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On the resurrection of *Persicaria puritanorum* (Polygonaceae)

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Abstract

Pond populations in Cape Cod described as *Persicaria puritanorum* (≡ *Polygonum puritanorum*) have been treated by recent authors as a synonym of *P. maculosa* (≡ *Polygonum persicaria*) based on gross morphology. However, consistent differences in leaf and inflorescence features raise the question as to whether this is an extreme phenotype of *P. maculosa*, an ecotype of that species, or a distinct taxon. In this study, we analyzed nuclear ITS region and chloroplast *mat*K region DNA sequences from 37 populations of 11 species within section *Eupersicaria* and counted the chromosome number of individuals from the pond populations in question. We also compared leaf size and shape of plants from these populations with those of the typical *P. maculosa* plants by measuring selected herbarium specimens. Phylogenetic trees inferred from sequence data indicate a separation of pond populations from *P. maculosa* suggesting to recover *P. puritanorum* as a distinct species. Chromosome counts of pond population plants (2n = 60) strongly support this result, and furthermore suggest the possibility of a hybrid polyploid origin of *P. puritanorum*. A leaf morphological trait (greater relative height of the maximum width point from the leaf base) also supports the separation of pond populations from *P. maculosa*. *P. punctata* allied to *P. hydropiper* in the ITS-based tree, and to pond populations as well as *P. hydropiperoides* and *P. opelousana* in the *matK*-based tree, suggesting the possibility of hybrid origin of *P. punctata*. Our ITS sequence analyses also reveal distinct two lineages in *P. lapathifolia* but the chloroplast *matK* region sequences of *P. pensylvanica* and two lineages of *P. lapathifolia* are almost identical suggesting cytoplasmic introgression or allopolyploid speciation sharing the maternal lineage.

Key words: Cape Cod, chromosome, hybrid, ITS, *mat*K, *Persicaria*, phylogeny, *Polygonum*, Polyploid

Introduction

Persicaria puritanorum (Fernald 1919: 141) Soják (1974: 154) [≡ *Polygonum puritanorum* Fernald (1919: 141–142)] is a species described on plants collected from sandy beaches around freshwater ponds on Cape Cod, Massachusetts. Fernald (1919) recognized it as a distinct indigenous species on the basis of its specialized distribution and the relatively smaller size of its inflorescences, flowers and leaves as compare to *P. maculosa* Gray (1821: 269) [≡ *Polygonum persicaria* Linnaeus (1753: 361)], a widespread and morphologically variable annual species naturalized from Europe (see e.g., Fernald 1919, 1950, Gleason & Cronquist 1963, 1991, Sultan *et al.* 1998, Kim & Donoghue 2008b). However, within *Persicaria* (Linnaeus 1753: 159) Miller (1754: no page number given) the range size, trichome density, leaf shape, and other morphological characters vary broadly and depend on moisture conditions (Mitchell 1971, 1976, Stanford 1925b). Consequently, many authors (e.g., Gleason & Cronquist 1991, Magee & Ahles 1999, Mitchell & Dean 1978) treated *P. puritanorum* as included in *P. maculosa*. Sultan & Bazzaz (1993a, 1993b, 1993c) compared the genotypes from one of the Cape Cod pond populations with a typical population of *P. maculosa* and found roughly similar patterns of phenotypic plasticity in response to light, moisture and nutrient variation. They also found that the peculiar upright leaf angle of the pond plants seen in the field was not expressed under greenhouse conditions. However, other distinctive traits were manifested in the pond plants such as more condensed short inflorescences and more acute leaf tips. In addition, these plants were occasionally infested with aphids and patrolled by ants in field and this fact is not known from *P. maculosa* populations in other habitats (Sultan pers. obs.).

All these data suggest several possibilities regarding the Cape Cod pond populations: (1) the plants might be phenotypes produced by the very plastic *Persicaria maculosa* under the harsh, low-nutrient conditions that characterize these beach sites (Sultan & Bazzaz1993b, Sultan *et al.* 1998), (2) they might represent a genetically distinct pond ecotype that evolved under local selection from *P. maculosa*, (3) they might represent an entirely distinct species, not directly related to *P. maculosa*, or (4) they might represent a hybrid between coexisting related species.

Because diagnostic morphological characters are limited and phenotypic plasticity in the *Persicaria maculosa* complex is high, molecular analyses, including potentially closely related species living in the same geographic region, might clarify the relationships of the pond populations to other *Persicaria* species. In this study, we analyzed nuclear ribosomal internal transcribed spacer (ITS) DNA sequences and chloroplast *matK* sequences. We also determined the chromosome number of pond plants.

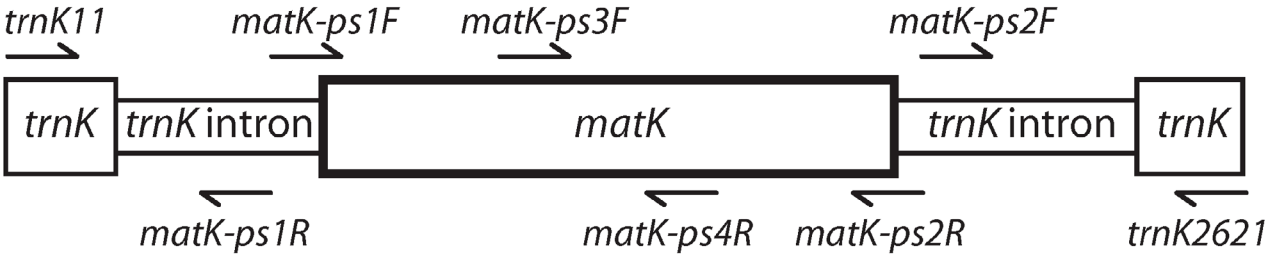
TABLE 1. Sampling used (specimens at YU). CT: Connecticut, MA: Massachusetts, NJ: New Jersey, NS: Nova Scotia, Canada, CL: inbred line cultivated in Wesleyan University. *: Voucher of root tip preparation for chromosome counting. **: reference to Kim & Donoghue (2008a).

