Plant Syst Evol (2018) 304:133–141
https://doi.org/10.1007/s00606-017-1447-2

SHORT COMMUNICATION

Evidence for genetic allopolyploidy in *Eutrema edwardsii* (Brassicaceae): implications for conservation

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Received: 20 December 2016 / Accepted: 19 July 2017 / Published online: 5 September 2017

Abstract *Eutrema edwardsii* R.Br. (*Brassicaceae)* is an arctic-alpine mustard with a circumpolar distribution. Its closest relative, *Eutrema penlandii* Rollins, is a federally listed, threatened species that is endemic to the Mosquito Range in the Southern Rocky Mountains of Colorado, USA. As part of a larger project addressing the systematics of this species complex in North America, we conducted chromosome counts, flow cytometry, and allozyme analysis to test the hypothesis that these taxa comprise an autopolyploid complex. Within that context, it should be noted that a chromosome count has not been reported previously for *E. penlandii*. Results obtained from mitotic counts obtained for two populations of *E. penlandii* reveal this taxon to be diploid. Diploidy was confirmed using flow cytometry for an additional 15 individuals representing four populations. Previously published chromosome counts for *E. edwardsii* reveal a polyploid complex of tetraploid, hexaploid, and octaploid populations for which an autopolyploid origin has been presumed. However, allozyme analysis revealed an allopolyploid origin for *E. edwardsii,* as evidenced from fixed heterozygosity at six loci. Although our data suggest that *E. penlandii* is a close relative of one of the progenitors of *E. edwardsii*, the taxonomic identity of the other progenitor(s) cannot be elucidated from these data. The data reported herein support the recognition of *E. penlandii* as taxonomically distinct, which has implications for conservation, and reveal cryptic variation within *E. edwardsii*.

Keywords Allopolyploidy · Allozyme · Cryptic variation · *Eutrema* · Fixed heterozygosity · Flow cytometry

Handling editor: Martin Lysak.

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Introduction

Although flowering plants are under-represented in the literature addressing cryptic species (Bickford et al. 2006), there is a growing body of research addressing these species in the flora of the arctic, in particular (e.g., Grundt et al. 2006). The underlying explanations proposed to explain this phenomenon include recent speciation, incomplete divergence, and reticulate evolution, with selection, inbreeding, and genetic drift proposed as mechanisms leading to cryptic variation. It is also clear that polyploidy—particularly autopolyploidy in plants—is an important and overlooked mechanism leading to the formation of cryptic species (Soltis et al. 2007). However, there has been a resistance to recognize multiple cytotypes as distinct—even when they confer reproductive isolation—as well as a reliance upon the morphological species concept, or some variant thereof (Soltis et al. 2007). Herein, we report the results of multidisciplinary research addressing the systematics of the *Eutrema edwardsii* R.Br. species complex (Brassicaceae), with a goal of contributing to a better understanding of *E. penlandii* Rollins, a federally listed Colorado endemic.

Until 1985, two *Eutrema* species were recognized from North America: *E. edwardsii* and *E. penlandii*, when Weber (1985) published the combination *E. edwardsii* R.Br. subsp.

penlandii (Rollins) W.A.Weber, thereby reducing the rank of this taxon to subspecies, although no rationale was provided. More recently, *E. penlandii* was subsumed into *E. edwardsii*, as part of a larger revision to the genus following a critical evaluation of *Neomartinella* Pilg., *Platycraspedum* O.E.Schulz, *Taphrospermum* C.A.Mey., and *Thellungiella* O.E. Schulz; this resulted in the aforementioned genera being subsumed into *Eutrema* (Al-Shehbaz and Warwick 2005; Warwick et al. 2006). Therein and subsequently, Al-Shehbaz (2010) claimed that the variation exhibited by *E. edwardsii* (e.g., plant height, leaf base shape, petal shape) could easily accommodate *E. penlandii*. As part of a larger study using allozyme analysis to address conservation genetics in Colorado populations of *E. penlandii* (Hardwick 1997), as well as the systematic relationship of *E. penlandii* to *E. edwardsii*, fixed heterozygosity was observed in populations of *E. edwardsii* suggesting to us an allopolyploid origin for the latter (e.g., Brochmann et al. 2004 and references therein).

Although it has long been assumed that the aforementioned two species form a polyploid complex, with autopolyploidy presupposed for *E. edwardsii* (e.g., Jørgensen, et al. 1958), no chromosome counts have been reported for *E. penlandii*, in particular. Herein, we take multiple approaches using chromosome counts, flow cytometry, and allozyme analysis to better understand the systematics of *E. edwardsii* s.l., and to test the hypothesis that *E. penlandii* and *E. edwardsii* form an autopolyploid complex. Whereas chromosome counts and flow cytometry are used to document ploidy for *E. penlandii*, allozyme analysis is used to elucidate the polyploid origin of *E. edwardsii*.

Materials and methods

Natural history

Al-Shehbaz and Warwick (2005) recognized 26 species comprising *Eutrema*, the majority of which occur in Central and East Asia. Several additional species have recently been described, also from Asia (Hao et al. 2015, 2016, Xiao et al. 2015). Only *Eutrema salsugineum* (Pall.) Al-Shehbaz & Warwick and *E. edwardsii* extend from Asia into North America where, like *E. salsugineum*, *E. penlandii* is highly disjunct in the Southern Rocky Mountains (Al-Shehbaz 2010; German and Koch 2017).

