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ORIGINAL ARTICLE

Evidence for multiple, autoploid origins of agamospermous populations in *Eupatorium sessilifolium* (Asteraceae)

Kunsiri C. Grubbs · Randall L. Small · Edward E. Schilling

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Abstract Comparative analyses were made of agamospermous populations of *Eupatorium sessilifolium*, which have previously been documented to be polyploid, to determine whether they are alloploid or autoploid in origin and to assess the possibility that they have arisen more than once. There was no variability in ITS sequences among seven agamospermous and eight sexual diploid populations of *E. sessilifolium*, which is consistent with morphological observations in suggesting that the agamospermous populations were autoploids. The ITS sequence characteristic of *E. sessilifolium* differs from all other North American species by a minimum of 15 changes, and heterogeneity or polymorphism would be expected if the agamospermous populations were alloploids. Analysis of the chloroplast-based *trn*C-*psb*M spacer region showed variability among both sexual diploid and agamospermous populations of *E. sessilifolium*, which suggested that the agamospermous populations stem from multiple origins. Analysis of ISSR data revealed considerable intraspecific variability within *E. sessilifolium*, and the distribution of variability, with agamospermous populations showing variability from one another, added further evidence for multiple origins of agamospermous populations. The results in conjunction with distributional evidence that the sexual diploid populations of *E. sessilifolium* are geographically restricted and uncommon suggest that monitoring of populations might be warranted to evaluate whether measures are needed to enhance their continued survival.

Keywords *Eupatorium* · Asteraceae · Autoploid · Polyploidy · ITS · ISSR

K. C. Grubbs
Department of Science, Kasetsart University,
Kamphangsaen Campus, Kamphangsaen,
Nakhon Pathom 73140, Thailand

R. L. Small · E. E. Schilling (✉)
Department of Ecology and Evolutionary Biology,
University of Tennessee,
Knoxville, TN 37996, USA
e-mail: eschilling@utk.edu

Introduction

A distinctive aspect of North American *Eupatorium* L. is the widespread presence of agamospermy, which in this genus is always associated with polyploidy (Sullivan 1972). In some cases, there are groups of polyploid populations that are morphologically distinct from any diploid, and have been recognized as taxonomically distinct. Examples for which genomic relationships have been established include *E. godfreyanum* Cronquist (Siripun and Schilling 2006a) and *E. novae-angliae* (Fernald) V. I. Sullivan ex A. Haines and Sorrie (Schilling et al. 2007). Other groups of polyploid populations are morphologically indistinguishable from associated sexual diploid populations and have been considered to be conspecific with them. Examples include *E. altissimum* L., *E. rotundifolium* L., and *E. sessilifolium* L. (Sullivan 1976). There are, however, still questions regarding whether the polyploids within a species are auto- or alloploid in origin, and whether they have single or multiple origins. Previous work that attempted to address these questions using flavonoids or isozymes (e.g. Sullivan 1972; Yahara and Sullivan 1986; Yahara et al. 1991) utilized markers that were not capable of decisively resolving among the alternatives.

Independent origins of polyploidy have been documented in various groups, and in fact form a recurrent theme where this has been investigated closely (Soltis et al.

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2003). For apomicts, though, the contrasting situation has sometimes been documented in which large numbers of populations may form essentially a single clone (Hollingsworth and Bailey 2000; Poulin et al. 2005; Xu et al. 2003). The generally low levels of variation for DNA sequence data have suggested that divergence in *Eupatorium* in eastern North America may be relatively recent (Siripun and Schilling 2006a), so it is possible that apomictic populations within species of *Eupatorium* may be relatively recent invaders of disturbed habitats that could stem from single origins.

Eupatorium sessilifolium is a morphologically uniform species that occupies a relatively wide range in eastern North America (Sullivan 1972; Siripun and Schilling 2006b; Fig. 1). It is distinguished from other members of the genus by its glabrous stems, and leaves that are sessile, truncate, relatively long (7–15 cm), and pinnately nerved. Sullivan (1972; 1976) documented that diploid, sexual populations are found only in a relatively restricted geographical area in the southern Blue Ridge Mountains, and the species is represented by agamospermous, polyploid (triploid where chromosome counts are available, Grant 1953; Montgomery and Fairbrothers 1970; Sullivan 1976) populations across the rest of its range. Other than the differences in presence or absence of viable pollen, the polyploids are indistinguishable both morphologically and by flavonoid chemistry (Sullivan 1972) from the diploids. This information suggests that the agamospermous populations of *E. sessilifolium* are autopolyploid. Hybridization within *Eupatorium* is frequent, however, and hybrids or hybrid-derived apomicts are known between *E. sessilifolium* and several other species, including *E. rotundifolium* (Siripun and Schilling 2006a), *E. perfoliatum* L. (Sullivan 1972), and *E. petaloideum* Britton, and *E. hyssopifolium* L. (Schilling, unpublished data). Thus, it is also possible that the polyploid populations of *E. sessilifolium* were derived via hybridization and are allopolyploid without this being reflected in the morphology or chemistry.

