Appendix B from M. C. Castellanos et al., “Anther Evolution: Pollen Presentation Strategies When Pollinators Differ”

(Am. Nat., vol. 167, no. 2, p. 000)

Images of *Penstemon* Anthers and Phylogenetic Relationships

“There are great variations within the genus *Penstemon* as regards the staminode, as well as with reference to the nectaries, and these are obviously related to the method of pollination.… In order to explain all these relations, and also the different methods of anther dehiscence, a comparative oecological research on numerous species is a desideratum” (Knuth 1906).

What follows is a block-by-block explanation of how the anthers contrast and the evidence behind the species pairs that represent separate origination of hummingbird pollination among penstemons. The letters correspond to the blocks as illustrated in figure B1. We frequently refer to the internal transcribed spacer (ITS) phylogeny shown in figure B2, which is a pruned version of the phylogeny for penstemons. A summary of the taxonomy is found in Lodewick and Lodewick (1999).

(A) Bee-pollinated *P. gentryi* anthers open much less widely than closely related bird-pollinated *P. kunthii* (both traditionally in subsection *Campanulati*). The phylogeny places these species together with the origination of hummingbird pollination as separate from all others.

(B) *Penstemon glabrescens*, *P. crandallii*, and many other bee-pollinated species in the same subsection (*Caespitosi)* open narrowly compared to the widely dehiscent bird-pollinated *P. pinifolius.* The ITS tree confirms the relationship of *P. pinifolius* to this group. Traditional taxonomists had separated *P. pinifolius* from *Caespitosi* and sometimes listed it with the likes of *P. fasciculatus*, but *Penstemon* specialist F. Crosswhite believed that it was never properly placed (Crosswhite and Crosswhite 1981).

(C) Both the ITS phylogeny and the traditional taxonomy place the bee-pollinated *P. patens* as related to the bird-pollinated *P. centranthifolius*. The exact relationship is not crucial since the anthers of *P. centranthifolius* open more widely than those of any bee-pollinated penstemon.

(D) *Penstemon rostriflorus* is the only bird-pollinated species in one of the traditional subgenera (*Saccanthera*), and it has anthers with a gaping mouth compared to *P. laetus* and all other bee-pollinated species except *P. neotericus* (not shown), which opens about as widely as *P. rostriflorus*. For statistical purposes, we compared *P. rostriflorus* to the mean of all bee-pollinated *Saccanthera* for which we had data. ITS indicates this origination as certainly separate from all others.

(E) The ITS tree confirms a separate origination of hummingbird pollination within another traditional subgenus (*Dasanthera*). It is debatable whether *P. newberryi* and the other hummingbird-pollinated species in the subgenus *P. rupicola* are monophyletic, so we only count one of them. In this group, the anthers are very woolly (possibly a relevant fact if pollen is secondarily presented on the hairs), and even the bee-pollinated *P. davidsonii* is widely dehiscent. However, there is more of an edge to the anthers of *P. davidsonii* than in the more bird-pollinated *P. newberryi*, and in *P. davidsonii* the anthers remain held together after dehiscence, whereas in *P. newberryi* the mature anthers are held more horizontally (Datwyler and Wolfe 2004).

(F) Based on close morphological similarity and biogeography, the bee-pollinated *P. confusus* seems to be related to bird-pollinated species such as *P. utahensis*, a species with anthers that open less widely. Unfortunately, we lack complete DNA data on *P. utahensis* despite many attempts, so it is not shown in figure B2, but it was found to be a separate origination by Wilson et al. (2005). This appears to be a second origination of hummingbird pollination in subsection *Centranthifolii*, aside from *P. centranthifolius*.

(G) All data agree that *Keckiella* is monophyletic and that hummingbird pollination must have arisen there separately from the originations within *Penstemon* itself. There are four species of bee-pollinated *Keckiella,* all of which have less open anthers than the three bird-pollinated species. The bee-pollinated *Keckiella rothrockii jacintensis* has markedly narrow dehiscence. Freeman et al. (2003) give a phylogeny with two originations of hummingbird pollination within *Keckiella*. Our studies with ITS allow for two equivocal ancestral character reconstructions, with either two originations of hummingbird pollination or an origination and a reversal. In this analysis, we conservatively lump all species in each pollinator type, using only one mean for each type.