Eutrema edwardsii and *E. penlandii* are perennial herbs that occupy a variety of imperfectly drained arctic and alpine tundra habitats, such as meadows, margins of ponds and stream banks, and solifluction slopes (Aiken et al. 2007). *Eutrema edwardsii* is widespread, with a circumpolar distribution (Aiken et al. 2007); it also occurs in the alpine of the White Mountains, Alaska Range, Chugach Mountains, Wrangell-St. Elias Mountains, and the Mackenzie Mountains of the northern Rocky Mountains in western North America (Fig. 1) and has been reported from the Altai Mountains, Tian Shan Mountains, Pamir Mountains, and Himalayas in Asia. In contrast, *E. penlandii* is a narrow endemic that is restricted in distribution to the Mosquito Range in the Southern Rocky Mountains of Colorado (USA), where typically it occupies alpine peatlands overlying calcareous substrates.

Relatively little is known about the natural history (e.g., reproductive biology) of either species. Although chromosome counts conducted for *E. edwardsii* have revealed tetraploid ($2n = 4x = 28$), hexaploid ($2n = 6x = 42$), and octaploid ($2n = 8x = 56$) populations, where $n = 7$, no counts have been reported for *E. penlandii* (Warwick and Al-Shehbaz 2006).

Field collections

Plant material, including leaf tissue for allozyme analysis and flow cytometry and seeds for chromosome counts, was collected between 1995 and 2014 (Table 1). As part of a larger study using allozyme analysis to characterize population genetic diversity and structure in *E. penlandii*, Hardwick (1997) collected leaf tissue from seven populations of the narrowly distributed Colorado endemic and three populations of its widespread congener *E. edwardsii* from Alaska. Bruederle subsequently collected leaf tissue from an additional five populations of *E. penlandii* and seven populations of *E. edwardsii* from Alaska and Yukon Territory. Population samples were collected from 7 to 34 individuals per population, depending on the size of the population, and maintained as such—samples were not bulked. Approval for tissue collection from *E. penlandii* was granted by the U.S. Fish and Wildlife Service under the authority of permits PRT-704930 and TE12513A-0.

Chromosome counts

Counts were obtained from squashes of root tips harvested from seedlings germinated specifically for this purpose. In the process of collecting tissue for population genetic and molecular systematics research, a small number of fruits were harvested. Seeds were germinated in 0.005 M gibberellic acid in a Percival environmental chamber set at 15 °C; attempts were made to cultivate those seedlings that were not harvested in a research greenhouse for subsequent use for these purposes. As such, either whole seedlings or root tips were harvested within 2–4 h after light conditions were restored and placed in a saturated solution of paradichlorobenzene for 2 h, after which they were fixed in Farmer's solution, in which they were stored. Squashes were prepared by macerating the root tip in a drop of 1% aceto-orcein on a microscope slide cleaned with 95% ethanol, heating for