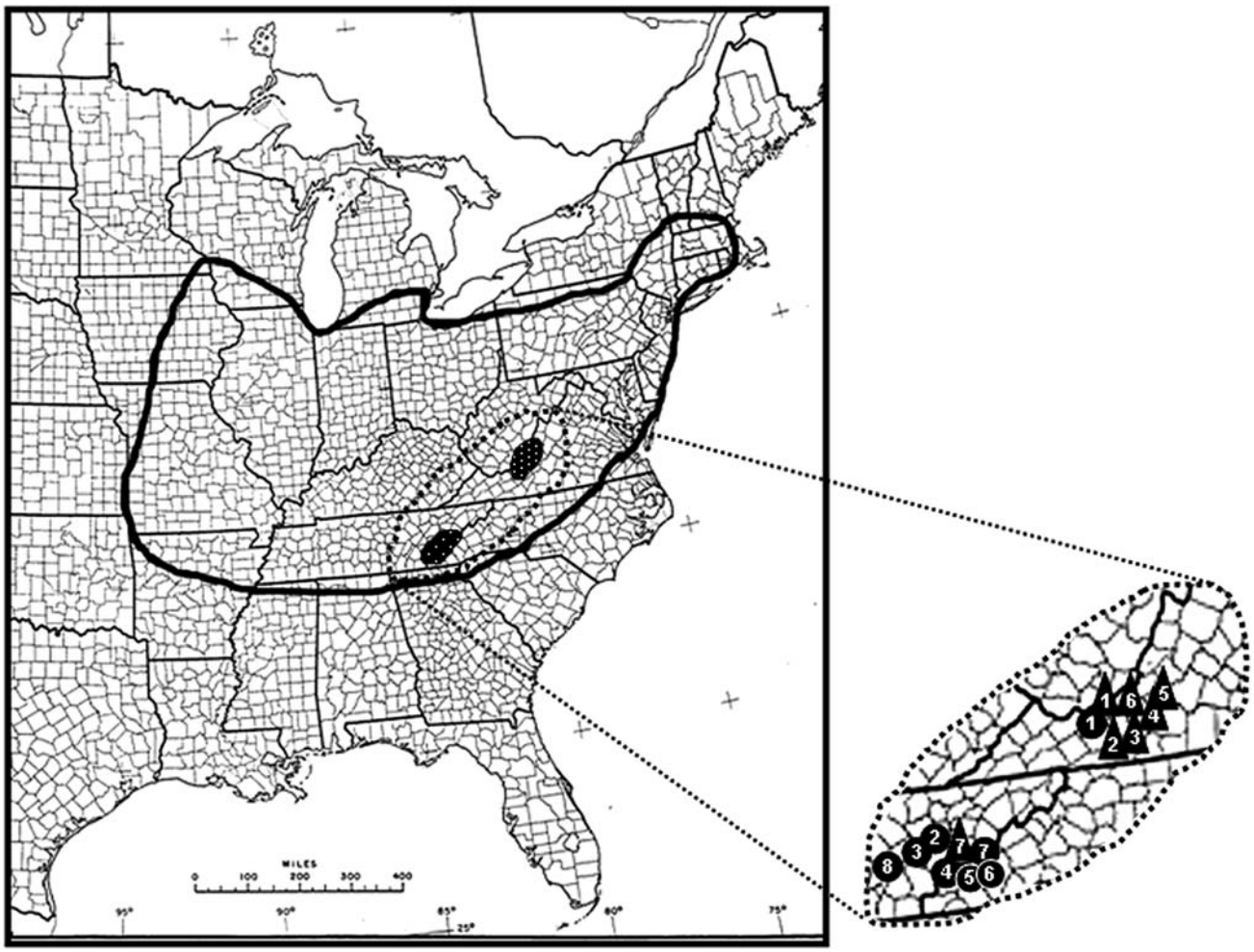

Fig. 1 Map of eastern North America showing the distribution range of *Eupatorium sessilifolium* (*solid line*) with the documented range of the diploid populations (*stippled areas*); enlargement shows approximate sources of populations sampled for molecular data, numbered as in Table 1 (*circles* sexual diploid populations, *triangles* agamospermous polyploid populations)

In this study we employed three different molecular approaches to examine the relationships of diploid and polyploid populations of *E. sessilifolium*. Two primary questions were addressed: (1) are the polyploid populations of *E. sessilifolium* allo- or autoploid in origin, and (2) are the polyploid populations the result of a single or multiple origins? Each of the three data sets provides unique insights into these questions.

Previous studies of ITS sequence variation in *Eupatorium* have shown that each diploid species has a unique ITS sequence type and that little intraspecific variation exists (Siripun and Schilling 2006a; Schilling et al. 2007). Further, studies of allopolyploid species and hybrids in *Eupatorium* have shown that ITS sequence types representative of all parents are maintained in allopolyploid and hybrid individuals (Siripun and Schilling 2006a; Schilling et al. 2007). We used ITS sequence data to determine whether polyploid *E. sessilifolium* populations were of allo- or autoploid origin. If polyploid *E. sessilifolium* populations are of alloploid origin then we expected to detect multiple different ITS sequence types within individuals. Alternatively, if *E. sessilifolium* populations are of autoploid origin then we expected to obtain a single ITS sequence type.

Because chloroplast DNA is maternally inherited (Sullivan et al. 1991), phylogenetic analyses of cpDNA sequences are expected to reflect only maternal lineages and can be used to address the issue of single versus multiple origins of polyploids. If polyploid *E. sessilifolium* originated once then we expect all polyploid populations to have a single cpDNA sequence type (if no mutations have occurred subsequent to polyploid formation), or to form a monophyletic group relative to sampled diploid populations. Alternatively, if polyploid *E. sessilifolium* populations have arisen through multiple origins then we expect different polyploid populations to have different cpDNA sequence types and for these to be more closely related to different diploid cpDNA sequence types.

ISSRs are anonymous, dominant, and biparentally inherited nuclear markers (Wolfe and Liston 1998). ISSRs have been used extensively to examine genetic variation within species given their ease of use and highly polymorphic nature (Reddy et al. 2002; Wolfe 2005). Within *E. sessilifolium* ISSRs were used to compare and contrast levels of variability between diploid and polyploid populations to address the issue of single versus multiple origins of polyploids. If polyploid *E. sessilifolium* originated once then we expect all polyploid populations to be genetically very similar to each other. Alternatively, if polyploid *E. sessilifolium* populations have arisen through multiple origins then we expect different polyploid populations to be genetically differentiated from each other.

