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INCONGRUENCE BETWEEN cpDNA AND nrITS TREES INDICATES EXTENSIVE HYBRIDIZATION WITHIN *EUPERSICARIA* (POLYGONACEAE)[1]

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Interspecific hybridization followed by polyploidization appears to have played a major role in plant diversification, but quantifying the contribution of this mechanism to diversification within taxonomically complex clades remains difficult. Incongruence among gene trees can provide critical insights, especially when combined with data on chromosome numbers, morphology, and geography. To further test our previous hypothesis on hybrid speciation in *Persicaria* (Polygonaceae), we performed molecular phylogenetic studies using three cpDNA regions and nuclear ITS sequences, with an emphasis on sampling within section *Eupersicaria*. Our analyses revealed major conflicts between the combined cpDNA tree and the nrITS tree; a variety of incongruence tests rejected stochastic error as the cause of incongruence in most cases. On the basis of our tree incongruence results and information on chromosome numbers, we hypothesize that the origin of 10 polyploid species involved interspecific hybridization. Our studies also support the recognition of several previously named species that have been treated as belonging within other species. Repeated allotetraploidy (as distinct from radiation at the tetraploid level) now appears to be the key mechanism governing the diversification of this taxonomically challenging group.

Key words: allopolyploidy; cpDNA; *Eupersicaria*; hybridization; incongruence test; ITS; *Persicaria*; Polygonaceae; *Polygonum*.

Hybridization between separately evolving species, followed by polyploidization, has long been appreciated to be a key mechanism in plant evolution (e.g., Stebbins, 1950; Grant, 1981; Arnold, 1997, 2006; Soltis and Soltis, 1999; Abbott and Lowe, 2004; Comai, 2005; Paun et al., 2007; Rieseberg and Willis, 2007). Because allopolyploidy can result in instant reproductive isolation, it has been viewed as driving diversification within some plant clades and especially as allowing new species to arise in sympatry. During the past two decades, molecular phylogenetic approaches have been brought to bear on this issue, and hybridization and allopolyploidy have now been carefully documented in a number of plant groups (e.g., *Cardamine*: Lihova et al., 2006; *Cerastium:* Brysting et al., 2007; *Glycine*: Doyle et al., 2004; *Senecio*: Abbott and Lowe, 2004; *Tragopogon*: Soltis et al., 2004). However, quantifying the extent to which allopolyploidy has been a factor in the evolution of taxonomically complex groups in which it may have played a role remains difficult. In such groups we may both underestimate the number of species (cf. Soltis et al., 2007) and misestimate the number of instances of ploypoid speciation. On one end of the spectrum, the number of such events could equal (or even exceed, in the cases of multiple origins; see Soltis et al., 2003; Hegarty and Hiscock, 2005) the number of polyploid species recognized in the group; that is, every polyploid species may have originated independently from diploid progenitors. At the other end, all the polyploid species in the group could have resulted from diversification at the polyploid level following just a single instance of allopolyploidy.

Here we attempt to assess the nature and extent of allopolyploidy in a major clade within *Persicaria* (Polygonaceae) by comparing gene trees based on nuclear and chloroplast markers, and by considering these results in the context of information on chromosome numbers, morphology, and geography. The *Eupersicaria* clade has long been recognized as being taxonomically complex (described in next section), and it contains numerous polyploid species. Hybridization has been suggested as a factor contributing to the pronounced morphological variability in the group, and our previous studies (focused on the higher-level phylogeny of *Persicaria*; Kim and Donoghue, 2008) have highlighted the possibility of allopolyploidy within *Eupersicaria*. However, until now this possibility of allopolyploidy has not been critically examined using molecular techniques and an extensive sample of relevant species. Our studies also provide a critical application of a battery of tests to explore the nature and extent of incongruence between chloroplast and nuclear data sets.

Eupersicaria within Persicaria and Polygonaceae—*Eupersicaria,* composed of ~70 species, was recognized as a section within *Persicaria* by Gross (1913). It includes species of *Polygonum* sections *Persicaria* and *Amblygonum* in the sense of Meisner (Meisner, 1826, 1856) or of *Persicaria* sect. *Persicaria* in the sense of Haraldson (1978). We use the name *Eupersicaria* for a clade identified by Kim and Donoghue (2008) that is nested within a more narrowly circumscribed *Persicaria* clade that corresponds to the genus *Persicaria* in the sense of Haraldson (1978). Within *Persicaria*, *Eupersicaria* is most closely related to two other named clades, *Tovara* and *Echinocaulon,*

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although the relationship among these three is not yet fully resolved (Lamb Frye and Kron, 2003; Kim and Donoghue, 2008). Here we do not assign formal ranks to these clades, but use *Persicaria* as the "genus" or "clade address" in referring to species of the *Eupersicaria* clade.

Plants in *Eupersicaria* are easily distinguished from other groups by narrow lanceolate leaves and spike-like racemes with densely or loosely arranged flower fascicles. Most species in *Eupersicaria* have been delimited based on gross morphology relating to vegetative and reproductive characters such as leaf shape, trichome type and density, ochrea shape and marginal trichomes, perianth number, color and venation pattern, style number, and achene shape. In addition to morphological characters, growth form and habitat preferences have been used in species circumscription. Although these morphological and ecological characters are useful in recognizing some species, many of them are highly variable, resulting in great taxonomic confusion. For instance, Greene (1904) described 38 new species, most of which are now generally considered to be part of the *Persicaria amphibia* complex (Mitchell, 1968; Mitchell and Dean, 1978; Hinds and Freeman, 2005). Many infraspecific entities have been segregated within some polymorphic species such as *P. lapathifolia*, *P. hydropiperoides*, and *P. punctata* (Danser, 1921; Stanford, 1926; Fassett, 1949; Fernald, 1950).

Owing to the high degree of morphological variation underlying such taxonomic confusion, some "species" of *Eupersicaria* have provided model systems for studies of phenotypic plasticity. Various moisture conditions relate to trichome, leaf, and ochrea variation in *P. amphibia* (Mitchell, 1968, 1976). Likewise, populations of *P. maculosa* grown under various conditions exhibit plasticity of ecological significance (Bell and Sultan, 1999; Sultan, 2001, 2003). Morphological variability in *Eupersicaria* has also been attributed to hybridization. Studies based on morphology (Stanford, 1925b; Timson, 1964), experimental fertilization (Timson, 1964; McDonald, 1980), and isozyme profiling (Consaul et al., 1991) have highlighted hybridization as a factor contributing to variability in *Eupersicaria*.

Assessing incongruence between nuclear and chloroplast data sets—Besides stochastic error, genealogical discordances may be caused by horizontal gene transfer, hybridization, lineage sorting, or heterogeneous rates of molecular evolution (e.g., Maddison, 1997). To assess the statistical significance of tree incongruence, many tests have been proposed (see Goldman et al., 2000; Planet, 2006, and references therein). Data congruence of separate matrices can initially be assessed by measuring the difference between the parsimony tree lengths inferred from randomly rearranged partitions and the tree length for the entire data set (e.g., incongruence length difference [ILD] test and its relatives; Farris et al., 1994, 1995; Lecointre et al., 1998; Thornton and DeSalle, 2000; Zelwer and Daubin, 2004). Although this matrix level approach has been criticized for its susceptibility to type I error (incorrectly rejecting the null hypothesis; Dolphin and Quicke, 2000; Yoder et al., 2001; Dowton and Austin, 2002; Hipp et al., 2004), it is useful as a starting point because of the low type II error rate (accepting the incorrect null hypothesis) when a sufficient number of informative sites are included (Darlu and Lecointre, 2002). So-called paired-sites tests (Felsenstein, 2004), focused on specific conflicts, have been developed for parsimony (e.g., the Templeton test; Templeton, 1983; Mason-Gamer and Kellogg, 1996) and likelihood (e.g., Kishino and Hasegawa [1989; K-H] and Shimodaira–Hasegawa [1999; S-H] tests). Finally, the approximately unbiased (AU) test has been developed to reduce biases in the tests assuming smoothness of the boundaries of the hypothesis regions (Shimodaira, 2002). The K-H, S-H, and AU methods use nonparametric bootstrapping, resampling estimated log-likelihoods (Kishino et al., 1990) to generate a distribution for testing the hypothesis, whereas the Templeton test employs a Wilcoxon signed-rank test (Templeton, 1983).

Although there are many methods to test the statistical significance of tree incongruence, we lack specific methods to distinguish among the underlying causes for incongruence between different data sets. Horizontal gene transfer, while common in bacteria and archaea (Doolittle et al., 2003), is fairly rare in plants, although examples have been reported recently for mitochondrial genes (see Richardson and Palmer, 2007, and references therein). Distinguishing between lineage sorting and hybridization is, in general, more difficult, although this distinction may be possible by taking into account branch lengths (Holder et al., 2001). In spite of the general difficulty in sorting out the causes, hybridization has often been favored when there are disparities between trees inferred from nuclear vs. chloroplast DNA sequences because chloroplasts are typically maternally inherited in angiosperms while nuclear genes are inherited from both parents. Hybridization has often been favored for polyploids (Arnold, 1997; Rieseberg, 1997; Sang et al., 1997; Soltis and Soltis, 1999; Otto and Whitton, 2000; Doyle et al., 2004; Soltis et al., 2004). In general, the most convincing explanations combine data on morphology, chromosome number, and distribution patterns or ecology, with incongruence between nuclear and cpDNA trees.

