

Appendix S5: Brownie Results

We analyzed numerous data sets using Brownie, as described in the Materials and Methods in the main text. When the nuclear data alone were analyzed, four replicate data sets delimited non-overlapping species, with the number of delimited species in data sets 1-3 ranging from 5-23 (Tables C-E). In data set 4, 2333 unique species trees were recovered, with 2007 separate delimited species. A majority rule consensus tree recovered one species including three individuals from populations 14 and 28 (Fig. J), but the other individuals were all placed into a basal polytomy. In all analyses, the delimited species did not include all the individuals from a population, and did not form geographically cohesive groups.

In a second set of analyses, we added mtDNA data to the nuclear data sets. For data set 1, all 500 runs recovered *P. kentucki* as a single species. In data set 2, two species were recovered, one including single individuals from populations 6, 9, and 17, and the other including all the other samples (Table F). The first species includes individuals from widely spaced populations, and does not include all the individuals from the included populations. In data set 3, 12618 trees were recovered from the 500 runs, including 8556 unique species tree topologies and 5018 delimited species. A majority rule consensus tree delimited three species, each with two individuals, while all other individuals formed a basal polytomy (Fig. K). Again, of the three delimited species, none formed a geographically cohesive group or included all of the individuals from their respective populations. In data set 4, two species were recovered in all 500 runs, one including a single individual from each of populations 7 and 19, and the other including all the remaining samples (Table G). The former does not form a geographically cohesive

group, or include all the individuals from the included populations. In summary, when we combined mtDNA and nuclear DNA, the delimited species varied widely among analyses. Only the run that delimited *P. kentucki* as a single species recovered a species that is geographically cohesive and does not split individuals from a single population into separate species.

Finally, we conducted an analysis of the diploid nuclear data, with both alleles from each individual included. This doubles the sample size, but does not account for the hierarchical structure of genetic material (i.e., that there are two alleles/individual). Niemiller et al. [1] found that including a phased nuclear data set increased the number of delimited species, as did increasing the number of individuals and loci sampled. In general, they recommended that a single allele per individual be used, but that analyses on phased data are valuable to determine if heterozygous individuals are split into separate species. Our analysis of the diploid data set recovered 24 unique species trees and 36 species (Table H). In every delimited species, alleles from a single individual were separated into separate species.

In sum, Brownie did not reliably delimit species. Vast differences were recovered when different data sets were used. The delimited species did not form contiguous geographic groups, and typically did not include all the individuals from single populations. This was true whether or not mtDNA was included. Thus, we did not further test the validity of these species.

Reference

1. Niemiller ML, Near TJ, Fitzpatrick BM. Delimiting species using multilocus data: diagnosing cryptic diversity in the southern cavefish, *Typhlichthys subterraneus* (Teleostei: Amblyopsidae). *Evolution*. 2011;66: 846–866. doi:10.1111/j.1558-5646.2011.01480.x

Table D. Species delimited by Brownie using data set 2. The results of 500 independent runs are shown here. Grey boxes indicate inclusion in a partition. 500 trees were recovered (1/run). The alleles in this set of runs and the set illustrated in Table S do not overlap for heterozygous individuals.

	Number	500	499	1	1	1		
	Specimen	Population	Freq	1.000	0.998	0.002	0.002	0.002
65589	2							
65590	2							
65666	3							
65667	3							
53378	4							
53379	4							
52628	5							
52629	5							
79575	6							
53307	7							
54812	7							
55951	8							
78206	9							
78479	9							
60245	10							
60246	10							
77587	11							
77588	11							
77616	12							
77617	12							
78215	13							
78216	13							
77591	14							
77595	14							
3197	15							
55651	16							
55652	16							
3203	17							
60231	19							
60233	19							
60215	20							
60216	20							
54111	21							
57300	21							
62899	22							
62902	22							
69455	23							
69834	24							
69838	24							
60257	25							
77577	26							
57058	27							
57064	27							
52677	28							
52683	28							
56194	29							
60255	30							
78178	31							
78179	31							
78217	32							
78218	32							
78198	33							
61393	34							
61396	34							
69433	35							
70699	36							
51859	37							
51866	37							
52769	38							
52780	38							
64610	39							
54117	40							
54118	40							
64622	41							
64623	41							
63677	42							
77607	43							
77608	43							