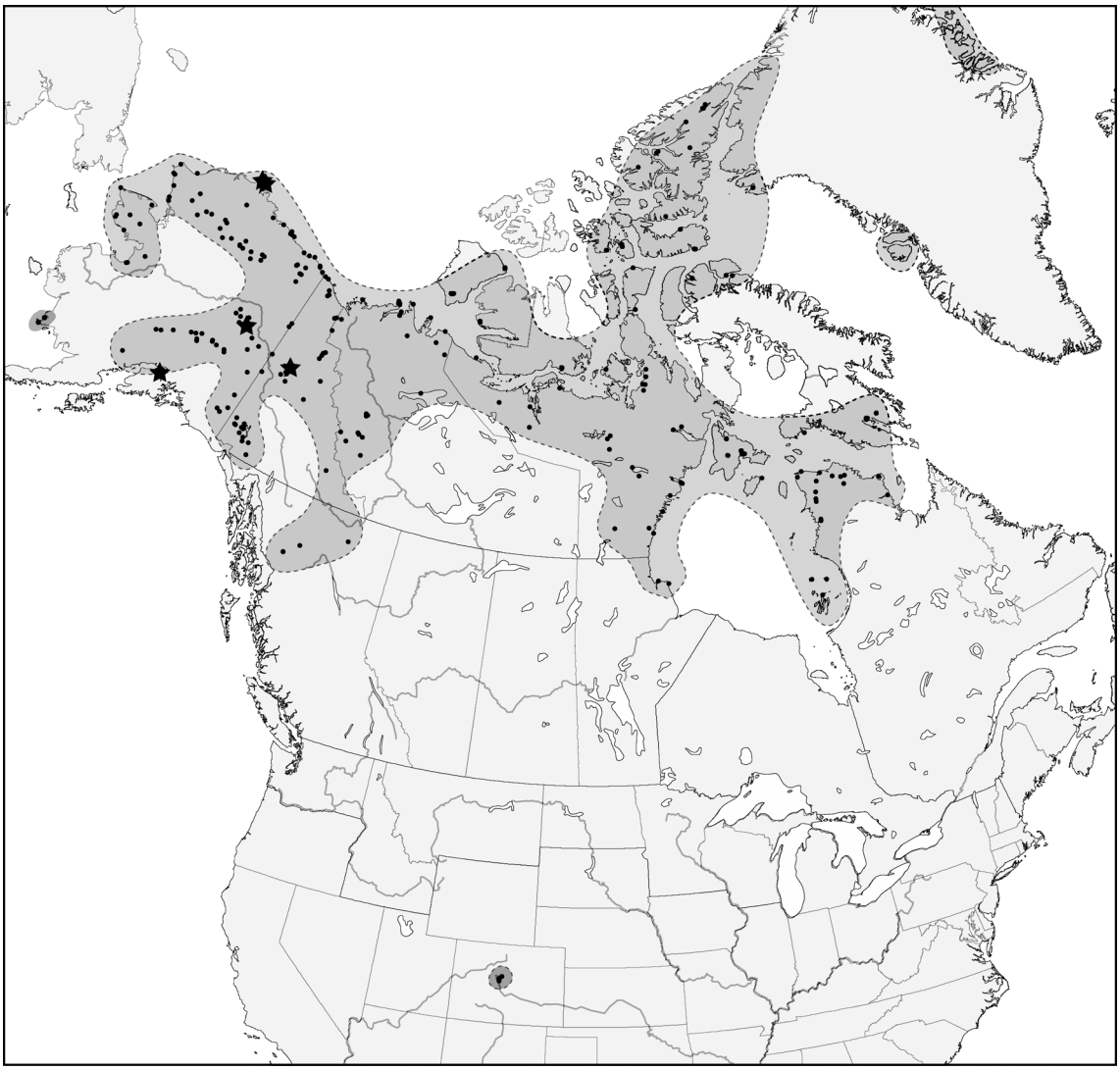

Fig. 1 Distribution of *Eutrema edwardsii* s.l. in North America

2 min at 50 °C, and squashing. Squashes were scanned at 100× and 400× for meiotic figures and photographed at 1000×. The chromosome count for *E. penlandii* was obtained from seed collected at the Treasure Vault Mountain site, for which a voucher has been deposited with the Katherine Kalmbach Herbarium (KHD) at the Denver Botanic Gardens.

Flow cytometry

Relative genome size was determined from silica gel-dried samples of leaf tissue to confirm ploidy for *E. penlandii* and *E. edwardsii* based upon chromosome counts, and to document DNA ploidy at sites across the range of *E. penlandii*. Flow cytometry methods followed Doležel et al. (2007) using a BD Accuri C6 flow cytometer and BD Accuri C6 software version 1.0.264.21.

Relative genome size was obtained for three individuals from each sampled population, depending upon the amount of dried leaf tissue that was available. Standards were grown in the research greenhouse at the University of Colorado Denver, with leaf tissue harvested and silica gel dried for subsequent use. *Solanum lycoppersicum* cv. Stupicke (2C = 1.96 pg DNA) was obtained from J Doležel (Institute of Experimental Botany, Czech Republic) to calculate relative DNA content of our samples. Standards were assayed externally for every 5–10 samples, and the ratio of G1 peaks on the FL2 channel (585/40 nm) was multiplied by the standard DNA content to calculate relative DNA content of the samples (Doležel et al. 2007).

Between 1–2 cm^2 of leaf tissue was minced using a razor blade in 1 ml of LB01 buffer solution pH 7.5 for 1–2 min. The homogenate was mixed using a pipettor and then incubated at 4 °C for 15 min. The solution comprising nuclei

Table 1 Locations for *Eutrema penlandii* and *E. edwardsii* populations. Coordinates for *E. penlandii* are withheld due to federal listing as threatened

	Site	Latitude	Longitude	Elevation (m)	Population size	Application
E. penlandii	Blue Lakes	NA	NA	3958	<10	2
	Cameron Amphitheater (3A)	NA	NA	3978–4039	~3000	3
	Cooney Lake	NA	NA	3767–3889	500–600	3
	Hilltop Mine (Hilltop Mine 13A)	NA	NA	3933	200–300	3
	Hoosier Ridge	NA	NA	3872	~300	3
	Peerless Mine (near Peerless Mine/Horseshoe Basin 15A)	NA	NA	3952.0	<50	2, 3
	Horseshoe Gulch (near Peerless Mine/Horseshoe Basin 15C)	NA	NA	3758	200–300	2, 3
	Kite Lake (4A)	NA	NA	3780–3812	200+	3
	Mosquito Pass (8A-E)	NA	NA	4009	1000+	3
	Mount Buckskin (North Mosquito Creek 16A-D)	NA	NA	3872	500+	3
	Mount Sheridan (14B)	NA	NA	3984	200+	3
	North London (American Flats/London Mountain 9A)	NA	NA	3857	1000+	3
	Treasurevault Mountain	NA	NA	3901	<100	1
	Weston Pass	NA	NA	3612–3627	100–200	2, 3
E. edwardsii	Barrow, East	71.23917	−156.3358	−1	500–1000	2, 3
	Barrow, West	71.26412	−156.8296	9.1	500–1000	2, 3
	Campbell Creek	61.06879	−149.6056	~823.9	~100	2, 3
	Dempster Highway	64.95168	−138.2713	933.6	~100	3
	Eagle Summit	65.4849	−145.404	1125.3	~100	2, 3

Application indicates populations sampled for *1* chromosome counts, *2* flow cytometry, *3* allozyme analysis

in suspension was filtered through 42 µm nylon mesh into a 1.5-ml Eppendorf tube (Doležel et al. 2007). RNase-A (0.25 µl at 100 mg/l) was added to 0.5 ml of the suspension and allowed to incubate on ice for 30 min. Propidium iodide (PI, 25 µl at 1 g/ml) was added to the suspension, which was gently mixed and incubated on ice for 3–5 min in the dark. The solution was then injected into the FCM with the sample rate set to "slow" and run until approximately 1000 events were recorded in a gated region of G1 nuclei to create a histogram and calculate mean peak value and coefficient of variation (CV). The nuclei suspension, without the addition of PI, could be kept in a refrigerator overnight without a decrease in the quality of flow cytometry output.

DNA ploidy was estimated recognizing that *E. penlandii* is diploid ($2n = 2x$), as documented through the aforementioned chromosome count, and comparing the relative DNA content of both taxa following Doležel et al. (2007).

Allozyme analysis

Although numerous molecular techniques exist that may be used to document polyploidy and infer inheritance (see Marhold and Lihová 2006 and references therein), we chose to use allozyme analysis, thereby building on research previously conducted on *Eutrema edwardsii* s.l. (Hardwick 1997). Soluble enzymatic proteins were extracted from two fresh leaves ground in 0.25 ml of a cold Tris–HCl grinding buffer modified by the addition of 4% w/v PVP-40 and 1% w/v 2-mercaptoethanol (Soltis et al. 1983); a small amount of sea sand facilitated grinding. Extracts were adsorbed onto 1.5 × 11 mm wicks cut from No. 17 Whatman chromatography paper and stored at −80 °C until electrophoresis.