MATERIALS AND METHODS

Taxon sampling—We included 63 accessions to represent 49 species: 40 species of *Eupersicaria* (54 accessions), four of *Echinocaulon*, and two of *Tovara*. Three species of the *Cephalophilon* clade were included for rooting purposes base on the results of Kim and Donoghue (2008). Appendix 1 provides voucher information on all accessions. Living samples were primarily collected by S.T.K. from fieldwork in China, Greece, Korea, and the United States during 2002–2005. Twenty-two populations from 19 species were sampled using herbarium specimens from the Harvard University Herbaria (GH), the University of New Hampshire Herbarium (NHA), and the Yale University Herbarium (YU). We included 17 accessions used in previous studies (Kim and Donoghue, 2008: GenBank accession numbers starting with EF in Appendix 1). For phylogenetic analyses, we used a data matrix with 60 terminals. Three populations of *Persicaria punctata* (P3–P5) were examined only in our exploration of nrITS polymorphism.

DNA extraction, PCR, and sequencing—Genomic DNA was extracted from fresh or dried leaf samples using a DNeasy Plant Mini Kit (Qiagen Valencia, California, USA). Because DNA extraction from relatively old herbarium specimens was inefficient compared to that from leaf samples dried in silica gel, we added a 20-h rocking incubation with proteinase K and 2-mercaptoethanol to the first step of the supplied protocol. Amplification of double-stranded DNA was carried out using standard polymerase chain reaction (PCR) in 25-μL reactions containing 1–10 ng DNA, 1.0 unit of *Taq* polymerase (Qiagen), 2.5 μL 10× buffer, 5 μL Q solution, and 1 μL $MgCl_2$ to make final concentrations of 2.5 mmol/L, 1.0 mmol/L dNTPs (NEB, Ipswich, Massachusetts, USA), 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 (Kim and Donoghue, 2008) 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); and "c" and "f" for the IGS between *trnL* and *trnF* and the *trnF* intron (*trnL-F*; Taberlet et al., 1991). PCR cycles followed those in Kim and Donoghue (2008). When gene regions were difficult to amplify from herbarium extractions, smaller fragments were amplified with internal sequencing primers (described later) using the conditions described.

Because the amplification of the smaller fragments obtained from some species did not yield enough product for direct sequencing, we cloned the PCR products using a TOPO TA cloning kit (Invitrogen, Carlsbad, California, USA) following the supplied protocol. PCR products of nrITS from five accessions of *P. punctata* were cloned to examine haplotype polymorphism; more than 36 colonies were picked for each accession and sequenced.

PCR products were purified using a QIAquick PCR Purification Kit (Qiagen) or the polyethylene glycol–NaCl precipitation method (Lis and Schleif, 1975). Sequencing was carried out using the amplification primers and additional internal primers, as follows: ITS2 (White et al., 1990) and ITS3b (the reverse sequence of ITS2) for the nrITS region, and "d" and "e" (Taberlet et al., 1991) for *trnL-F*. Cycle sequencing followed the protocol provided with the ABI PRISM Dye Primer Cycle Sequencing Ready Reaction Kit (Revision B, August 1995, Perkin-Elmer, Foster City, California, USA) and was visualized using a Base-Station 510 (MJ Research, Sauk City, Wisconsin, USA), an ABI 377, or an ABI 3100 automated DNA sequencer. Sequencing was conducted in part at the W. M. Keck Facility and the Science Hill DNA Analysis Facility at Yale University.

Alignments and phylogenetic analyses—Sequences were aligned using the program CLUSTAL_X (Thompson et al., 1997), T-coffee version 1.35 (Notredame, Higgins, and Heringa, 2000), or MUSCLE version 3.6 (Edgar, 2004) and adjusted by eye to resolve minor conflicts. Our aligned data matrices and the trees published here are available in TreeBASE (SN3727, http://www.treebase.org) or upon request from the first author. Phylogenetic analyses were conducted using a nrITS data set and a combined data set of three chloroplast gene regions using PAUP* version 4.0b10 (Swofford, 2002) and MrBayes version 3.1 (Huelsenbeck and Ronquist, 2001). Maximum parsimony (MP) searches were performed using heuristic search methods with tree-bisection-reconnection (TBR) branch swapping, collapse of zero maximum branch lengths, MULTREES option in effect, and equal weighting of all characters. Analyses were repeated 500 times with a random order of sequence addition in an attempt to sample multiple islands of most parsimonious trees. Bootstrap tests (Felsenstein, 1985) were carried out to evaluate node support using 1000 replicates. Heuristic search settings were identical to those for the original search for the combined cpDNA data set, whereas the MULTREES option was not in effect for the nrITS data set to avoid inappropriate rearrangements (Debry and Olmstead, 2000). We determined the best-fit model of sequence evolution in a series of hierarchical likelihood ratio tests (hLRT) using MODELTEST version 3.7 (Posada and Crandall, 1998). Maximum likelihood (ML) searches were carried out in PAUP* using the models selected by hLRT for each data set (Appendix S1, see Supplemental Data with online version of this article.). 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 a random order of sequence addition. Bootstrap tests were performed using 500 replicates with nearest neighbor interchange (NNI) branch swapping. Parameters for bootstrap tests were fixed to values estimated from the maximum likelihood tree. Bayesian inferences were conducted using the models selected using MODELTEST. Also, for the combined data set of three chloroplast gene regions, we applied different models selected from MODELTEST for each partition. Five million generations were run to estimate parameters relating to sequence evolution and likelihood probabilities using a Markov chain Monte Carlo (MCMC) method. Trees were collected every 100th generation. After removing 25% of the generations (125 000 generations) as burn in, a 50% majority rule consensus tree was calculated to generate a posterior probability for each node.

Incongruence tests—Tree incongruence was assessed using the five different approaches noted in the introduction. (1) The ILD test was performed using the partition homogeneity test implemented in PAUP* with simple taxon addition, TBR branch swapping, and heuristic searches of 1000 repartitions of the data. Pairwise tests between each data set and between the combined cpDNA and nrITS data sets were carried out to assess how much the original partitions differed from random partitions in parsimony tree length. (2) Templeton tests were performed using PAUP* to assess the contribution of specific nodes to the conflict between trees. A "test tree," the strict consensus of the most parsimonious trees inferred from a given data set, was compared to two types of "rival trees": (1) the strict consensus of the most parsimonious trees inferred from another data set and (2) modified "test trees" with constrained nodes where topological conflict was observed ("test" and "rival" are used here as in the sense of Mason-Gamer and Kellogg, 1996). For example, where a particular conflict in tree topology existed between the strict consensus trees from nrITS and the combined cpDNA data sets, we specifically modified the nrITS tree to reflect each conflicting relationship suggested by the cpDNA tree and then compared the nrITS strict consensus tree (test tree) to each modified trees (rival tree) for the nrITS data set. Likewise, we tested rival trees suggested by the nrITS using the cpDNA data set. The last three tests—(3) the K-H test, (4) the S-H test, and (5) the AU test—used the likelihood-based δ statistic to test competing hypotheses postulated by different tree topologies. The null distribution was generated by nonparametric boostrapping using the RELL method (Kishino and Hasegawa, 1989; Kishino et al., 1990). We calculated log likelihood scores of trees constrained by topological conflicts using PAUP* and test values including *P* values using the program CONSEL (Shimodaira and Hasegawa, 2001).

RESULTS

Aligned DNA sequences—Information on the sequences, including total sequence length and % GC content for our four gene regions are presented in Appendix S1. Sequence lengths varied in the noncoding regions of each gene region, and indels were introduced for alignment. The aligned sequence length for the nrITS region was 687 bp and for the combined cpDNA regions (cp-combined) was 2023 bp. The cp-combined data set contained more parsimony-informative sites (PIS) than nrITS, but showed slightly less variability in the ratio of PIS to variable sites (Appendix S1, see Supplemental Data with online version of article). We did not include indels in our phylogenetic analyses because most data sets contained inconsistent, variously overlapped indels of questionable phylogenetic significance. There were 21-bp short inversions attaching inverted repeats of 17 bp (complementary reversed regions) in *psbA* sequences, as reported by Kim and Donoghue (2008) for five species belonging to *Cephalophilon* and *Echinocaulon*. Three additional species of *Eupersicaria*, *Persicaria densiflora*, *P. hydropiperoides*.P2, and *P. kawagoeana* had the same inversions in *psbA*. These fragments were inverted for alignment and included in our phylogenetic analyes as in Kim and Donoghue (2008). We also conducted phylogenetic analyses without these regions; the same tree topologies were recovered with only slight differences in confidence values (data not shown).

Phylogenetic analysis of the combined cpDNA data set—Our phylogenetic analyses of cpDNA focused on the combined data set as pairwise ILD tests indicated that the three cpDNA regions were not significantly different from one another (Table 1). The strict consensus tree from 416 most parsimonious trees (tree length [TL] = 48; consistency index [CI] = 0.867; retention index [RI] = 0.938) and the maximum likelihood tree from heuristic searches (model selected = K81uf + G; –log likelihood = 5921.876) are presented in Fig. 1A, and the 50% majority rule consensus tree from Bayesian inference (model selected for each data set: *psbA* = F81 +G, p-*matK* = K81uf + G, *trnLF* = F81 + G; mean –log likelihood = 6585.224*)* is presented in Fig. 2A. The monophyly of *Eupersicaria* is strongly supported in MP and ML analyses with 100% bootstrap (BP) support and 1.00 posterior probability (PP; Figs. 1 and 2). Our

TABLE 1. *P* values from pairwise incongruence length difference (ILD) test.

