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NOTES AND COMMENTS

Characterization of diverse ploidy in the arctic-alpine *Arenaria ciliata* species complex (Caryophyllaceae) using shoot meristem staining and flow cytometry analysis of archived frozen tissue

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Abstract

Ploidy levels were analyzed in 21 European populations of the *Arenaria ciliata* complex using baseline chromosome counts derived from Feulgen staining of HCl-treated shoot meristems and calibrated flow-cytometry analysis of fresh and archival frozen tissue. Calibration with two to three control samples of different ploidy facilitated rapid identification of ploidy states in unknown samples. Observed ploidy levels varied from 2N = 40–200, with the majority of populations showing 2N = 40–80. High-altitude populations collectively showed the full range of ploidy states, but at low elevations only lower ploidy levels were observed. Populations with the highest observed ploidy contained the greatest observed phylogenetic diversity in the western and eastern Alps. Multiple polyploidization events are inferred in the continental European metapopulation, with lower, more stable ploidy characteristic of the west and north. The method deployed provides an effective approach to ploidy analysis for archival desiccated/frozen tissue samples from biogeographic collections.

Keywords: chloroplast, Holocene, phylogeography, polyploidy, postglacial.

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Introduction

The arctic-alpine *Arenaria ciliata* species complex (Caryophyllaceae) comprises a group of poorly differentiated herbaceous taxa exhibiting a range of overlapping morphological and ploidy identities (Wyse-Jackson & Parnell 1987; Tutin *et al*. 1993; Dang 2012; Berthouzoz *et al*. 2013; Howard-Williams 2013; Walker *et al*. 2013). The group has a wide but fragmented distribution across Europe, extending from high alpine habitats in the mountains of central Europe and Iberia to sea-level habitats in the arctic. Morphometric analysis implies three intergrading taxa within the complex, *A. ciliata* s.s. L., *A. gothica* Fr. and *A. norvegica* Gunn., comprising multiple subspecific identities, (including *A. ciliata* subsp. *pseudofrigida* Ostenf. & O. C. Dahl, subsp. *bernensis* Favarger and *A. gothica* subsp. Moehringioides (Murr) M.B. Wyse Jacks. & J. Parn.); however, the morphological traits that distinguish these taxa are continuous rather than discrete, and no taxon displays floral, fruit or ploidy traits that uniquely set it apart (Wyse-Jackson & Parnell 1987).

This lack of taxonomic distinction is not unusual for arctic-alpine taxa: many species show weak morphological differentiation among biogeographic types, a characteristic often associated with repeated refugial isolation and migrant mixing during Quaternary glacial cycles (Bennett 2004). Ploidy levels are also highly variable among arctic-alpine species, with a higher proportion of polyploids across multiple familial lineages compared

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with other ecogeographic plant categories (Bliss 1971; Brochmann *et al*. 2004). This phenomenon has long been viewed as an outcome of selective pressure during post-glacial recolonization, where polyploid identities (combined in part with apomixis) are seen to facilitate adaptive leading-edge population dispersal into novel habitats (Bliss 1971), and there is now some empirical support for this proposition (Schinkel *et al*. 2016).

In their *Flora Europea* account of *Arenaria,* Tutin *et al*. (1993) note the lack of clarity regarding the *A. ciliata* complex, and among the taxa they recognize in the group, they report intergrading ploidy ranges of 2N = 40, 80, 100, 120, 160, 200 and 240. Recent molecular analysis of chloroplast haplotype identities in populations across Europe has revealed that *A. ciliata* group taxa share a complex matrilineal history that does not reconcile with this taxonomic and ploidy account, with multiple inferred migration and hybridization events between biogeographic subpopulations (Dang 2012; Dang *et al*. 2012). Because differential ploidy levels represent a similar reproductive barrier between populations as spatial and elevational isolation, validated ploidy estimates are necessary to allow a clear resolution of biogeographic history in the group.