(H) The bee-pollinated *P. dasyphyllus* opens by a small crack, whereas the bird-pollinated *P. lanceolatus* has anthers that nearly turn inside out. ITS data agrees on the traditional alliance (in section *Chamaeleon*) between these two species.

(I) The bee-pollinated *P. gentianoides* opens less widely than the more bird-pollinated *P. hartwegii* (traditionally in subsection *Fasciculi*). See comments under block P for doubts about the distinctness of the origination.

The following four pairs are closely related and belong to the traditional subgenus *Habroanthus*, a group with 43 bee-pollinated species and seven bird-pollinated species. ITS phylogenies suggest multiple originations of bird pollination in this subgroup, but it is not clear which bee-pollinated species are most appropriate for each comparison, and we lack complete data on many of the species. Using the species we have studied the most, we did four pairings for illustrative purposes of bee- and bird-pollinated species (pairs J–M). For statistical purposes, we compare each of the four independent bird-pollinated species with the mean of all bee-pollinated species in the subgenus. All are similar in how widely their anthers open, and the differences in how they open make assessing the differences in dispensing difficult.

(J) Contrary to our dehiscence ranks, the velvet data suggested that the bee-pollinated *P. strictus* dispenses more gradually than the bird-pollinated *P. barbatus*, so we could count this pair either as following predictions or as a tie, depending on whether the velvet data or the rank morphology is viewed as more definitive. Notice that recognizing fewer blocks and pooling with block M would actually make our results more significant.

(K) Mostly bee-pollinated *P. speciosus* has anthers that open narrowly compared to those of the bird-pollinated *P. labrosus*. ITS indicates that *P. labrosus* is a separate origination in bootstrap trees, and constraining it to be with other bird-pollinated species lengthens the best tree.

(L) For the third pair, the bee-pollinated *P. neomexicanus* has unique anthers with a deep trough but widely flared edges. Based on the gape measurement, it may present pollen more openly than the molecularly and biogeographically similar bird-pollinated *P. cardinalis*, although the latter species presents its pollen rather openly. It is hard to judge whether the pair follows or contradicts prediction. *Penstemon cardinalis* is not shown in figure B2, but it was a separate origination in the earlier data set of Wilson et al. (2005).

(M) The fourth pair, contrasting the bee-pollinated *P. alpinus* and the bird-pollinated *P. eatonii*, is close but in favor of the prediction, especially because *P. eatonii* has anthers that open more widely than most bee-pollinated species in the subgenus. The distinctness of the origination from that of *P. barbatus* is equivocal in the ITS data set (see comment in block J).