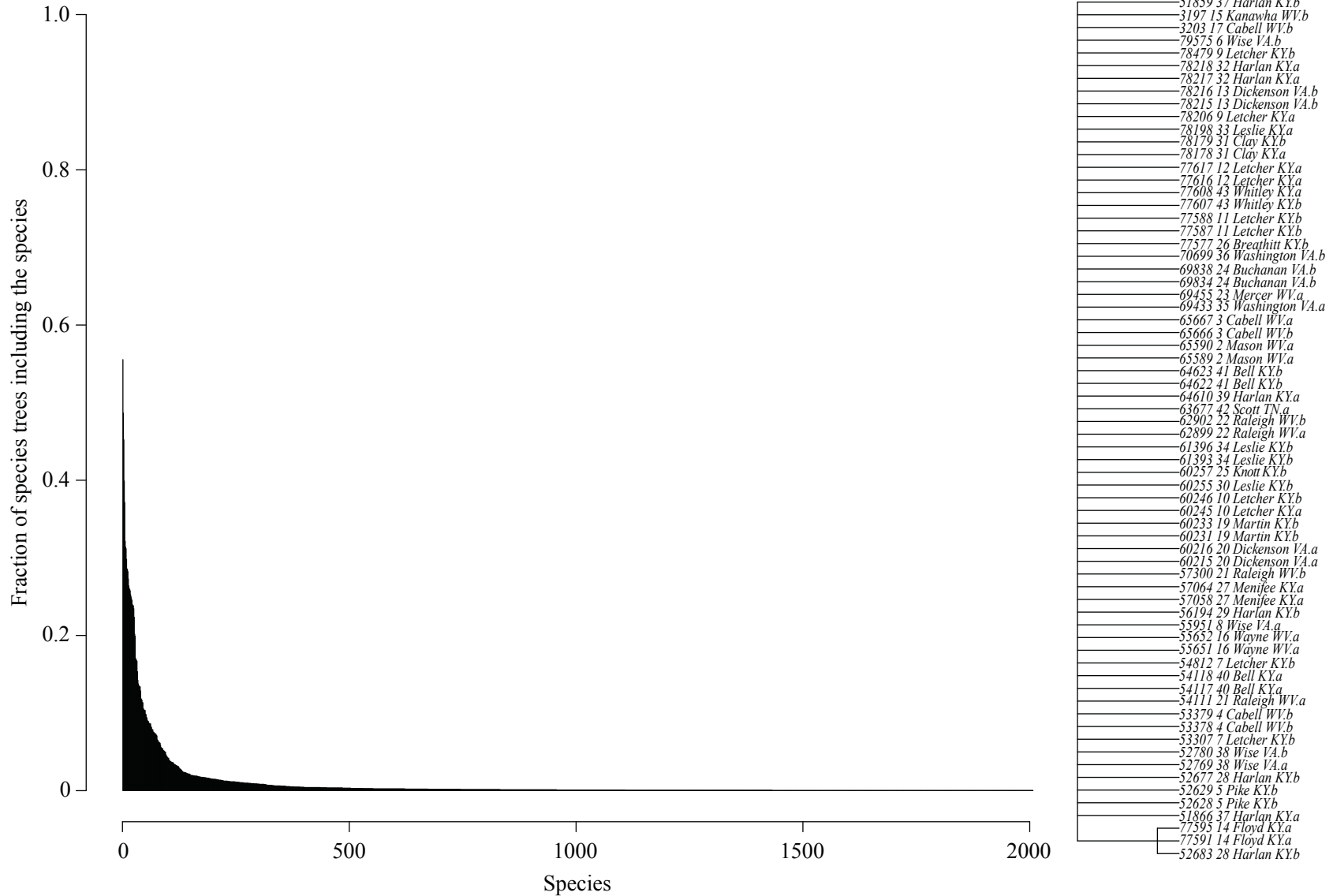


Fig. J. Summary of 500 replicate runs in Brownie with data set 4. 2333 unique species tree topologies were recovered, including 2007 delimited species. The graph shows that most of these species were recovered in a small number of trees. A majority rule consensus tree is provided in the right portion of the figure, showing the one species that was recovered in >50% of trees.