Taxon (*P. = Persicaria*)	**Accession locality (population code)**	**Voucher**	**GenBank number (nrITS, *matK*)**
P. amphibia	Spring Lake, Mercer Co. NJ	*Kim 600***	EF653701, KY978013
P. longiseta	Towle Paddock, section 1, Dover, MA (TP1)	CL	KY978049, KY978016
	Arch Road, Leeds, MA (ARL)	CL	KY978047, KY978014
	Katherine Ordway Preserve, Weston, CT (ORD)	CL	KY978048, KY978015
	Weir Farm, Wilton, CT (WEIR)	CL**	EF653701, KY978017
P. hydropiper	Mount Herman Farm, Northfield, MA (MHF)	CL	KY978051, KY978019
	Dewitt Fish Farm, Amherst, MA (DFF)	CL	KY978050, KY978018
	Towle Paddock, section 2, Dover, MA (TP2)	CL	KY978052, KY978021
	New Haven, CT (NHC)	Kim 570**	EF653702, KY978020
P. hydropiperoides	New Haven, CT (NHC)	*Kim 565***	EU196881, KY978022
	New London Co., CT (NLC)	*Kim 670*	KY978053, KY978023
P. lapathifolia	Daniel Webster Sanctuary, Marshfield, MA (M)	CL	KY978056, KY978027
	Adams Ditch, Newington, CT (AD)	CL	KY978054, KY978024
	Northfield Barn Lot, Northfield, MA (NBL)	CL	KY978057, KY978028
	East Granby Site, E. Granby CT (EG)	CL**	EF653704, KY978026
	New Haven, CT (NHC)	*Heschel & Sultan s.n.*	KY978058, KY978029
	Brewster Co., MA (BCM)	*Kim, Donoghue & Sultan 23*	KY978055, KY978031
	New London Co., CT (NLC)	*Kim 665.*	KY978059, KY978030
P. opelousana	Keeler Pond Brewster Co., MA (BCM)	*Lundgren & Kim s.n.** *	EU196897, KY978031
P. maculosa	Daniel Webster Sanctuary, Marshfield, MA (M)	CL	KY978063, KY978036
	Mount Herman Farm, Northfield, MA (MHF)	CL	KY978064, KY978037
	Towle Paddock, section 1, Dover, MA (TP1)	CL	KY978066, KY978039
	Natick Farmyard, Natick, MA (NAT)	CL	KY978065, KY978038
	Dewitt Fish Farm, Amherst, MA (DFF)	CL	KY978062, KY978035
	Roadside, Brewster Co., MA (BCM)	*Kim, Donoghue & Sultan 11***	EU196892, KY978034
	Sydney Mines, Cape Breton Co. NS (NSC)	*Bissel & Linder 21085*	KY978061, KY978040
P. puritanorum	Long Pond, Brewster Co., MA (LP)	**Kim 511*, CL	KY978061, KY978040
	Snake Pond, Brewster Co., MA (SP)	**Kim 512*, CL	KY978070, KY978044
	Cliff Pond, Brewster Co., MA (CP)	*Kim, Donoghue & Sultan 5*	KY978068, KY978042
	Grand Lake Annapolis Co. NS (NSC)	*Fernald, Bartram, Long & Fassett 23801*	KY978067, KY978041
P. pensylvanica	Long Pond, Brewster Co., MA (LP)	*Kim, Donoghue & Sultan 14***	EF653705, KY978032
	New Haven, CT (NHC)	*Kim 545.*	KY978060, KY978033
P. punctata	Long Pond, Brewster Co., MA (LP)	*Kim, Donoghue & Sultan 19***	EU196909, KY978045
P. virginiana	New Haven, CT (NHC)	*Kim 575*	KY978046, KY978012
	Pearl st. Middletown, CT (MC)	*Sultan s.n.***	EF653698, KY978011

Material and Methods

Samples

Samples for DNA extraction were mainly collected from 18 living inbred lines cultivated in Wesleyan University: 4 populations for *Persicaria longiseta* (Bruijn 1854: 307) Kitagawa (1937: 322) [≡ *Polygonum caespitosum* var. *longisetum* (Bruijn 1854: 307) Steward (1930: 67)] and *P. lapathifolia* (Linnaeus 1753: 360) Gray (1821: 270), 3 populations for *P. hydropiper* (Linnaeus 1753: 361) Spach (1841: 536), 7 populations (including 2 pond populations of *P. puritanorum*) for *P. maculosa*. Other 14 living samples were collected from fieldworks made in Connecticut, Massachusetts, and New Jersey, US during the period of years from 2002 to 2004 (Table 1). We also used two herbarium specimens collected in Nova Scotia, Canada for DNA extraction. One individual was identified and annotated as *P. puritanorum* and the other as a variety of *P. maculosa* [= *Polygonum persicaria* var. *ruderale* Meisner (1856: 118)]. *Persicaria virginiana* (Linnaeus 1753: 360) H. Gross (1913: 321) was chosen as an outgroup species because the section *Tovara* (Adanson 1763: 276, 612) H. Gross (1913: 27), which includes *P. virginiana*, is considered to be the most closely related to the section *Eupersicaria*, which includes the studied species (Haraldson 1978, Ronse Decraene & Akeroyd 1988, Suh *et al.* 1997, Kim & Donoghue 2008a). Fifty-nine herbarium specimens of *Persicaria maculosa* (44) and *P. puritanorum* (15) were measured (leaf characters) (see Appendix).



*trnK 11**	CTCAACGGTAGAGTACTCG
*trnK 2621**	AACTAGTCGGATGGAGTAG
matK-ps1F	GTGAATCCTAACTATTAAC
matK-ps1R	GTTAATAGTTAGGATTCAC
matK-ps2F	TGTGCTAGAACGTTGGCTC
matK-ps2R	GAGCCAACGTTCTAGCACA
matK-ps3F	CAAATGTATCAACAGAATCA
matK-ps4R	GTATAACCCGAAATGCTTA

FIGURE 1. Simple diagram of plastid *mat*K (black box), *trn*K 5' intron (left side line), and *trn*K 3' intron region (right side line) including amplification and sequencing primers used in this study. Amplification primers are *trn*K 11 and *trn*K 2621. Primer sequences are listed 5' to 3'. * see Young *et al*. 1999.

DNA extraction, Amplification, and sequencing

The total DNA was extracted from fresh or dried materials with silica-gel using DNeasy Plant Mini Kit (Qiagen Valencia, CA). For the extraction from the herbarium specimen we added 20 hours' rocking incubation with Proteinase K and 2-mercapto-ethanol for the first step in the Kit protocol. Amplification of double stranded DNA was performed using standard polymerase chain reaction (PCR) in 25 µL reactions contained 5µL of 5X Buffer C (PCR Optimizer Kit, Invitrogen, CA) including 1.5 mmol/L final concentration of $MgCl_2$, 1.0 mmol/L dNTPs, 1.0 µmol/L primers; ITSLeu (Baum *et al.* 1998) and ITS4 (White *et al.* 1990) for ITS, and *trnK*-11 and *trn*k-2621 (Young *et al.* 1999) for *matK* region (Fig. 1), 1.0 unit AmpliTaq DNA polymerase (Applied Biosystems, Foster City, CA), and 1–10 ng DNA. PCR was carried out with these steps; ITS pre-denaturation 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 final extension for 7 min. *matK* pre-denaturation at 94°C for 90 sec., 35 cycles of denaturation at 94°C for 30 sec., annealing at 62°C for 45 sec., extension at 72°C for 90 sec., and concluding with a final extension for 4 min. Because there were difficulties in amplifying whole gene regions with herbarium extractions smaller fragment was amplified using primer for sequencing (ITS2, ITS3b, or *matK*-ps2F). PCR products were purified with QIAquick PCR Purification Kit (Qiagen Valencia, CA). Sequencing of ITS region was accomplished using the amplification primers with two additional primers, ITS2 (White *et al.* 1990) and ITS3b (the reverse sequence of ITS2) for ITS region and six additional primers, specifically designed for the genus *Persicaria*, for *matK* and *trnK* intron region (Fig. 1). 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 510 (MJ Research, Sauk City, WI) or an ABI 377 (or 3100) automated DNA sequencer.

Sequence alignment and Phylogenetic analyses. Sequences were primarily aligned using Sequencer 4.1 (Gene Code Corp. Ann Arbor, MI) and finally examined by eyes. The sequence boundaries of ITS 1, 5.8S coding region and ITS 2 were determined by comparison with other published sequences (Baldwin 1993, Suh *et al.* 1997). The sequences of the chloroplast *trnK* 3' and 5' end introns and *matK* gene were compared with reported sequences on GenBank to determine the boundary of coding region of *matK*. These aligned data sets are available in TreeBase (http://www.treebase.org) or upon request from the first author.

Phylogenetic analyses were conducted using PAUP* 4.0b10 (Swofford 2001) and MrBayes 3.1 (Heulsenbeck & Ronquist 2001) with ITS and *matK* data sets respectively. Maximum parsimony searches were performed using heuristic search methods with tree bisection reconnection (TBR) branch swapping, collapse of zero maximum branch length, and equal weighting of all characters. The analyses were repeated 500 times with random order of sequence addition to minimize problems of multiple islands of most parsimonious trees. We confirmed the node stability with bootstrap tests (Felsenstein 1985) estimated using 1000 replicates with heuristic search settings identical to those of the original search except analysis with ITS data set. In bootstrap analysis using ITS data we restricted the time for rearrangement to 300 seconds per replicate in order to avoid unpredictable searching due to possible polytomy caused by many identical sequences.

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 & Crandall 1998). Maximum likelihood searches were carried out in PAUP* using each model suggested for each data set (Table 2). 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 500 replicates with nearest neighbor interchange (NNI) branch swapping. Parameters for bootstrap tests were fixed to values estimated from the maximum likelihood tree.