Materials and methods

Sources of plant material

Samples of *E. sessilifolium* were collected from field populations in the southeastern USA in the area where both sexual diploid and agamospermous polyploid populations have been documented previously (Table 1; Fig. 1). At each site, multiple individuals were sampled. Ploidy level and type of reproduction of populations was inferred from the presence or absence of pollen coupled with the presence of filled cypselae. Sullivan (1972, 1976) demonstrated the absolute correlation within *Eupatorium* between male sterility and polyploidy, and the further correlation between polyploidy and agamospermy, and the presence of uniformly filled cypselae in heads in which the flowers lacked not only pollen but also anthers provided further evidence of their agamospermous nature. Measurements were made of a number of characters (leaf length and width, phyllary length and width; corolla length, cypsela and pappus length) for comparisons.

Isolation of DNA

DNA samples were extracted principally from young leaves with the CTAB protocol of Doyle and Doyle (1987). To decrease the interference from phenolic compounds, DNA extractions were cleaned up using the Wizard Kit protocol (Promega, Madison, WI, USA). The purified DNA extracts were quantified and the concentrations were adjusted to 25–100 ng/μl.

Molecular methods

For ITS sequence studies, only one individual from each population was analyzed because of the overall lack of intraspecific variability that was revealed for this marker. ITS amplifications were performed in 20 μl reactions using 10–20 ng of genomic DNA, 10X PCR buffer (Promega), 1.8–2.25 mM $MgCl_2$, 0.2 mM each dNTP, 1.25 units of *Taq* polymerase, and 0.2 μM each primer. Primers used were "ITS-4" (5′-TCCTCCGCTTATTGATATGC-3′) and "ITS-5" (5′-GGAAGTAAAAGTCGTAACAAGG-3′; White et al. 1990). PCR was performed with the following protocol: 94°C for 2 min; 40 cycles of 94°C for 1 min, 50°C for 1 min, and 72°C for 1 min; and a final extension of 72°C for 3 min. For the cpDNA sequence studies, only a single individual from each population was analyzed. Selection of a cpDNA region to serve as a marker relied on data from an earlier study (Shaw et al. 2005), which suggested that the *trn*C-*psb*M region was the most variable of 21 non-coding cpDNA regions screened for a three species subset of *Eupatorium*. This region was amplified and sequenced

Table 1 Collections of *Eupatorium sessilifolium* and related taxa analyzed for molecular data. Vouchers at TENN except *Sorrie 8228* at NCU

Taxon	Collection	Location	GenBank #	
Eupatorium L.			ITS	*trn*C-*psb*M
E. sessilifolium L.				
Diploid populations				
SD1	*Siripun 859*	Montgomery Co, VA	DQ236179	FJ395160
SD2	*Siripun 874*	Blount Co, TN	DQ236180	FJ395161
SD3	*Siripun 884*	Blount Co, TN	DQ236181	FJ395162
SD4	*Siripun 895*	Blount Co, TN	FJ395145	FJ395163
SD5	*Siripun 902*	Graham Co, NC	FJ395146	FJ395164
SD6	*Siripun 904*	Graham Co, NC	FJ395147	FJ395165
SD7	*Siripun 910*	Swain Co, NC	FJ395148	FJ395166
SD8	*Siripun 1094*	Bledsoe Co, TN	FJ395149	FJ395167
Polyploid populations				
SP1	*Siripun 1072*	Craig Co, VA	DQ236182	FJ395168
SP2	*Siripun 824*	Floyd Co, VA	DQ236183	FJ395169
SP3	*Siripun 829*	Franklin Co, VA	DQ236184	FJ395170
SP4	*Siripun 847*	Botetourt Co, VA	FJ395150	FJ395171
SP5	*Siripun 854*	Bedford Co, VA	FJ395151	FJ395172
SP6	*Siripun 871*	Roanoke Co, VA	FJ395152	FJ395173
SP7	*Siripun 915*	Jefferson Co, TN	FJ395153	FJ395174
E. album L.	*Schilling 06-17*	Wayne Co, GA	EU646472	FJ395175
E. altissimum L.	*Schilling 04-56*	Marion Co, AR	DQ236178	FJ395176
E. capillifolium (Lamarck) Small	*Siripun 869*	Orange Co, NC	DQ415733	AY727124
E. compositifolium Walter	*Siripun 1129*	Levy Co, FL	FJ395154	FJ395177
E. hyssopifolium L.	*Siripun 870*	Orange Co, NC	DQ236177	AY727125
E. lancifolium (Torrey & A. Gray) Small	*Godfrey 68059*	Pike Co, AR	DQ236175	FJ395178
E. leucolepis (DC.) Torrey & A. Gray	*Godfrey 80849*	Wakulla Co, FL	DQ415736	FJ395179
E. linearifolium Walter	*Kral 51191*	Henry Co, AL	DQ236199	FJ395180
E. mikanioides Chapman	*Godfrey 84685*	Wakulla Co, FL	DQ415739	FJ395181
E. mohrii Greene	*Schilling 05-04*	Berrien Co, GA	FJ395155	FJ395182
E. paludicola E. E. Schill. & Leblond	*Schilling 04-17*	Hoke Co, NC	DQ236202	FJ395183
E. perfoliatum L.	*Schilling 04-32*	Hot Springs Co, AR	DQ236191	FJ395184
E. petaloideum Britton	*Godfrey 80839*	Leon Co, FL	DQ236201	FJ395185
E. pilosum Walter	*Schilling 05-11*	Charlton Co, GA	DQ236198	FJ395186
E. resinosum Torrey ex DC.	*Sorrie 8228**	Moore Co, NC	DQ415742	FJ395187
E. rotundifolium L.	*Siripun 997*	Brunswick Co, NC	DQ236193	AY727126
E. semiserratum DC.	*Schilling 06-08*	Bamberg Co, SC	FJ395156	FJ395188
E. serotinum Michaux	*Schilling 06-20*	Knox Co, TN	FJ395157	FJ395189
Eutrochium Rafinesque (Outgroup)				
E. fistulosum (Barratt) E. E. Lamont	*Schilling* s.n.	Knox Co, TN	FJ395158	FJ395190
E. purpureum (L.) E. E. Lamont	*Schilling 95-11*	Knox Co, TN	FJ395159	FJ395192