Protein extracts were applied to each of three different starch gel and electrode buffer systems (Nielsen and Johansen 1986): lithium borate, gel buffer pH 8.3/electrode buffer pH 8.1; histidine-HCl-tris, gel buffer pH 7.5/electrode buffer pH 7.5; tris-citrate, gel buffer pH 7.8/electrode buffer pH 8.7. Electrophoresis was conducted at 4 °C until a bromophenol blue marker had migrated approximately 12 cm.

Following electrophoresis, gels were sliced horizontally into approximately 1.5-mm-thick slices, with interior slices incubated in substrate specific stains (Soltis et al. 1983) previously revealed to be informative with respect to the polyploid origin of *E. edwardsii* (Bruederle unpublished data). The lithium borate gel was stained for glucose-6-phosphate isomerase (GPI, Enzyme Commission Number 3.5.1.9), superoxide dismutase (SOD, EC 1.15.1.1), and triose-phosphate isomerase (TPI, EC 5.3.1.1); the histidine-HCL gel was stained for malate dehydrogenase (MDH, EC 1.1.1.37)

and phosphogluconic dehydrogenase (PGD, EC 1.1.1.44); and the tris-citrate gel was stained for aspartate aminotransferase (AAT, EC 2.6.1.1).

Designation of loci and alleles was based on relative mobility of proteins following Hardwick (1997), with the most rapidly migrating loci receiving sequentially higher number and the alleles at a locus similarly assigned sequential higher lowercase letters. No formal genetic analyses (e.g., controlled crosses) were performed to document patterns of inheritance. Putative genetic loci and genotypes were inferred from the known substructure and intracellular compartmentalization of enzymes, as well as electrophoretic patterns observed in individuals presumed heterozygous at polymorphic loci. For each population of *E. penlandii* ($n = 12$) and *E. edwardsii* ($n = 5$), data were collected as individual genotypes ($n = 7$–34) from which ploidy was extrapolated based upon dosage with support provided using flow cytometry. For each allozyme locus, observed heterozygosity (H_o) was calculated based upon a direct count from each population ($H_o = n_{hets}/N$), while expected heterozygosity (H_e) was calculated based upon allele frequencies estimated for each population ($H_e = 2pq$). Deviations from Hardy–Weinberg expectations assuming polysomic inheritance were tested using Chi-square analysis ($x^2 = \sum((\text{obs} - \text{exp})^2/\text{exp})$), with the model modified for tetraploidy and hexaploidy by polynomial expansion. All calculations were performed using Microsoft Excel version 14.7.3.

Results

Chromosome counts, DNA content, and ploidy

The chromosome count reported here from the Treasurevault Mountain site revealed *E. penlandii* to be diploid, with $2n = 2x = 14$ and $n = 7$ (Fig. 2). Relative DNA content for *E. penlandii* ranged from 0.68 to 0.90 pg, with a mean of 0.74 pg (Table 2). By contrast, relative DNA content for *E.* edwardsii populations ranged from 1.32 to 2.68 pg, suggesting that our population samples included both tetraploid and hexaploid individuals (Fig. 3), whereas three populations from the Arctic Coastal Plain in northern Alaska and the Chugach Range in southern Alaska were tetraploid, those populations sampled from Central Alaska and Yukon were hexaploid. CV for these measurements were between 3 and 5%; typically, 500–2000 nuclei were detected in each run.

Although field observations, such as relatively tall habit and large leaf morphology observed in some individuals, suggested infrequent autopolyploidy in some populations of *E. penlandii*, this was not substantiated by flow cytometry—all individuals of *E. penlandii* sampled here were found to be diploid. In contrast, *E. edwardsii* was exclusively polyploid,

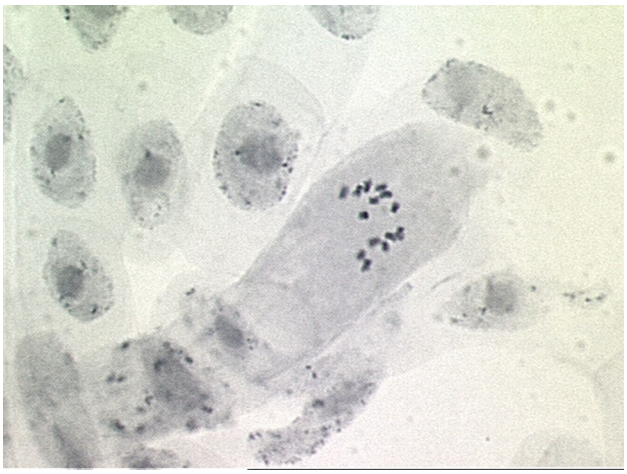

Fig. 2 Mitotic count of $2n = 2x = 14$ for *Eutrema penlandii* obtained from a root squash stained fixed in Farmer's solution and stained with 1% aceto-orcein

as expected based upon counts previously reported in the literature; populations sampled here are either tetraploid or hexaploid, with no evidence of diploidy. Although octaploids have been reported in the literature, none were observed as part of the present study.

Comparisons between populations of *E. penlandii* and *E. edwardsii* provided evidence supporting polyploidy in the latter. *Eutrema edwardsii* populations sampled for this study were tetraploid or, more commonly hexaploid. Furthermore, relative genome size in hexaploid *E. edwardsii* was greater than three times that of *E. penlandii,* which could be the result of genome modification after polyploidization. Alternatively, this could be the result of another, unknown, cryptic taxon with a slightly larger genome being one of the other progenitors of *E. edwardsii*.

Allozyme analysis

Six loci exhibited fixed heterozygosity in one or more of the five *E. edwardsii* populations ($N = 115$–129 individuals): *Aat-2*, *Mdh-1*, *Pgd-3*, *Gpi-2*, *Sod-1*, and *Tpi-1*. Chi-squared analyses revealed that genotypic frequencies at all six loci deviated from expectations of independent assortment of chromosomes ($p < 0.05$), refuting the hypothesis of an autopolyploid origin for *E. edwardsii*. Although recently diverged species that have undergone polyploidization may appear to be autopolyploid, disomic inheritance still occurs (Brochmann et al. 2004).