TABLE 2. Summary of maximum parsimony, maximum likelihood, and Bayesian analyses of nrITS and chloroplast *mat*K sequence data sets.

Characteristics	ITS	*matK*
Aligned sequence length	653	2477
No. of variable characters	127	137
No. of parsimony-informative characters	116	108
No. of most parsimonious trees	8	21
Length of most parsimonious trees	167	139
Consistency Index (CI)	0.868	1.000
CI excluding uninformative characters	0.859	1.000
Retention Index (RI)	0.951	1.000
No. of most likelihood trees	2	8
Log likelihood of the most likelihood trees	-1819.845	-4268.428
Model chosen by hLRT implemented in 'Modeltest'	TrN + G	K81uf + G
Gamma distribution shape parameter for ML search	0.3322	0.4223
Average Log likelihood in Bayesian analysis	-1886.086	-4326.865

Bayesian inferences were conducted using the GTR+G 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 500,000 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 between the ITS and *matK* data sets, we carried out the incongruence length difference (ILD) test (Farris *et al.* 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.

Chromosome counting

To count chromosome numbers for two pond populations of *Persicaria maculosa* (≡ *Polygonum puritanorum*; Table 1) we basically used the root-tip squash technique modified by Bailey and Stace (1992) and examined and took photographs on Zeiss Axiophoto microscope.

Morphological characters

We chose one or two fully matured leaves (67 leaves out of 59 herbarium specimens) to measure the length (Character 1), the maximum width (Ch. 2), and the distance from the leaf base to the point of the maximum width (Ch. 3) in each specimen examined (see Appendix 1). The ratio characters were calculated to assess the leaf shape such as the ratio of the length to the maximum width (Ch. 4) and that of the distance from the leaf base up to the point of the maximum width to the length (Ch. 5).

Results

Sequence characteristics

The entire sequence length of the ITS region ranged from 644 to 650 bp excepting for the partial sequences of *Persicaria maculosa* NSC population (*P. puritanorum* NSC) due to the difficulty in amplifying the entire fragment from herbarium extraction. *P. lapathifolia* and *Polygonum persicaria* var. *ruderale* (*P. maculosa* NSC) showed the length variation in ITS2, and *P. amphibia* (Linnaeus 1753: 361) Gray (1821: 268) and the outgroup species, *P. virginiana*, in ITS1 and 2. The 5.8S subunit was 164 bp long in all species examined. Aligned data matrix of ITS region, required eight 1-bp and one 3-bp indels, consisted of 653 aligned base pairs, of which 127 were variable and 116 were parsimony informative (Table 2). GC content ranged from 58.3 % to 65.2 % in the entire ITS region sequenced. The entire size of chloroplast *mat*K gene and *trn*K introns sequenced in this study ranged from 2414 to 2429 bp except for partial sequences of *P. maculosa* NSC and *P. puritanorum* NSC, which were sequenced by only partial region of *mat*K and 5' side *trn*K intron; 605, 402 bp for each. *P. amphibia* and *P. virginiana* mainly showed length variation in *trn*K 5' intron (739, 743 bp for each), *mat*K (1530, 1527 bp), and *trn*K 3' intron (189, 166 bp) regions. Aligned data matrix of *mat*K and the *trn*K intron region required various size of indels (1–9 bp) and it consisted of 2442 bp, of which 137 were variable and 108 parsimony informative (Table 2). GC content of *mat*K and *trn*K intron region ranged from 34.1 % to 34.5 %. A partition homogeneity test suggested that the data from ITS and *mat*K region were significantly incongruent ($p = 0.001$).

Phylogenetic relationships

Maximum parsimony analysis (MP; Fig. 2) using the ITS region sequences resulted in eight most parsimonious trees of 167 steps (CI = 0.868, RI = 0.951; trees not shown). Eight different relationships in ITS MP trees were produced by the combination with two different relationships associated with the placement of *P. longiseta* WEIR and four of *P. puritanorum* NSC. ITS MP analysis suggested that *P. longiseta* WEIR was the sister to either the clade including *P. maculosa* BCM ~ *P. longiseta* TP (Fig. 2) or the whole clade including *P. maculosa* BCM ~ *P. longiseta* ARL. Also, four different relationships associated with the placement of *P. puritanorum* NSC included whether *P. puritanorum* NSC was nested in or the sister to either the clade including *P. puritanorum* CP, SP, and LP populations or the clade C (Fig. 2). MP analysis using the *mat*K region sequences produced 21 most parsimonious trees of 139 steps (CI = 1.000, RI = 1.000; trees not shown). Those 21 MP *mat*K trees were resulted from different relationships caused by the combination with several different placements of *P. puritanorum* NSC and *P. maculosa* NSC. Similarly in ITS MP trees, those various relationships in *mat*K MP trees barely affected the total relationship maintaining the clade A (including *taxa* in box A; Fig. 2) and the clade E [including *P. punctata* Small (1903: 379), LP ~ *P. puritanorum* LP; Fig. 2).