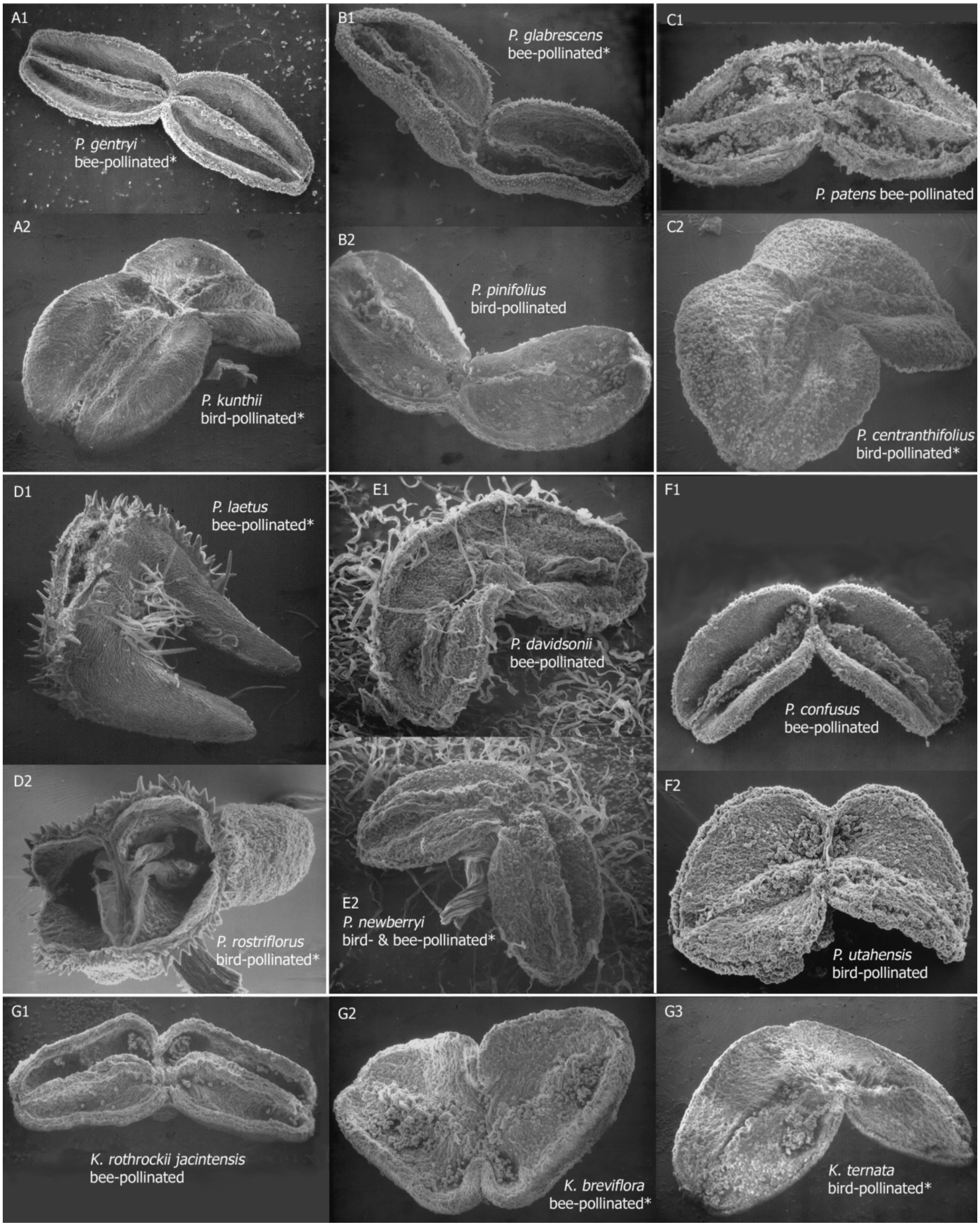
(N) *Penstemon spectabilis* seems to be more recalcitrant in presenting pollen than bird-and-bee-pollinated *P. pseudospectabilis*. The ITS phylogeny allows these two species as close relatives among many other choices, so our pairing in this case is based on taxonomy (traditionally subsection *Peltanthera*). In any case, the exact choice of a bee-pollinated species would not affect the result for this block.