Partitions	*p-matK*	*PsbA*	*trnL-F*	nrITS
p-matK	—	0.078	1.000	0.001*
psbA		—	0.381	0.001*
trnL-F			—	0.001*
nrITS				—
CP combined				0.001*

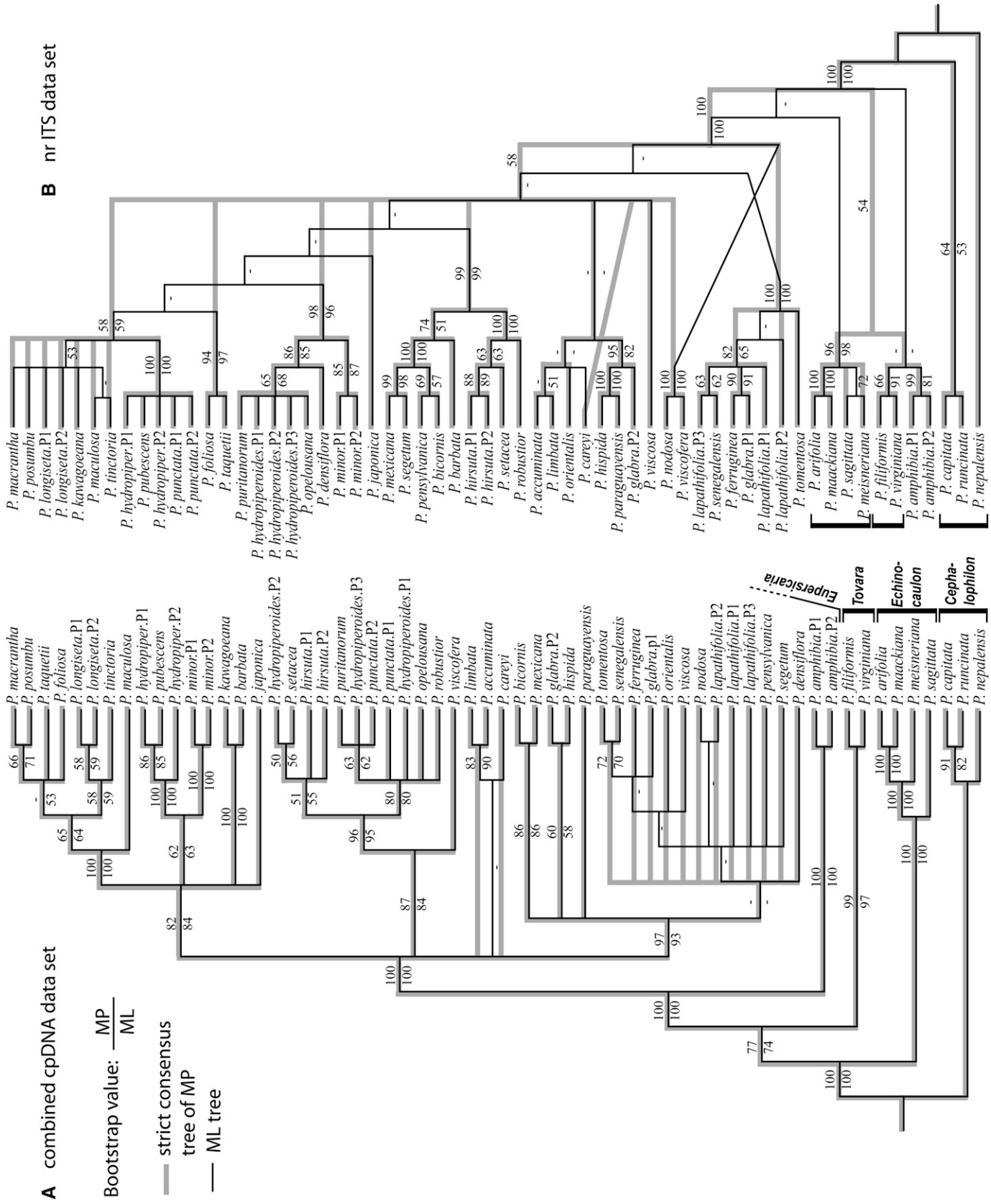


Fig. 1. Maximum likelihood tree (solid line) and strict consensus parsimony tree (gray line) using the combined data set of three cpDNA regions (A) and the nrITS data set (B). Numbers above branches are parsimony bootstrap values; numbers below branches are maximum likelihood bootstrap values.

cpDNA combined data analyses show strong support for *P. amphibia* as sister to the rest of *Eupersicaria*.

Three major groupings within core *Eupersicaria* were recovered with moderate to strong support (Figs. 1A and 2A): (1) *P. macrantha* ~ *P. japonica* ("~ " signifies the inclusion of all species between the two on our trees); (2) *P. hydropiperoides*.P2 ~ *P. viscofera*; and (3) *P. bicornis* ~ *P. densiflora*. Within the first clade in Fig. 1A, two strongly supported clades and one with weaker support (stronger in Bayesian analysis, Fig. 2A) were suggested, although relationships among these were not well resolved in any analysis. *Persicaria kawagoeana* and *P. barbata* form a clade with strong supports in all analyses. Interspecific relationships within the other strongly supported clade (*P. macrantha* ~ *P. maculosa*; Fig. 1A) were ambiguous in both MP and ML analyses but well resolved in the Bayesian analysis (Fig. 2A). In contrast, relationships within the weakly supported clade (*P. hydropiper*.P1 ~ *P. minor*.P2; Fig. 1A) were well resolved, with *P. hydropiper* and *P. pubescens* forming a clade. The second major clade (*P. hydropiperoides*.P2 ~ *P. viscofera*; Fig. 1A) was mostly composed of American species except the East Asian *P. viscofera*. Relationships within this clade were resolved with moderate to weak support in MP and ML analyses, but with relatively strong support in the Bayesian analysis (Figs. 1A and 2A). Our three accessions of *P. hydropiperoides* did not appear together. One formed a clade with *P. setaceae* and *P. hirsuta* and another with *P. puritanorum* and *P. punctata*.P2; the position of the third accession remained unresolved (Figs. 1A and 2A). *Persicaria viscofera* appears to be sister to the remaining species in the second major clade (Figs. 1A and 2A).

Relationships within the third major clade revealed in every analysis (*P. bicornis* ~ *P. densiflora*) were not well resolved except for several clades with moderate confidence: *P. bicornis* with *P. mexicana*, *P. glabra*.P2 with *P. hispida*, and *P. tomentosa* with *P. senegalensis* (Figs. 1A and 2A). Parsimony analyses also suggested an unresolved clade including *P. tomentosa* ~ *P. densiflora,* but with less than 50% bootstrap support. Likelihood analysis resolved more relationships within this clade but again with bootstrap values below 50% (Fig. 1A). In contrast, some relationships in the Bayesian analysis were well supported (Fig. 2A).

The clade including *P. limbata* and *P. accuminata* (MP/ML/PP = 80/90/1.00), and *P. careyi*, were linked as sister to the three major clades discussed in parsimony and Bayesian analyses. Likelihood analysis suggested a close relationship of *P. careyi* to the clade including *P. limbata* and *P. accuminata,* but with less than 50% bootstrap support (Fig. 1A).

Phylogenetic analysis of the nrITS data set—Information on our phylogenetic analyses using nrITS data are summarized in Appendix S1. A strict consensus tree from 305 most parsimonious trees (TL = 594; CI = 0.647; RI = 0.827) and the ML tree from heuristic search (model selected = TrN + G; –log likelihood = 4200.498) are presented in Fig. 1B, and the 50% majority rule consensus tree from the Bayesian inference (model selected = TrN + G; mean –log likelihood = 4290.123) is shown in Fig. 2B. The monophyly of all *Eupersicaria* except for *P. amphibia* was strongly supported in all three analyses (MP/ML/PP = 100/100/1.00). However, basal relationships inferred from the nrITS data set differ from those in our cpDNA trees. The close relation of *P. amphibia* to the two representatives of *Tovara* (*P. filiformis* and *P. virginiana*) was consistent, despite low support values (MP/ML/PP = <50/<50/0.81). As in Kim and Donoghue (2008), relationships among the sectional level clades are not confidently resolved. *Echinocaulon* (*P. arifolia*, *P. maackiana*, *P. sagittata*, and *P. meisneriana*) is seen to be more closely related to *Eupersicaria* excluding *P. amphibia* (Figs. 1B and 2B) in ML and Bayesian analyses, but with weak support (ML/PP = <50%/0.53), whereas *Echinocaulon* appears as sister to the clade including *Tovara* and *P. amphibia* in MP analyses, again with a weak bootstrap support (54%; Fig. 1B).

