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Molecular phylogeny of *Persicaria* (Persicarieae, Polygonaceae)

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***Abstract*—**Relationships within the Polygonaceae have been recently examined using *rbcL* sequences, with an emphasis on *Polygonum* and its segregates. Here we test these results with respect to *Polygonum* (*sensu lato*) with an expanded dataset, including additional species and gene regions. Specifically, we focus on inferring the relationships of *Eupersicaria* (*Polygonum* sect. *Persicaria* in many prior treatments), using the chloroplast genes *rbcL*, *trnL–F*, *trnK* intron–*matK*, and *psbA–trnH* IGS, and nuclear ribosomal ITS sequences. We conclude that *Eupersicaria* is monophyletic and most closely related to *Tovara* and *Echinocaulon*. In turn, this clade is most closely related to *Cephalophilon*. The sister group of this entire *Persicaria* clade contains *Bistorta* and a clade including *Aconogonon* and *Koenigia,* which supports the monophyly of the Persicarieae. Within *Eupersicaria* there appears to be a deep split between *P. amphibia* and the remaining species, and there is strong conflict regarding the placement of *P. punctata*. These results set the stage for a more detailed phylogenetic analysis of *Eupersicaria*.

***Keywords*—**chloroplast DNA, *Eupersicaria*, ITS, *Polygonum*, Templeton test.

Polygonum, in the broad Linnaean sense, has presented a great taxonomic challenge. A wide variety of classification systems have been proposed over the years and the same names have been applied to quite different groups and at different taxonomic ranks (Haraldson 1978). Communication has become difficult to the point that most botanists are content to sidestep the underlying issues by reference to *Polygonum sensu lato*. In attempting to sort out phylogenetic relationships in this group, we have adopted a set of names and circumscriptions that we hope will cause the least confusion. In making these choices we have been guided principally by the desire to conform to standard usage as much as possible, to have names for the smallest (least inclusive) taxa that have been recognized, and to have a unique name for each taxon under discussion.

Among modern systems, the classification of Haraldson (1978) comes the closest to achieving these objectives. Along with most modern workers, Haraldson recognized a genus *Persicaria*, comprised of some 150 species of annual or perennial herbs or vines distributed around the northern temperate and into tropical regions (Brandbyge 1993). These plants are characterized by many-flowered spicate or capitate panicles (Stanford 1925a); usually entire, ciliate or pectinate ochrea; 4–5 tepals with trifid venation; and 4–8 stamens (Haraldson 1978; Ronse Decraene and Akeroyd 1988). Within *Persicaria* Haraldson (1978) recognized four sections: *Cephalophilon* (ca. 16 species), *Echinocaulon* (ca. 21 species), *Persicaria* (ca. 60 species), and *Tovara* (ca. 3 species). She considered *Aconogonon* (ca. 25 species), *Bistorta* (ca. 50 species), and *Koenigia* (ca. 9 species) to be closely related genera in her *Persicarieae*. *Persicarieae* is distinguished from the other major tribe *Polygoneae* (including *Polygonum* sensu stricto, *Polygonella*, *Oxygonum*, *Fallopia*, *Pteropyrum*, *Atraphaxis*, and *Calligonum*) by having nondilated filamentous stamens with the number reduced mostly in the inner whorl, the presence of nectaries, trifid tepal venation, and mostly rectangular to elongated tepal epidermis cells (Ronse Decraene and Akeroyd 1988; Ronse Decraene et al. 2000). There is a difference of opinion regarding the placement of *Fagopyrum* (ca. 16 species). Haraldson (1978) excluded it from her *Persicarieae* on the basis of petiole anatomy and life-form while Ronse Decraene and Akeroyd (1988) included it in *Persicarieae* based on floral characters.

Although most of these taxa have been recognized since the major treatments by Meisner (1826, 1856), there has been great disparity in the taxonomic ranks assigned to them (Haraldson 1978). For our present purposes these rank assignments are irrelevant, and we will use these names only as designations for putative clades. Thus, for example, we will simply refer to *Tovara* or to *Bistorta*, rather than to "section *Tovara*" or to "genus *Bistorta*". This approach is unproblematic except in the case of the name *Persicaria* itself, since this name has been applied both to a genus-level taxon and to a section-level taxon. We will use *Persicaria* for the more inclusive, genus-level taxon, in keeping with standard usage. To avoid any confusion, for purposes of this paper we have adopted the name *Eupersicaria* for the less inclusive, section-level taxon. The choice of the name *Eupersicaria* is based on Gross (1913a, b), who used it for what most authors have referred to as section *Persicaria* (although exact circumscriptions have varied somewhat from worker to worker).

With these names in hand, the purpose of our paper becomes easy to describe. Our aim is to test the monophyly of each of the aforementioned taxa and to infer their relationships to one another using DNA sequence data. Our primary focus is on relationships within *Persicaria*, and on the identification of its closest relatives. Most especially we are interested in assessing the monophyly and relationships of *Eupersicaria* as a prelude to more detailed phylogenetic and evolutionary studies within this group. The data used here are from the chloroplast genes *rbcL*, *trnL–F*, partial *matK* with *trnK* intron, and *psbA–trnH* IGS, and from the nuclear ribosomal ITS region. Our analyses build upon and test the results of a study of Polygonaceae by Lamb Fyre and Kron (2003) based on *rbcL* sequences alone.

Materials and Methods

***Taxon Sampling*—**Appendix 1 provides voucher specimen information on the 26 accessions we used to represent 24 species: eight accessions represent six species of *Eupersicaria* (two accessions each were included of *P. amphibia* and *P. hydropiper*); four species of *Echinocaulon* were included, three of *Cephalophilon*, two each of *Tovara* and *Bistorta*, and one of *Aconogonon* and each of the remaining six groups. A *Rumex* species was included for rooting purposes. This sample was chosen to represent all of the major clades within *Persicaria* sensu Haraldson (1978) and its presumed close relatives; it also largely corresponds to the sample in Lamb Frye and Kron (2003). Most of our samples were collected in the field by STK between 2002–2004 in North America, Crete, China, and Korea, but

a living sample of *P. amplexicaulis* was obtained from the New York Botanical Garden in 2006. All field-collected specimens were identified by STK and are available in the Yale University Herbarium (YU) of the Peabody Museum of Natural History. Samples of *P. arifolia* and *Koenigia islandica* were obtained from specimens in the Yale University Herbarium; *P. meisneriana* was obtained from the herbarium of the University of New Hampshire (NHA).