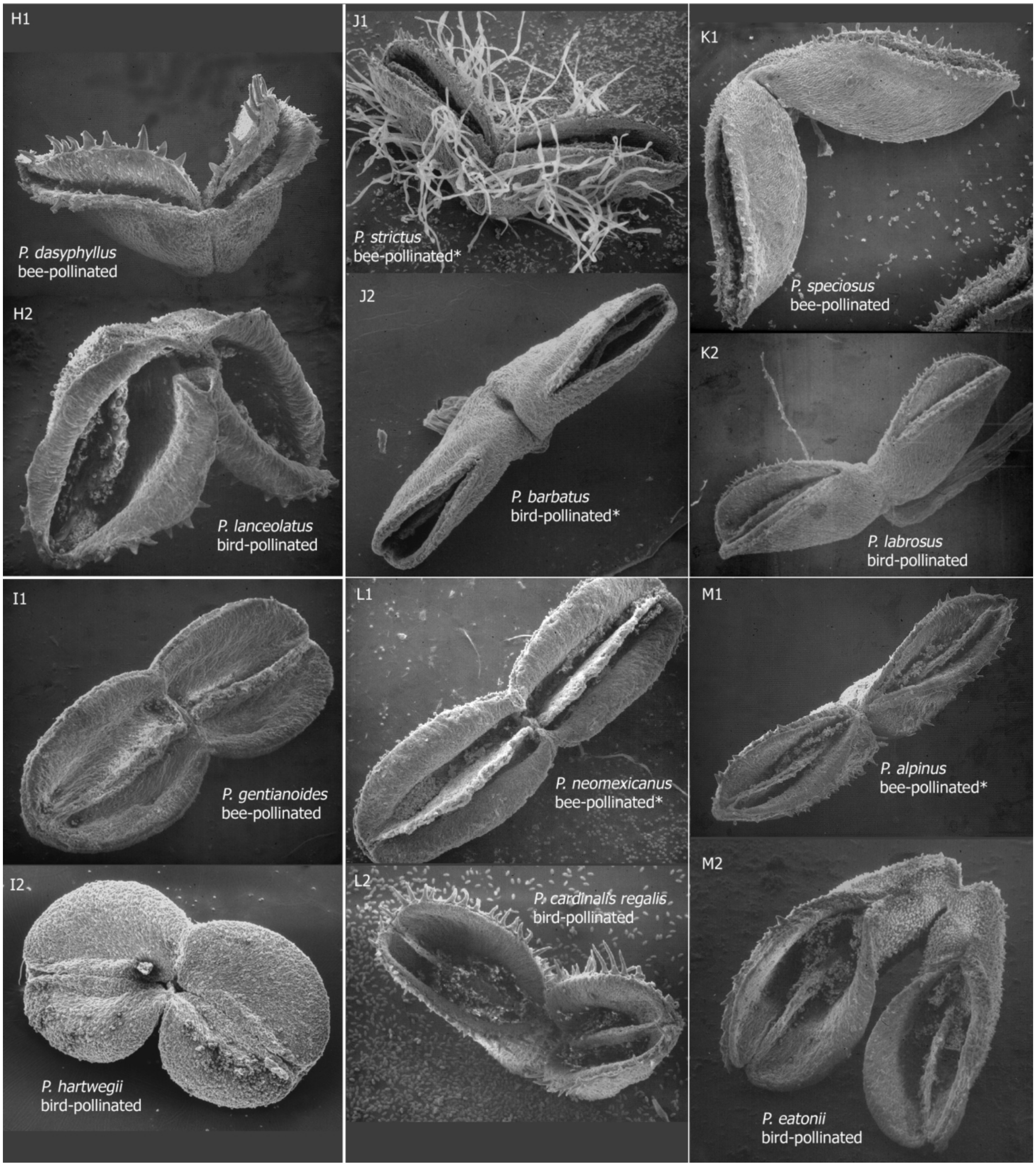
(O) Traditional taxonomy places *P. havardii* in a separate subsection from *P. centranthifolius* (block C), though one might conceive that they are descended from a common ancestor that was hummingbird pollinated. However, the ITS tree dissuades us of this possibility, in which case *P. havardii* represents a separate origination of hummingbird pollination from those listed above, with the greatest doubt actually being that it could be nested within a group with narrow dehiscence (*Habroanthus*). It has anthers that open more widely than any bee-pollinated penstemon. Which one is used for comparison does not matter, and we show *P. buckleyi* only as an example in figure B1 (similar to *P. acuminatus*, which is shown on the ITS tree). For our statistics, we compare *P. havardii* to all bee-pollinated species of penstemons.

(P) Bird-pollinated *P. fasciculatus*, which again has anthers that open more widely than any bee-pollinated penstemon, could conceivably be a descendant from the same origination of hummingbird pollination as *P. hartwegii* (block I), but it is very dissimilar and more extreme in its reliance on hummingbirds. DNA data suggest it may be compared to the bee-pollinated *P. amphorellae*. For statistical purposes, we compare it to all bee-pollinated species.

(Q) We have no match for the bird-pollinated *P. baccharifolius*. The way the anthers open along the connective suggests a relationship with species in block D (*Saccanthera*), but the DNA data argue strongly against such an alliance. It seems fairly deeply rooted in all DNA studies, but no firm conclusions can be made. For our statistics, we compare *P. baccharifolius* to all bee-pollinated penstemons.