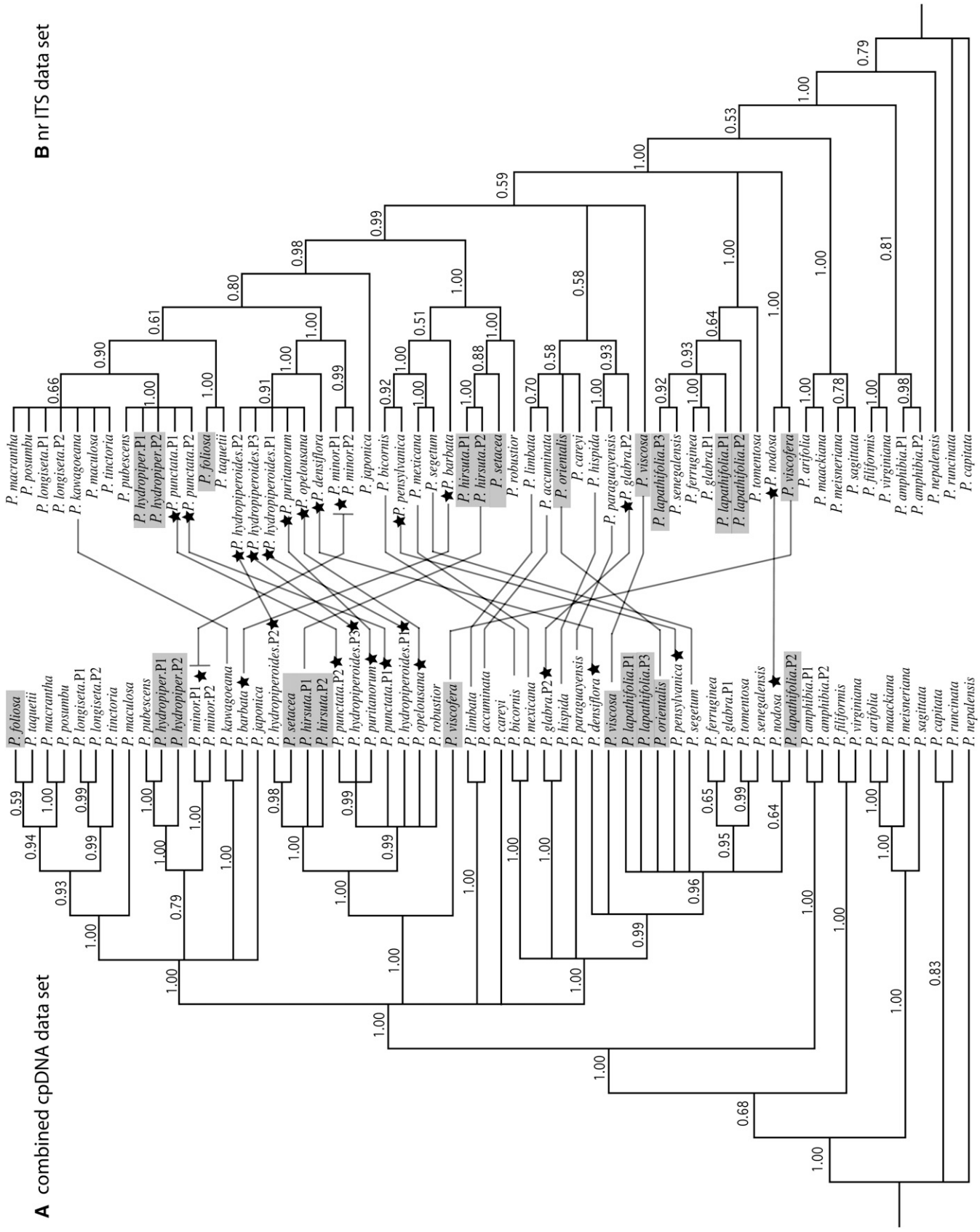
All three analyses using nrITS revealed seven major clades within *Eupersicaria* excluding *P. amphibia*, of which five were strongly supported and two were weakly supported (Figs. 1B and 2B). Relationships among these clades were not resolved in MP analyses except that the *P. lapathifolia*.P3 ~ *P. tomentosa* clade is weakly supported (58% BP; Fig. 1B) as sister to the other six clades and three other species (*P. japonica*, *P. viscosa*, and *P. careyi*). Relationships among these clades were resolved in ML and Bayesian analyses, although confidence values in the ML analysis were below 50% (Fig. 1B). The strongly supported clade *P. hydropiper*.P1 ~ *P. punctata*.P2 (Fig. 1B) appears as sister to the *P. macrantha* ~ *P. tinctoria* clade with weak to moderate support (MP/ML/PP = 58/59/0.9). However, relationships within these two clades were not resolved except for a weakly supported relationship between *P. maculosa* and *P. tinctoria* (Figs. 1B and 2B). Nuclear ITS analyses suggest rather different placements of *P. kawagoenan* and *P. punctata* compare to our cpDNA analyses. Also, nrITS analyses suggest that *P. foliosa* and *P. taquetii*, seen as close relatives of *P. macrantha* and *P. posumbu* in the cpDNA analyses, are linked as sister group to *P. macrantha* ~ *P. punctata*.P2 (Fig. 1B). Within another strongly supported clade, *P. puritanorum* ~ *P. minor*.P2 (MP/ML/PP = 98/96/1.00), the clade including *P. hydropiperoides*, *P. puritanorum*, and *P. opelousana* is most closely related to *P. densiflora*, and, in turn, to two accessions of *P. minor* (Fig. 1B). In marked contrast, *P. densiflora* joined *P. lapathifolia* and its relatives in our cpDNA analyses, and *P. minor* linked with *P. hydropiper* and *P. pubescens* (Fig. 1A).

The *P. mexicana* ~ *P. robustior* clade includes an interesting combination of taxa and relationships in comparison with our cpDNA analyses. One nrITS group, consisting of *P. hirsuta*, *P. setacea*, and *P. robustior,* appears to be closely related to *P. hydropiperoides* and *P. punctata* in our cpDNA trees, whereas the later are spread apart in our nrITS trees (Figs. 1B and 2B). Moreover, the *P. mexicana* ~ *P. bicornis* clade (MP/ML/PP = 100/100/1.00; Figs. 1B and 2B) includes species that do not appear to be at all closely related based on cpDNA.

Our nrITS results also provided quite a different view of specific relationships within the *P. accuminata* ~ *P. glabra*.P2 clade. Although weakly supported in nrITS analyses, the monophyly of *P. accuminata* and *P. limbata* does correspond to the cpDNA analysis. However, these taxa are seen to be more closely related to *P. orientalis* in the nrITS analyses. The monophyly of *P. hispida*, *P. paraguayensis*, and *P. glabra*.P2

→

Fig. 2. The 50% majority-rule consensus tree inferred from Bayesian analysis using (A) the combined data set of three cpDNA regions and (B) the nrITS data set. Number above a branch is the posterior probability. Shaded taxa are known diploids. Gray lines connect the taxa that have significant conflicts (see Tables 2 and 3). Asterisk represents proposed allopolyploid species.


A combined cpDNA data set
B nr ITS data set
P. foliosa
P. taquetii
P. macrantha
P. posumbu
P. longiseta.P1
P. longiseta.P2
P. tinctoria
P. maculosa
P. pubescens
P. hydropiper.P1
P. hydropiper.P2
P. minor.P1
P. minor.P2
P. kawagoeana
P. barbata
P. japonica
P. hydropiperoides.P2
P. setacea
P. hirsuta.P1
P. hirsuta.P2
P. punctata.P2
P. hydropiperoides.P3
P. puritanorum
P. punctata.P1
P. hydropiperoides.P1
P. opelousana
P. robustior
P. viscofera
P. limbata
P. accuminata
P. careyi
P. bicornis
P. mexicana
P. glabra.P2
P. hispida
P. paraguayensis
P. densiflora
P. viscosa
P. lapathifolia.P1
P. lapathifolia.P3
P. orientalis
P. pensylvanica
P. segetum
P. ferruginea
P. glabra.P1
P. tomentosa
P. senegalensis
P. nodosa
P. lapathifolia.P2
P. amphibia.P1
P. amphibia.P2
P. filiformis
P. virginiana
P. arifolia
P. maackiana
P. meisneriana
P. sagittata
P. capitata
P. runcinata
P. nepalensis

is strongly supported by nrITS, but the relationship of *P. hispida* directly to *P. paraguayensis* differs from the cpDNA for which *P. hispida* is more closely related to *P. glabra*.P2 (Figs. 1A and B).

The monophyly of seven accessions (*P. lapathifolia*.P3 ~ *P. tomentosa*; Figs. 1B and 2B) is supported in nrITS analyses with 100% BP and 1.00 PP. These are all part of the *P. tomentosa* ~ *P. densiflora* clade in the cpDNA tree (Fig. 1A), but relationships among them differ in the two trees. South American *P. ferruginea* and *P. glabra*.P1 are strongly clustered and more closely related to the clade including *P. senegalensis* and *P. lapathifolia*.P3 in our nrITS trees (Figs. 1B and 2B).

ML analysis suggest that the *P. lapathifolia*.P3 ~ *P. tomentosa* clade is sister to the entire *P. macranth* ~ *P. viscosa* clade (Fig. 1B) and that the clade consisting of *P. nodosa* and *P. viscofera* is sister to all other core *Eupersicaria* (i.e., excluding *P. amphibia*). However, these results are only weakly supported.

Incongruence tests—Our molecular analyses using nrITS and a combined cpDNA data set indicate many topological conflicts, some of which appear to be quite strong judging by support values. We conducted a battery of incongruence tests to evaluate the significance of these conflicts. Results from our Templeton tests are summarized in Table 2. Most constraints, in both directions, were rejected with >95% confidence, indicating that those differences are most likely not the result of stochastic error—8 of 13 cpDNA clades are rejected by the nrITS data, and 15 of 20 of the nrITS clades are rejected by cpDNA. Templeton tests indicate that the differing placements of *P. foliosa* and *P. taquetii*, and whether *P. senegalensis* is more closely related to *P. tomentosa* or to *P. lapathifolia*.P3, are not significant (constraints A, c-1, and I; Table 2, Fig. 1). Conflicts associated with the relationship among *P. amphibia*, *Echinocaulon*, and *Tovara* were not rejected by nrITS using cpDNA constraints, but were rejected by the cpDNA tree using nrITS constraints (constraints L, M, and q, r; Table 2, Fig. 1). Clades i and m from the nrITS tree are not significantly rejected by cpDNA (Table 2).

Three incongruence tests using likelihood also indicate that most of the conflicts seen between the cpDNA and nrITS trees are statistically significant. Test results and *P* values are presented in Table 3. All three tests rejected the null hypothesis that the nrITS tree does not differ from cpDNA constrained trees in 18 of 21 cases using the AU and K-H tests, and 16 using the S-H test. All three tests agree that differences in the placement of the clade including *P. foliosa* and *P. taquetii*, and in the placement of *P. japonica*, are not significant (constraints i and iii-2; Table 3). Likewise, different placements of *P. senegalensis* and relationships among *P. amphibia*, *Echinocaulon*, and *Tovara* are not significant (constraints viii, xiv, and xv; Table 3).