DNA Extraction, Amplification, and Sequencing—Genomic DNA was extracted from leaf samples dried in silica-gel or from herbarium specimens using a DNeasy Plant Mini Kit (Qiagen, Valencia, California). Amplification of double stranded DNA was performed using standard polymerase chain reaction (PCR) in 25 μL reactions contained 1–10 ng DNA, 1.0 unit of taq polymerase (Qiagen); 2.5μL of 10× buffer, 5μL of Q solution, 1μL of $MgCl_2$ to make final concentrations of 2.5 mmol/L, 1.0 mmol/L dNTPs (New England Biolabs, Ipswich, Massachusetts), and 1.0 μmol/L amplification primers. The following primers were used: ITSLeu (Baum et al. 1998) and ITS4 (White et al. 1990) for the internal transcribed spacer region, including the 5.8S rRNA coding region (nrITS); *matK*-PA1F (5′-GTTCCAATTATGCCTCT GG-3′) and *trnk*-2621 (Young et al. 1999) for the 5′ *trnK* intron and partial *matK* region (p-*matK*); *psbA*F and *trnH*R for the *psbA–trnH* IGS (*psbA*; Sang et al. 1997); "c" and "f" for IGS between *trnL* and *trnF* and the *trnF* intron (*trnL–F*; Taberlet et al. 1991); and 1FS (Lamb Frye and Kron 2003) and *rbcL*-1425R (5′-CAAAGTATCCATTGCT-TGGA-3′) for the *rbcL* coding region (*rbcL*). Polymerase chain reactions were carried out as follows for the ITS region: predenaturation at 97°C for 1 min followed by 40 cycles of denaturation at 97°C for 10 sec., annealing at 48°C for 30 sec., extension at 72°C for 20 sec. increasing 4 sec. with each cycle, and a final extension for 7 min. For the chloroplast markers we used the following: predenaturation at 94°C for 90 sec., 35 cycles of denaturation at 94°C for 30 sec., annealing at 54–62°C for 45 sec., extension at 72°C for 90 sec., and a final extension for 10 min. There were difficulties in amplifying nrITS, *rbcL*, and *trnL–F* from the herbarium extraction of *Koenigia islandica*, so smaller fragments were amplified with additional primers for sequencing (see below) using the conditions described above. Because the amplification of the smaller fragments obtained from *Koenigia islandica* usually did not yield enough product for direct sequencing, we cloned the PCR products using a TOPO TA cloning kit (Invitrogen, Carlsbad, California) following the supplied protocol. Eight colonies were picked and screened using primers M13For and M13Rev, and at least two PCR products were selected for sequencing using the steps below. PCR products were purified using a QIAquick PCR Purification Kit (Qiagen). Sequencing was carried out using the amplification primers and additional primers, as follows: ITS2 (White et al. 1990) and ITS3b (the reverse sequence of ITS2) for the nrITS region, d and e (Taberlet et al. 1991) for *trnL–F*, and *rbcL*-PS1F (5′-AGGMCATTACTTGAATGC-3′) and *rbcL*-PS1R (the reverse sequence of *rbcL*-PS1F) for *rbcL*. Dye terminator cycle sequencing followed the protocol specified by the ABI PRISM Dye Primer Cycle Sequencing Ready Reaction Kit (Revision B, August 1995, Perkin-Elmer) and was visualized using a BaseStation 51 (MJ Research, Sauk City, Wisconsin), an ABI 377, or an ABI 3100 automated DNA sequencer.

Alignments and Phylogenetic Analyses—Sequences were primarily aligned using Clustal X (Thompson et al. 1997) and adjusted by eye to achieve slight improvements. Aligning noncoding regions such as *psbA–trnH* IGS, *trnL–F* intron and IGS, and the nrITS region was difficult and required gaps. In these cases we also used T-COFFEE (Notredame et al. 2000, Ver. 1.35) and MUSCLE (Edgar 2004, Ver. 3.6) for alignment, again adjusted by eye. Our aligned data sets (and the trees published here) are available in TreeBASE (study number S1816) or upon request from the first author. Phylogenetic analyses of nrITS, the chloroplast data sets, and our combined data sets, were conducted using PAUP* 4.0b10 (Swofford 2002) and MrBayes 3.1 (Huelsenbeck and Ronquist 2001). Maximum parsimony searches were performed using heuristic search methods with tree bisection reconnection (TBR) branch swapping, collapse of zero maximum branch lengths, and equal weighting of all characters. The analyses were repeated 100 times with a random order of sequence addition in an attempt to sample multiple islands of most parsimonious trees. We evaluated node support with the bootstrap (Felsenstein 1985) using 1,000 replicates with heuristic search settings identical to those for the original search. A series of hierarchical likelihood ratio tests (hLRT) was performed to determine which model of sequence evolution best fit the data using the program Modeltest version 3.7 (Posada and Crandall 1998). Maximum likelihood searches were carried out in PAUP* using models selected by hLRT and alternative models using the Akaike Information Criterion (AIC) for each data set (Table 1). Parameters for each search were simultaneously estimated via maximum likelihood for all datasets. Heuristic search methods were used with TBR branch swapping and collapse of zero-length branches. Analyses were repeated 100 times with the "random addition" option. Bootstrap tests were performed using 1,000 replicates with nearest neighbor interchange (NNI) swapping. Parameters for bootstrap tests were fixed to values estimated from the maximum likelihood tree. Bayesian inferences were conducted using the GTR + G or GTR + G + I models. Five million generations were run to estimate parameters relating to sequence evolution and likelihood probabilities using MCMC. Trees were collected every 100th generation. After removing 100,000 generations as a "burn in" a 50% consensus tree was calculated to generate a posterior probability for each node. To assess the level of congruence among data sets from different gene markers we carried out the incongruence length difference (ILD) test (Farris et al. 1994, 1995) implemented in PAUP* as the partition homogeneity test. We used simple taxon addition, TBR branch swapping, and heuristic searches with 999 repartitions of the data. The ILD test was carried out with pairwise partitions for each gene data set as well as with the combined data set. When ILD tests suggested a significant differences between data sets we conducted a Wilcoxon signed-ranks test (WSR test; Templeton 1983) as implemented in PAUP* to assess the level of contribution of specific nodes that might be responsible for the conflict between trees. We compared a "test tree," the strict consensus tree inferred from a given data set, to two types of "rival trees:" (1) the strict consensus tree inferred from another data set, and (2) several modified "test trees" constrained at a node where topological conflict was observed ('test' and 'rival' are used here as in Mason-Gamer and Kellogg 1996).

Results

Aligned DNA Sequences—The range of sequence length and %GC content for five gene markers are presented in Table 1. Sequences of coding regions such as *rbcL*, a part of *matK*, and the 5.8S region of nrITS are conserved in sequence length and alignments were therefore straightforward. In contrast, sequences of noncoding regions showed length variation and it was necessary to introduce indels in the alignment. *Koenigia islandica* showed the shortest sequences in most noncoding regions; differences from the longest sequences were 221 bp in *psbA* (*P. pensylvanica*: 393 bp), 134 bp in *trnL–F* (*Rumex* sp.: 963 bp), and 117 bp in nrITS (*P. lapathifolia*: 650 bp). The aligned sequence length of each marker ranged from 577 bp to 1335 bp. The nrITS region was the most variable, with 30% parsimony-informative (PI) sites in the aligned data set; *rbcL* was least variable, containing 9% PI sites. The proportion of PI to variable sites, however, was similar in each gene marker (Table 1). We did not include indel information in phylogenetic analyses, as most data sets except *rbcL* (with no indels) contained indels that were not identical or variously overlapped, rendering their phylogenetic significance unclear. Short inversions of 21 bp attaching inverted repeats of 17 or 18 bp in *psbA–trnH* IGS were found in *Bistorta pleacea*, *Persicaria capitata*, *P. nepalensis*, *P. arifolia*, *P. maackiana*, *P. meisneriana* (Type II in Fig. 1 A). These fragments were inverted for alignment and included in our phylogenetic analyses (see Discussion for details). We also conducted an analysis with these inversion removed.