Flow-cytometry methodology has greatly improved the acquisition of ploidy data, allowing expanded surveys using standard samples of known ploidy to calibrate genome size in fresh and desiccated samples of unknown identity according to metaphase stages G_1 and G_2 (Galbraith *et al*. 1983; Suda *et al*. 2007). Baseline ploidy counts for calibration are first achieved *via* microscopy staining, and in some cases this can be a significant bottleneck in the procedure, depending on the species. Root meristems are the preferred source as they provide an ideal supply of cells at appropriate stages in the cell-division cycle (Ahloowalia 1965); however, root tips, by nature hard and durable, can be very difficult to disrupt for certain taxa without excessive damage to cellular integrity. In the case of the *Arenaria ciliata* complex, as for other species, the alternative is to use shoot meristems (Kitajima *et al*. 2001). Tissue disruption and exposure of dividing cells is usually achieved after pretreatment with either enzymatic digestion (Schwarzacher & Leitch 1994) or hydrochloric acid (Ma *et al*. 1996), followed by staining using fuchsin, Fuelgen, DAPI or propidium iodide.

Here we present a new evaluation of ploidy levels across the *A. ciliata* complex, based on a novel composite protocol modified from several sources, specific to individuals with known genetic, population and morphological identities (Dang *et al*. 2012) and for which frozen desiccated tissue samples are stored in archive. Baseline ploidy counts for control samples have been achieved using HCl-mediated tissue digestion of shoot meristems (after Ma *et al*. 1996; Kitajima *et al*. 2001), followed by Feulgen cell staining and visualization using confocal microscopy (Hepler & Gunning 1998). Flow cytometry analysis has utilized propidium iodide staining of unknown and calibrated control samples, with samples drawn from living and archival frozen tissue collections (after Galbraith *et al*. 1983; Suda *et al*. 2007).

Materials and methods

Sample populations

Sampling from *A. ciliata* complex populations comprised two tissue sources: (i) fresh tissue from collected wild plants and germinated wild seedlings growing at Maynooth University plant growth facilities, and (ii) archival plant tissue previously desiccated with silica gel and stored at −20°C in the years 2008–2012 (Table 1). The 21 sampled populations and 52 individual plants (Table 1) covered the core geographic range of the species complex in Europe from Iberia to Svalbard and from Iceland to Austria, with the exception of isolated populations in Greenland to the extreme northwest and the Carpathian mountains to the southeast, and included, following the taxonomy of Wyse-Jackson and Parnell (1987), *A. ciliata* s.s., *A. ciliata* subsp. *pseudofrigida*, *A. ciliata* subsp. *bernensis*, *A. gothica* subsp. *moerhingioides* and *A. norvegica* subsp. *norvegica*.

Confocal microscopy

Newly developed shoots were cut from living plants (*A. ciliata* s.s., *A. norvegica* and *A. ciliata* subsp. *Bernensis*; Table 2) and stored in distilled sterile water for 24 h at 4°C. For pretreatment, axillary and apical buds were then cut from the tissue using a dissecting razor and placed in 1.5-mL tubes containing 0.002 mol/L 8-hydroxyquinoline solution (Sigma, Arklow, Ireland) for 4 h at 20°C. Fixation was carried out in a mixture of 98% 3:1 absolute ethanol: glacial acetic acid (Carnoy's solution) for at least 1 h at 4°C. Buds were then washed with distilled water for 5 min. Bud hydrolysis was completed with a solution of 1N HCl (Sigma) at 60 C for 5–10 min. Following a 2-min rinse in distilled water, buds were incubated in 50% Schiff's reagent (Feulgen stain) (VWR Chemicals, Leuven, Belgium) for 20 min at room temperature, and then washed with 45% acetic acid three times for 5 min each time. Buds were then transferred to a clean slide and covered with 45% acetic acid to prevent drying, and from this stock, one or two buds were placed on a new glass slide and covered with a small drop of acetic acid. Under a dissecting microscope, epidermis cells were carefully removed by using forceps and a scalpel blade. Using a teasing needle and scalpel, exposed meristem cells were then separated out as much as possible to form a single layer to enable clear identification of individual cells upon squashing, and then a cover slip was applied. A piece of filter paper was placed over the cover slip and then

Table 1 Individual and population samples from the *A. ciliata* complex included in the present study. Taxonomic identities follow Wyse-Jackson and Parnell (1987)