Tests using cpDNA data constrained by nrITS clades also indicate that most conflicts are statistically significant. The AU and K-H tests gave similar results except that the monophyly of *P. maculosa* and *P. tinctoria*, and that of *P. hispida* ~ *P. glabra*. P2, while these were rejected in the AU test but not in the K-H test. The S-H test showed very conservative behavior, as only 6 of 21 constraints were rejected, compared to the rejection of all constraints by the AU test, and 19 of 21 constraints by the K-H test (Table 3).

Nuclear ITS polymorphism in P. punctata—We found nrITS polymorphism in one of five *P. punctata* accessions. A minimum of 36 colonies were picked and sequenced for each accession, and seven sequences from 48 colonies from the Florida accession (P2) showed differences. When we added 10 more sequences into our 60 tip nrITS analyses, representing each *P. punctata* accession, one of the two types of sequences from the Florida accession strongly linked with the clade including *P. hirsuta*, *P. setaceae*, and *P. hydropiperoides*.P2, while the type from Florida, along with all other *P. punctata* accessions, remained within the clade including *P. hydropiper* and *P. pubescens* (tree not shown).

DISCUSSION

Incongruence tests and their implications—Our results demonstrate major conflicts between cpDNA and nrITS data for *Eupersicaria* (Fig. 2). The incongruence tests strongly reject the possibility that these conflicts are simply the result of statistical uncertainty (Tables 1–3). Most of the conflicts, involving 23 species of the 40 species examined, appear to be statistically significant in both parsimony and likelihood-based tests (Tables 2 and 3). Interestingly, results of the S-H tests differed in suggesting fewer strong conflicts. The S-H test was proposed to compensate for the K-H test's bias in multiple tree tests (Shimodaira and Hasegawa, 1999; Goldman et al., 2000), but it is known to be more conservative when the number of trees increases (Buckley et al., 2001; Buckley, 2002). As expected, the S-H test was especially conservative in testing cpDNA using constraints from the nrITS ML tree (Table 3). There is an asymmetry in the results using the S-H test, with more rejections when nrITS is constrained by cpDNA clades than vice versa. This asymmetry may be due to the need for apparently bigger changes in the nrITS tree in setting up the constraints, as opposed to rather minor or weakly supported relationships in constraining the cpDNA tree. Although we cannot completely rule out statistical error, especially where relationships are poorly resolved, we conclude from these analyses that there is evidence of strong genealogical discordance in numerous cases.

Hybridization as a cause of tree incongruence—Our analyses support nonstochastic processes underlying the observed conflicts. It is difficult, however, to distinguish between underlying processes—horizontal gene transfer, incomplete lineage sorting, or hybridization. Although we cannot totally rule out horizontal transfer or lineage sorting, we favor hybridization as the main cause of the conflicts in *Eupersicaria*. Horizontal transfer is least likely, perhaps, because we know of no special mechanisms of transfer (viral or bacterial vectors or parasitism) in this group of plants (Richardson and Palmer, 2007). It is much more difficult to distinguish between incomplete lineage sorting and hybridization because these processes can produce almost identical outputs at the level of tree discordances (Rosenberg, 2002; Rokas et al., 2003; Doyle et al., 2004; Linder and Rieseberg, 2004). However, on the basis of several lines of evidence discussed later, we suggest that hybridization is the more likely process in this case.

Aside from the strong incongruence documented here between maternally inherited cpDNA and biparentally inherited nrITS, the most important evidence in support of hybridization is that many of the species involved in strong conflicts are polyploids. Excluding five known diploid species (*P. hirsuta*, *P. setaceae*, *P. orientalis*, *P. viscofera*, and *P. viscosa*), 10 of

TABLE 2. Templeton tests for the significance of tree conflicts. The constraints listed below were used one at a time. Boldfaced values indicate rejection of the null hypothesis with 95% confidence. TL: tree length, *N*: sum of steps gained and lost in the constrained tree as compared to the unconstrained tree. ~: clade including these two species and all species situated between them in the parsimony tree in Fig. 1.

Constraints	TL	*N*	*P*
nrITS analysis constrained by the following clades from the cpDNA tree			
(A) *P. macrantha* ~ *P. foliosa*	624	0	—
(B) *P. macrantha* ~ *P. maculosa*	625	8	0.7630
(C) *P. hydropiper*.P1 ~ *P. minor*.P2	639	20	**0.0018**
(D) *P. kawagoeana* and *P. barbata*	642	16	**0.0008**
(E) *P. hydropiperoides*.P2 ~ *P. hirsuta*.P2	650	29	**<0.0001**
(F) *P. puritanorum*, *P. hydropiperoides*.P3, and *P. punctata*.P2	645	24	**0.0001**
(G) *P. hydropiperoides*.P2 ~ *P. robustior*	731	39	**<0.0001**
(H) *P. viscofera* and clade (G)	750	48	**<0.0001**
(I) *P. senegalensis* and *P. tomentosa*	629	11	0.1317
(J) *P. tomentosa* ~ *P. densiflora*	761	72	**<0.0001**
(K) *P. bicornis* ~ *P. densiflora*	674	32	**<0.0001**
(L) *Eupersicaria*; *P. macrantha* ~ *P. amphibia*.P2	632	15	0.0593
(M) *Eupersicaria* and *Tovara; P. macrantha* ~ *P. virginiana*	631	18	0.1266
Strict consensus tree from nrITS MP analysis, unconstrained	626	8	0.4795
Strict consensus tree of 416 MP trees from cp combined data set	953	108	**<0.0001**
cpDNA analysis constrained by the following clades from the nrITS tree			
(a) *P. hydropiper*.P1 ~ *P. punctata*.P2	522	23	**<0.0001**
(b) *P. macrantha* ~ *P. punctata*.P2	500	14	**0.0002**
(c-1) *P. foliosa* and *P. taquetii*	486	0	—
(c-2) clade (c-1) is the sister to the clade *P. macrantha* ~ *P. punctata*.P2	497	11	**0.0009**
(d) *P. puritanorum* ~ *P. opelousana*	492	5	**0.0339**
(e) *P. puritanorum* ~ *P. densiflora*	499	15	**0.0008**
(f) *P. puritanorum* ~ *P. monor*.P2	499	13	**0.0003**
(g) *P. mexicana* and *P. segetum*	490	4	**0.0455**
(h) *P. pensylvanica* and *P. bicornis*	490	4	**0.0455**
(i) *P. mexicana* ~ *P. bicornis* (the clade (g) plus the clade (h))	492	4	0.0633
(j) *P. mexicana* ~ *P. barbata*	506	18	**<0.0001**
(k) *P. mexicana* ~ *P. robustior*	522	29	**<0.0001**
(l) *P. accuminata* ~ *P. orientalis*	492	6	**0.0143**
(m) *P. hispida* ~ *P. glabra*.P2	488	2	0.1573
(n) *P. accuminata* ~ *P. glabra*.P2 (the clade (l) plus the clade (m))	498	8	**0.0097**
(o) *P. nodosa* and *P. viscofera*	494	8	**0.0047**
(p) The placement of *P. viscosa*; *P. macrantha* ~ *P. viscosa*	490	6	0.1025
(q) *P. filiformis* ~ *P. amphibia*.P2	499	13	**0.0003**
(r) *P. arifolia* ~ *P. amphibia*.P2	500	22	**0.0028**
(s) *P. hirsuta* P1 ~ *P. robustior*	489	3	0.0833
Strict consensus tree from cpDNA MP analysis, unconstrained	486	0	—
Strict consensus tree of 305 MP trees from nrITS data set	655	84	**<0.0001**

the 16 conflicting species are polyploids (Kim, 2008); chromosome numbers for the remaining six have not been reported. We suggest (further discussed later) that the tetra- and hexaploid *Eupersicaria* species involved in tree incongruence are allopolyploids stemming from hybridization, as proposed for many other groups, e.g., *Cardamine* (Lihova et al., 2006), *Glycine* (Doyle et al., 2004), *Tragopogon* (Soltis et al., 2004), and *Triticeae* (Kellogg et al., 1996).

Additional evidence in favor of hybridization as the cause comes from previous studies in *Eupersicaria*. Plants in *Eupersicaria* are notoriously polymorphic in morphology, which has been attributed to hybridization (Stanford, 1925b, 1926, 1927; Fernald, 1950). More importantly, artificial crosses have been successful among several North American species at the same ploidal level (e.g., between diploid *P. hirsuta* and *P. setacea* and between tetraploid *P. hydropiperoides* and *P. opelousana*; McDonald, 1980). These experiments are critical in demonstrating the potential for hybridization (incomplete reproductive isolation) in plants that are quite often cleistogamous (Stanford, 1925a; Simmonds, 1945a). Finally, Consaul et al. (1991) examined isozyme profiles in the *P. lapathifolia* complex and proposed a hybrid origin for *P. maculosa*. Specifically, they suggested that the tetraploid *P. maculosa* was an allopolyploid species having the diploid *P. lapathifolia* as one possible parent. Interestingly, this particular origin is not evident in our tree incongruence results. That is, the placement of *P. maculosa* does not strongly conflict between the cpDNA and nrITS trees (Fig. 1). If *P. maculosa* is of hybrid origin, there must have been concerted evolution in *P. maculosa* nrITS sequences toward the maternal source lineage. In general, this homogenization in nrITS sequences will lead to an underestimation of hybridization events. Other approaches, such as the use of low copy nuclear genes, are needed to reveal such cases.