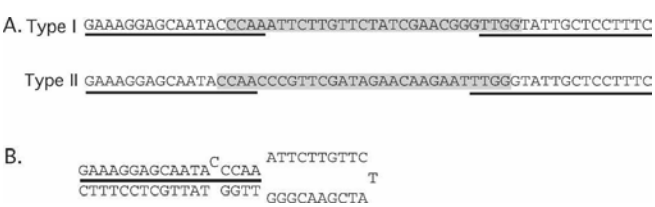

FIG. 1. A. Inversion sequence types in the *psbA–trnH* IGS region. The sequence of Type I is from *P. sagittata,* and that of Type II from *P. meisneriana*. Type II was present in *Bistorta pleacea*, *Persicaria capitata*, *P. nepalensis*, *P. arifolia*, *P. maackiana*, and *P.meisneriana.* Shaded portion indicates inversion. Inverted complementary sequences are indicated by solid lines. B. Possible stem and loop structure of *P. sagittata* sequences.

Analyses of cpDNA Gene Data Sets—Statistics associated with our maximum parsimony, maximum likelihood, and Bayesian analyses of each chloroplast gene data set are summarized in Table 1 and the resulting gene trees for *rbcL* and *trnL–F* are presented in Fig. 2. Very similar trees for *psbA* and p-*matK* are discussed below, but are not shown. The monophyly of *Eupersicaria* was well supported in analyses with *psbA* and *trnL–F* data sets (over 89% bootstrap support and a PP of 1.00; Fig. 2 B) but weakly supported in p-*matK* (MP/ML/PP = 67/62/0.9) and unresolved in *rbcL* analyses (owing to *P. amphibia;* Fig. 2 A). The clades representing *Tovara*, *Echinocaulon*, and *Cephalophilon* were also strongly supported (bootstrap values over 77%; PP over 0.99) except for *Tovara* in *psbA* analyses (MP/ML/PP = 59/53/0.72). The monophyly of *Persicaria* was strongly supported in every cpDNA analysis. Relationships of the clades within *Persicaria*, however, remained uncertain in *rbcL* analyses and were poorly resolved in other data sets with the exception of the clear resolution of a sister relationship between the *Cephalophilon* clade and the clade including *Euperscaria*, *Echinocaulon* and *Tovara. Aconogonon* and *Koenigia* were strongly linked in each tree except in the p-*matK* tree where *Koenigia* was not included in our analyses. The *Aconogonon-Koenigia* clade was linked with *Bistorta* with high support (MP/ML/PP = 99/99/1.00) in *rbcL* analyses (Fig. 2 A), whereas the clade including *Aconogonon*, *Bistorta,* and *Koenigia* (A.B.K. clade) received less support in *psbA*, p-*matK,* and *trnL–F* (Fig. 2 B) analyses. Strong support for the A.B.K. clade being sister to the *Persicaria* clade was found in Bayesian analysis (with PP 0.93–1.00), but not in MP and ML analyses (Fig. 2 A, B). *Fagopyrum* was placed as a sister to the *Persicaria*-A.B.K. clade with high support only in *psbA* tree, but generally this relationship remained unresolved. *Polygonum* s. str. and *Polygonella* formed a clade with strong support (bootstraps over 97%; PP = 1.00) in *rbcL*, or moderate support (bootstraps 63–72%; PP = 0.9) in p-*matK* and *trnL–F* analyses. *Fallopia* is strongly (100/100/1.00) linked with the *Polygonum* s. str.-*Polygonella* clade in *rbcL* and *trnL–F* (Fig. 2 A, B), and moderately (78/82/0.99) so in p-*matK* analyses. Relationships among *Polygonum* s. str., *Polygonella*, and *Fallopia* were less clear in the *psbA* analyses.

Analyses of a Combined cpDNA Data Set and an nrITS Data Set—Incongruence length difference tests with a combined data set of the four chloroplast genes found no significant differences among the gene partitions (P = 0.372). Pairwise tests with each chloroplast gene also indicated that the data set were combinable (Table 2). Compared to the separate gene analyses, combined cpDNA analyses provided improved phylogenetic resolution with higher node confidences (Fig. 2 C). However, relationships among *Eupersicaria*, *Echinocaulon,* and *Tovara* were ambiguous and unresolved in the 50% consensus trees from three analyses. Relationship among *Eupersicaria*, *Echinocaulon*, and *Tovara* were also problematic in nrITS sequence analyses, where *Tovara* appeared (with bootstrap support of 66%) within the *Eupersicaria* clade in the MP analysis. Bayesian analysis suggested *Eupersicaria* paraphyly, as the two *P. amphibia* populations plus *Tovara* formed a clade with moderate support (PP = 0.91). Some topological differences between the nrITS and the combined cpDNA data set concerned the A.B.K. clade and the placement of *Cephalophilon* clade. In general, however, the nrITS results were not well supported except in the Bayesian analysis (Fig. 2 D). In contrast to the cpDNA tree, the A.B.K. clade did not appear in our nrITS analyses; instead *Bistorta* was linked with a clade including the four major groups within *Persicaria*, although the *Persicaria* clade was well supported only in the Bayesian analysis. Several differences between the nrITS and cpDNA analyses concerned species relationships within the named clades (Fig. 2 C, D). *Persicaria punctata* was placed as sister to all other taxa of *Eupersicaria* (excluding *P. amphibia*) in the cpDNA combined analysis, but was linked with *P. hydropiper* in the nrITS analysis. *Persicaria meisneriana* formed a clade with *P. sagittata* in the nrITS analysis, but was strongly placed as sister to other species of *Echinocaulon* in the cpDNA combined analysis. Similarly, *P. nepalensis* and *P. runcinata* formed a weakly supported clade within *Cephalophilon*, whereas *P. nepalensis* appeared as sister to the clade including *P. runcinata* and *P. capitata* in the cpDNA tree.

Tree Incongruence and Analyses of a Data Set Including All Gene Markers—Incongruence length difference tests using two partitions (cpDNA versus nrITS) rejected the null hypothesis of no significant difference between two data sets (P < 0.0001). Pairwise ILD tests with the nrITS versus each of the four cpDNA markers indicated that p-*matK* and *psbA* were not significantly different from nrITS (Table 2). Results

TABLE 1. Aligned sequence information and the summary of maximum parsimony, maximum likelihood, and Bayesian analyses of various datasets. Note: [1] intergenic spacer region between *psbA* and *trnH*; [2] partial *matK* and *trnK* 5′ intron; [3] intergenic spacer region between *trnL* and *trnF* and *trnL* intron; [4] combined dataset with *psbA*, p-*matK*, *rbcL*, and *trnL–F*; [5] combined dataset with *psbA*, p-*matK*, *rbcL*, *trnL* and nrITS; PIS = parsimony-informative sites; MP = most parsimonious; G = general time reversible; g = among site rate variation modeled to fit a discrete gamma distribution; i = invariable sites; ML = maximum likelihood; BA = Bayesian analysis. * Arithmetic mean of two runs after burn in.