Seventeen alleles were observed at the six polymorphic loci in *E. edwardsii*—four alleles comprising *Tpi-1* and *Mdh-1*, three alleles at *Aat-2*, and two alleles each for the other loci (Table 3). *Eutrema penlandii* shared nine of the alleles found in *E. edwardsii*, suggesting a progenitor–derivative

Table 2 Flow cytometry data for *Eutrema penlandii* and *E. edwardsii* populations sampled for ploidy determination

Taxon Population (site)	#/Inds.	Relative DNA Content (pg)	DNA Ploidy	Estimated Ploidy
E. edwardsii				
Barrow East, AK (0721201001)	3	1.59	4.3	4
Barrow West, AK (0722201001)	3	1.39	3.7	4
Campbell Creek, AK	3	1.59	4.3	4
Denali Hwy, AK (0704201101)	3	2.43	6.6	6
Eagle Summit, AK (0706201101a)	3	2.26	6.1	6
12 Mile Summit (0705201101)	3	2.56	6.9	6
E. penlandii				
Blue Lake (906201302)	3	0.70	1.9	2
Weston Pass (0906201301)	3	0.71	1.9	2
Horseshoe Gulch (09052011)	2	0.69	1.9	2
Peerless Mine 1 (0818201101)	2	0.85	2.3	2
Peerless Mine 2 (0818201102)	2	0.79	2.1	2

Relative DNA content is calculated by comparison with a standard (*Solanum lycopersicum* cv. Stupicke 2C DNA = 1.96 pg). DNA ploidy is calculated with a ratio of DNA content by comparison to the sample average of diploids

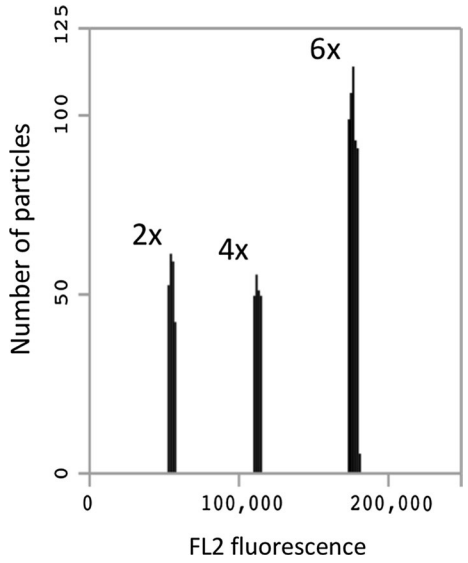

Fig. 3 Histogram of fluorescence for three *Eutrema* cytotypes: diploid *E. penlandii* and tetraploid and hexaploid *E. edwardsii*

relationship for this species. For example, whereas *E. penlandii* has the b, c, and d alleles, tetraploid *E. edwardsii* show fixed heterozygosity for the "b" and "c" alleles (i.e., bbcc), with all of the hexaploids also having the "a" allele (e.g., aabbcc), presumably contributed by a third progenitor.

We also found evidence for multiple origins of *E. edwardsii* based upon variability for dosage at loci that exhibited fixed heterozygosity. Hexaploid populations of *E. edwardsii* exhibited as many as eight different heterozygous genotypes per locus (i.e., *Tpi-1*), suggesting that the progenitors to this taxon were polymorphic at these loci.

Although these data are not unequivocal, *E. penlandii* or a close relative appears to have contributed one genome to tetraploid and to hexaploid *E. edwardsii*. Furthermore, the allozyme data suggest that one or more extinct or extant, and possibly yet to be described diploid *Eutrema* s.l. species contributed the remaining genome(s) through multiple origins from polymorphic populations.

Discussion

Here, we present genetic evidence documenting the allopolyploid origin of tetraploid and hexaploid populations of *E. edwardsii* from northwestern North America. In tetraploids having an allopolyploid origin, hybridization between two individuals with divergent genomes is followed by chromosome doubling resulting in polyploid progeny characterized by two complete genomes (Soltis and Soltis 2000). In contrast to autopolyploidy, where chromosomes segregate and pair independently during meiosis (polysomic inheritance), chromosomes are inherited disomically with allopolyploidy; that is, chromosomes of the same genome pair during meiosis, which typically results in fixed heterozygosity at loci that were divergent in the parental taxa (Soltis et al. 2014 and references therein). The five tetraploid and hexaploid populations of *E. edwardsii* studied here exhibited fixed heterozygosity at each of six loci from which we inferred disomic inheritance, although no progeny analyses were conducted to confirm this.

Polyploid species may result from multiple events of polyploidization involving either the same parental progenitors (polytropic), or different progenitors (polyphyletic) (Brochmann et al. 2004). Polyploids involving multiple

Table 3 Allele frequencies at six polymorphic loci in populations of *Eutrema penlandii* and *E. edwardsii*, where n = sample size and H_o = observed heterozygosity