Taxon identity	Population	Country	Location	Latitude	Longitude	Elevation (m)
A. ciliata subsp. ciliata	Ac1	Ireland	King's Mountain, Co. Sligo	N54°20.672′	W08°27.373′	490
	Ac2	Ireland	Gowlaun Valley, Co. Sligo	N54°21.353′	W08°27.290′	475
	Ac3	Ireland	Glencarbury Mine, Co. Sligo	N54°21.549′	W08°24.044′	475
	Ac6	Italy (W. Alps)	Refugio Mongioe, Piemote	N44°09.864′	E07°47.201′	2057
	Ac8	Italy (W. Alps)	Colle delle'Angelo, Piemonte	N44°40.709′	E06°59.484′	2475
	Ac16	Austria (E. Alps)	Niedere Tauren, Steiermark	N47°16.270′	E14°21.210′	2135
	Ac17	Austria (E. Alps)	Karawanken, Karten	N46°30.200′	E14°29.120′	2100
	Ac19	Switzerland (W. Alps)	Daubensee, Bernese Alps	N46°25.165	E07°37.478′	2257
	Ac20	Austria (E. Alps)	Schneealp, Styria	N47°41.570′	E15°36.36	1720
A. ciliata spp. bernensis	Ab1	Switzerland (W. Alps)	Les Galeres, Canton Fribourg	N46°53.630′	E07°15.363′	2175
	Ab2	Switzerland (W. Alps)	Dent de Brenleire, Canton Fribourg	N46°55.263′	E07°17.358′	2100
A. ciliata spp. pseudofrigida	Ap1	Norway	Svalbard	N78°54.480′	E12°04.70′	200
	Ap3	Norway	Svalbard	N78°53.410′	E16°21.50′	100
A. gothica subsp. moehringioides	Ac10	Spain (Iberia)	Valle de Benasque, Aragon	N42°40.957′	E00°36.010′	1804
	Ac13	Spain (Iberia)	Hospital de Benasque, Aragon	N42°41.408′	E00°36.950′	1834
	Ac14	Spain (Iberia)	Cabana Veronica, Picos de Europa, Cantabria	N43°10.644′	W04°49.967′	2325
	Ac15	Spain (Iberia)	Corarrobres, Picos de Europa, Cantabria	N43°09.374′	W04°48.213′	1933
A. norvegica	An1	Ireland	Black Head, Co. Clare	N53°08.243′	W09°16.048′	60
	An5	Scotland	Inchnadamph, Highlands	N58°07.493′	W04°55.374′	380
	An8	Iceland	Fjallsarlon, Hofn	N64°00.431′	W16°22.200′	20
	An9	Norway	Tromso, Troms	N69°38.570′	E18°57.190′	14

Table 2 Confocal microscopy and flow cytometry-recorded ploidy levels in the *A. ciliata* group

Taxon identity	Cited ploidy†	Population	*n*	Ploidy analysis‡			Recorded ploidy level					
				Microscopy Source tissue	Flow cytometry Source tissue	Internal control	40	60	80	120	160	200
A. ciliata subsp. ciliata	*40, 80, 120, 160*	Ac1	*2*		frz	Ac3 [40]	2					
		Ac2	*4*		frz	Ac3 [40]	4					
		Ac3	*7*	f x 4	f/ frz	An9 [80], Ab1 [200]	7					
		Ac6	*4*		frz	Ac3 [40]	3		1			
		Ac8	*1*		frz	An9 [80]	1					
		Ac16	*3*		frz	Ab2 [200]					1	2
		Ac17	*3*		frz	Ab2 [200]				1	2	
		Ac19	*2*		frz	Ac3 [40], Ab1 [200]	2					
		Ac20	*1*		f	Ab2 [200]						1
A. ciliata subsp. bernensis	*200, 240*	Ab1	*1*	f	f/ frz	Ac3 [40], An9 [80]						1
		Ab2	*2*		f/ frz	Ab1 [200]						2
A. ciliata subsp. pseudofrigida	*40*	Ap1	*2*		f/frz	Ac3 [40], An9 [80]	2					
		Ap3	*1*		f	Ac3 [40]	1					
A. gothica subsp. moehringioides	*100*	Ac10	*3*		frz	Ac3 [40], An9 [80]	2		1			
		Ac13	*2*		frz	Ac3 [40], An9 [80]	2					
		Ac14	*2*		frz	Ac3 [40], An9 [80]	2					
		Ac15	*4*		frz	Ac3 [40], An9 [80]	2		2			
A. norvegica	*80*	An1	*3*	f	f/ frz	An9 [80]			3			
		An5	*1*		frz	An1 [80], An9 [80]			1			
		An8	*1*		frz	Ac3 [40], Ab1 [200]		1				
		An9	*4*		f/frz	Ac3 [40], Ab1 [200]			4			
Total							*30*	*1*	*12*	*1*	*3*	*6*	***52***