	psbA[1]	p-*matK*[2]	*rbcL*	*trnL–F*[3]	comb-cpDNA[4]	nrITS	comb-all[5]
Sequence length (bp)	172–393	557–626	1324–1328	829–963		533–650	
%GC content (all sites)	23.6–33.8	32.6–36.9	443.9–45.1	32.6–35.6		57.6–67.7	
Aligned seq. length (bp)	577	648	1335	1107	3667	753	4420
No. of variable sites	265	174	189	403	1030	356	1386
No. of PIS	155	103	115	233	608	229	837
PIS/aligned seq. length (%)	27	16	7	21	17	30	19
PIS/variable sites (%)	58	59	61	58	60	64	60
No. of MP trees	28	2	6	18	2	1	2
length of MP trees	409	269	317	623	1626	836	2481
Consistency Index (CI)	0.822	0.766	0.700	0.822	0.785	0.650	0.734
CI (excl. invariant sites)	0.739	0.675	0.603	0.741	0.697	0.572	0.644
Retention Index (RI)	0.844	0.817	0.792	0.839	0.822	0.704	0.776
Model selected	G+g+i	G+g	G+g+i	G+g	G+g+i	G+g	G+g+i
-ln L in ML	2637.860	2406.142	3840.002	4862.155	14224.537	4969.158	19634.516
Mean -ln L in BA	2667.459	2441.061	3878.535	4894.937	14251.055	4977.958	19665.026

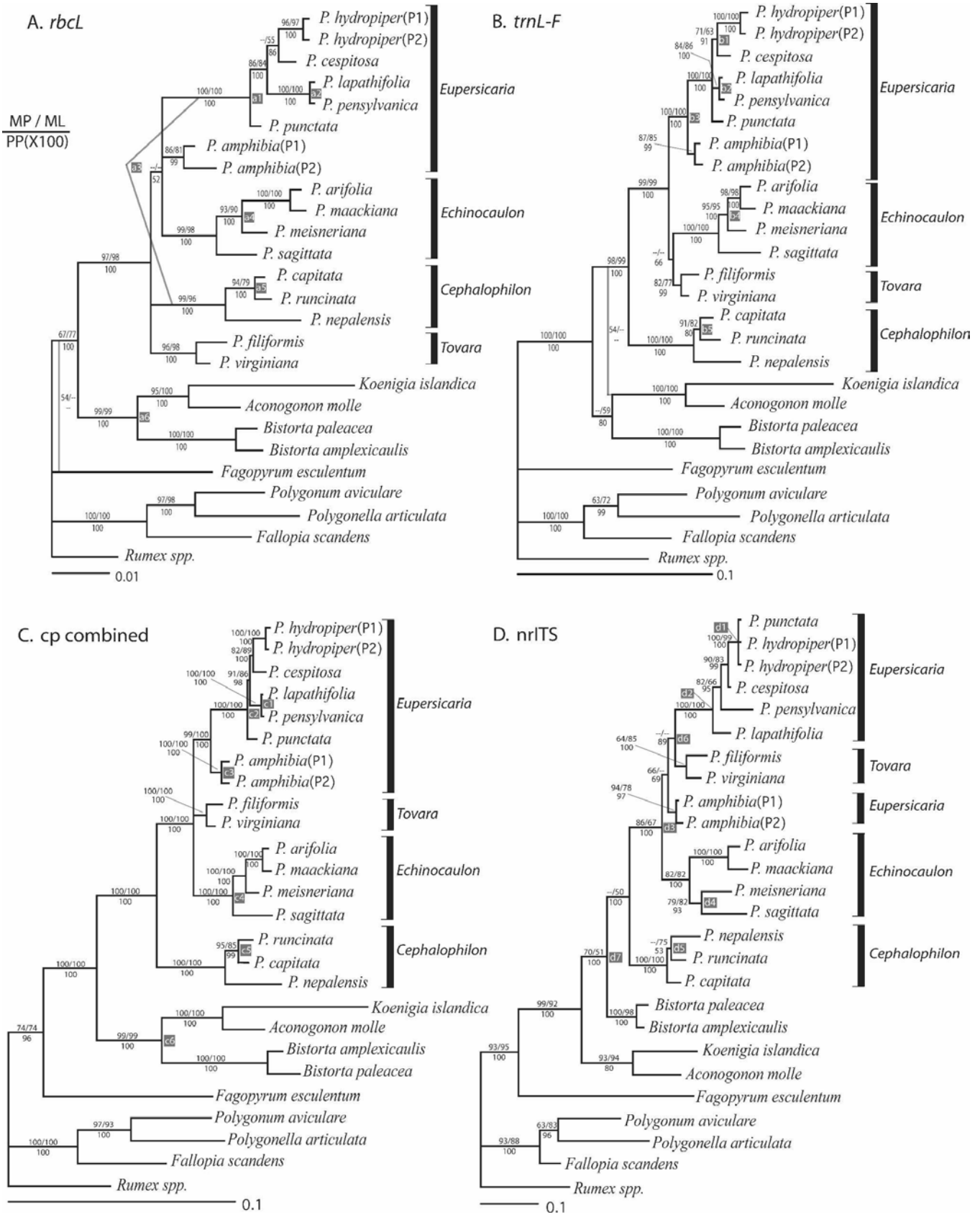

FIG. 2. 50% consensus trees from Bayesian inferences for A. *rbcL*, B. *trnL–F*, C. the combined data set for four cpDNA genes (*psbA*, p-*matK*, *rbcL*, *trnL–F*), and D. the nuclear ribosomal ITS sequence data set. Posterior probabilities are presented under the branches. Bootstrap values for maximum parsimony / maximum likelihood analyses are presented above the branches. Support of less than 50% bootstrap value or 0.5 posterior probability is indicated by --. Symbols representing some nodes indicate constraints used in Wilcoxon signed-ranks tests (see Table 3).

TABLE 2. *p* value obtained from pairwise ILD test between each gene (above diagonal). Significant difference between datasets suggesting not-combinable in ILD test is presented by *.

	psbA	p-*matK*	*rbcL*	*trnL–F*	nrITS
psbA	—	0.301	0.412	0.811	0.076
p-*matK*		—	0.392	0.897	0.073
rbcL			—	0.479	0.001*
trnL–F				—	0.007*

of the WSR test with nrITS versus *rbcL*, *trnL–F*, and the combined cpDNA data are presented in Table 3. Specifically, we investigated conflicts concerning the placement of *P. punctata* (a1, b1, c1 and d1 in Fig. 2 and Table 3), the existence of a *P. lapathifolia* plus *P. pensylvanica* clade (a2, b2, c2 and d2 in Fig. 2 and Table 3), the existence of a *P. sagittata* and *P. meisneriana* clade (a4, b4, c4 and d4 in Fig. 2 and Table 3), and relationships among the three species of *Cephalophilon* (a5, b5, c5 and d5 in Fig. 2 and Table 3). At the "section" level we studied conflicts concerning the relationship of *Cephalophilon* to *Eupersicaria* excluding *P. amphibia* (a3 and d3 in Fig. 2 and Table 3), the placement of *Bistorta* (a6, c6 and d7 in Fig. 2 and Table 3), and the placement of *Tovara* (b3, c3 and d6 in Fig. 2 and Table 3). Many tests contrasting rival strict consensus trees rejected the null hypothesis. The placement of *P. punctata* is seen to contribute significantly to the conflict between cpDNA and nrITS. None of the other conflicts in Table 3 showed significance in tests carried out in both directions. For example, *Cephalophilon* is the sister to *Eupersicaria* excluding *P. amphibia* in the *rbcL* strict consensus tree, but it is sister to the clade including *Eupersicaria*, *Echinocaulon*, and *Tovara* in the most parsimonious tree inferred from nrITS. When the nrITS was constrained by the *rbcL* tree, the WSR showed a significant difference (P = 0.0015). In contrast, when the *rbcL*

TABLE 3. Templeton (Wilcoxon signed-ranks) test for incongruence between data sets (see Fig. 2). Gain (G), loss (L) and net represent additional or reduced steps that the data set under consideration is constrained by the incongruent topology in the 'rival tree'. Approximate probability (P) evaluates the null hypothesis of no difference between the two trees with two-tailed test. Asterisk indicates the significant difference at $p < 0.05$.