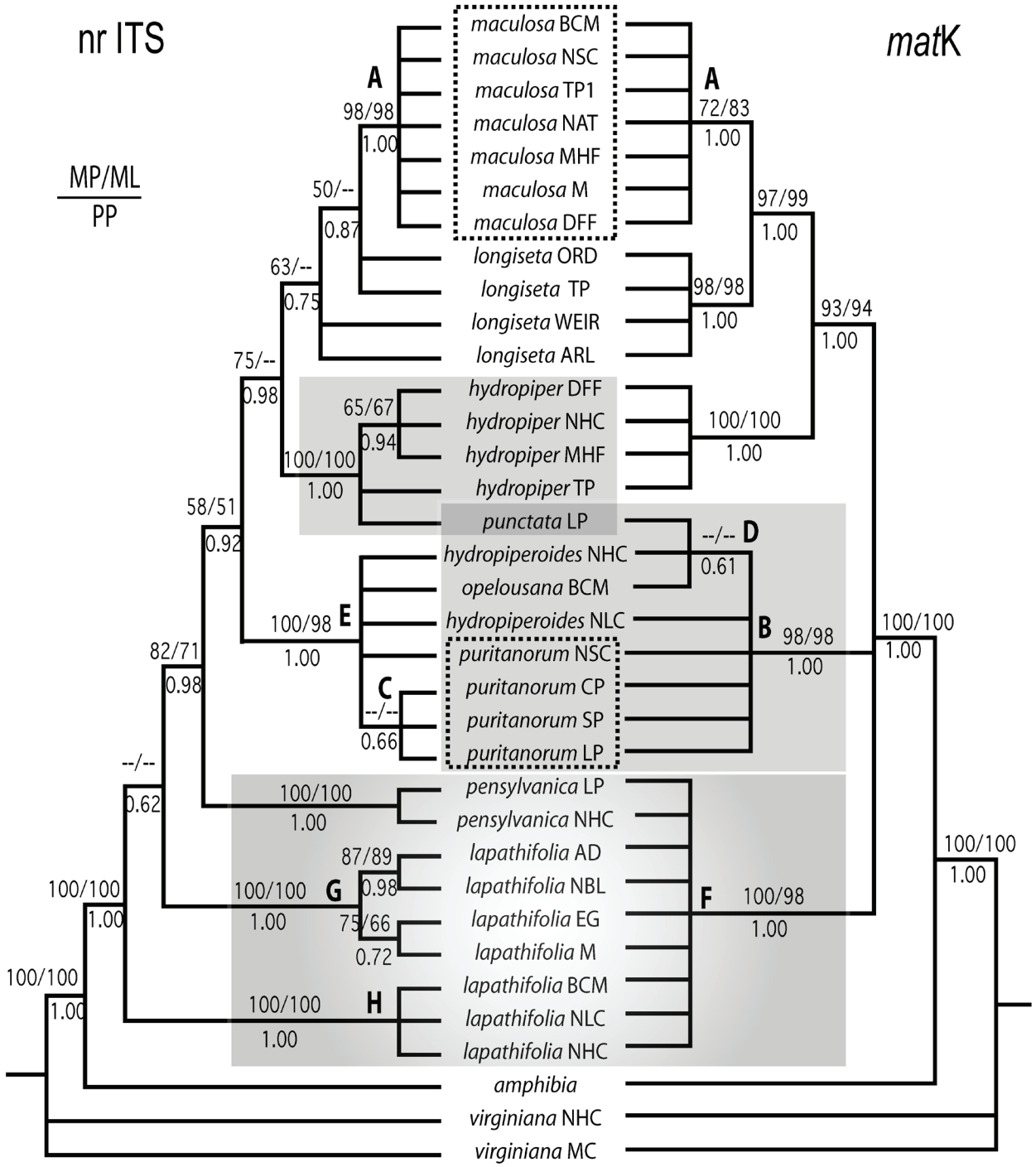


FIGURE 2. 50 % major-rule consensus tree from Bayesian inference for nrITS (left side) and cp *matK* and partial *trnK* intron region (right side). Posterior probabilities are presented under the branches. Bootstrap values for maximum parsimony / maximum likelihood analyses are presented above the branches. N-dash (-) indicates support of less than 50 % bootstrap value or 0.5 posterior probability.

Hierarchical likelihood ratio test (hLRT) suggested that the optimal model for the ITS region sequence data was the TrN+G model (Tamura & Nei 1993), which allowed for independent rates of transition but the same rate of transversion and allowed rate heterogeneity among sites to be approximated by a gamma distribution with a single shape parameter, alpha (Table 2). Maximum likelihood analysis of the ITS region sequence data under this model produced two trees (not shown) with a log likelihood score of -1819.845. ITS ML analysis suggested that *P. puritanorum* NSC either formed a clade with other *P. maculosa* pond populations (*P. puritanorum* CP, LP, and SP) or is the sister to the clade including three *P. maculosa* pond populations (Fig. 2). The hLRT of the *mat*K region sequence suggested K81uf+G model (Kimura 1981) as a best fitting model, which allowed three different substitution rates and

unequal base frequencies, rate heterogeneity among sites to be approximated by a gamma distribution with a single shape parameter, alpha (Table 2). ML search of the *mat*K region under this model resulted in eight trees with–lnL value of 4268.428. Eight different relationship suggested in matK ML analysis were resulted from the combination of two cases whether *P. maculosa* NAT and *P. maculosa* NSC formed a clade or not, and four cases that *P. puritanorum* NSC (1) formed a clade with *P. punctata* LP, (2) was nested in the clade including *P. punctata* LP ~ *P. opelousana* (Riddell 1853: 611) Small (1903: 378) BCM, (3) was the sister to the clade including *P. punctata* LP ~ *P. opelousana* BCM or (4) was just nested in the clade E as in Fig. 2.

Our results strongly suggested the separation of four pond populations (*P. puritanorum*) from other populations representing *P. maculosa* (Fig. 2). Seven *P. maculosa* populations excluding four pond populations formed a clade with strong bootstrap supports and posterior probability in both ITS and *mat*K analysis (clade A in Fig. 2). On the other hand, four pond populations of *P. maculosa* formed a clade (clade C in Fig. 2) with two populations of *P. hydropiperoides* Small (1903: 378) and *P. opelousana* in ITS sequence analysis (with an addition of *P. punctata* in *mat*K analysis; clade E in Fig. 2) with strong supports (MP/ML/PP = 100/98/1.00 and 98/98/1.00 in ITS and *mat*K analysis respectively). *P. maculosa* (excluding pond populations) was most closely related to *P. longiseta* with strong support in *mat*K analyses (97/99/1.00) whereas weak supports in ITS analyses (63/<50/0.75), and the clade including *P. maculosa* and *P. longiseta* was more closely related to the clade representing *P. hydropiper* with strong or moderate supports (100/100/1.00 in *mat*K or 75/<50/0.98 in ITS; Fig. 2). *P. punctata* was clustered with *P. hydropiper* in ITS analysis but nested in clade E in *mat*K analysis, which was strongly supported with high bootstrap values and posterior probability. DNA sequences of chloroplast *mat*K region from two accessions of *P. pensylvanica* (Linnaeus 1753: 362) M. Gomez (1896: 278) and seven accessions of *P. lapathifolia* were almost identical to result in forming a clade unresolving relationship between two species. However, ITS sequence analyses indicated the paraphyletic relationship of two species and additionally suggested two separate lineages in *P. lapathifolia* accessions; one representing clade H and the other clade I (Fig. 2). Our sequence analyses strongly supported the monophyly of the sect. *Eupersicaria* (Kim & Donoghue 2008) and *P. amphibia* was the sister to the rest of *Eupersicaria*. Although the relationship was not fully resolved in *mat*K analyses and weakly supported in ITS analyses our results indicated that the clade including *P. maculosa* (excluding pond populations), *P. longiseta*, and *P. hydropiper* was more closely related to the clade D (including *P. hydropiperoides*, *P. opelousana*, pond populations of *P. maculosa*), the *P. pensylvanica* clade, and *P. lapathifolia* clade in turn.

Chromosome numbers

The chromosome number counted from the two pond populations (LP and SP) of *P. maculosa* (*P. puritanorum sensu* Fernald), was 2n = 60 (Fig. 3). This hexaploid number is different from that cunted for *P. hydropiper* (2n = 20, or 22, Jaretzky 1928, Mulligan 1957), *P. maculosa* (2n = 40 or 44, Graham & Wood 1965, Jaretzky 1928), for *P. lapathifolia* (2n = 44, from Japan, Hara 1952), and *P. hydropiperoides*, *P. opelousana*, and *P. punctata* (2n = 40, McDonald 1980, Subranmanian 1980).

Leaf size and shape

The total length of leaf (Character 1) ranged typically from 51.1 to 125.9 mm (average = 84.1 mm) *P. maculosa*, while in pond populations of *P. maculosa* (*P. puritanorum*) from 31.0 to 72.4 mm (average = 51.3 mm). The maximum width (Ch. 2) and the distance from the base to the point of the maximum width (Ch. 3) is 9.1–31.0 mm (average = 17.2 mm) and 18.0–46.5 mm (average = 32.7 mm) in the typical *P. maculosa* whereas it is 7.6–15.0 mm (average = 10.5 mm) and 15.6–36.5 mm (average = 24.4 mm) in pond populations. The ratio of the total length to the maximum width (Ch. 4 = Ch. 1 / Ch. 2) was very similar in typical *P. maculosa* and pond populations of *P. maculosa* respectively ranging from 3.97 to 6.95 (average = 5.01) and from 2.92 to 7.02 (average = 4.94). In contrast, the ratio of the total length to the distance from the base to the point of the maximum width (Ch. 5 = Ch.1 /Ch. 3) showed differences [range 2.17–3.00 (average = 2.58) in typical *P. maculosa*, 1.82–2.35 (average = 2.10) in pond populations of *P. maculosa* (Fig. 4)].