following protocols of Shaw et al. (2005), which involved making a single amplification with the *trn*C and *psb*M primers. The resulting PCR product was subsequently sequenced using two forward primers, *trn*C (ca. 600 bp) and *ycf*6 (ca 490 bp), with an ca. 50 bp overlap. Sequences were prepared utilizing the ABI Prism Dye Terminator Cycle Sequencing reaction kit and run at the University of Tennessee Automated Sequencing Facility on an ABI 3100 DNA sequencer (Perkin-Elmer Inc., Foster City, CA, USA). For ISSR studies, each individual in each population was analyzed. After an initial screening of 21 candidate primers, four were chosen based on a combination of reliable amplification and presence of multiple bands that exhibited variability between samples: 808 $(AG)_8$-C, 810 $(GA)_8$-T,

811$(GA)_8$-C (800 series, University of British Columbia primer set #9) and MANNY $(CAC)_4$-RC (Dr. Andrea Wolfe's ISSR web page, http://www.biosci.ohio-state.edu/~awolfe/ISSR.html). ISSR amplifications were performed in 15 µl reactions using 10–100 ng of genomic DNA, 1X PCR buffer (Promega), 2.5 mM $MgCl_2$, 0.2 mM each dNTP, 0.5 units of *Taq* polymerase, 2% v:v formamide, and 0.33 µM primer. PCR was performed with the following protocol: 94°C for 1.5 min; 35 cycles of 94°C for 45 s, 45°C for 45 s, and 72°C for 1.5 min; and a final extension of 72°C for 5 min. PCR products were run on 2% agarose gels, which contained 0.5 µg/ml ethidium bromide, at ~96–98 V for 2–2.5 h or until the blue marker dye migrated approximately 8 cm. Two replicate PCR reactions were conducted for each primer/population pair. Only bands that appeared in both replicates were scored. Bands were manually scored and sized by comparing the migration distances with those of a standard molecular weight marker (100 bp ladder, Fisher BioReagents, Atlanta, GA, USA) that was run in two lanes on each gel.

Data analysis

The initial sequence data text files were edited following comparison with the same data displayed in four-color electropherograms before they were analyzed further. For comparative purposes, data sets were assembled that included ITS and *trn*C-*psb*M sequences for all diploid species of *Eupatorium*, as well as sequences for the sister genus *Eutrochium* utilized as the outgroup. GenBank accession numbers for all samples analyzed are provided in Table 1. Sequence alignment was performed manually. Phylogenetic relationships were analyzed using maximum parsimony, implemented using PAUP 4.0b10* (Swofford 2003), with gaps treated as missing data, using a heuristic search with 1,000 random addition replicates and with TBR branch swapping. Bootstrap analysis (Felsenstein 1985) was performed with 10,000 replicates using the FAST-STEP search option. For ISSR data, amplified bands were scored as present or absent for each individual. A neighbor-joining analysis was implemented in PAUP 4.0b10 using Nei and Li (1979) restriction site distance, an algorithm that counts only positive band matches (shared bands) and ignores negative band matches (shared absence). The resulting tree was mid-point rooted.

Results

Morphology

Although there was some variability among samples for each of the morphological features that were measured, there was extensive overlap between sexual diploid and apomictic populations for each (data not shown). Comparison of sample means failed to reveal any character for which variation between diploid and apomictic populations was statistically significant at the 0.05 level.

ITS sequences

ITS sequences obtained for samples of *E. sessilifolium* were consistent with previous reports for the species in the lengths of the ITS-1, ITS-2, and 5.8S regions. There was no variation for ITS sequence among any of the populations of *E. sessilifolium*, and none of the ITS sequences showed any evidence of either indel or base pair polymorphisms that characterize interspecific hybrid combinations in the genus (Siripun and Schilling 2006a; Schilling et al. 2007; Schilling, unpublished data). Phylogenetic analysis of the ITS sequence data gave overall results (Fig. 2) that were consistent with previous reports for the genus (Schmidt and Schilling 2000; Siripun and Schilling 2006a; Schilling et al. 2007). The monophyly of the genus relative to *Eutrochium* was strongly supported, and there was a basal dichotomy between the dog fennel group (*E. capillifolium*, *E. compositifolium*) and the rest of the genus. Only one other multispecies group, formed by *E. album* and *E. petaloideum*, received bootstrap support. All populations of *E. sessilifolium* were placed in a single strongly supported clade characterized by seven base pair changes. Pair-wise comparisons showed that ITS sequences of *E. sessilifolium* differed from those of other North American *Eupatorium* species by 12–23 bp changes and 0–4 indels.

cpDNA sequences

The *trn*C-*psb*M region includes two spacers surrounding the *pet*N (*ycf*6) gene, and in species of *Eutrochium* and *Eupatorium* the region varied in length from 1,086 to 1,216 bp. In most species, the *trn*C-*pet*N spacer was 625 bp, the *pet*N coding region was consistently 90 bp, and the *pet*N-*psb*M spacer was 493 bp. Alignment of sequences required a total of eight indels and produced a matrix of length 1,234. Two insertions (4, 6 bp) were present in all species of *Eupatorium* but absent in the three samples of *Eutrochium*; two deletions (12, 107 bp) were present in the two species of dog fennels, *E. capillifolium* and *E. compositifolium*; there was a 1 bp insertion in the two samples of *E. perfoliatum*, a 9 bp insertion in the sample of *Eutrochium maculatum*, and a 9 bp insertion in one of the samples of *E. rotundifolium*. The final indel was a 6 bp insertion which was present in three of the diploid samples of *E. sessilifolium*. In addition to the indels, the samples differed for a relatively large inversion in the *trn*C-*pet*N