Data set / Constraints in "rival tree"	G	L	Net	P-value
nrITS vs. *rbcL*				
nrITS strict consensus tree/*rbcL* strict consensus tree	50	16	34	< 0.0001*
(a1) *P. punctata* is the sister to the rest of *Eupersicaria* excluding *P. amphibia*	18	0	18	< 0.0001*
(a2) Clade of *P. lapathifolia* and *P. pensylvanica*	6	4	2	0.5271
(a3) Monophyly of *Cephalophilon* plus *Eupersicaria* excluding *P. amphibia*	25	7	18	0.0015*
(a4) Clade of *P. arifolia, P. maackiana,* and *P. meisneriana*	6	3	3	0.3173
(a5) Clade of *P. capitata* and *P. runcinata*	2	1	1	0.5637
(a6) Monophyly of *Bistorta, Koenigia,* and *Aconogonon*	10	5	5	0.1967
rbcL / nrITS strict consensus tree	25	4	21	< 0.0001*
(d1) Clade of *P. punctata* and *P. hydropiper*	7	0	7	0.0082*
(d2) *P. lapathifolia* is the sister to the rest of *Eupersicaria* excluding *P. amphibia*	9	0	9	0.0027*
(d3) Monophyly of *Echinocaulon, Tovara,* and *Eupersicaria*	3	2	1	0.6547
(d4) Clade of *P. sagittata and P. meisneriana*	3	1	2	0.3173
(d5) Clade of *P. capitata* and *P. nepalensis*	4	0	4	0.0455*
(d6) Monophyly of *Tovara* and *Eupersicaria* excluding *P. amphibia*	2	2	0	1.0000
(d7) Monophyly of *Bistorta* and *Persicaria*	8	0	8	0.0047*
nrITS vs. *trnL–F*				
nrITS / *trnL-F* strict consensus tree	44	8	36	< 0.0001*
(b1) Clade of *P. hydropiper* and *P. cespitosa*	18	0	18	< 0.0001*
(b2) Clade of *P. lapathifolia* and *P. pensylvanica*	6	4	2	0.5271
(b3) Monophyly of *Eupersicaria*	4	0	4	0.0082*
(b4) Clade of *P. arifolia, P. maackiana*, and *P. meisneriana*	6	3	3	0.3173
(b5) Clade of *P. capitata* and *P. runcinata*	2	1	1	0.5637
trnL-F /nrITS strict consensus tree	21	5	16	0.0016*
(d1) Clade of *P. punctata* and *P. hydropiper*	9	0	9	0.0027*
(d2) *P. lapathifolia* is the sister to the rest of *Eupersicaria* excl. *P. amphibia*	2	0	2	0.1573
(d4) Clade of *P. sagittata* and *P. meisneriana*	3	0	3	0.0833
(d5) Clade of *P. capitata* and *P. nepalensis*	2	0	2	0.1573
(d6) Monophyly of *Tovara* and *Eupersicaria excluding P. amphibia*	6	1	5	0.0588
(d7) Monophyly of *Bistorta and Persicaria*	0	0	0	-
nrITS vs. cp DNA combined				
nrITS / cp DNA combined data set strict consensus tree	36	16	20	0.0006*
(c1) *P. punctata* is the sister to the rest of *Eupersicaria* excluding *P. amphibia*	18	0	18	< 0.0001*
(c2) Clade of *P. lapathifolia* and *P. pensylvanica*	6	4	2	0.5271
(c3) Monophyly of *Eupersicaria*	7	0	7	0.0082*
(c4) Clade of *P. arifolia, P. maackiana,* and *P. meisneriana*	6	3	3	0.3173
(c5) Clade of *P. capitata* and *P. runcinata*	2	1	1	0.5637
(c6) Monophyly of *Bistorta, Koenigia,* and *Aconogonon* clade	10	5	5	0.1967
cp DNA combined / nrITS strict consensus tree	75	9	66	< 0.0001*
(d1) Clade of *P. punctata* and *P. hydropiper*	20	0	20	< 0.0001*
(d2) *P. lapathifolia* is the sister to the rest of *Eupersicaria* excluding *P. amphibia*	12	0	12	0.0005*
(d4) Clade of *P. sagittata* and *P. meisneriana*	10	1	9	0.0067*
(d5) Clade of *P. capitata* and *P. nepalensis*	7	0	7	0.0082*
(d6) Monophyly of *Tovara* and *Eupersicaria* excluding *P. amphibia*	13	5	8	0.0593
(d7) Monophyly of *Bistorta* and *Persicaria*	17	4	13	0.0046*

data were constrained by the nrITS tree the WSR test was not significant (P = 0.6547).

Because the main focus of our study is on section level relationship and we found no significant conflicts between cpDNA and nrITS at this level, we combined these datasets for further analyses. Also because we found no topological differences at this level in trees inferred with or without *P. punctata* (data not shown), we retained *P. punctata* in these analyses. Clearly, its placement in the combined results must be treated with caution in view of the significant conflict we have documented. In analyses of the combined cpDNA-ITS data set, most clades found in the cpDNA combined data set were maintained with high support values (Fig. 3). *Tovara* was linked directly with *Eupersicaria*, but this relationship was weakly supported, with less than 50% bootstrap values and only 0.64 PP in Bayesian analyses.

Discussion

Short Inversion Sequences—There are inversions situated between short inverted repeats of 17 or 18 bp in aligned sequence of *psbA* between nucleotide sites 80 and 109, suggesting a possible stem-loop structure (Fig. 1). Several types of inversions have been reported in the same region from *Paeonia* (Sang et al. 1997) and in other genes (e.g. *trn*K intron and *trn*C-*rpo*B spacer) from *Fagopyrum* (Ohsako and Ohnishi 2000). Large scale inversions in the chloroplast genome have been considered useful characters for phylogenetic analyses at higher taxonomic levels (Jansen and Palmer 1987; Raubeson and Jansen 2005; Kim et al. 2005), although homoplasy has been documented in such inversions in Chenopodiaceae and Cactaceae (Downie and Palmer 1994), Ranunculaceae (Hoot and Palmer 1994), and Campanulaceae (Cosner et al. 2004), and intrapopulational polymorphism has been shown in conifers (Tsumura et al. 2000). In contrast, short inversions usually associated with stem-loop or hairpin structures have been viewed as problematic in phylogenetic analyses owing to the difficulty of determining the order of inversion events and base mutations and possible heterogeneous evolutionary rates compared to other nucleotide sites in the same gene (Kelchner and Wendel 1996; Sang et al. 1997; Ohsako and Ohnishi 2000). In our analyses, we did not code the inversion events themselves as characters; instead we inverted these sequences to match the others in the dataset and included them. We found that the inverted sequences (adjusted as necessary to the complimentary strand) were identical with those from other species in the same "section," with the exception of one autapomorphy in *P. capitata* within *Cephalophilon*. Inverting and including these sequences made a difference within *Cephalophilon*: without inverting we obtained a *psbA* tree in which *P. capitata* and *P. nepalensis* were directly linked, whereas from the dataset including the inverted sequence we obtained a *P. capitata* plus *P. runcinata* clade, consistent with the other cpDNA markers (Fig. 2 A, B). Analyses with these inversions removed yielded the same topology as when the sequences were inverted, with only minor differences in the confidence values.