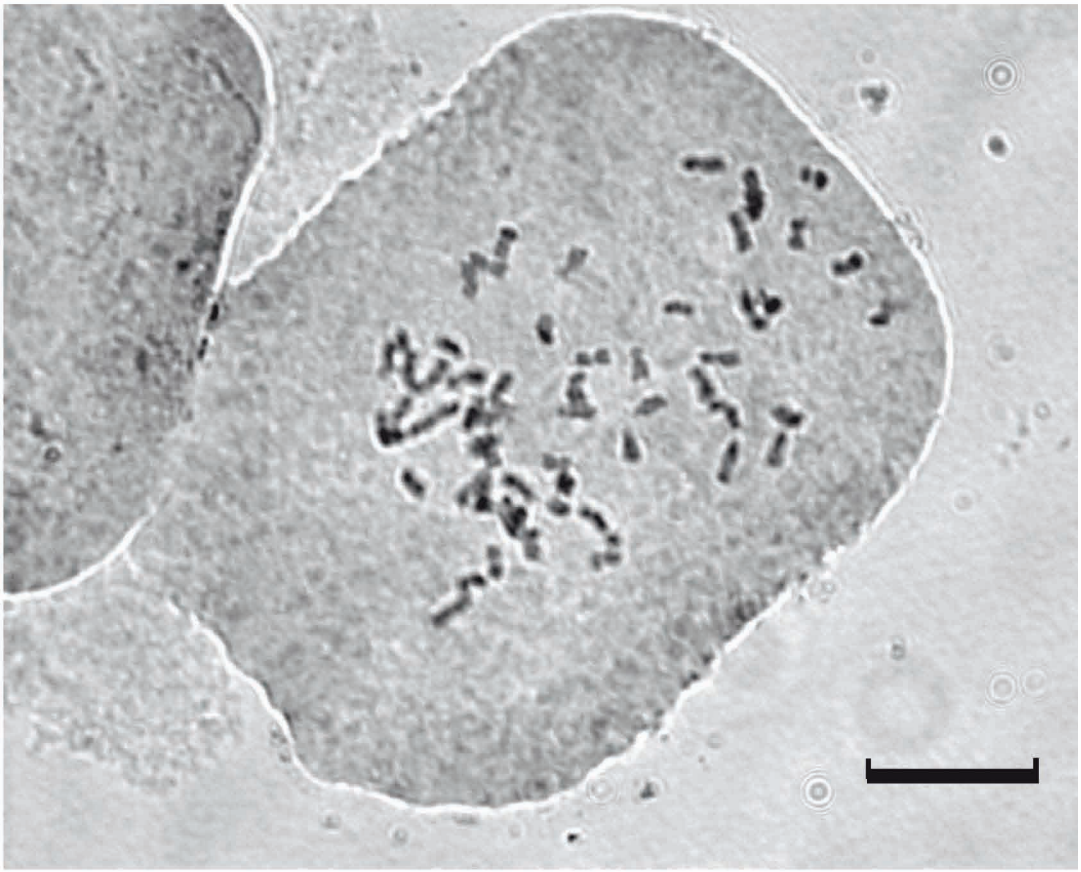


FIGURE 3. Somatic chromosomes of *Persicaria puritanorum*. It is counted into 2n = 60. Scale bar is 10 μm.

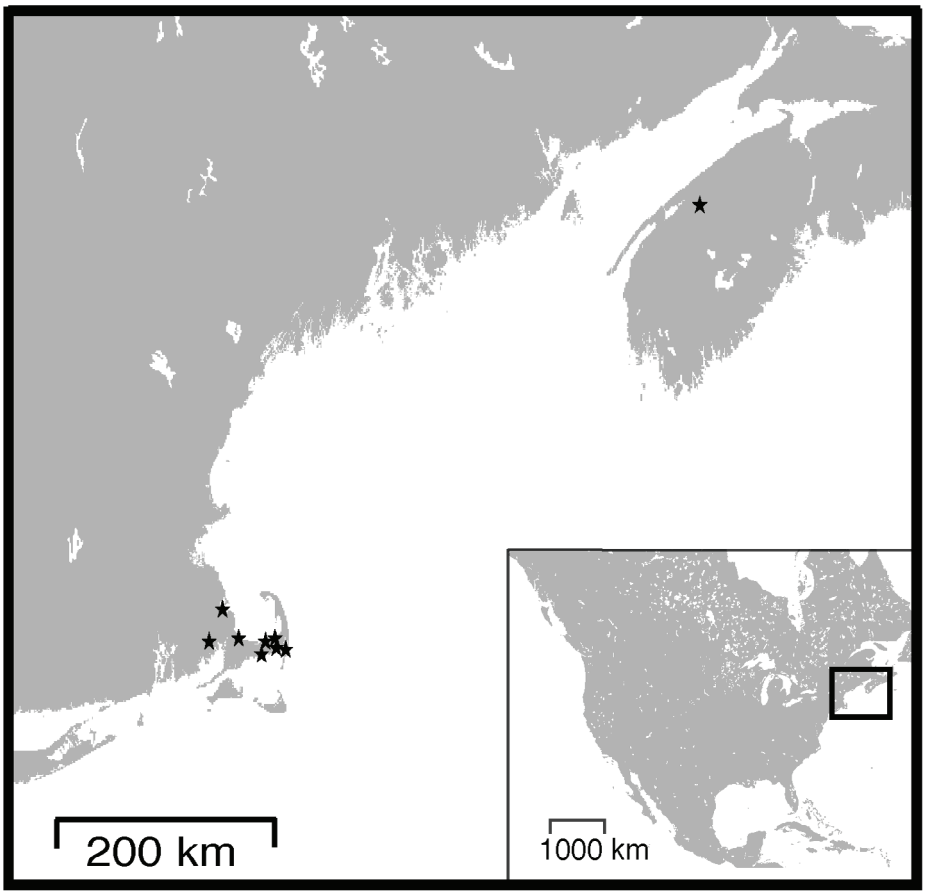


FIGURE 4. Distribution map of *Persicaria puritanorum*. See Appendix 1 for more detailed localities. Some localities are overlapped.

Discussion

We find that pond populations of Cape Cod is neither a plasticity-based phenotypes nor a locally differentiated ecotypic population of *Persicaria maculosa*, but rather a separate group which should be named as *P. puritanorum*. Our ITS and *mat*K sequence analyses strongly support the separation between these two species: the clade including 7 accessions from *P. maculosa* is more closely related to *P. longiseta* and *P. hydropiper*, while 4 accessions of *P. puritanorum* are strongly clustered with *P. hydropiperoides* and *P. opelousana* (Figure 2). On the other hand, an accession of *Polygonum persicaria* var. *ruderale*, which is morphologically very similar to *P. puritanorum* by the short inflorescences, prostrate growth form and smaller leaves, was clustered with other accessions of *P. maculosa* with strong support values (MP/ML/PP = 98/98/1.00). All these results strongly indicate that *P. puritanorum* is not an extreme variant of *P. maculosa*. The higher chromosome number of *P. puritanorum* (2n = 60), when compared to that of tetraploid *P. maculosa* (2n = 40 or 44), confirms the segregation of *P. puritanorum* from *P. maculosa*. Furthermore, the relatively smaller leaf size of *P. puritanorum*, and the more abruptly tapering leaf shape from the point of the maximum width to the leaf tip (Fig. 4), also support separating *P. puritanorum* from *P. maculosa*. Although morphological characters in *Polygonum s.lat.* are known to be highly variable (see e.g., Fassett 1949, Greene 1904, Mitchell 1971, Stanford 1925b, Wilson 1990, Kim & Donoghue 2008a, 2008b) our observations are strongly consistent with those given in the original description by Fernald (1919). The latter author noted that morphological differences from *P. maculosa* include shorter inflorescences with less densely arranged flower fascicles, semi-prostrate growth form, and smaller leaf size as well as a specialized ecological distribution on Cape Cod pond shores (Fernald 1919, 1950).