Locus	Allele	*E. edwardsii* ($N = 5$) site (DNA ploidy)					*E. penlandii* ($N = 12$) site (DNA ploidy)											
		Barrow East, AK (4*x*)	Barrow West, AK (4*x*)	Campbell Creek, AK (4*x*)	Dempster Highway, AK (6*x*)	Eagle Summit, AK (6*x*)	Cameron Amphitheater, CO (2*x*)	Cooney Lake, CO (2*x*)	Hilltop Mine, CO (2*x*)	Hoosier Ridge, CO (2*x*)	Horseshoe Gulch 1, CO (2*x*)	Horseshoe Gulch 2, CO (2*x*)	Kite Lake, CO (2*x*)	Mosquito Pass, CO (2*x*)	Mt Buckskin, CO (2*x*)	Mt Sheridan, CO (2*x*)	North London Mine, CO (2*x*)	Weston Pass, CO (2*x*)
Aat-2	a	0.000	0.000	0.412	0.597	0.556	1.000	1.000	1.000	1.000	na	1.000	1.000	1.000	1.000	na	1.000	1.000
	b	1.000	0.990	0.588	0.403	0.444	0.000	0.000	0.000	0.00na	0.000	0.000	0.000	0.000	na	0.000	0.000	0.000
	c	0.000	0.010	0.000	0.000	0.000	0.000	0.000	0.000	0.000	na	0.000	0.000	0.000	0.000	na	0.000	0.000
	n	25	25	17	24	24	25	25	22	26	na	17	7	25	25	na	14	25
	H_o	0.000	0.040	1.000	1.000	1.000	0.000	0.000	0.000	0.000	na	0.000	0.000	0.000	0.000	na	0.000	0.000
Mdh-1	a	0.000	0.000	0.000	0.590	0.667	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
	b	0.750	0.750	0.750	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
	c	0.000	0.000	0.000	0.410	0.333	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000
	d	0.250	0.250	0.250	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
	n	25	22	34	24	24	25	25	24	26	19	17	8	25	25	8	14	25
	H_o	1.000	1.000	1.000	1.000	1.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
Pgd-3	Ø	0.000	0.000	0.000	0.000	0.000	0.000	0.030	0.000	0.025	0.000	0.000	0.000	0.000	0.040	0.000	0.000	0.000
	a	0.000	0.000	0.500	0.167	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
	b	1.000	1.000	0.500	0.833	1.000	1.000	0.970	1.000	0.975	1.000	1.000	1.000	1.000	0.960	1.000	1.000	1.000
	n	25	22	34	20	24	25	25	24	20	19	17	8	25	25	8	14	25
	H_o	0.000	0.000	1.000	0.500	0.000	0.000	0.120	0.000	0.050	0.000	0.000	0.000	0.000	0.080	0.000	0.000	0.000
Gpi-2	a	0.500	0.500	0.500	0.333	0.292	0.000	0.000	0.000	0.058	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
	b	0.500	0.500	0.500	0.667	0.708	1.000	1.000	1.000	0.942	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000
	n	25	25	34	25	24	25	25	24	26	19	17	8	25	25	8	14	25
	H_o	1.000	1.000	1.000	0.500	0.875	0.000	0.000	0.000	0.115	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
Sod-1	a	0.50	0.50	0.50	0.67	0.67	1.00	1.00	1.00	1.00	1.00	na	1.00	1.00	1.00	1.00	1.00	1.00
	b	0.50	0.50	0.50	0.33	0.33	0.00	0.00	0.00	0.00	0.00	na	0.00	0.00	0.00	0.00	0.00	0.00
	n	25	25	26	25	18	25	25	24	26	19	na	8	25	25	8	14	25
	H_o	1.000	1.000	1.000	1.000	1.000	0.000	0.000	0.000	0.000	0.000	na	0.000	0.000	0.000	0.000	0.000	0.000
Tpi-1	a	0.000	0.000	0.000	0.333	0.333	0.000	0.000	0.000	0.000	0.000	na	0.000	0.00	0.000	0.000	0.000	0.00
	b	0.500	0.500	0.500	0.333	0.333	0.000	0.000	0.042	0.000	0.605	na	0.000	0.020	0.600	0.625	0.214	0.000
	c	0.500	0.500	0.500	0.333	0.333	0.360	0.000	0.000	0.019	0.395	na	1.000	0.00	0.000	0.000	0.000	1.000
	d	0.000	0.000	0.000	0.000	0.000	0.640	1.000	0.958	0.981	0.000	na	0.000	0.980	0.400	0.375	0.786	0.000
	n	25	25	25	25	25	25	25	24	26	19	na	8	25	25	8	14	25
	H_o	1.000	1.000	1.000	1.000	1.000	0.000	0.000	0.000	0.038	0.053	na	0.000	0.020	0.000	0.250	0.000	0.000

progenitors can be difficult to discern; likewise, polyploidy resulting from a single event that with subsequent recombination, gene flow between polyploids or diploids, or mutations can cause an overestimate of the number of events of polyploidization (Wallace 2003). However, variation in dosage of alleles at fixed heterozygous loci is the result of multiple polyploidization events from a polymorphic progenitor (Wyatt et al. 1989). Although our data reveal multiple origins for *E. edwardsii,* they also suggest that tetraploids and hexaploids arose from hybridization events involving the same two or three progenitors, respectively.

We also provide evidence suggesting that *E. penlandii* is the closest living descendant of one of the diploid progenitors of *E. edwardsii* (Paun et al. 2009). Although we are unable to identify the closest living descendant(s) of the other progenitor(s), attempts at genome reconstruction coupled with the number of loci at which fixed heterozygosity was observed suggest that they were genetically divergent representatives of *Eutrema* s.l. Given the morphological similarity of *E. penlandii* and *E. edwardsii*, it seems likely that the progenitors were morphologically cryptic, despite genetic differentiation, as has been demonstrated in other genera, such as *Draba* (Grundt et al. 2005). Although phylogenetic analyses conducted by Hao et al. (2015) recovered a clade comprising *Eutrema heterophyllum* (W.W. Sm.) H.Hara, *Eutrema racemosum* Al-Shehbaz, G.Q.Hao & J.Q.Liu, *E.* edwardsii, and *Eutrema schulzii* Al-Shehbaz & Warwick, these results do not inform the present study. Regardless, given the genetic differentiation that we see among the progenitors, it seems likely that hybridization resulted in increased unreduced gamete formation in the hybrids, with whole genome duplication resulting from the fusion of these unreduced gametes (Paun et al. 2009). Different factors have been correlated with unreduced gamete formation (Ramsey and Schemske 1998; Mason and Pires 2015), several of which may have contributed to the formation of *E. edwardsii* (e.g., hybridization, stress).