Data Combinability and Incongruence—Despite controversy over the utility of the ILD test as a measure of combinability (Dolphin et al. 2000; Darlu and Lecointre 2002; Dowton and Austin 2002; Barker and Lutzoni 2002; Yoder et al. 2001; Hipp et al. 2004), there appears to be a low chance of concluding that significantly different data sets are homogeneous enough to combine (low Type II error rate) when data sets have a sufficient number of informative sites (Darlu and Lecointre 2002). Our pairwise ILD tests indicated that the cpDNA markers are not significantly different from one another, consistent with the observation of overall congruence between the trees obtained from the different markers (with the exception of several differences within *Persicaria* and the placement of *Fagopyrum*; Fig. 2). In contrast, we found strong conflicts in comparing nrITS to our cpDNA data sets, which our WSR tests showed were mainly due to differences in the placement of *P. punctata*. Conflicts concerning higher-level relationships, such as the relationships of *Bistorta*, *Cephalophilon*, and *Tovara*, were few and showed significance only in one direction. On this basis, we carried out analyses of a combined data set. Analyses with and without *P. punctata* resulted in the same topology for the remainder of the species. We have shown the tree with *P. punctata* included in Fig. 3, but caution that its position may be misleading. The most likely explanation for the incongruence associated with *P. punctata* may be that it originated through hybridization. Reticulation in *Eupersicaria* has been proposed previously (Stanford 1925b; Timson 1964; McDonald 1980; Consaul et al. 1991), and we have been exploring the possible parentage of *P. punctata* using intron sequences of a single copy nuclear gene (Kim and Donoghue, MS in prep.). It is possible that some other, more minor, discordances between cpDNA and nrITS, such as those involving *Echinocaulon*, *Cephalophilon*, and *Bistorta*, also reflect hybrid speciation and/or cytoplasmic introgression. This possibility is supported by polyploidy in these groups and throughout *Polygonum* in the broad sense (Jaretzky 1928; Löve and Löve 1956), and by examples of hybridization in groups related to *Persicaria*, such as in *Fallopia* (Bailey and Stace 1992; Hollingsworth et al. 1999) and *Fagopyrum* (Nishimoto et al. 2003). However, we cannot yet rule out the possibility that at least some of the observed incongruence is a result of sampling error (de Queiroz et al. 1995; Cunningham 1997). Clarification of these issues will require additional markers, especially nuclear low copy genes not affected by rapid concerted evolution.

The Monophyly of Persicaria and Relationship within Persicarieae—Results from our analyses of the separate and combined data sets strongly support the monophyly of *Persicaria* (Fig. 2, 3). This is the group that Haraldson (1978) recognized as the genus *Persicaria*, composed of the sections *Persicaria* (here *Eupersicaria*), *Tovara*, *Echinocaulon*, and *Cephalophilon*. Likewise, there is strong support for a sister group relationships between *Persicaria* and a well-supported clade including *Aconogonon*, *Bistorta*, and *Koenigia* (the A.B.K. clade; Fig. 3). The genus *Persicaria* is sometimes circumscribed so as to include *Aconogonon* and *Bistorta*, but to exclude *Koenigia* (i.e. Ronse Decraene and Akeroyd 1988). Based on our results this would not be a monophyletic group. *Koenigia,* with nine species, has often been segregated as a separate genus on the basis of its predominant distribution in alpine and arctic areas, unique spinulose-spheroidal pollen grains, and a base chromosome number of seven (Hedberg 1946, 1997; Löve and Sarkar 1957; Federov 1969). However, a close affinity between *Koenigia* and *Aconogonon* has been suggested based on floral and vegetative similarities (Haraldson 1978; Ronse Decraene and Akeroyd 1988; Hong et al. 1998; Ronse Decraene et al. 2000), and was supported in a previous phylogenetic analysis of *rbc*L sequences (Lamb Frye and Kron 2003).