(R) Finally, the widespread bee-pollinated *P. palmeri* may be a sister species to the endemic *P. floridus*, which is pollinated by both birds and bees. The ITS tree and the strong vegetative similarities confirm it. *Penstemon palmeri* has slightly less, not more, widely open anthers and thereby is a good candidate for defying the general pattern. This group needs further study.


A1
P. gentryi
bee-pollinated*
B1
P. glabrescens
bee-pollinated*
C1
P. patens bee-pollinated
A2
P. kunthii
bird-pollinated*
B2
P. pinifolius
bird-pollinated
C2
P. centranthifolius
bird-pollinated*
D1
P. laetus
bee-pollinated*
E1
P. davidsonii
bee-pollinated
F1
P. confusus
bee-pollinated
D2
P. rostriflorus
bird-pollinated*
E2
P. newberryi
bird- & bee-pollinated*
F2
P. utahensis
bird-pollinated
G1
K. rothrockii jacintensis
bee-pollinated
G2
K. breviflora
bee-pollinated*
G3
K. ternata
bird-pollinated*


H1
P. dasyphyllus
bee-pollinated
H2
P. lanceolatus
bird-pollinated
J1
P. strictus
bee-pollinated*
J2
P. barbatus
bird-pollinated*
K1
P. speciosus
bee-pollinated
K2
P. labrosus
bird-pollinated
I1
P. gentianoides
bee-pollinated
I2
P. hartwegii
bird-pollinated
L1
P. neomexicanus
bee-pollinated*
L2
P. cardinalis regalis
bird-pollinated
M1
P. alpinus
bee-pollinated*
M2
P. eatonii
bird-pollinated