Particular cases of allopolyploid speciation in Eupersicaria—*Persicaria punctata* likely originated from hybridization between the diploid species *P. hydropiper* and either *P. hirsuta* or *P. setacea*. This is clearly supported by the nrITS polymorphism; *P. punctata* had two different types of nrITS sequence, and each type was shared with a form in an inferred diploid parental species. More specifically, *P. hydropiper* may have provided the paternal parent based on the nrITS tree, and

TABLE 3. *P* values obtained from the AU (approximately unbiased), K-H (Kishino–Hasegawa), and S-H (Shimodaira–Hasegawa) tests. The constraints listed were used one at a time. Boldfaced values indicate rejection of the null hypothesis with 95% confidence. ~: Clade including these two species and all species situated between them in the maximum likelihood tree in Fig. 1.

Constraints	−ln likelihood	∂	AU	K–H	S–H
nrITS analysis constrained by the following clades from the cpDNA maximum likelihood tree					
ML tree of nrITS, unconstrained	4200.498		0.972	0.813	1.000
(i) *P. macrantha* ~ *P. foliosa*	4207.032	6.534	0.084	0.112	0.918
(ii) *P. hydropiper*.P1 ~ *P. minor*.P2	4228.433	27.935	**0.004**	**0.010**	0.415
(iii-1) *P. macrantha* ~ *P. japonica*	4257.293	56.795	**<0.001**	**<0.001**	**0.031**
(iii-2) *P. macrantha* ~ *P.barbata*	4249.177	48.679	**<0.001**	**<0.001**	0.056
(iii-2) *P. macrantha* ~ *P. japonica* without *P. kawagoeana* and *P. barbata*	4207.983	7.485	0.076	0.075	0.933
(iv) *P. hydropiperoides*.P2 ~ *P. hirsuta*.P2	4292.762	92.264	**0.004**	**0.010**	**<0.001**
(v) *P. puritanorum* ~ *P. robustior*	4349.139	148.641	**<0.001**	**<0.001**	**<0.001**
(vi) *P. hydropiperoides* ~ *P. robustrior*	4284.947	84.449	**<0.001**	**<0.001**	**0.002**
(vii-1) *P. hydropiperoides*.P2 ~ *P. viscofera*	4387.997	187.499	**<0.001**	**<0.001**	**<0.001**
(vii-2) *P. hydropiperoides*.P2 ~ *P. viscofera* without clade (v)	4305.798	105.300	**<0.001**	**<0.001**	**<0.001**
(viii) *P. tomentosa* and *P. senegalensis*	4212.088	11.590	0.143	0.115	0.841
(ix-1) *P. tomentosa* ~ *P. orientalis*	4275.286	74.788	**<0.001**	**<0.001**	**0.009**
(ix-2) *P. tomentosa* ~ *P. viscosa*	4277.322	76.824	**<0.001**	**<0.001**	**0.005**
(x) *P. nodosa* and *P. lapathifolia*.P2	4318.180	117.682	**<0.001**	**<0.001**	**<0.001**
(xi-1) *P. tomentosa* ~ *P. pensylvanica*	4354.155	153.657	**<0.001**	**<0.001**	**<0.001**
(xi-2) *P. tomentosa* ~ *P. segetum*	4373.952	173.454	**<0.001**	**<0.001**	**<0.001**
(xii) *P. tomentosa* ~ *P. densiflora*	4258.809	58.311	**<0.001**	**<0.001**	**0.017**
(xiii-1) *P. bicornis* ~ *P. densiflora* without *P. glabra*.P2 and *P. hispida*	4278.056	77.558	**<0.001**	**<0.001**	**0.009**
(xiii-2) *P. glabra*.P2 ~ *P. densiflora*	4264.830	64.332	**<0.001**	**<0.001**	**0.022**
(xiv) *P. macrantha* ~ *P. amphibia*.P2	4202.974	2.476	0.245	0.186	0.984
(xv) *P. macrantha* ~ *P. virginiana*	4202.943	2.445	0.346	0.187	0.984
cpDNA analysis constrained by the following clades from the nrITS maximum likelihood tree					
ML tree of combined CP (not constrained)	5921.876		0.998	0.894	1.000
(1) *P. maculosa* and *P. tinctoria*	5932.301	10.425	**0.030**	0.106	0.889
(2) *P. macrantha* ~ *P. tinctoria*	5999.277	77.401	**<0.001**	**<0.001**	**0.036**
(3) *P. hydropiper*.P1 ~ *P. punctata*.P2	6121.801	199.924	**<0.001**	**<0.001**	**<0.001**
(4) *P. macrantha* ~ *P. taquetii*	5971.080	49.204	**<0.001**	**0.002**	0.185
(5) *P. puritanorum* ~ *P. opelousana*	5945.668	23.792	**0.005**	**0.031**	0.606
(6) *P. puritanorum* ~ *P. densiflora*	5988.511	66.635	**<0.001**	**<0.001**	**0.035**
(7) *P. puritanourm* ~ *P. minor*.P2	5975.149	53.273	**<0.001**	**0.001**	0.093
(8) *P. mexicana* and *P. segetum*	5949.789	27.913	**0.001**	**0.024**	0.490
(9) *P. pensylvanica* and *P. bicornis*	5949.776	27.901	**0.003**	**0.024**	0.488
(10) *P. mexicana* ~ *P. bicornis*	5966.156	44.280	**0.002**	**0.030**	0.251
(11–1) *P. mexicana* ~ *barbata* without maintaining clades (8) and (9)	5999.836	77.960	**<0.001**	**<0.001**	**0.025**
(11–2) *P. mexicana* ~ *P. barbata*	6044.103	122.227	**<0.001**	**<0.001**	**0.003**
(12) *P. mexicana* ~ *P. robustior*	6059.820	137.944	**<0.001**	**<0.001**	**0.001**
(13) *P. accuminata* ~ *P. careyi*	5965.643	43.767	**<0.001**	**0.003**	0.212
(14) *P. hispida* ~ *P. glabra*.P2	5933.691	11.816	**0.017**	0.088	0.863
(15) *P. accuminata* ~ *P. glabra*.P2	5960.821	38.945	**<0.001**	**0.006**	0.296
(16) *P. nodosa* and *P. viscofera*	5973.786	51.910	**<0.001**	**0.002**	0.129
(17) *P. macrantha* ~ *P. viscosa*	5953.468	31.592	**0.024**	**0.025**	0.437
(18) *P. macrantha* ~ *P. tomentosa*	5973.786	51.910	**<0.001**	**0.002**	0.129
(19) *P. filiformis* ~ *P. amphibia*.P2	5964.833	42.958	**<0.001**	**0.003**	0.257
(20) *P. macrantha* ~ *P. meisneriana*	5966.745	44.869	**<0.001**	**0.001**	0.229

P. hirsuta or *P. setacea* may have served as the maternal parent based on the cpDNA tree if we consider allopolyploid speciation involving only known diploid parents (Figs. 1 and 2). Distingushing between *P. hirsuta* and *P. setacea* is difficult because these appear to be very closely related (Figs. 1 and 2). *Persicaria hirsuta* can be distinguished from *P. setacea* by hairs throughout the plant and more or less pink-colored perianth, but the two are very similar in other morphological traits. Experimental crosses between the two species showed that fully fertile seeds in the F1 progeny were produced only when *P. hirsuta* was used as the maternal lineage (McDonald, 1980). Morphologically, *P. punctata* shows intermediacy between *P. hydropiper* and *P. hirsuta*/*setacea*. Distinct glands on the perianth, peppery taste, and relatively glabrous stems and leaves in *P. punctata* are shared with *P. hydropiper*. On the other hand, *P. punctata* is similar to *P. hirsuta*/*setacea* in having relatively distinct and long inflorescences.

Our results might also be consistent with a different interpretation of the origin of *P. punctata* given the support values in our cpDNA tree. *Persicaria punctata* belongs to a clade with *P. hydropiperoides*, *P. opelousana*, *P. puritanorum*, and *P. robustior*, with little resolution among them (Figs. 1A and 2A). It is possible, therefore, that another tetraploid species, *P. hydropiperoides* or *P. opelousana,* provided the maternal parent for *P. punctata,* but only if *P. hydropiper* supplied an unreduced paternal gamete (Appendix S2, see Supplemental Data with

online version of article). The *P. hydropiperoides* complex itself, including *P. hydropiperoides* and *P. opelousana*, may also have originated via allopolyploid speciation. The diploid maternal parent most likely belonged to the *P. hirsuta*/*setacea* lineage, while the paternal parent is unclear. In the nrITS tree, the clade including the *P. hydropiperoides* complex and *P. puritanorum* is not directly linked with any diploid lineage.

The hexaploid *P. densiflora* is most closely related to the *P. hydropiperoides* complex in the nrITS tree, whereas it is clustered with the clade that includes *P. lapathifolia* in the cDNA tree (Figs. 1 and 2). On this basis, *P. densiflora* may have originated by a cross between a tetraploid paternal parent from the *P. hydropiperoides* complex or an unknown diploid species and a diploid maternal parent among *P. lapathifloia*, *P. viscosa*, or *P. orientalis*. Because *P. viscosa* and *P. orientalis* were originally distributed in Asia and are densely hairy throughout, the widely distributed *P. lapathifolia*, which is usually glabrous except for glandular trichomes in the inflorescence, is perhaps a more likely diploid parent for *P. densiflora*, which is glabrous and distributed in the New World.

The tetraploid *P. minor*, which is sister to the *P. hydropiperoides* complex and *P. densiflora* in the nrITS tree, may have arisen through hybridization between an unknown diploid lineage or possibly a tetraploid in the *P. hydropiperoides* complex and the diploid *P. hydropiper*. It is not entirely clear that *P. hydropiper* served as the maternal parent, however, because the relationship between *P. hydropiper* and *P. minor* is somewhat weakly supported in the cpDNA tree (Figs. 1A and 2A). Morphologically, *P. minor* is more similar to the diploid *P. foliosa,* which is nested in a clade including *P. macrantha* ~ *P. maculosa* and other tetraploid species (Fig. 1A). Inasmuch as the *P. macrantha* ~ *P. maculosa* clade (Fig. 1A) is closely related to the clade including *P. hydropiper* and *P. minor* (Fig. 1A), *P. foliosa* should also be considered as a possible diploid maternal lineage for *P. minor*.