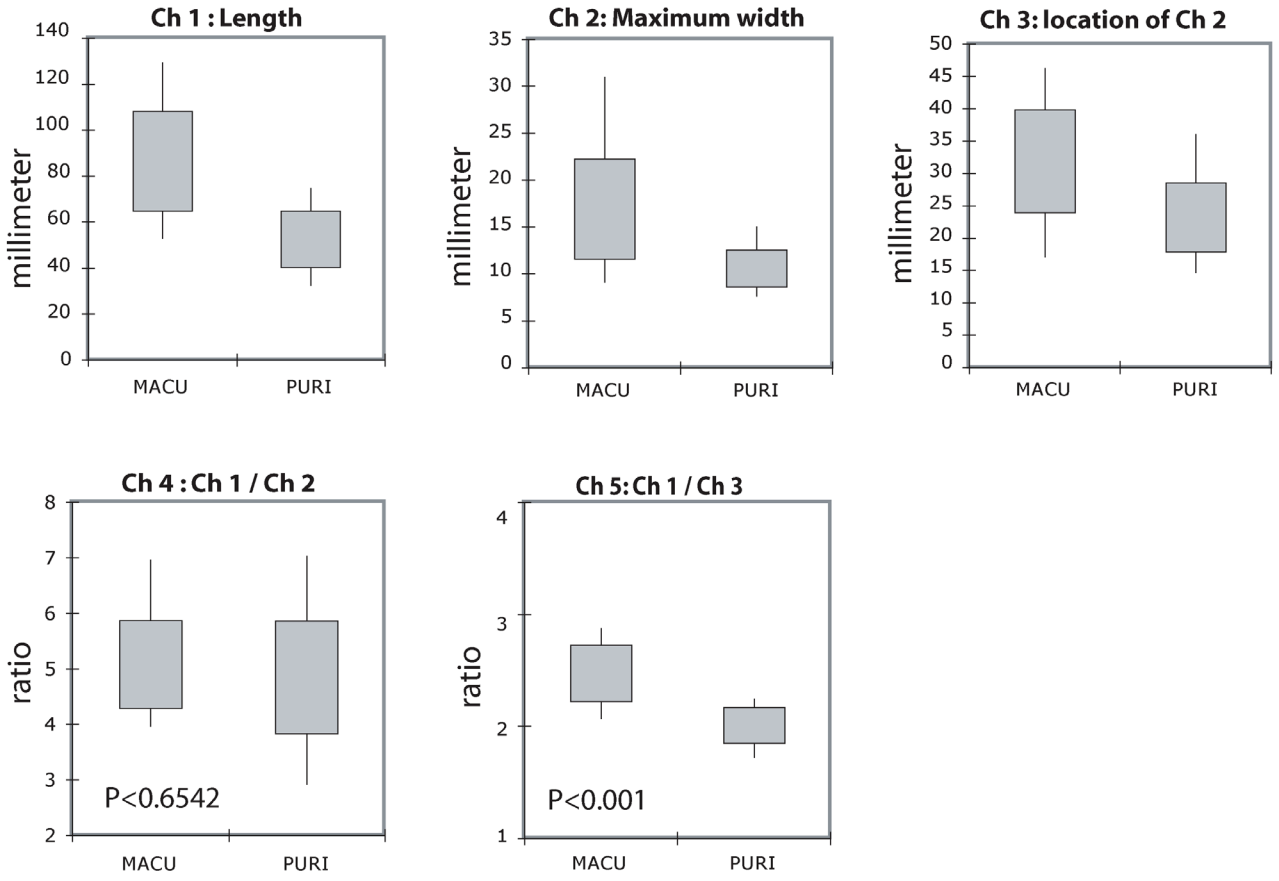


FIGURE 5. Leaf character measurement from *Persicaria maculosa* (MACU) and *P. puritanorum* (PURI). P value from t-test. See methods for more details.

Interestingly, in addition to the separation of *Persicaria puritanorum* as a distinct lineage from *P. maculosa*, our sequence analyses also indicated the strong relatioship of *P. puritanorum* with *P. hydropiperoides* or *P. opelousana* (Figure 2). The close relationship between *P. hydropiperoides* and *P. opelousana* is expected, because these two tetraploid species, [which along with two diploid species, *P. setacea* Small (1903: 379) and *P. hirsuta* (Walter 1788: 132) Small (1903: 379) are among the four species comprising the *P. hydropiperoides* complex], are not fully established as distinct due to their morphological affinity (Stanford 1926, Dalci 1974, Mitchell 1978) and incomplete reproductive

isolation (McDonald 1980). *P. hydropiperoides* can be recognized by its thin inflorescences and adventitious roots and its distribution mainly in very moist habitats, but *P. hydropiperoides* plants are morphologically variable in leaf shape, inflorescence size and arrangement, and flower colour. Based on its narrower leaves and achenes that protrude from the perianth at maturity, *P. opelousana* has been considered either a distinct species or a variety of *P. hydropiperoides* depending upon use of those characters for taxonomic delimitation. The morphological similarity between *P. hydropiperoides* and *P. opelousana* and their incomplete reproductive isolation explain their clustering and lack of phylogenetic resolution in our phylogenetic trees. However, our finding that *P. puritanorum* is clustered with the *P. hydropiperoides* complex is unexpected because the morphological affinity between the *P. hydropiperoides* complex and *P. puritanorum* (or indeed *P. maculosa*) is relatively low. Rather, the *P. hydropiperoides* complex shows some degree of morphological affinity with *P. hydropiper*, a species that is more closely related to *P. maculosa* and *P. longiseta*.

There are several possible explanations of this result: (1) *P. puritanorum* and the *P. hydropiperoides* complex may comprise a single, highly variable species, (2) *P. puritanorum* and the *P. hydropiperoides* complex may have undergone very recent diversification and are not yet fully genetically distinct, or (3) this group of taxa may be entangled by allopolyploid hybrid speciation. Although we cannot rule out the possibility that *P. puritanorum* and the *P. hydropiperoides* complex are the same species, this explanation is the least likely, due to the higher degree of morphological differentiation as well as the hexaploid chromosome number of *P. puritanorum* compared with the tetraploid number of *P. hydropiperoides* and *P. opelousana*. Hypothesis (2) seems more likely to explain the low genetic differentiation of *P. puritanorum* from the *P. hydropiperoides* complex in both nrITS and *mat*K sequences. Rapid diversification of a lineage is often not linked with sufficient genetic differentiation to solve phylogenetic relationships (Richardson *et al.* 2001a, 2001b, Wang *et al.* 2005). Although the geographic range of *P. puritanorum* is restricted to a relatively small area (Cape Cod and Nova Scotia, see Fig. 5) this range overlaps with that of *P. hydropiperoides* complex. However, these taxa grow in different habitats: *P. puritanorum* is restricted to sandy, nutrient-poor freshwater beaches of glacier kettle ponds (Fernald 1919, Sultan *et al.* 1998), whereas the *P. hydropiperoides* complex occurs in relatively high-nutrient marsh areas near creeks or ditches (Stanford 1926, McDonald 1980, Mitchell 1971, Kim pers. obs.). Possibly this habitat difference has promotee the recent speciation of *P. puritanorum*. Nevertheless this hypothesis remains speculative, because we cannot rule out the possibility that we chose to study a slow-evolving gene region which did not permit phylogenetic resolution, or that we encountered statistical artifacts due to limited sample size. A more direct test of the rapid diversification hypothesis needs including a larger sample of gene regions and of local populations.