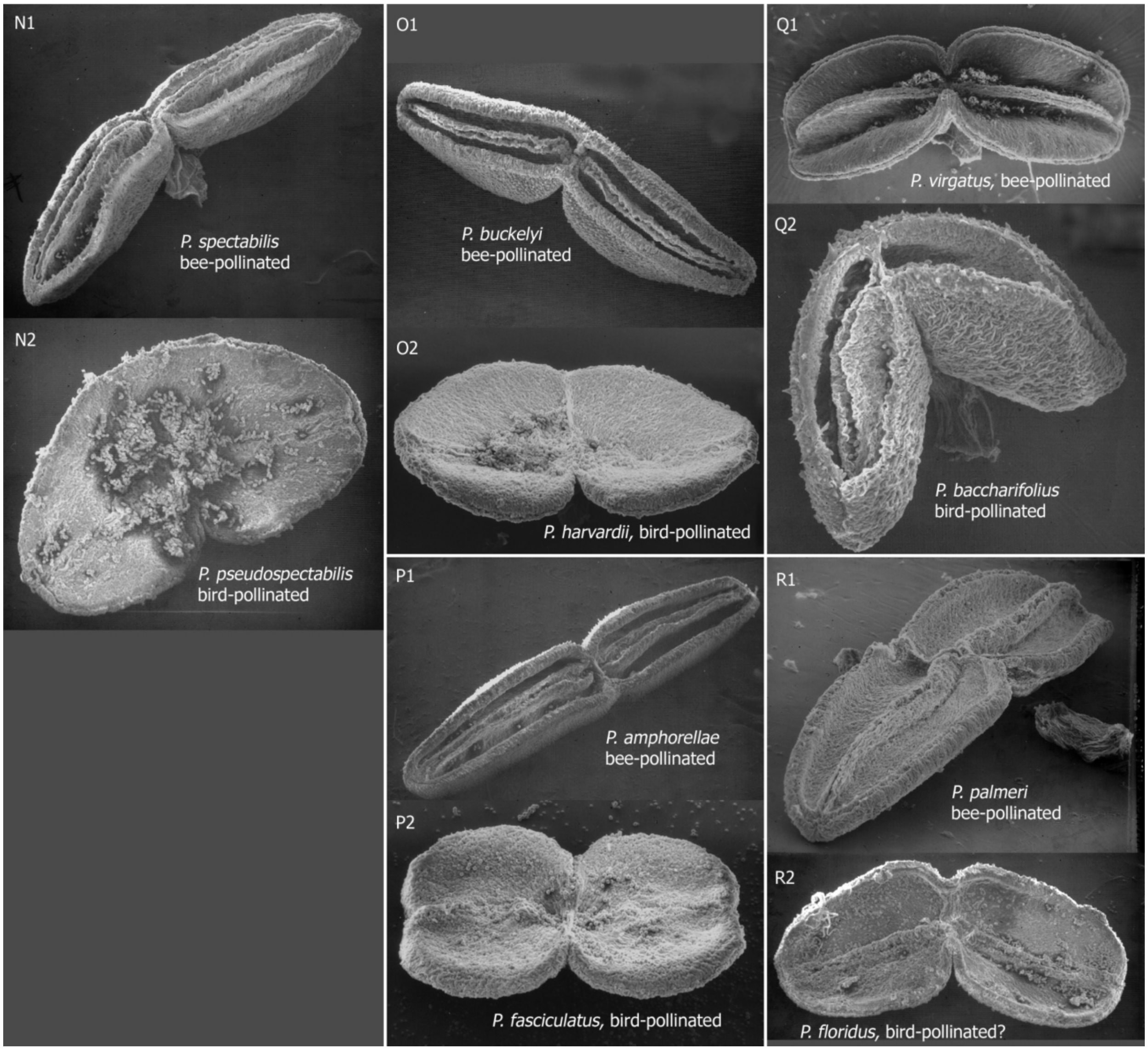


Figure B1: Scanning electron microscope images of fully dehisced *Penstemon* and *Keckiella* anthers, blocked by phylogenetic group. For the micrographs, pollen grains were removed with a paintbrush for better visibility of anther morphology. The anthers were coated with gold, 20 nm thick, using a Hummer II sputter coater, and photographed with a Jeol JSM 5400 scanning electron microscope. Asterisks mark species studied by the velvet method described in the text. Phylogenetic groups are separated by thick white lines. The letters correspond to the pairs in table 2 and to the phylogenetic blocks discussed below.