† Tutin *et al.* (1993). ‡ Tissue sample preparation: fresh (f) or frozen (frz).

pressed firmly with the thumb to flatten cells and remove excess acetic acid. Using an Olympus FV1000 confocal microscope (Olympus Europa GMBH, Hamburg) under standard PI (propidium iodide) excitation settings, Fuelgen-stained chromosomes were then counted by reviewing layered three-dimensional cell-section images, an approach that minimizes halation-related miscounting.

Flow cytometry

Following the protocol of Galbraith *et al*. (2001), 10 mg of fresh leaf tissue or 0.3 mg dried tissue from each sample was cut and placed in a sterile Petri dish; 0.4 mL of cooled 4°C homogenization buffer was added to this for every 10 mg wet or 0.3 mg dried tissue, and using a single-edge razor blade, tissues were homogenized to release the nuclear material. Following filtration through a 20-μm nylon mesh, the resulting suspension was incubated for 5 min at room temperature with 1 mg/mL stock solution of Type IIA RNAse A (Sigma) to a final sample concentration of 10 μg/mL, to remove RNA, after which propidium iodide stock solution (1 mg/mL) was added to each sample to a final concentration of 100 μg/mL, and samples were incubated for a further 10 min at room temperature for staining prior to flow cytometric analysis.

Three internal size standard samples, validated using confocal microscopy chromosome counts, were used to calibrate each run. Depending on anticipated ploidy levels for the unknown sample, each analysis run included either three or five samples: the unknown sample, either one or two individual internal size standard samples (for anticipated ploidy levels $<$ or $>$ 2N = 80, respectively) and one or two mixed samples that combined the unknown sample and the size standard samples. Prior to analysis it was established (using multiple tissue samples harvested from the same individual plant) that 0.3 mg of frozen desiccated tissue yielded equivalent quality and quantity of PI staining of nuclear material compared with the protocol-recommended fresh tissue starting weight of 10 mg, and this ensured that comparable levels of nuclear staining were possible for all test and standard samples in each run. To maximize accuracy, standard calibration samples were desiccated and frozen prior to use in analysis of archive samples. Between-run consistency was checked and where necessary re-established, using repeat analyses of test samples.

Results

Direct counting of chromosome numbers in control samples using confocal microscopy

Storage of excised bud tissue for 4–24 h at 4°C prior to pretreatment positively affected the metaphase index in the meristem (aiding chromosome staining), with the maximum gain evident after 24 h. This protocol yielded superior results compared with multiple alternative treatments, including modified HCl and/or enzymatic digestion preparations, and the use of propidium iodide, DAPI, acetic-orcein and aceto-carmine staining (after the protocols of Ahloowalia 1965; Kitajima *et al*. 2001; García-Fernandez *et al*. 2012; Kirov *et al*. 2014). Preparation of root meristem tissue from the target taxa using the same array and permutation of techniques failed to produce effective chromosome staining, due to the durable hardness of root tissue and also a low metaphase index in any exposed stained cells, despite cold temperature pretreatment.