Polyploidy—both autopolyploidy and allopolyploidy—is extremely common in the Brassicaceae, with well over one-third of all species in the family purportedly polyploid, ranging from paleopolyploids to neopolyploids (Warwick and l-Shehbaz 2006, Marhold and Lihová 2006). Polyploidy is also extremely common in the flora of the arctic, where fluctuations in climate have resulted in cycles of fragmentation and range expansion (Brochmann et al. 2004). Genetic drift, exacerbated by selfing, would have resulted in population differentiation, which has been correlated with an increased ability to colonize new sites associated with deglaciation (Brochmann et al. 2004).

As previously discussed, taxonomic treatments of *E. penlandii* differ markedly, with the recent treatments of Al-Shehbaz and Warwick (2005), Al-Shehbaz (2010), and Weber and Wittmann (2012) subsuming *E. penlandii* into *E. edwarsdsii*. However, genetic data presented herein reveal that the taxonomic boundaries between *E. edwardsii* and *E. penlandii* are obscured due to a progenitor–derivative relationship involving allopolyploidy and morphologically cryptic progenitors.

Eutrema penlandii is purportedly a relictual Arcto-Tertiary element in the Colorado flora (Weber 2003), with the greatest species diversity within the genus found in Asia. Given the strong evidence for an allopolyploid origin for *E. edwardsii*, the diploid progenitors were presumably sympatric in high latitudes at the end of the Tertiary and during the Pleistocene, when hybridization coupled with unreduced gamete formation—we found no evidence of somatic doubling (e.g., mixoploidy)—and selfing resulted in whole genome duplication and the cytotypes observed in the polymorphic, polyploid *E. edwardsii*. Given the genetic differences deduced from the allozyme data, the progenitor taxa were genetically well differentiated, much more so than expected for sibling species. While morphological boundaries between *E. penlandii* and *E. edwardsii* are admittedly subtle, morphometric analyses of 24 continuous, quantitative vegetative and reproductive characters (e.g., plant height, cauline leaf size, fruit size) have revealed differences that are inconsistent with a null hypothesis of no difference between the two species (Fayette and Bruederle 2001, E Pansing and LP Bruederle unpublished data). This uncoupling between morphological and genetic differentiation highlights the importance of recognizing cryptic species, especially with regard to polyploid complexes. Polyploidization between genetically divergent species has immediate consequences for genetic diversity of the progeny. Fixed heterozygosity can preserve genetic diversity when drift occurs due to founder effect or when plants self (Abbott and Brochmann 2003). Populations of autopolyploids, however, will be more susceptible to loss of genetic diversity due to polysomic inheritance coupled with drift or selfing. Regardless, multiple forms of evidence, in addition to the cytotype differences reported herein—molecular sequence data, morphology, and distribution—reveal *E. penlandii* to be distinct. Furthermore, it is reproductively isolated within *Eutrema edwardsii* s.l. and, as such, we propose recognition of this taxon at the species level, despite it being morphologically cryptic.

Finally, although we studied only tetraploid and hexaploid populations of *E. edwardsii*, we cannot exclude the possibility that additional taxa were involved in the formation of this taxon, nor can we make conclusions regarding the origin of octaploid populations. Although octaploidy has been reported for this taxon (Knaben 1968), no octaploid populations were included as part of this research and, as such, we are unable to address its origin here.

Ongoing research addressing polyploidy in *E. edwardsii* s.l. includes: (1) screening populations of *E. penlandii* for polyploid individuals which, if present, would be autopolyploid in

origin; (2) mapping cytotypes across the range of *E. edwardsii*; (3) ecological niche modeling of cytotypes comprising the *E. edwardsii* species complex to detect ecological differentiation; and (4) developing a phylogeny for the complex, recognizing that with allopolyploidy it is expected to reticulate. Although our focus has been on the *E. edwardsii* species complex, specifically, we recommend that future systematic research addressing the genus be informed by documentation of ploidy for the taxa under consideration and, in the case of polyploid taxa, documentation of their mode of origin.

Acknowledgements We acknowledge James Salmen (Director of Facilities and Laboratory Manager, University of Colorado Denver), who provided technical support with respect to flow cytometry, and Eileen Yakish, Hannah Tystad, and Daniel Harper, then undergraduates at CU Denver, who collected preliminary allozyme data for this study. The research was supported by a contract awarded to LPB by the U.S. Fish and Wildlife Service (F10AC00603).

Conflict of interest The authors declare that they have no conflict of interest.

References

Abbott RJ, Brochmann C (2003) History and evolution of the arctic flora: in the footsteps of Eric Hultén. Molec Ecol 12:299–313

Aiken SG, Dallwitz MJ, Consaul LL, McJannet CL, Boles RL, Argus GW, Gillett JM, Scott PJ, Elven R, LeBlanc MC, Gillespie LJ, Brysting AK, Solstad H, Harris JG (2007) Flora of the Canadian arctic archipelago: descriptions, illustrations, identification, and information retrieval. NRC Research Press, National Research Council of Canada, Ottawa. Available at: http://nature.ca/aaflora/data. Accessed 19 Apr 2017

Al-Shehbaz IA (2010) *Eutrema* R. Brown. In: Flora of North America Editorial Committee (eds) Flora of North America North of Mexico, vol. 7. Oxford University Press, New York, pp 555–556

Al-Shehbaz IA, Warwick SI (2005) A synopsis of *Eutrema* (Brassicaceae). Harvard Pap Bot 10:129–135

Bickford D, Lohman DL, Sodhi NS, Ng PKL, Meier R, Winker K, Ingram KK, Das I (2006) Cryptic species as a window on diversity and conservation. Trends Ecol Evol 22:148–155. doi:10.1016/j.tree.2006.11.004