Table F. Species delimited by Brownie using data set 2 with mtDNA. The results of 500 independent runs are shown here. Grey boxes indicate inclusion in a delimited species. Two species were consistently delimited in this analysis

Specimen	Number Freq Population	500 1.0	500 1.0
65589	2		Grey
65590	2		Grey
65666	3		Grey
65667	3		Grey
53378	4		Grey
53379	4		Grey
52628	5		Grey
52629	5		Grey
79575	6	Grey	Grey
53307	7		Grey
54812	7		Grey
55951	8		Grey
78206	9		Grey
78479	9	Grey	Grey
60245	10		Grey
60246	10		Grey
77587	11		Grey
77588	11		Grey
77616	12		Grey
77617	12		Grey
78215	13		Grey
78216	13		Grey
77591	14		Grey
77595	14		Grey
3197	15		Grey
55651	16		Grey
55652	16		Grey
3203	17	Grey	Grey
60231	19		Grey
60233	19		Grey
60215	20		Grey
60216	20		Grey
54111	21		Grey
57300	21		Grey
62899	22		Grey
62902	22		Grey
69455	23		Grey
69834	24		Grey
69838	24		Grey
60257	25		Grey
77577	26		Grey
57058	27		Grey
57064	27		Grey
52677	28		Grey
52683	28		Grey
56194	29		Grey
60255	30		Grey
78178	31		Grey
78179	31		Grey
78217	32		Grey
78218	32		Grey
78198	33		Grey
61393	34		Grey
61396	34		Grey
69433	35		Grey
70699	36		Grey
51859	37		Grey
51866	37		Grey
52769	38		Grey
52780	38		Grey
64610	39		Grey
54117	40		Grey
54118	40		Grey
64622	41		Grey
64623	41		Grey
63677	42		Grey
77607	43		Grey
77608	43		Grey