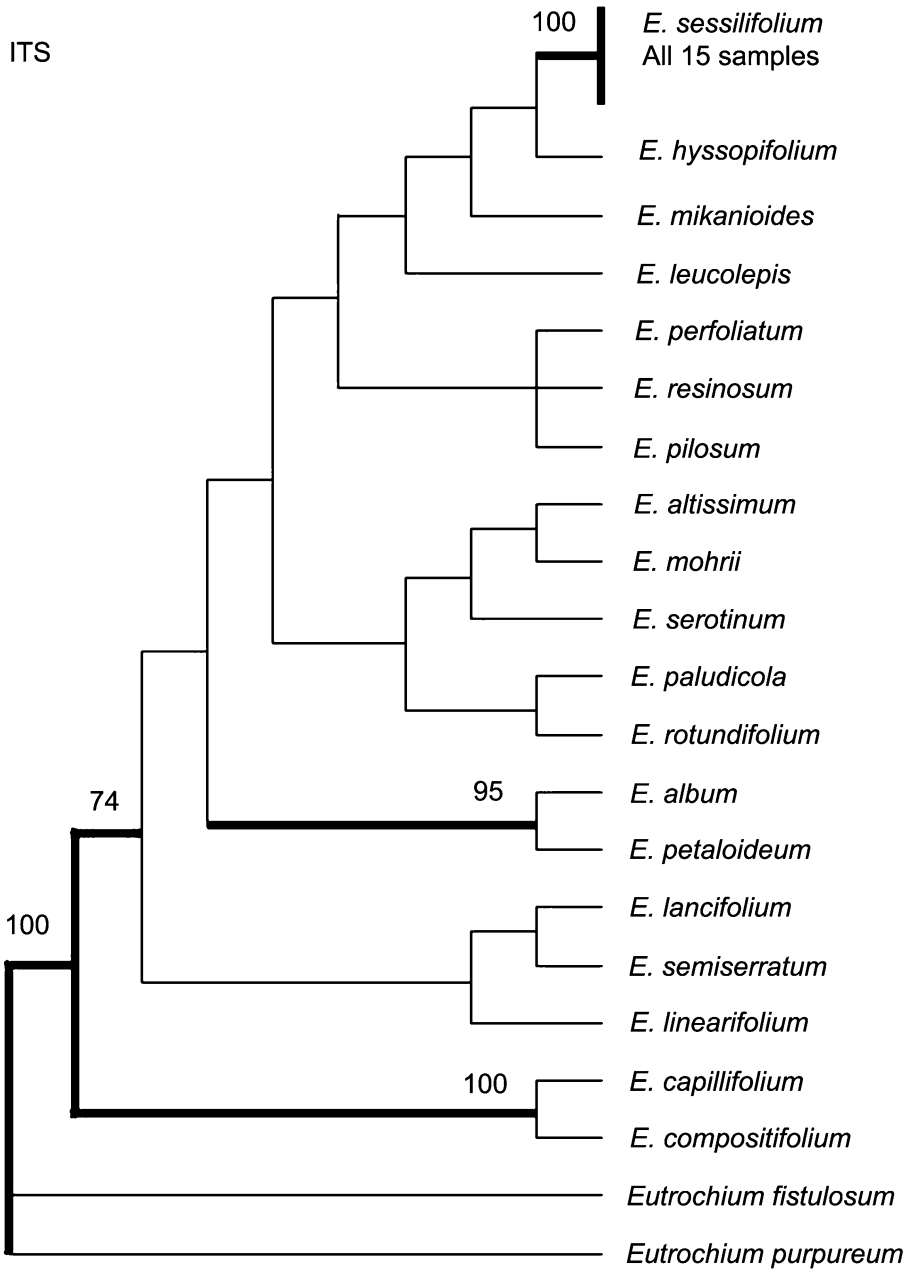

Fig. 2 Strict consensus of two minimum length trees based on ITS sequence data showing relationships of *Eupatorium sessilifolium* to diploid North American species of *Eupatorium*. Nodes receiving bootstrap support above 50% highlighted in bold. *Eutrochium* species were designated as the outgroup

spacer (the shortest resolution was 23 bp, but it could also be resolved as a 56 bp inversion). Samples of the outgroup, *Eutrochium*, and of *Eupatorium capillifolium*, *E. compositifolium*, *E. album*, *E. hyssopifolium*, *E. leucolepis*, *E. mohrii*, *E. paludicola*, *E. perfoliatum*, and *E. pilosum* shared one configuration, which was also present in Liatrinae, a more distantly related outgroup (Shaw et al. 2005). The other configuration of the inversion was found in the other species of *Eupatorium* that were sampled, including *E. sessilifolium*, and thus formed a synapomorphy for this group. For the phylogenetic analysis, the indel regions were excluded, and the inversion region was recoded as a single presence/absence character.