Based on our findings to date, hybrid speciation (allopolyploid speciation) may provide the best explanation for the clustering of *P. puritonorum* with the *P. hydropiperoides* complex (Kim & Donoghue, 2008b, Kim *et al.* 2008). Although this need not contradict a history of rapid, recent diversification, it provides a plausible mechanism for reproductive isolation of *P. puritanorum.* In the view of the close relationship between the hexaploid *P. puritanorum* and tetraploid species of the *P. hydropiperoides* complex, we hypothesize a possible allopolyploid event either between one of tetraploid species in *P. hydropiperoides* complex and an unknown diploid species, or between two unknown diploid species (Fig. 6A, B). Although allopolyploid hybrid speciation can produce a hexaploid in several ways, viable reproduction is more likely to be achieved by chromosomal doubling in the F1 hybrid than the other possible hybrid origins (i.e., union of two unsegregated gametes from tetraploid and diploid parents (Fig. 6C); union of an unsegregated gamete from the tetraploid and a normal gamete from tetraploid (Fig. 6D), union of two normal gametes from tetraploid and octaploid (Fig. 6E), or union of an unsegregated gamete from the diploid and anormal gamete from octaploid species (Fig. 6F). Hybrid speciation via alloploryploidy that results in an immediate reproductive isolation is considered the most widespread and feasible mechanism of plant species diversification (Arnold 1997, Grant 1981, Riesberg 1997, Mallet 2007, Soltis & Soltis 2000, Wendel 2000). Hybrid speciation through polyploidy may lead to rapid sympatric diversification or to highly developmentally plastic genomes (Otto 2003, Adams & Wendel 2005, Comai 2005). Approaches such as morphological comparison (Dalci 1974, Timson 1964), experimental fertilization (McDonald 1980, Timson 1963) and isozyme profiling (Consaul *et al.* 1988) have been applied to investigate hybridization in the genus *Persicaria* as a possible cause of the high morphological variability in this group (Stanford 1925a). Polylpoidy and possible hybridization have also been reported for several groups which are closely related to *Persicaria* that also show high phenotypic plasticity such as *Fallopia* Adanson (1763: 274) (Bailey and Stace 1992; Tiébré *et al.* 2007), *Polygonum s. str.* (Löve and Löve 1956), and *Fagopyrum* Miller (1754: no page numbers given) (Nishimoto *et al.* 2003). The possible hybrid origin of *P. puritanorum* adds to the ecological developmental information regarding these plants, and raises additional questions about the roles of plasticity and of hybridization in the origin of new species. We conclude that *P. puritanorum* should be resurrected as an independent species with its own unique evolutionary trajectory.

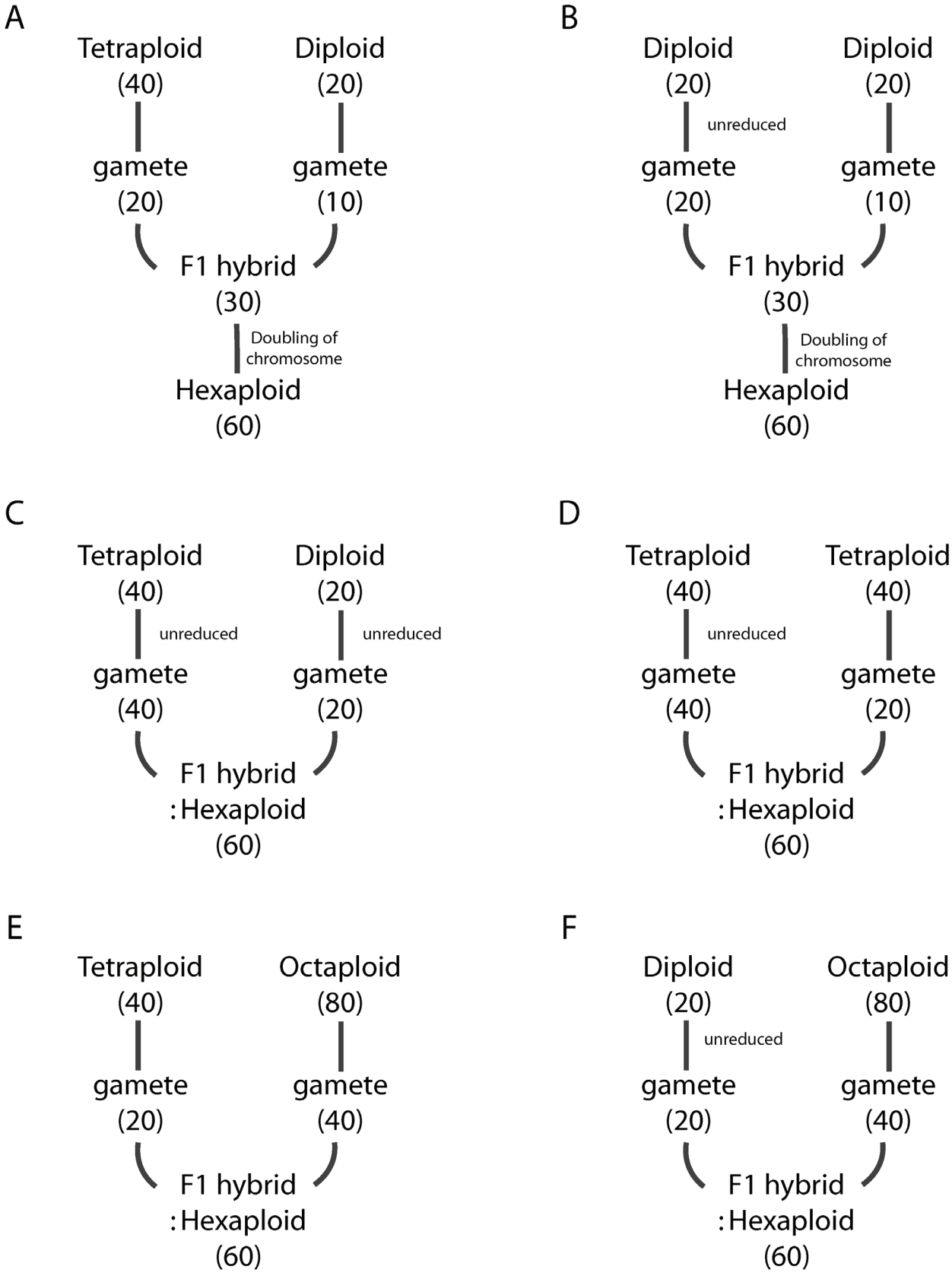


FIGURE 6. Hypothetical diagrams of possible scenarios presenting allopolyploid hybrid speciation. The number in parenthesis represents diploid chromosome number (2n). More details see discussion.

In addition to its taxonomic significance, this result has four major implications of conservation, ecological, and evolutionary interest. First, we now know that *P. puritanorum* is in fact a rare New England species endemic to a specific habitat (Fig. 4), and it deserves legal protection. Such protection is particularly pressing since the sites at which it occurs are quite limited in area and are subject to strong pressures for residential development. Second, having recognized this as a distinct species, we will need better information on its distribution. Is it restricted, as Fernald (1919) believed, to the freshwater ponds of Cape Cod and Nova Scotia, or does it occur in similar habitats across a wider geographic range? Third, the ecology of *P. puritanorum* demands further study in order to understand the unique role of this taxon in freshwater beach communities. These pond-side habitats are characterized by sparse vegetation, extremely high insolation and reflectance, and severely low-nutrient substrates, as well as variable moisture conditions (Sultan *et al.* 1998). Together with specialized amphibious herbs such as *Gratiola aurea* Muhenberg (1813: 2) and *Cyperus dentatus* Torrey (1823: 61), *P. puritanorum* is one of very few herbaceous species to form populations in these extreme sites; in particular the ecological relationship of *P. puritonorum* plants with patrolling ants merits further investigation (S. E. Sultan, pers. obs.). Fourth, it will be important to resolve phylogenetic relationships within these *Persicarian* plants to clarify the origin of this taxon and identify potential hybrid parents. The possibility of hybrid origin may explain the species shared plasticity with *P. maculosa*, and resolving phylogenetic history may illuminate the roles of both plasticity and hybridization in the origin of new species (West-Eberhard 1989).

Conclusion

Our molecular phylogenetic study strongly indicates that *P. puritanorum* has been erroneously considered a synonym of the common and plastic species *P. maculosa*, but is instead a distinct species representing an independent evolutionary lineage. This finding raises important issues regarding allopolyploid hybrid speciation in the genus *Persicaria* to explain the origin of the hexaploid ecological specialist *P. puritanorum*.

Persicaria puritanorum (Fernald) Soják (1974: 154)

=Basionym: *Polygonum puritanorum* Fernald (1919: 141).

Type—USA: Massachusetts, Barnstable County, Harwich, East end of Long Pond, Bare sandy beach, 14 August 1918, *M. L. Fernald & B. H. Long 16756* (holotype GH-00036748!, image of the holotype available at http://kiki.huh.harvard.edu).