Staining of chromosomes in mid-late metaphase shoot meristem cells was most successful in the early spring growth period (February to May), prior to extensive tissue bud expansion. Compared with DAPI, which generated very poor staining profiles (data not shown), Feulgen stain generated high light emission and clear color contrast in the 555–655 nm (propidium iodide) wavelength, and was selected for staining. Chromosome counts were confirmed by reviewing the layered three-dimensional cell-section images generated on the Olympus FV-1000. Staining was completed for *A. ciliata* (Ireland, four samples), *A. norvegica* (Norway, one sample) and *A. ciliata subsp. bernensis* (Switzerland, one sample), revealing ploidy levels of 2N = 40, 80 and 200, respectively (Table 2, Fig. 1); this provided validated chromosome counts that covered a range of expected ploidy levels across the *A. ciliata* complex as indicated in the literature.

Calibrated flow cytometry analysis of ploidy across the A. ciliata *group*

Ploidy levels were estimated in a total of 52 samples from 21 populations (Table 2). Thirteen populations were sampled solely from archived frozen tissue samples, whereas the remaining eight populations were sampled from fresh tissue or a combination of fresh and frozen tissue (Table 2). Internal calibration facilitated ploidy estimation in all tested samples (Fig. 2a–e), in some cases following re-analysis with additional calibration where the ploidy level inferred for a test sample from metaphase G_1 and G_2 peaks was equivocal. Some variation in peak amplitude emissions for metaphase G_1 and G_2 peaks was recorded in individual samples between repeat runs, due mainly to differences in final sample concentration; however, shifts in peak emission position between runs were minimized, and were always consistent with shifts in the position of calibration sample peaks. Fresh and frozen tissue generated near-identical G_1 and G_2 peak positions for all samples (Fig. 2a,b), with

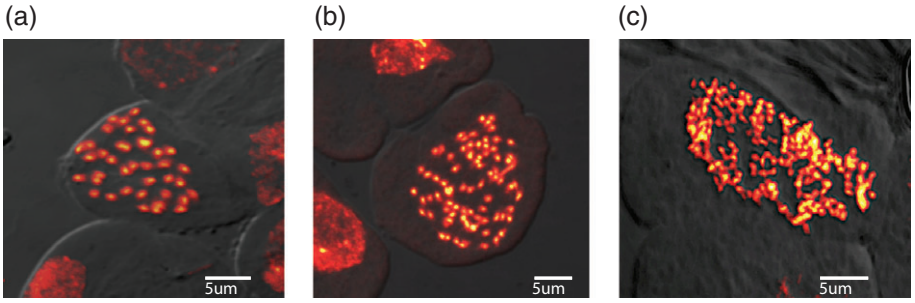

Fig. 1 Confocal micrograph images of Fuelgen-stained late metaphase chromosomes in (a) *Arenaria ciliata* subsp. *ciliata*, (b) *Arenaria norvegica* and (c) *Arenaria ciliata* subsp. *bernensis*. Image scale 1000×, produced on an Olympus FV1000 Confocal Microscope suite at 543-nm laser excitation wavelength.

mean CV (coefficient of variance) values of 3.70 for fresh samples and 2.81 for frozen samples.

Recorded ploidy levels varied from 2N = 200 to 2N = 40 (Table 2, Figs 2–3). Among the analyzed taxa, *A. ciliata* subsp. *pseudofrigida* (2N = 40) and subsp. *bernensis* (2N = 200) were invariant, whereas *A. gothica* subsp. *moehringioides* and *A. norvegica* both showed two ploidy states (40, 80 and 60, 80, respectively), and *A. ciliata* subsp. *ciliata* showed five ploidy states (40, 80, 120, 160, 200) (Table 2). Among populations, 16 showed only one ploidy state (three of which were single samples); the remaining five showed two ploidy states (Table 2, Fig. 3a). Testing for association between sampling variables, the strongest correlation was observed between observed ploidy and site longitude (Rho, −0.44; $P < 0.05$; Spearman rank-order correlation test). Latitude and ploidy showed the weakest correlation (Spearman's Rho, 0.12; $P > 0.05$), whereas altitude and ploidy showed a stronger, but non-significant, correlation (Spearman's Rho; 0.26; $P > 0.05$).