Another possible allopolyploid species is *P. nodosa*. *Persicaria nodosa* has generally been considered a synonym of the diploid *P. lapathifolia* based on morphology (Mitchell and Dean, 1978; Gleason and Cronquist, 1991; Yang and Wang, 1991; Hinds and Freeman, 2005), but our nrITS sequence data indicate that *P. nodosa* is distinct from *P. lapathifolia* and instead is closely related to the diploid *P. viscofera* (Figs. 1 and 2). This observation, combined with our confirmation that *P. nodosa* is a tetraploid, leads us to hypothesize that *P. nodosa* originated via hybridization between *P. lapathifolia* and *P. viscofera*. However, this scenario raises a question regarding geographic distributions because *P. viscofera* populations are confined to East Asia, whereas *P. nodosa* populations are found in Europe and North America (Danser, 1921; Simmonds, 1945b). Perhaps *P. nodosa* moved into Europe and North America following allopolyploid speciation in East Asia between the widely distributed *P. lapathifolia* and the East Asian *P. viscofera* (cf. Sang et al., 1997).

It is difficult to evaluate the possibility of allopolyploid speciation for taxa whose chromosome numbers have not yet been determined. Chromosome numbers for three species (*P. mexicana*, *P. segetum*, and *P. bicornis*) have not been reported. Although the chromosome number for *P. pensylvanica* has been reported as $2n = 22$ (Löve and Löve, 1982) or $2n = \sim 80$ (Gervais, 2000), our preliminary count indicates that this species is possibly an octaploid. These four species form a strongly supported clade in the nrITS tree and in turn are linked with the clade that includes the diploid species *P. hirsuta* and *P. setacea*. Their paternal contribution may therefore have come from this lineage. The maternal contribution is unclear based on lack of resolution in the cpDNA tree (Figs. 1 and 2).

Implications for relationships and taxonomy in Eupersicaria—In view of the strong disagreements between trees inferred from the nrITS and the combined cpDNA sequences and our tests indicating that these are mostly not due to stochastic error, we have not combined these data sets and will resist the temptation to discuss phylogenetic relationships within *Eupersicaria*. Instead, in this section we discuss several specific cases that bear on taxonomic treatments. In general, our analyses support the view that the number of species has been underestimated in polyploidy complexes (Soltis et al., 2007).

On the basis of a partial sampling of North American species, we proposed the resurrection of the New England endemic, *P. puritanorum*, which had frequently been synonymized with *P. maculosa*. *Persicaria puritanorum*, a hexaploid, does not appear to be closely related to the tetraploid *P. maculosa*, but instead likely originated via hybridization involving at least one parent from the *P. hydropiperoides* complex (Kim, 2008).

The circumscription of *P. lapathifolia* has caused difficulty. Timson (1963), following up on cultivation experiments by Danser (1921), delimited 12 lineages within the *P. lapathifolia* complex based on several morphological characters. He and many authors since (Mitchell and Dean, 1978; Gleason and Cronquist, 1991; Yang and Wang, 1991; Hinds and Freeman, 2005) have recognized *P. lapathifolia* in the broad sense, thus synonymizing *P. nodosa*, *P. tomentosa*, and others. Our analyses strongly support the resurrection of *P. nodosa,* which is a tetraploid (unpublished data) that probably originated through allopolyploid speciation, as suggested in earlier taxonomic treatments (Meisner, 1856; Danser, 1921; Steward, 1930; Britton, 1933). *Persicaria nodosa* can be distinguished from the typical *P. lapathifolia* by red spots on the stems, the red-purple perianth near the base, and more or less coarse trichomes covering the whole plant (cf. Simmonds, 1945c).

Surprisingly, our incongruence tests indicated that differences in the placement of *P. tomentosa* (Figs. 1 and 2) might be due to stochastic error (Table 3: I, p; Table 4: viii), which may support the view that it represents a phenotypic extreme within *P. lapathifolia* (Danser, 1921; Timson, 1963; Mitchell and Dean, 1978; Gleason and Cronquist, 1991; Yang and Wang, 1991; Hinds and Freeman, 2005). However, we favor the recognition of *P. tomentosa* as a separate species on the basis of our observation that it is a tetraploid (S.-T. Kim, unpublished data) and possibly of allopolyploid origin and that it consistently has dense hairs on the undersides of the leaves. Confirming the allopolyploid origin in this group clearly requires additional work, including the use of low copy nuclear markers and further morphological analyses. The use of population genetic approaches, sampling from multiple populations, would also shed light on this issue.

Another case in need of attention relates to *Persicaria macrantha* and *P. japonica*. Meisner (1856) originally described these two species (as *Polygonum macranthum* and *Polygonum japonicum*), contrasting glandular dots on the perianth and larger flowers in the former with the lack of glands on the perianth and relatively small and more flowers in the later. Later, Nakai (1908) proposed the recognition of two varieties in *P. japonica*, one representing Meisner's *P. macrantha* and the

other representing Meisner's *P. japonica* but with glands on the perianth. Steward (1930) restored *P. macrantha* and *P. japonica,* but named a new variety in *P. macrantha* with glands on the perianth. In the most recent taxonomic treatment, Li et al. (2003) recognized two varieties in *P. japonica*. Our analyses strongly indicate that *P. macrantha* and *P. japonica* are distinct lineages (Figs. 1 and 2). It seems apparent from previous studies that there may be three possible entities, corresponding to *P. macrantha*, *P. japonica* with glands, and *P. japonica* without glands. The sample of *P. japonica* used in this study has glandular dots on the perianth. Further evaluation will require the inclusion of *P. japonica* individuals without these glands. In any case, however, our results strongly support the resurrection of *P. macrantha*.

Lastly, we suggest that *P. densiflora* be treated as a distinct species, which possibly also originated via allopolyploidy. *Persicaria densiflora*, described by Meisner (1856) based on American plants, has been merged into *P. glabra* mainly based on their sharing a glabrous plant body (Wilson, 1990; Li et al., 2003; Hinds and Freeman, 2005). Our analyses support three independent lineages based on the separation in our trees of *P. densiflora* from North America, and of the accessions of *P. glabra* from India and from Bolivia (Figs. 1 and 2). The morphological resemblances among these three lineages could reflect shared parentage in their allopolyploid origins or the convergence in loss of trichomes, more or less glandular dots on the perianth, and relatively longer petioles.

Summary—The strong conflicts we have shown between cpDNA and nrITS, combined with information on chromosome numbers and geography, support the view that there has been extensive hybridization and allopolyploid speciation in *Eupersicaria*. Specifically, our studies have identified 23 cases of conflict between nrITS and the combined cpDNA gene trees. We propose allopolyploidy as the mechanism underlying 10 known polyploid species of the 16 cases involving known polyploids that appear to be statistically meaningful in our congruence tests (Fig. 2). We cannot completely rule out the possibility that we have overestimated the number of allopolyploid events because other mechanisms can underlie discordance, but the concordance of different lines of evidence favors our interpretation. In fact, we are very likely to have underestimated allopolyploidy owing to limitations on the sample examined here and especially to concerted evolution in the nrITS. This problem is most evident in our finding nrITS polymorphism within *P. punctata*.

Additional studies are needed, employing different approaches (e.g., low copy nuclear markers; Sang, 2002; Álvarez and Wendel, 2003; Small et al., 2004), and with a focus on geographic and ecological patterns and the origin and spread of the important weedy species in this group. In the meantime, however, it now seems clear that multiple instances of hybridization and allopolyploidy have occurred in this group and that this has contributed to the taxonomic difficulties. It also appears that much of the diversification of this group has been driven by separate events of hybridization among diploids and allotetraploidy, as opposed to speciation following allotetraploidy (which would have resulted in clades of polyploid species). Finally, because many of these events are likely to have taken place relatively recently, more targeted comparisons in this group may be especially useful in elucidating the initial molecular and ecological impacts of allopolploidy (Paun et al., 2007).

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APPENDIX 1. Voucher information and GenBank accession numbers for taxa used in this study. Voucher specimens are deposited in the following herbaria: YU = Yale University, GH = Gray Herbarium, Harvard University, NHA = University of New Hampshire.

Taxon and author(s) (specifier for accession if any)—GenBank accessions: nrITS (additional clone sequence if any), p-*matK*, *psbA-trnH*, *trnL-F*; Locality; *Voucher specimen* (Herbarium).