The results of phylogenetic analysis of the aligned *trn*C-*psb*M sequence data are shown in Fig. 3. The outgroup samples of *Eutrochium* differed by a total of 18 bp changes from all samples of *Eupatorium*. Within *Eupatorium* there was relatively little resolution, although many of the species had at least one distinctive character. The two samples of the dog fennel group, *E. capillifolium* and *E. compositifolium*, formed a well supported group that differed by 12 bp changes in addition to the two deletions noted above from all other members of the genus. A well supported node separated the samples with the unique inversion configuration from the other species of the genus, with three additional single bp characters

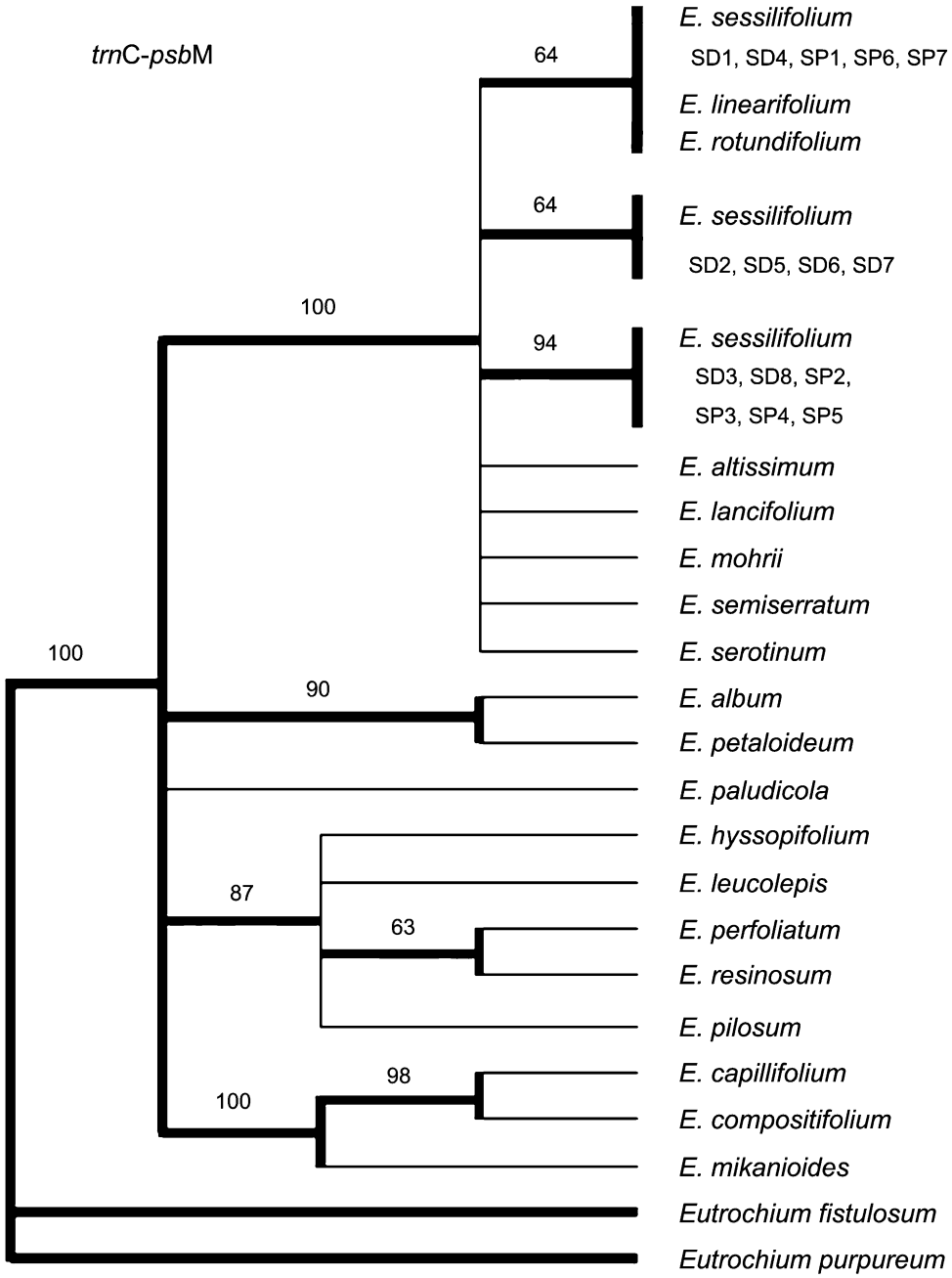

Fig. 3 Minimum length tree based on *trn*C-*psb*M sequence data showing relationships of populations of *Eupatorium sessilifolium* (labeled as in Table 1; SD designates sexual diploid populations; SP designates agamospermous polyploid populations) to one another and to diploid North American species of *Eupatorium*. Bootstrap support values shown above nodes

correlating with the inversion. Although it was not monophyletic, all samples of *E. sessilifolium* were placed in a single clade, within which they were separated into three groups supported by one (plus an insertion), one, and three characters, respectively. One of the groups contained only diploid samples of *E. sessilifolium*, but the other two contained a mixture of diploid and polyploid samples. The samples of *E. linearifolium* and *E. rotundifolium* were also placed in one of these groups, and samples of *E. altissimum*, *E. lancifolium*, *E. mohrii*, *E. semiserratum*, and *E. serotinum* were also placed in the larger clade containing *E. sessilifolium*.

ISSRs

In contrast to the ITS sequence data, considerable variability was observed in the results of the ISSR amplifications, both between as well as within populations of *E. sessilifolium*. The four primers that were used gave the following results: primer 808 gave 37 total, scorable bands, 335–1,970 bp in estimated lengths; primer 810 gave 32 bands, 322–2,500 bp; primer 811 gave 46 bands, 300–2,200 bp; primer MANNY gave 42 bands, 219–2,200 bp. Individual samples produced 4–13 bands for a given primer. There were differences in both the number of bands

and the variability between individuals that were clearly observable in comparing sexual diploid and agamospermous polyploid populations. The total number of bands in diploid populations was higher than in polyploids, and variation between individuals was clearly higher in sexual diploid populations than in agamospermous polyploid ones. The mean pairwise identity of individuals within diploid populations of *E. sessilifolium* was 0.92–1.0, with an overall average of 0.96, whereas for polyploid ones the range was 0.98–1.0, with an overall average of 0.99. In diploid populations, almost every individual had a distinctive band pattern. In contrast, five of the seven sampled agamospermous populations were basically monomorphic for ISSR markers, containing only one or two multi-locus genotypes. Additionally, the pairwise genetic divergence among polyploid populations (average 0.87; range 0.81–0.90) was nearly as great as was observed among diploid populations (average 0.83; range 0.73–0.90).