When compared with the known rps16 haplotype identity for each individual sample (after Dang *et al.* 2012; Fig. 3b; Genbank Accessions 394309514-531), there is no significant correlation with observed ploidy (Spearman's Rho, 0.07; $P > 0.05$). At the population level, the high ploidy levels recorded for eastern Alps *A. ciliata* individuals (2N = 120–200) are associated with the widest phylogenetic variability in chloroplast haplotypes (Clade Ia, Ib and III, Fig. 3b) for any population group. A similar wide phylogenetic pattern is observed for the 2N = 200 *A. ciliata* subsp. *bernensis* population in the Swiss (western) Alps, with recorded haplotypes from Clade Ia and II. High-elevation populations in western continental Europe show lower ploidy levels and reduced phylogenetic variation compared with those in the east: in Iberia, *A. gothica* subsp. *moehringioides* individuals showing 2N = 80 belong to the Ib clade only, those showing 2N = 40 belong to either the Ia or Ib clades, whereas *A. ciliata* subsp. *ciliata* in the western Alps (2N = 40–80) belongs only to Clade II. Similarly, the northwestern 2N = 40–80 populations of *A. norvegica*, *A. ciliata* subsp. *ciliata* and subsp. *pseudofrigida* with typically lower elevation, appropriate to latitude (Fig. 3a), represent a much narrower phylogenetic range of haplotypes from Clade Ia, Ib or Ic only (Fig. 3b). Among haplotype lineages, the greatest range of observed host-plant ploidy levels is for Clade Ib Ac012 (2N = 80 and 200) and Clade II Ac009 (2N = 40, 80 and 200), in both cases the higher ploidy level being recorded from a biogeographically distinct population compared with the lower.

Discussion

In optimizing an array of previously tested approaches the methodology presented here facilitates a rapid *de-novo* appraisal of ploidy levels in a polyploid species complex. Utilization of HCl-treated shoot meristems and Feulgen staining enabled effective initial chromosome counting using three-dimensional confocal microscopy cell imaging, and frozen tissue from an archived collection assembled for a previous study contributed substantially and reliably to flow-cytometry data acquisition for the wider biogeographic distribution of the group. Relative positioning of sample and control peaks, and peak CV values, were consistent across flow cytometry runs, showing equivalence between fresh and desiccated/frozen samples, and in this sample set there were no observed instances of anomalous peaks that could be attributed to aneuploidy or endreduplication.

The recorded variation in ploidy levels across the *A. ciliata* complex are broadly in keeping with older published accounts; however, there appears to be a clear substructure in genetic identity across the continent (Table 2). This is especially evident when ploidy, haplotype, geographic location and elevation are considered in tandem (Fig. 3). In general, populations in the Alps are home to the greatest variation in ploidy levels, whereas western and northern populations are less variable. This east–west trend is also reflected in observed variation in haplotypes among different population and ploidy groups (Fig. 3b), where elevated ploidy levels in the east correspond with the highest within-population phylogenetic diversity.

In terms of addressing taxonomic classification and biogeographic identity within the group, these data