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APPENDIX

HERBARIUM VOUCHER RECORDS FOR LEAF CHARACTER MEASUREMENTS.

Persicaria maculosa

CANADA NEW BRUNSWICK CHARLOTTE CO.: Grand Manan, North Head, moist ground by old road, 13 Aug 1927, *Weatherby 5795* NEWFOUNDLAND: Avalon Peninsula, Trepassey, weed about garden, 18 Aug 1924, *Fernald, Long, and Dunbar 26635,* Bonne Bay, Middle Brook, bushy margin of cultivated clearing near mouth of, 21 Aug 1929, *Fernald, Long, and Fogg, Jr. 1662*, Sandy shore of Lake St. John, 30 Aug 1904, *Wright 765* NOVA SCOTIA: Sable Island, weed at Main station, 20 Aug 1913, *St. John 1214* ONTARIO THUNDER BAY DIST.: Red Pine I., Sagana L., Thunder Bay-Minnesota Boundary, barnyard, rocky soil, wet places, 16 Aug 1950, *Garton and Campbell 1267* QUEBEC MATANE CO.: Little Metis, 3 Sep 1907, *Fowler s.n*.
U.S.A. CALIFORNIA, BUTTE CO.: Hills 8 miles north of Oroville in cultivated ground where it occurs sparingly in low, moist ground, 30 Sep, *Heller 12663* San Bernardino Co.: 1 mile south of Oak Glen, alt. 4500 ft., 17 Jul 1924, *Munz and Johnston 8697* CONNECTICUT NEW HAVEN CO.: , Milford, 25 Aug 1893 *Eames s.n*., Milford, gravelly waste, 21 Sep 1937, *Eames 11884* IDAHO BONNER CO.: wet places at north end of lake Pend d'Oreille, 25 Aug 1892, *Sandberg 960* KENTUCKY ROCKCASTLE CO.: South of Livingston, Brookbed, 9 Jul 1937, *Smith and Hodgdon 3740* LOUISIANA RAPIDES CO.: The vicinity of Alexandria, infrequent in low ground, 2 Jun 1899, *Ball 532* MAINE HANCOCK CO.: Seal Harbor, dry gravelly road, 17 Aug 1905, *Batchelder 986*. Mt. Desert Island, Northeast harbor, 4 Aug 1910, *William s.n*. WALDO CO.: Lincolnville, Pitcher Pond, Miller's Farm, barnyard, Damp, nitrogenous soil, 24 Aug1935, *Rossbach 345* WASHINGTON CO.: Meddybemps, wet open woods, 16 Aug 1983, *Knowlton s.n*., YORK CO.: North Berwick, cultivated ground, Jul 1903, *Parlin s.n*. MASSACHUSETTS BARNSTABLE CO.: Eastham, roadside, 10 Aug 1907, *Collins 450*, Eastham, wetground, 1 Sep 1907, *Collins 555* Harwich, Long Pond, Bare sandy beach, east end of Long Pond, 14 Aug 1918, *Fernald and Long 16751* DUKES CO.: The Elizabeth islands, W. end of island, small pondhale, 2 Sep 1927, *Fogg, Jr. 3017* HAMPSHIRE CO.: Amherst, Aug 1873, *Blanchard s.n*., Greenwich, 14 Aug 1931, *Goodale, Potsubay and St. John s.n*., Nouthington, open ground about dwellings barns, alt. 450 ft., 13 Aug 1912, *Robinson 605* MIDDLESEX CO.: Wakefield, 3 Sep 1946, *Bean s.n*. Westford, no specific date, *Fletcher s.n*. NORFOLK CO.: roadside, 14 Sep 1897, *Harris s.n*. Tinth Pond, 13 Oct 1932, *Kidder s.n*. PLYMOUTH CO.: Scituate, Barnyard, 13 Sep 1914, *Knowlton s.n*. WORCESTER CO.: rough meadow, pasture, 29 Jul 1935, *Batchelder s.n*. MISSOURI JEFFERSON CO.: limestone slopes and flats along Parker Creek, ca. 0.35 mi NNE of its confluence with Big River, 6.5 mi W of DeSoto, near lat. 38º07'30''N, long. 90º40'30''W. Township 39N. Range 3 E., Elev. Ca. 560 ft. Annual; perianth pale pink, 27 Jul 1985, *Raven and Raven 26807* NEBRASKA CHERRY CO.: 7 miles south of Valentine, Schlagle creek, 20 Aug 1936, *Tolstead 726* NEVADA ORMSBY CO.: Eagle Valley, alt. 1446 m, 3 Jul 1902, *Baker 1398* NEW HAMPSHIRE CHESHIRE CO.: Hinsdale, among rank weeds in barnyard, 13 Sep 1926, *Batchelder s.n*. NEW YORK WASHINGTON CO.: North of Hudson Falls, Vaughns, R.C.Burnham's yard, 29 Aug 1912, *Burnham s.n*. NORTH CAROLINA NEW HANOVER CO.: Wilmington, military cut-off between US routes 17 and 74, sandy flatwoods, flowers pink, 19 May 1973, *Boufford and Ahles 8845* NORTH DAKOTA RANSOM CO.: North Dakota Maple river, ca. 2.5 mi. of Enderlin Bank and "mud" bar of meandering stream woods of oaks, elms, etc., 31 Aug 1968, *Weedon 4486* PENNSYLVANIA THILAR CO.: Wissahickon Ravine, 15 Aug 1924, *Lang 354* RHODE ISLAND PROVIDENCE CO.: Providence, Aug 1884, *Collins s.n*. VERMONT ADDISON CO.: Bristol, Weed in cornfield, 24 Jul 1937, *Knowlton s.n*. CALEDONIA CO.: Groton, Along Wells River gravelly strand with Sagina procumbens L., 25 Aug 1959, *Seymour 18323* WEST VIRGINIA CABELL CO.: Roland Park, roadside ditch, 30 Oct 1932, *Gilbert 244*

Persicaria puritanorum

CANADA NOVA SCOTIA ANNAPOLIS CO.: beach of Grand Lake, in sand or gravel or among granite boulders, 19 Jul 1921, *Fernald, Bartram, Long and Fassett 23081* (HUH)
U.S.A. MASSACHUSETTS BARNSTABLE CO.: Marston mills, Hamblin pond, 6 Sep 1978, *Coddington s.n*., Sandwich, Triangle Pond, gravelly beach, 26 Aug 1928, *Fernald 898*, Harwich, Buck Pond, sandy shore, 8 Jul 1918, *Fernald 16752*, Harwich, Hawk's Nest Pond, sandy and gravelly beach, 28 Jul 1918, *Fernald 16754,* Half-way Pond, dry sandy and gravelly beach, 13 Sep 1919, *Fernald 18399*, Brewster, Long pond, wet sandy beach of Long pond (east of Cliff Pond), 27 Aug 1918, *Fernald and Long 16758*, Half-way Pond, wet sandy or gravelly lower beach, 4 Sep

1918, *Fernald and Long 16759*, Harwich, Seymour Pond, sandy beach, 19 Sep 1918, *Fernald and Weatherby 16762*, Brewster, Cliff pond, inundated sandy border of, 26 Oct 1919, *Fernald and Wiegand s.n*., Dennis, sandy strand of pond, 1 Oct 1915, *Weatherby s.n*. PLYMOUTH CO.: Plymouth, Little Sandy Pond, gravelly and sandy beach, 7, 8 Aug 1918, *Fernald and Clark 16755*, Plymouth, Great South Pond, damp sandy beach of, 6 Sep 1913, *Fernald, Hunnewell and Long 9401*, Plymouth, Clear Pond, sandy shore, 30 Aug 1913, *Fernald, Hunnewell and Long 9402*, Plymouth, Little South Pond, wet sandy shore, 4 Sep 1910, *Knowlton s.n*