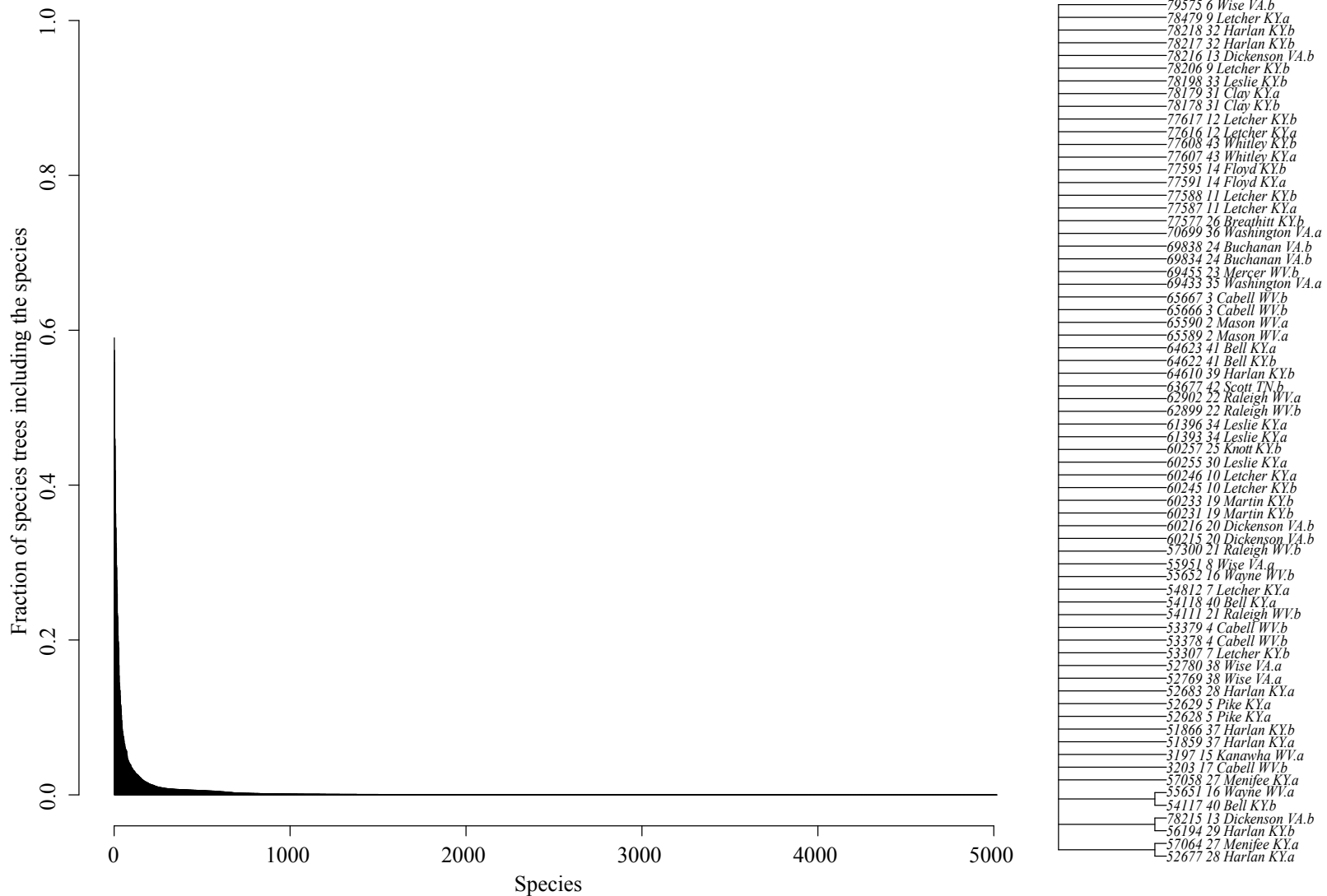


Fig. K. Summary of 500 replicate runs in Brownie, using data set 3 with mtDNA. 8556 unique species tree topologies were recovered, including 5018 delimited species. The graph shows that most of these species were recovered in a single tree. A majority rule consensus tree is provided in the right portion of the figure, showing the three species that were recovered in >50% of trees.

Table G. Species delimited by Brownie using data set 4 with mtDNA. The results of 500 independent runs are shown here. Grey boxes indicate inclusion in a delimited species. Two species were consistently delimited in this analysis

Specimen	Number	500	500
Population	Freq	1.0	1.0
65589	2		
65590	2		
65666	3		
65667	3		
53378	4		
53379	4		
52628	5		
52629	5		
79575	6		
53307	7		
54812	7		
55951	8		
78206	9		
78479	9		
60245	10		
60246	10		
77587	11		
77588	11		
77616	12		
77617	12		
78215	13		
78216	13		
77591	14		
77595	14		
3197	15		
55651	16		
55652	16		
3203	17		
60231	19		
60233	19		
60215	20		
60216	20		
54111	21		
57300	21		
62899	22		
62902	22		
69455	23		
69834	24		
69838	24		
60257	25		
77577	26		
57058	27		
57064	27		
52677	28		
52683	28		
56194	29		
60255	30		
78178	31		
78179	31		
78217	32		
78218	32		
78198	33		
61393	34		
61396	34		
69433	35		
70699	36		
51859	37		
51866	37		
52769	38		
52780	38		
64610	39		
54117	40		
54118	40		
64622	41		
64623	41		
63677	42		
77607	43		
77608	43		