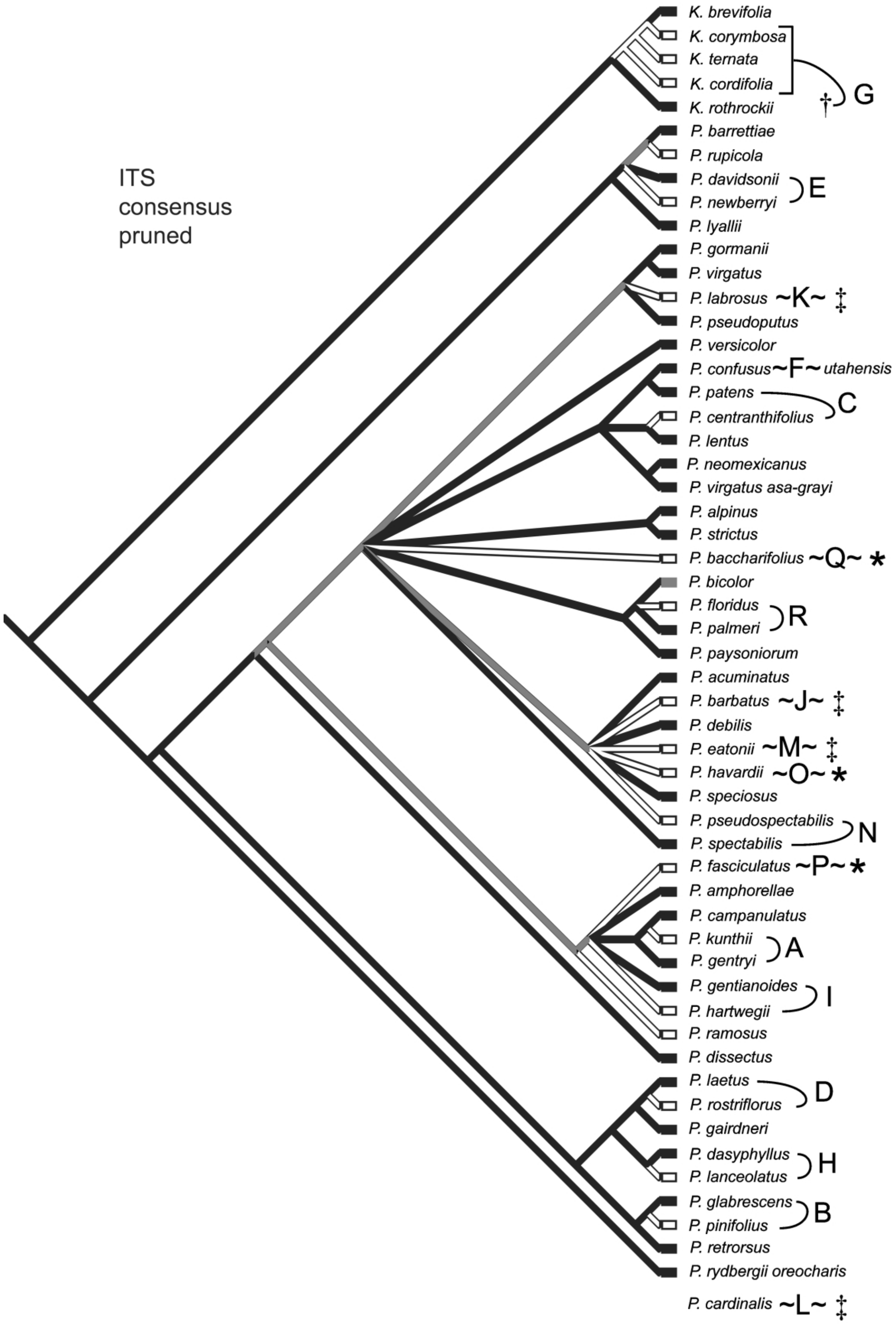

ITS consensus pruned
K. brevifolia
K. corymbosa
K. ternata
K. cordifolia
K. rothrockii
† G
P. barrettiae
P. rupicola
P. davidsonii
P. newberryi
E
P. lyallii
P. gormanii
P. virgatus
P. labrosus ~K~ ‡
P. pseudoputus
P. versicolor
P. confusus~F~utahensis
P. patens
P. centranthifolius
C
P. lentus
P. neomexicanus
P. virgatus asa-grayi
P. alpinus
P. strictus
P. baccharifolius ~Q~ *
P. bicolor
P. floridus
P. palmeri
R
P. paysoniorum
P. acuminatus
P. barbatus ~J~ ‡
P. debilis
P. eatonii ~M~ ‡
P. havardii ~O~ *
P. speciosus
P. pseudospectabilis
P. spectabilis
N
P. fasciculatus ~P~ *
P. amphorellae
P. campanulatus
P. kunthii
P. gentryi
A
P. gentianoides
P. hartwegii
I
P. ramosus
P. dissectus
P. laetus
P. rostriflorus
D
P. gairdneri
P. dasyphyllus
P. lanceolatus
H
P. glabrescens
P. pinifolius
B
P. retrorsus
P. rydbergii oreocharis
P. cardinalis ~L~ ‡

Figure B2: Phylogeny based on nuclear internal transcribed spacer (ITS) sequence data. A heuristic parsimony search was done on data including many more taxa. An Adams consensus was computed of 2,000 equally parsimonious trees, and irrelevant taxa were pruned out. Mapped onto the phylogeny is hummingbird pollination (*white*) versus insect pollination (*black*) based on flower color (there are several species that are intermediate one way or the other if one codes based on more floral characters). Equivocal lineages are shown in gray. Character mapping was done by parsimony of unordered character states. Although this is a pruned diagram and greatly underestimates the number of originations of hummingbird pollination among all penstemons, it is helpful in judging phylogenetic blocks for species whose anthers were studied (see table 2). Arcs show the species pairs used for the analysis. Asterisks indicate comparisons using all bee-pollinated *Penstemon* species. Dagger indicates the comparison of the hummingbird-pollinated *Keckiella* species collectively to all bee-pollinated *Keckiella* species collectively. Double daggers indicate comparisons using all bee-pollinated species in the subgenus *Habroanthus. Penstemon cardinalis* and *Penstemon utahensis* are shown for the sake of completeness, but their ITS data was not included in constructing the phylogeny (cf. Wilson et al. 2005).