Brochmann C, Brysting AK, Alsos IG, Borgen L, Grundt HH, Scheen AC, Elven R (2004) Polyploidy in arctic plants. Biol J Linn Soc Lond 82:521–536

Doležel J, Greilhuber J, Suda J (2007) Estimation of nuclear DNA content in plants using flow cytometry. Nat Protoc 2:2233–2244. doi:10.1038/nprot.2007.310

Fayette K, Bruederle LP (2001) Morphometric analyses support specific status for *Eutrema penlandii* Rollins (Brassicaceae). In: Botany 2001, Abstracts, 12–16 Aug 2001, Albuquerque, New Mexico. Botanical Society of America, St. Louis, p 112

German DA, Koch MA (2017) *Eutrema salsugineum* (Cruciferae) new to Mexico: a surprising generic record for the flora of Middle America. PhytoKeys 76:13–21. doi:10.3897/phytokeys.76.9731

Grundt HH, Obermayer R, Borgen L (2005) Ploidal levels in the arctic-alpine polyploidy *Draba lacteal* (Brassicaceae) and its low-ploid relatives. Bot J Linn Soc 147:333–347

Grundt HH, Kjølner S, Borgen L, Rieseberg LH, Brochmann C (2006) High biological species diversity in the arctic flora. Proc Natl Acad Sci USA 103:927–975. doi:10.1073/pnas.0510270103

Hao G, Al-Shehbaz IA, Wang Q, Liang Q, Liu J (2015) *Eutrema racemosum* (Eutremeae, Brassicaceae), a new tetraploid species from southwest China. Phytotaxa 224:185–195

Hao G, Bi H, Li H, He Q, Ma Y, Guo X, Ma T (2016) The whole chloroplast genomes of two Eutrema species (Brassicaceae). *Mitochondrial* DNA Part A 27:3727–3728. doi:10.3109/19401736.2015.1079877

Hardwick RC (1997) Population genetics of the rare alpine endemic *Eutrema penlandii* Rollins (Brassicaceae). MS Thesis, University of Colorado Denver, Denver

Jørgensen CA, Sørensen T, Westergaard M (1958) The flowering plants of Greenland: a taxonomical and cytological survey. Kongel Danske Vidensk Selsk Biol Skr 9:1–172

Knaben G (1968) Chromosome numbers of flowering plants from central Alaska USA polyploidy. Nytt Mag Bot 15:240–254

Marhold K, Lihová J (2006) Polyploidy, hybridization and reticulate evolution: lessons from the Brassicaceae. Pl Syst Evol 259:143–174. doi:10.1007/s00606-006-0417-x

Mason AS, Pires JC (2015) Unreduced gametes: meiotic mishap or evolutionary mechanism. Trends Genet 31:5–10. doi:10.1016/j.tig.2014.09.011

Nielsen G, Johansen HB (1986) Proposal for the identification of barley varieties based on the genotypes for 2 hordein and 39 isoenzyme loci of 47 reference varieties. Euphytia 35:717–728

Paun O, Forest F, Fay ME, Chase MW (2009) Hybrid speciation in angiosperms: parental divergence drives ploidy. New Phytol 182:507–518. doi:10.1111/j.1469-8137.2009.02767.x

Ramsey J, Schemske DW (1998) Pathways, mechanisms, and rates of polyploidy formation in flowering plants. Annual Rev Ecol Syst 29:467–501

Soltis PS, Soltis DE (2000) The role of genetic and genomic attributes in the success of polyploids. Proc Natl Acad Sci USA 97:7051–7057

Soltis DE, Haufler CH, Darrow DC, Gastony GJ (1983) Starch-gel electrophoresis of ferns—a compilation of grinding buffers, gel and electrode buffers, and staining schedules. Amer Fern J 73:9–27

Soltis DE, Soltis PE, Schemske DW, Hancock JF, Thompson JN, Husband BC, Judd WS (2007) Autopolyploidy in angiosperms: have we grossly underestimated the number of species. Taxon 56:13–30

Soltis PS, Liu X, Marchant DB, Visger CJ, Soltis DE (2014) Polyploidy and novelty: Gottlieb's legacy. Phil Trans Roy Soc B 369:20130351. doi:10.1098/rstb.2013.0351

Wallace LE (2003) Molecular evidence for allopolyploid speciation and recurrent origins in *Platanthera huronensis* (Orchidaceae). Int J Pl Sci 164:907–916

Warwick SI, Al-Shehbaz IA (2006) Brassicaceae: chromosome number index and database on CD-Rom. Pl Syst Evol 259:237–248. doi:10.1007/s00606-006-0421-1

Warwick SI, Al-Shehbaz IA, Sauder CA (2006) Phylogenetic position of Arabis arenicola and generic limits of Aphragmus and Eutrema (Brassicaceae) based on sequences of nuclear ribosomal DNA. Canad J Bot 84:269–281. doi:10.1139/B05-161

Weber WA (1985) New names and combinations, principally in the Rocky Mountain USA Flora V. Phytologia 58:382–384

Weber WA (2003) The Middle Asian Element in the Southern Rocky Mountain flora of the western United States: a critical biogeographical review. J Biogeogr 30:649–685

Weber WA, Wittmann RC (2012) Colorado Flora: eastern slope, 4th edn. A field guide to the vascular plants. University Press of Colorado, Boulder

Wyatt R, Stoneburner A, Odrzykoski IJ (1989) Bryophyte isozymes: systematics and evolutionary implications. In: Soltis DE, Soltis PS (eds) Isozymes in plant biology. Dioscorides Press, Portland, pp 221–240

Xiao Y, Li C, Hsieh TY, Tian DK (2015) *Eutrema bulbiferum* (Brassicaceae), A new species with bulbils from Hunan, China. Phytotaxa 219:233–242