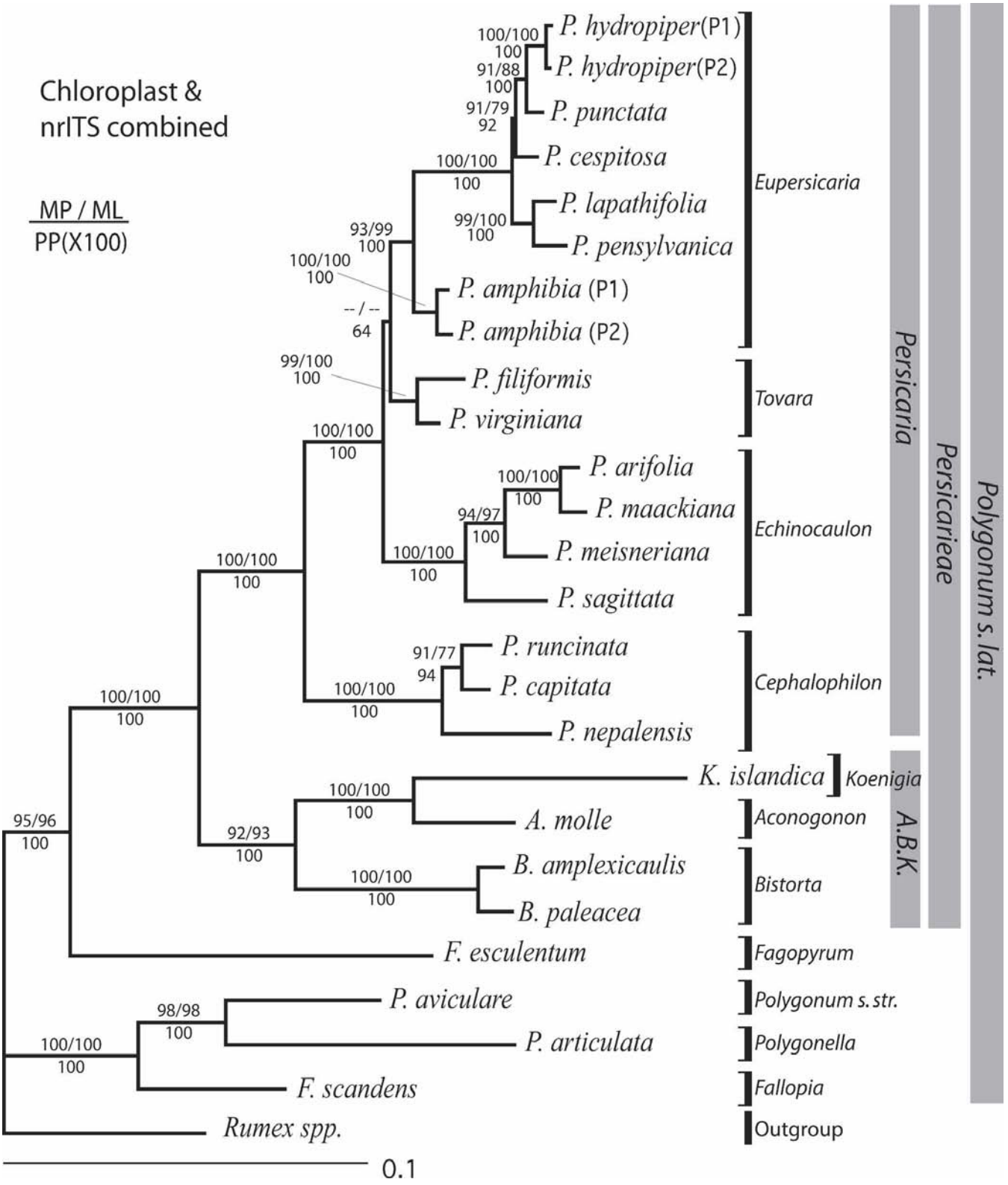

FIG. 3. 50% consensus tree from Bayesian inferences for the combined data set of four cpDNA markers and nrITS sequences. Posterior probabilities are presented under the branches. Bootstrap values for maximum parsimony / maximum likelihood analyses are presented above the branches. Support of less than 50% bootstrap values or 0.5 posterior probability is indicated by --.

The *Persicarieae* was established by Haraldson (1978) to include *Persicaria*, *Aconogonon*, *Bistorta* and *Koenigia* (commonly recognized as genera), and this has been supported by morphological evidence (Ronse Decraene and Akeroyd 1988; Hong et al. 1998; Ronse Decraene et al. 2000) and *rbc*L sequence analysis (Lamb Frye and Kron 2003). Our results strongly support a monophyletic group comprised of these lineages (Fig. 3). Studies of floral characters have suggested the transfer of *Fagopyrum* (which Haraldson put in the *Polygoneae*) into *Persicarieae* (Ronse Decraene and Akeroyd 1988;

Hong et al. 1998; Ronse Decraene et al. 2000), but this was not supported in the *rbcL* study (Lamb Frye and Kron 2003). Our analyses do support a sister relationship between *Fagopyrum* and the *Persicaria* plus A.B.K. clade (Fig. 3). Consequently, the name *Persicarieae* could be applied either to a less inclusive clade (including *Persicaria*, *Aconogonon*, *Bistorta* and *Koenigia*) or to a more inclusive clade (also including *Fagopyrum*). We favor the application of *Persicarieae* to the less inclusive clade both because this was the initial circumscription and because this clade is better supported at the present time. If the position of *Fagopyrum* is upheld in future analyses it would be useful to coin a new name for the more inclusive clade.

The Placement of Eupersicaria within Persicaria—In the *rbcL* tree of Lamb Frye and Kron (2003) *Eupersicaria* appeared to be paraphyletic, owing to a well-supported placement of *P. sagittata* (of *Echinocaulon*) within it. In contrast, our own analyses of *rbcL* sequences place *P. sagittata* with other species of *Echinocaulon*, well outside of *Eupersicaria*, and this coincides with its placement based on all of our other markers. This discrepancy may represent a case of misidentification, or it may reflect the relatively high number of ambiguous base pairs in the *rbcL* sequence of *P. sagittata* deposited in GenBank.

Overall, our results provide higher levels of resolution and support for relationships among the "sections" within *Persicaria* than previous studies. Thus, *Cephalophilon* appears to be the sister group of a clade comprised of the other three lineages. This relationship was strongly supported in analyses of three of our chloroplast genes and in the combined trees, the exceptions being in *rbcL,* where these relationships were unresolved, and in nrITS, where the *Persicaria* clade itself was not strongly resolved in MP and ML analysis (Fig. 2, 3). However, relationships among *Eupersicaria*, *Tovara,* and *Echinocaulon* remain poorly resolved. In p-*matK* analyses *Eupersicaria* was most closely related to *Tovara*, and *Echinocaulon* was sister to this clade. On the other hand, in *psbA* and *trnL–F* analyses *Eupersicaria* was weakly supported as sister to a clade including *Echinocaulon* and *Tovara*. Nuclear ribosomal ITS analyses suggested that *Eupersicaria* is more closely related to *Tovara* than it is to *Echinocaulon*, but this was very weakly supported. However, it should be noted the nrITS sequences also show the two populations of *P. amphibia* as sister of *Tovara* plus the remainder of *Eupersicaria*. This possible paraphyly of *Eupersicaria* was only suggested in Bayesian analysis, and then only with very weak support. Our combined analyses strongly support the monophyly of *Eupersicaria* and weakly connect *Eupersicaria* with *Tovara* (Fig. 3).

Morphological characteristics are distributed in a mosaic fashion across our best trees, and the evolution of these traits is difficult to interpret at present. Here we briefly highlight the distinctive characteristics of the major lineages within *Persicaria*, and several traits that are shared between them.

Tovara, composed of three species (Hara 1962; Suh et al. 1997) disjunct between eastern Asia (*P. filiformis* and *P. neofiliformis*) and eastern North America (*P. virginiana*), may have been in existence since the middle Tertiary (Hara 1962). This group is distinctive in having two long, persistent styles, which are hooked at the tip (presumably as a fruit dispersal mechanism), and whip-like, interrupted racemes having remotely arranged fascicles of 1–3 flowers, each with four perianth parts (Steward 1930; Small 1933; Li 1952; Haraldson 1978; Ronse Decraene and Akeroyd 1988). Pollen is of the *Tovara*-type, with 12 oblong pores and a delicate reticulum of exine ridge.

Plants of *Eupersicaria* are distinguished from other *Persicaria* by spike-like inflorescences with many flowers and lanceolate simple leaves. Plants of *Eupersicaria* and *Tovara* have similar inflorescences, simple elliptic leaves with acute apices, and similar trichomes (Haraldson 1978). Perianth number is quite variable in *Eupersicaria*, even within an individual; however, some species of *Eupersicaria*, such as *P. senegalensis*, consistently produce four perianth parts. The pollen grains of *Eupersicaria* are of the *Persicaria*-type, having 20 circular pores and a reticulum (Hedberg 1946), but here too there is a hint of a close relationship to *Tovara*, as *P. amphibia* exhibits an intermediate pollen form (Hedberg 1946).

Plants of *Echinocaulon* are readily distinguished from other *Persicaria* by their capitate inflorescences and sagittate or hastate to triangular leaves. Most species also have spiny and/or stiff stellate trichomes. However, there are several similarities to *Eupersicaria*. *Echinocaulon* pollen grains are of the *Persicaria*-type; polyporate and with a rod-like structure to the reticulum (Hedberg 1946). Some species of *Echinocaulon* lack the characteristic spiny prickles or stellate trichome and have similar inflorescences to those found in *Eupersicaria* (Haraldson 1978; Park 1988). For instance, *P. bungeana*, which has spicate inflorescences and simple leaves, is difficult to distinguish from species of *Eupersicaria* except for the presence of recurved prickles on the stem.

Finally, *Cephalophilon* plants are characterized by broadly elliptic to ovate leaves with subtending lobes and winged petioles. They share the capitate inflorescence form with *Echinocaulon*, and a close alliance between *Cephalophilon* to *Echinocaulon* has been proposed based on gross morphology (Dammer 1893; Danser 1927). However, members of *Cephalophilon* lack the multicellular stiff trichomes found in *Echinocaulon* (Haraldson 1978), and these groups differ markedly in their pollen grains. *Cephalophilon* grains are of a third type, being tricolpate with narrow furrows and bearing a reticulum of relatively higher exine ridges (Hedberg 1946). The pollen grains of *Cephalophilon* are similar to those of *Aconogonon* in being tricolpate (and to *Bistorta* with tricolporate grains), but are similar to others in *Persicaria* in that their exines have larger and fewer columellae (Hedberg 1946; Nowicke and Skvarla 1977).