The overall pattern of distribution of ISSR markers in *E. sessilifolium* can be visualized in the results of the neighbor-joining analysis shown in Fig. 4. All of the individuals from a given population clustered together. In contrast to diploid populations, most of which exhibited considerable variability, there was little or no variability within agamospermous populations. Diploid and polyploid populations were intermixed in the tree, and there was no indication that the polyploid populations collectively formed a monophyletic group.

Discussion

The results of the comparative molecular analyses of populations of *E. sessilifolium* suggested that the agamospermous polyploid populations were autoploids and had multiple origins. The results of morphological comparisons agreed with previous reports in failing to uncover any significant morphological differences between diploid and apomictic populations of the species. The results of analysis of ITS sequence data were consistent with an autoploid origin of the agamospermous polyploid populations by failing to reveal any evidence of sequence polymorphism that would be an indication that a second species was involved in their origin. The analysis of the chloroplast-based *trn*C-*psb*M sequence data showed variation among populations of *E. sessilifolium* for both sexual diploid and agamospermous populations, suggesting that the agamospermous populations stem from multiple origins. The ISSR analysis revealed considerably more variation than did the ITS results, and there were significant differences in the distribution of variability within and between populations. Lack of variability between individuals suggested that agamospermous populations of *E. sessilifolium* are largely clonal. In contrast, variability for ISSR markers among populations adds evidence for multiple origins of the agamospermous polyploids.

The evidence from ITS sequence data that polyploids in *E. sessilifolium* are autoploid is strengthened by two distinctive aspects of the genus. One is the relatively high level of variability in ITS sequence data, particularly the frequent occurrence of indels (insertions and deletions), that has been observed to occur between species of the genus (Siripun and Schilling 2006a; Schilling et al. 2007.) And, in contrast to some other recently described examples (e.g. Flagel et al. 2008), examination of multiple populations has shown that individual diploid species of *Eupatorium* are fixed for a characteristic ITS sequence type (Fig. 2; Siripun and Schilling 2006a; Schilling et al. 2007; Schilling unpublished data). Thus, it can be readily detected if an individual is of interspecific hybrid origin, based on the presence in ITS sequence data showing multiple polymorphisms, and these are particularly obvious when they involve indels (Siripun and Schilling 2006a; Schilling et al. 2007). The second is that in apomicts the genomes are subject to little or no recombination, so that divergent ITS sequences will not be homogenized by gene conversion. Failure to detect multiple ITS sequences in any of the polyploid agamospermous populations of *E. sessilifolium* contrasts to the ease of detection of ITS sequence polymorphisms in samples of the genus that have been documented to be of alloploid (Siripun and Schilling 2006a; Schilling et al. 2007) or hybrid (Schilling unpublished data) origin. Thus, the polyploid cytotype of *E. sessilifolium* can be added to the growing list of examples of natural autoploids (Soltis et al. 2003).

The chloroplast-based *trn*C-*psb*M sequence data set showed only limited phylogenetic resolution within *Eupatorium*, but there was variability within *E. sessilifolium*. Although the data matrix included 58 variable positions for the *trn*C-*psb*M region, only 46 were potentially parsimony informative, and of these 18 separated the ingroup of *Eupatorium* from the outgroup *Eutrochium* and a further 12 were apomorphic for the morphologically distinctive dog fennel group. Other than for *E. sessilifolium*, extensive intraspecific sampling for this marker was not undertaken, so it was not possible to assess the extent to which species might be fixed for a particular cpDNA sequence type. The most significant aspect of the *trn*C-*psb*M sequence data was to show variability among both sexual diploid and agamospermous populations of *E. sessilifolium*. The presence of two different *trn*C-*psb*M sequences, which differed from one another by four bp changes, in the agamospermous populations suggested that agamospermous lineages have arisen more than once in *E. sessilifolium*. The presence of sequences in diploid populations that were identical to those in agamospermous ones was consistent with the