Persicaria accuminata (Kunth) M. Gómez—EU196870, EU196923, EU196966, EU197009; Cercado, Bolivia; [a]*Ritter 3876* (NHA). ***P. amphibia*** (L.) Gray (P1)—EF653699, EF653724, EF653750, EF653802; New Jersey, US; *Kim 600* (YU). (P2)—EF653700, EF653725, EF653752, EF653803; KyungSangNam, Korea; *Kim & Kim Ch-Ko-91* (YU). ***P. arifolia*** (L.) Haraldson—EF653693, EF653718, EF653744, EF653796;Connecticut, US; [a]*Magee 89-56* (YU). ***P. barbata*** (L.) Hara—EU196871, EU196924, EU196967, EU197010;Pingtung, Taiwan; [a]*Ou & Kao 9463* (GH). ***P. bicornis*** (Raf.) Nieuwl.—EU196872, EU196925, EU196968, EU197011; Kansas, US; [a]*Lathrop 1290* (GH). ***P. capitata*** (Buch.-Ham. ex D. Don) H. Gross—EF653690, EF653715, EF653741, EF653793; Yunnan, China ; *Kim & Deng Ch-Ko-48* (YU). ***P. careyi*** (Olney) Greene—EU196873, EU196926, EU196969, EU197012;Connecticut, US; [a]*Souther 7-18-92* (YU). ***P. densiflora*** Moldenke—EU196874, EU196927, EU196970, EU197013; Florida, US; *Kim, Koh & Yoo 50* (YU). ***P. ferruginea*** (Weddell) Soják—EU196875, EU196928, EU196971, EU197014; Cercado, Bolivia; [a]*Ritter & Ritter 3297* (NHA). ***P. filiformis*** (Thunb.) Nakai—EF653697, EF653748, EF653800,EF653722; KyungGi, Korea; *Kim & Kim Ch-Ko-102* (YU). ***P. foliosa*** (H. Lindb.) Kitag.—EU196876, EU196929, EU196972, EU197015; KyungSangNam, Korea; *Kim & Kim Ch-Ko-99* (YU). ***P. glabra*** (Willd.) M. Gómez (P1)—EU196877, EU196930, EU196973, EU197016; Florida, Bolivia; [a]*Ritter, Crow & Crow 4080* (NHA). (P2)—EU196878, EU196931, EU196974, EU197017; Karnataka, India; [a]*Lundal 10* (GH). ***P. hirsuta*** Small (P1)—EU196879, EU196932, EU196975, EU197018; Florida, US; [a]*Sorrie 9886* (NHA). (P2)—EU196880, EU196933, EU196976, EU197019; Florida, US; *Kim, Koh & Yoo s.n.* (YU). ***P. hispida*** (Kunth) M. Gómez—EU196881, EU196934, EU196977, EU197020; Florida, Bolivia; [a]*Ritter & Ritter 2941* (NHA). ***P. hydropiper*** (L.) Spach (P1)—EF653702, EF653727, EF653753, EF653805; Connecticut, US; *Kim 570* (YU). (P2)—EF653703, EF653728, EF653754, EF653806; Yunnan, China; *Kim & Ma Ch-Ko-37* (YU). ***P. hydropiperoides*** (Mich.) Small (P1)—EU196882, EU196935, EU196978, EU197021; Connecticut, US; *Kim 565* (YU). (P2)—EU196883, EU196936, EU196979, EU197022; Florida, US; *Kim, Koh & Yoo s.n.* (YU). (P3)—EU196884, EU196937, EU196980, EU197023; Massachusetts, US ; *Kim & Chae s.n*. (YU). ***P. japonica*** (Meisn.) Nakai—EU196885, EU196938, EU196981, EU197024; KyungSangNam, Korea; *Kim & Kim Ch-Ko-92* (YU). ***P. kawagoeana*** (Makino) Nakai—EU196886, EU196939, EU196982, EU197025; Yunnan, China; *Kim & Deng Ch-Ko-74* (YU). ***P. lapathifolia*** (L.) Gray (P1)—EF653704, EF653729, EF653755, EF653807; Connecticut, US; [b]*EG.3.* (P2)—EU196887, EU196940, EU196983, EU197026; Yunnan, China; *Kim & Ma Ch-Ko-23* (YU). (P3)—EU196888, EU196941, EU196984, EU197027; Yunnan, China; *Kim & Ma Ch-Ko-40* (YU). ***P. limbata*** (Meisn.) Hara—EU196889, EU196942, EU196985, EU197028; Gauteng, South Africa; [a]*Kok 60* (GH). ***P. longiseta*** (Bruijn) Moldenke (P1)—EF653701, EF653726, EF653752, EF653804; Connecticut, US; [b]*WEIR.8.* (P2)—EU196890, EU196943, EU196986, EU197029; KyungGi, Korea; *Kim & Kim Ch-Ko-84* (YU). ***P. maackiana*** (Regel) Nakai ex T. Mori—EF653694, EF653719, EF653745, EF653797; KyungSangNam, Korea; *Kim & Kim Ch-Ko-89* (YU). ***P. macrantha*** (Meisn.) Haraldson—EU196891, EU196944, EU196987, EU197030; Yunnan, China; *Kim & Ma Ch-Ko-41* (YU). ***P. maculosa*** Gray—EU196892, EU196945, EU196988, EU197031; Massachusetts, US; *Kim, Donoghue & Sultan 11* (YU). ***P. meisneriana*** (Cham. & Schlechtend.) M.Gómez—EF653695, EF653720, EF653746, EF653798; Florida, Bolivia; [a]*Ritter, Crow & Crow 4083* (NHA). ***P. mexicana*** Small—EU196893, EU196946, EU196989, EU197032; Durango, Mexico; [a]*Fernandez 1090* (GH). ***P. minor*** (Huds.) Opiz (P1)—EU196894, EU196947, EU196990, EU197033; Honshu, Japan; [a]*Tanaka 5020* (GH). (P2)—EU196895, EU196948, EU196991, EU197034; Massachusetts, US; *Kim & Chae s.n.* (YU). ***P. nepalensis*** (Meisner) H. Gross—EF653691, EF653716, EF653742, EF653794; Yunnan, China; *Kim & Deng Ch-Ko-50* (YU). ***P. nodosa*** (Pers.) Opiz—EU196896, EU196949, EU196992, EU197035; Connecticut, US; *Sultan & Heschel 7-18-01* (YU). ***P. opelousana*** (Riddell) Small—EU196897, EU196950, EU196993, EU197036; Massachusetts, US; *Kim & Lundgren s.n.* (YU). ***P. orientalis*** (L.) Spach—EU196898, EU196951, EU196994, EU197037; Yunnan, China; *Kim & Ma Ch-Ko-34* (YU). ***P. paraguayensis*** (Wedd) Kim & Donoghue **comb. nov.**—EU196899, EU196952, EU196995, EU197038; Luis Calvo, Bolivia ; [a]*Ritter 3919* (NHA). ***P. pensylvanica*** (L.) M. Gómez—EF653705, EF653730, EF653756, EF653808; Massachusetts, US; *Kim, Donoghue & Sultan 14* (YU). ***P. posumbu*** (Buch.-Ham. ex D. Don) H. Gross—EU196900, EU196953, EU196996, EU197039; Yunnan, China ; *Kim & Deng Ch-Ko-46* (YU). ***P. pubescens*** (Blume) Hara—EU196901, EU196954, EU196997, EU197040; Yunnan, China ; *Kim & Deng Ch-Ko-75* (YU). ***P. punctata*** (Elliott) Small (P1)—EF653706 (EU196902), EF653731, EF653757, EF653809; Connecticut, US; *Kim 560* (YU). (P2)—EU196903 (EU196904, EU196905, EU196906, EU196907, EU196908), EU196955, EU196998, EU197041; Florida, US; *Kim, Koh & Yoo s.n.* (YU). (P3)—EU196909, —, —, —; Massachusetts, US; *Kim, Donoghue & Sultan 19* (YU). (P4)—EU196910, —, —, —; Massachusetts, US; *Kim, Donoghue & Sultan 20* (YU). (P5)—EU196911 (EU196912), —, —, —; Bolivia; [a]*Ritter 1761* (NHA). ***P. puritanorum*** (Fern.) Soják—EU196913, EU196956, EU196999, EU197042; Massachusetts, US; [b]*LP.6.* ***P. robustior*** (Small) E. P. Bicknell—EU196914, EU196957, EU197000, EU197043; Connecticut, US ; [a]*Eames s.n.* (YU). ***P. runcinata*** (Buch.-Ham. ex D. Don) H. Gross—EF653692, EF653717, EF653743, EF653795; Yunnan, China; *Kim & Deng Ch-Ko-62* (YU). ***P. sagittata*** (L.) H. Gross—EF653696, EF653721, EF653747, EF653799; Connecticut, US; *Kim 650* (YU). ***P. segetum*** (Kunth) Small—EU196915, EU196958, EU197001, EU197044; Guanacaste, Costa Rica ; [a]*Crow 5967* (NHA). ***P. senegalensis*** (Meisn.) Soják—EU196916, EU196959, EU197002, EU197045; Basse-Casamance, Senegal ; [a]*Berghen 9693* (GH). ***P. setacea*** (Baldwin) Small—EU196917, EU196960, EU197003, EU197046; Florida, US ; *Kim, Koh & Yoo s.n.* (YU). ***P. taquetii*** (H. Lev.) Koidz.—EU196918, EU196961, EU197004, EU197047; Honshu, Japan ; [a]*Murata & Terao 1461* (GH). ***P. tinctoria*** (Ait.) H. Gross—EU196919, EU196962, EU197005, EU197048; Seoul, Korea; *Kim Ch-Ko-88* (YU). ***P. tomentosa*** E. P. Bicknell—EU196920, EU196963, EU197006, EU197049; Crete, Greece; *Kim & Chae Cr-40* (YU). ***P. virginiana*** (L.) Gaertn.—EF653698, EF653723, EF653749, EF653801; Connecticut, US; *Sultan s.n.* (YU). ***P. viscofera*** (Makino) H. Gross ex Nakai—EU196921, EU196964, EU197007, EU197050; KyungGi, Korea; *Kim Ch-Ko-85* (YU). ***P. viscosa*** (Buch.-Ham. ex D. Don) H. Gross ex Nakai—EU196922, EU196965, EU197008, EU197051; Yunnan, China; *Kim & Ma Ch-Ko-35* (YU).

[a] Extraction from herbarium specimen

[b] Inbred line cultivated at Wesleyan University (see Sultan et al., 1998) without voucher specimen.

APPENDIX 2. A new combination.

Persicaria paraguayensis (Wedd.) Kim and Donoghue comb. nov.

= *Polygonum paraguayense* Wedd., *Ann. Sci. Nat., Bot*. 3: 253 (1849).

Type: *H. A. Weddell 3155*, Lectotype in P.