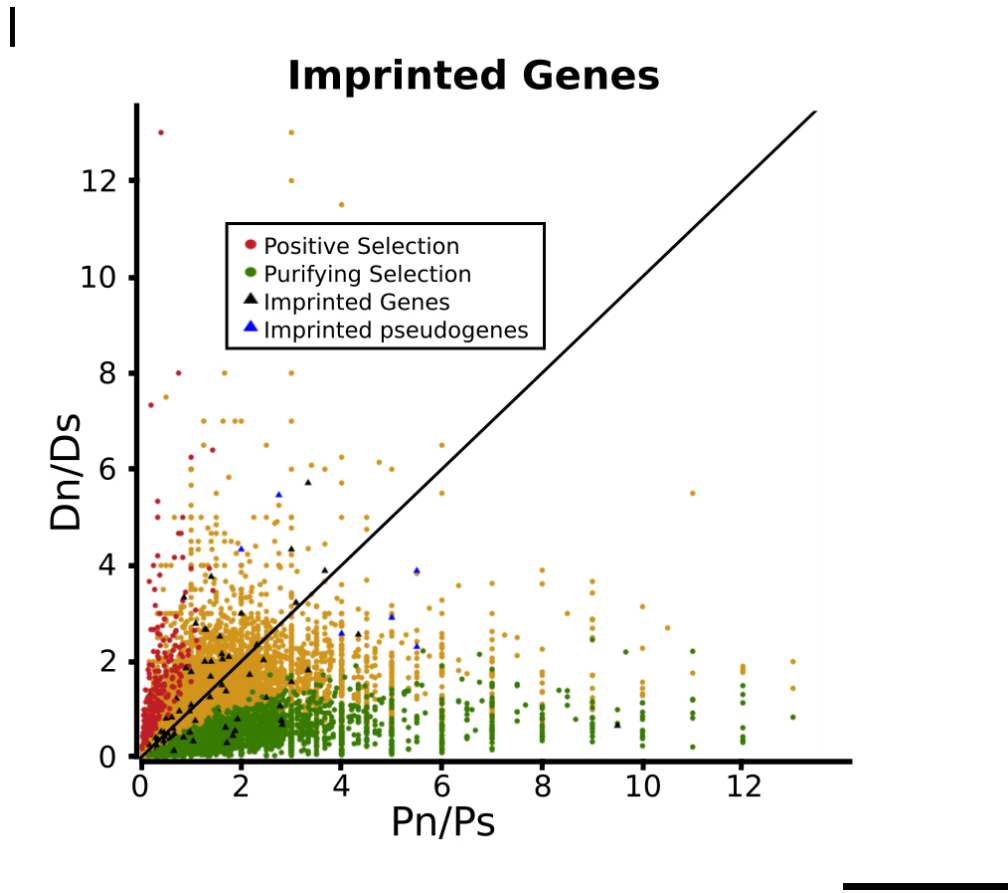


Figure 5