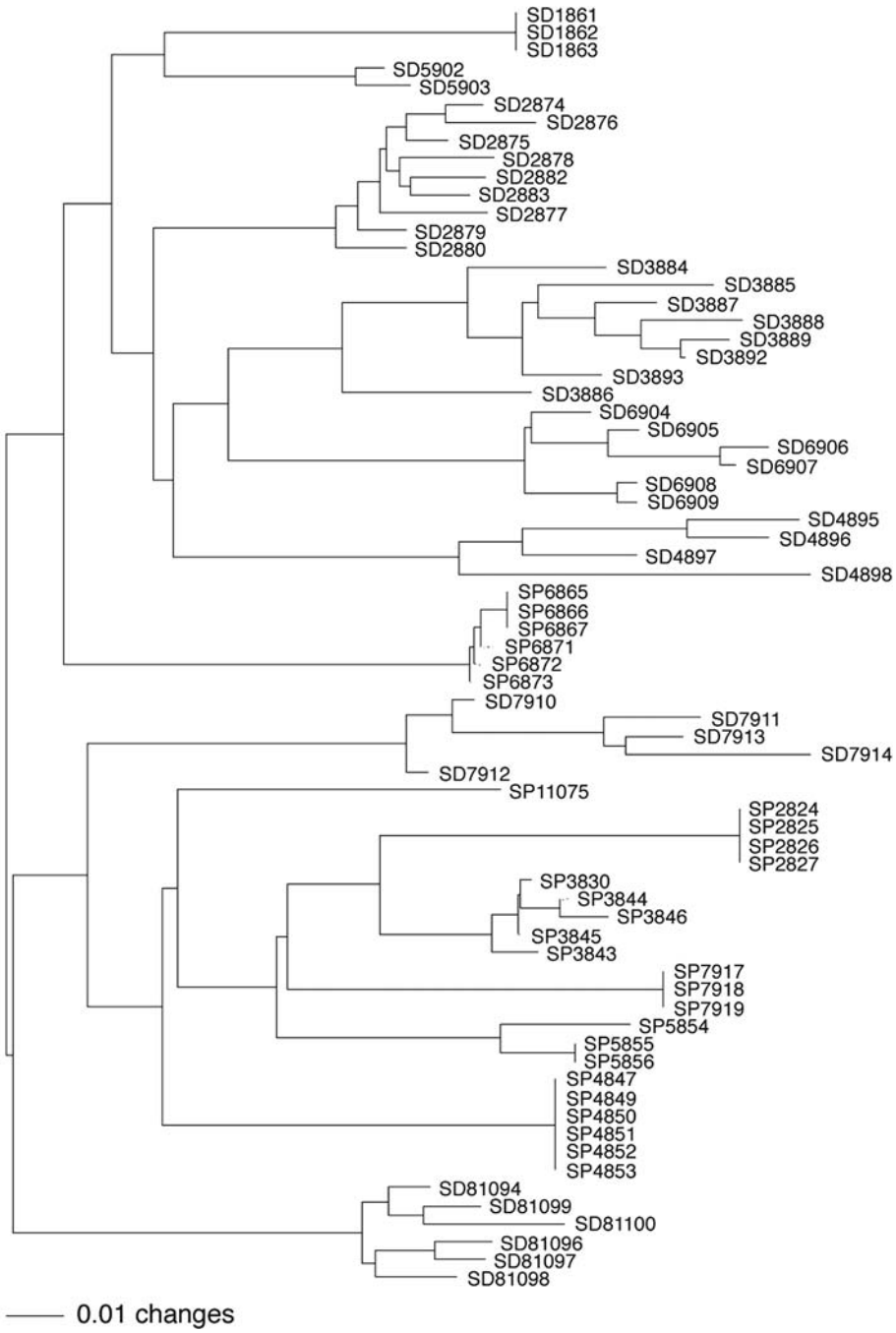

Fig. 4 Results of neighbor-joining analysis with midpoint rooting showing relationships among individuals and populations of *Eupatorium sessilifolium* analyzed with ISSR data. Each individual labeled to show population (first three digits; SD designates sexual diploid populations; SP designates agamospermous polyploid populations; Table 1) and individual sample numbers (final three digits)

autoploid origin of the latter inferred from the ITS sequence data.

The ISSR data revealed a higher level of variability than did the ITS sequence data, from which interpretations of the origin and relationships of diploid and polyploid populations can be made. For sexual diploid populations, almost every individual had a distinctive ISSR banding pattern when all four primer combinations were considered. In contrast, little or no variation between individuals was observed for most of the agamospermous polyploid populations, which would be consistent with their establishment within a limited area as a single clone. Significant variation in ISSR markers was observed between polyploid populations, however, as well as among and between diploid and polyploid populations (Fig. 4). This suggests that polyploids are likely to have had multiple origins, involving different diploid progenitors. It also suggests that the origins of individual polyploids likely involved hybridization between genetically different diploid individuals, a phenomenon termed interracial autopolyploidy by Grant (1981).

The geographic distribution patterns of sexual diploid and agamospermous populations of *E. sessilifolium* can be considered in light of the molecular data. The diploid populations occur in the southern Appalachian Highland area that has been proposed to be a glacial refugium (Soltis et al. 2006). The diploid populations appear to be restricted to the refugial region, whereas the polyploid populations have attained a relatively widespread distribution (Fig. 1). The polyploid populations as a whole appear to mimic to at least some extent the variation to be expected in a sexually reproducing species, not because of segregation and recombination, but rather because they have multiple origins from varying parental combinations. Thus, while polyploid populations contain little variation within them, there is significant divergence among them due to multiple origins, giving the polyploid entity a higher level of genetic variation than would be expected if it were the result of a single origin.

Some consideration might be given to whether the diploid and polyploid populations of *E. sessilifolium* should be separated taxonomically, despite their overall morphological resemblance. The polyploids can be distinguished from diploids based on presence or absence of anthers and pollen. No additional features have been noted, however, that would separate populations of diploids from those of polyploids. Soltis et al. (2003) bemoan the fact that lack of taxonomic recognition tends to suppress detailed studies of species that are represented by multiple cytotypes, and Soltis et al. (2007) go further in strongly advocating taxonomic recognition of autoploids. Within *Eupatorium*, there may also be biological considerations that would suggest taxonomic separation of the cytotypes. In *E. sessilifolium*, as well as in the other species of the genus that have been suggested to have diploid and autopolyploid cytotypes, the agamospermous polyploids have achieved a much greater geographical distribution than the sexual diploids (Fig. 1). However, theory (e.g. van Dijk and Vijverberg 2005) suggests that it is the diploids that contain the greatest (and perhaps exclusive) evolutionary potential within a species with both sexual diploid and agamospermous polyploid populations. Thus, when the different cytotypes are considered to be conspecific, there is the possibility that the species may be endangered even if it is widespread. In *E. sessilifolium* the diploids are found in distinctive high elevation habitats where they do not appear to be common, and might warrant further investigation to evaluate whether any measures need to be adopted to enhance their continued survival. Ironically, the places where *E. sessilifolium* has been listed as an endangered, threatened, or species of special concern are at the geographical margins of its total range (e.g. Indiana, Iowa, Michigan, Minnesota, New Hampshire, Vermont, Wisconsin) where the species is probably represented only by agamospermous populations.

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