Establishing the direction of evolution and the nature of homoplasy in these traits will require a broader, more detailed morphological analysis. However, our trees suggest that the first *Persicarieae* may have had paniculate inflorescences, flowers with five perianth parts, and tricolpate pollen grains. Capitate inflorescences may have evolved independently in *Cephalophilon* and in *Echinocaulon*, and spike-like inflorescences appear to have originated in *Eupersicaria* and *Tovara*. Four perianth parts may have been derived in *Tovara* and possibly again within *Eupersicaria*. Finally, polyapeturate pollen may have evolved independently in *Koenigia* (Hong and Hedberg 1990) and in the *Eupersicaria-Tovara-Echinocaulon* clade.

In summary, our molecular studies support previous morphological work (Haraldson 1978; Ronse Decraene and Akeroyd 1988; Hong et al. 1998; Ronse Decraene et al. 2000) in confirming the monophyly of *Eupersicaria*, *Tovara*, *Echinocaulon*, and *Cephalophilon*, and the existence of a *Persicaria* clade comprised of these four lineages. In turn, it now appears that the sister group of *Persicaria* is a previously unnamed clade

that includes *Aconogonon*, *Bistorta* and *Koenigia*. Together, these form a clade that corresponds to the *Persicarieae* of Haraldson and other authors, which in turn is linked with *Fagopyrum*.

Within *Persicaria* our analyses support an initial split into *Cephalophilon* and a clade made up of *Echinocaulon*, *Tovara*, and *Eupersicaria*, but relationships among these three remain uncertain. Within *Eupersicaria* there appears to be a deep split between *P. amphibia* and the rest, though our sampling is still limited. Finally, there is significant conflict between our cpDNA markers and nrITS sequences in the placement of *P. punctata*, which may reflect hybridization in its ancestry. These analyses set the stage for more detailed studies of the role of hybridization and polyploidy in the evolution of *Eupersicaria* and its relatives.

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Appendix 1. Voucher information for DNA extractions used in the present study. Taxon; collector(s) and number, locality; Genbank accession number for nrITS, *psbA*, p-*matK*, *rbcL*, *trnL–F*. Specimens are deposited at YU unless another herbarium is given. NJ = New Jersey, CT = Connecticut, MA = Massachusetts, NY = New York, CO = Colorado. Notes: * Inbred line in Wesleyan University see Sultan et al. (1998) for more specific locality; ** DNA extraction was done from herbarium specimen; *** Sample from New York Botanical Garden living collection.

***Genus Persicaria*—**Sect. *Persicaria Persicaria amphibia* (L.) S. F. Gray; (P1) *Kim 600*, NJ, USA; EF653699, EF653750, EF653724, EF653776, EF653802. (P2); *Kim & Kim Ch-Ko-91*, KyungSangNamDo, Korea; EF653700, EF653751, EF653725, EF653777, EF653803. *P. cespitosa* (Blume) Nakai; **WEIR.8*, CT, USA; EF653701, EF653752, EF653726, EF653778, EF653804. *P. hydropiper* (L.) Opiz; (P1) *Kim 570*, CT, USA; EF653702, EF653753, EF653727, EF653779, EF653805. (P2); *Kim & Ma Ch-Ko-37*, Yunnan, China; EF653703, EF653754, EF653728, EF653780, EF653806. *P. lapathifolia* (L.) Gray; **EG.3*, CT, USA; EF653704, EF653755, EF653729, EF653781, EF653807. *P. pensylvanica* (L.) M. Gómez; *Kim, Donoghue & Sultan 14*, MA, USA; EF653705, EF653756, EF653730, EF653782, EF653808. *P. punctata* Small; *Kim 560*, CT, USA; EF653706, EF653757, EF653731, EF653783, EF653809. Sect. *Echinocaulon P. arifolia* (L.) K. Haraldson; ***Magee 89-56*, CT, USA; EF653693, EF653744, EF653718, EF653770, EF653796. *P. maackiana* Nakai; *Kim& Kim Ch-Ko-89*, KyungSangNamDo, Korea; EF653694, EF653745, EF653719, EF653771, EF653797. *P. meisneriana* (Cham. & Schlechtend.) M. Gómez; ***Ritter, Crow & Crow 4083*, Bolivia (NHA); EF653695, EF653746, EF653720, EF653772, EF653798. *P. sagittata* (L.) H. Gross; *Kim 650*, CT, USA; EF653696, EF653747, EF653721, EF653773, EF653799. Sect. *Cephalophilon P. capitata* (Buch.-Ham ex D. Don) H. Gros; *Kim & Ma Ch-Ko-48*, Yunnan, China; EF653690, EF653741, EF653715, EF653767, EF653793. *P. nepalensis* (Meisn.) H. Gross; *Kim & Ma Ch-Ko-50*, Yunnan, China; EF653691, EF653742, EF653716, EF653768, EF653794. *P. runcinata* (Buch.-Ham. ex D. Don) Masam.; *Kim & Deng Ch-Ko-62*, Yunnan, China; EF653692, EF653743, EF653717, EF653769, EF653795. Sect. *Tovara P. filiformis* Nakai; *Kim & Kim Ch-Ko-102*, KyunggiDo, Korea; EF653697, EF653748, EF653722, EF653774,EF653800. *P. virginiana* (L.) Gaertn.; *Sultan s.n.*, CT, USA; EF653698, EF653749, EF653723, EF653775, EF653801.

Other genera*—Aconogonon molle* (D. Don) Hara; *Kim & Deng Ch-Ko-54*, Yunnan, China; EF653687, EF653738, EF653712, EF653764, EF653790. *Bistorta amplexicaulis* (D. Don) Greene; *Kim 710* (****Acc. Num. 1797/94*), NY, USA EF653688, EF653739, EF653713, EF653765, EF653791. *Bistorta paleacea* (Wall. ex Hook. f.) Yonek. & Hiroyoshi Ohashi; *Kim & Ma Ch-Ko-8*, Yunnan, China; EF653689, EF653740, EF653714, EF653766, EF653792. *Fagopyrum esculentum* Moench.; *Kim & Kim Ch-Ko-96*, KyungSangNamDo, Korea; EF653685, EF653736, EF653711, EF653762, EF653788. *Fallopia scandense* (L.) Holub; *Kim 504*, CT, USA; EF653682, EF653733, EF653708, EF653759, EF653785. *Koenigia islandica* L.; ***Lutz s.n.*, CO, USA; EF653686, EF653737,——, EF653763, EF653789. *Polygonella articulata* Meisn.; *Kim & Lundgren s.n.*, MA, USA; EF653683, EF653734, EF653709, EF653760, EF653786. *Polygonum aviculare* L.; *Sultan & Heschel s.n.*, CT, USA; EF653684, EF653735, EF653710, EF653761, EF653787. *Rumex spp*; *Kim & Chae Crete-39*, Crete, Greece; EF653681, EF653732, EF653707, EF653758, EF653784.