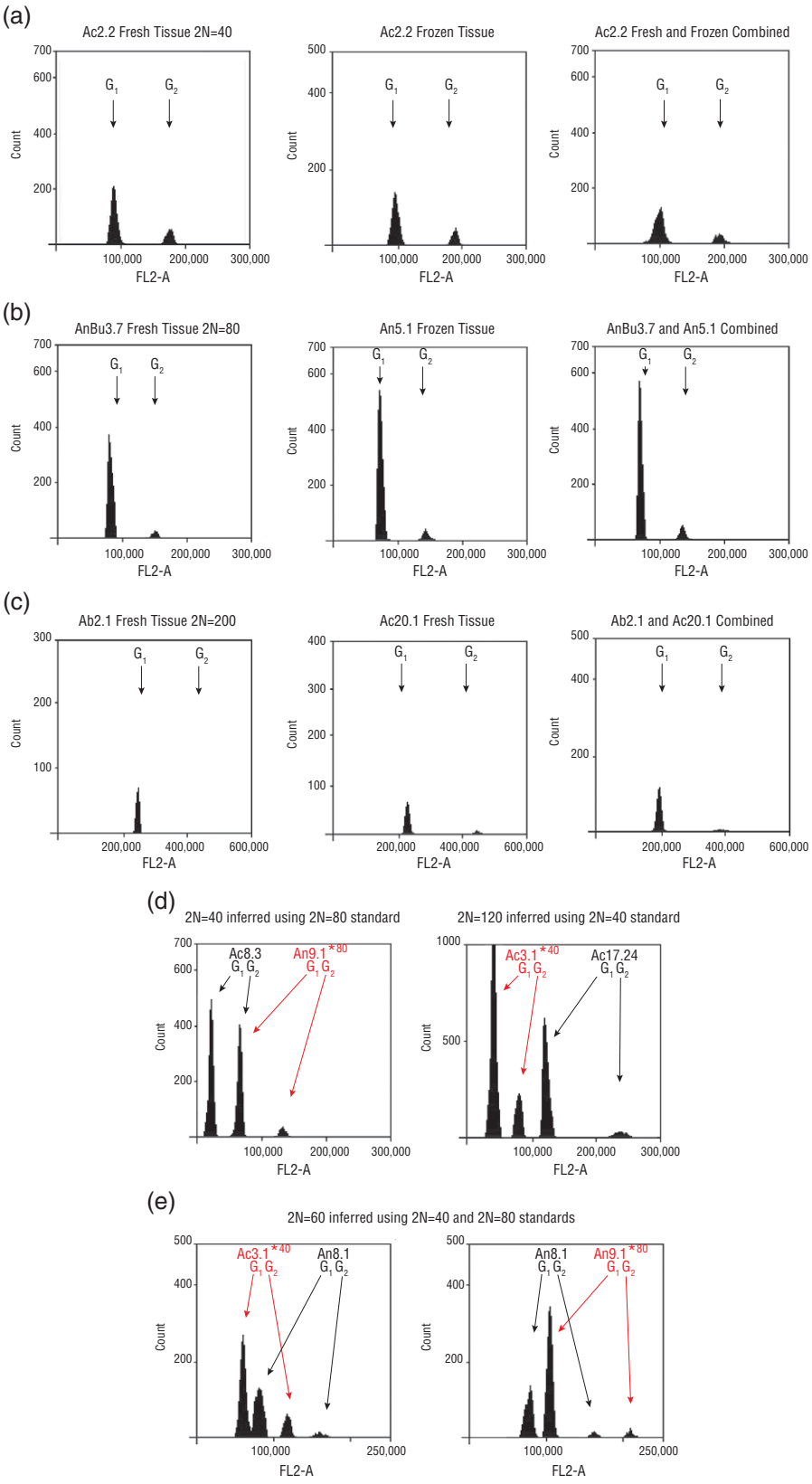

Fig. 2 Flow cytometry identification of ploidy levels in samples from the *A. ciliata* complex. G_1 and G_2 metaphase peaks in unknown samples are isolated in the FL2-A spectrum and compared with equivalent peak positions in known calibration standards. (a) Fresh and frozen tissue from *A. ciliata* subsp. *ciliata* Ac2.2 (population2.sample2) (Ireland), 2N = 40. (b) Fresh and frozen tissue from *A. norvegica* An1.7 (Ireland) and *A. norvegica* An5.1 (Scotland), both 2N = 80. (c) Fresh tissue standard *A. ciliata* subsp. *bernensis* Ab 2.1 (Switzerland) and fresh tissue sample *A. ciliata* subsp. *ciliata* Ac20.1 (Austria), both 2N = 200. (d) (left panel) Frozen tissue standard *A. norvegica* An9.1 (Norway) and frozen sample *A. ciliata* subsp. *ciliata* Ac8.3 (Italy), 2N = 80 and 40, respectively; (right panel) frozen tissue standard *A. ciliata* Ac3.1 (Ireland) and frozen sample *A. ciliata* subsp. *ciliata* Ac17.24 (Austria), 2N = 40 and 120, respectively. (e) Determination of intermediate ploidy state 2N = 60 in frozen *A. norvegica* An8.1 (Iceland), using frozen Ac3.1 (left panel) and frozen An9.1 (right panel).

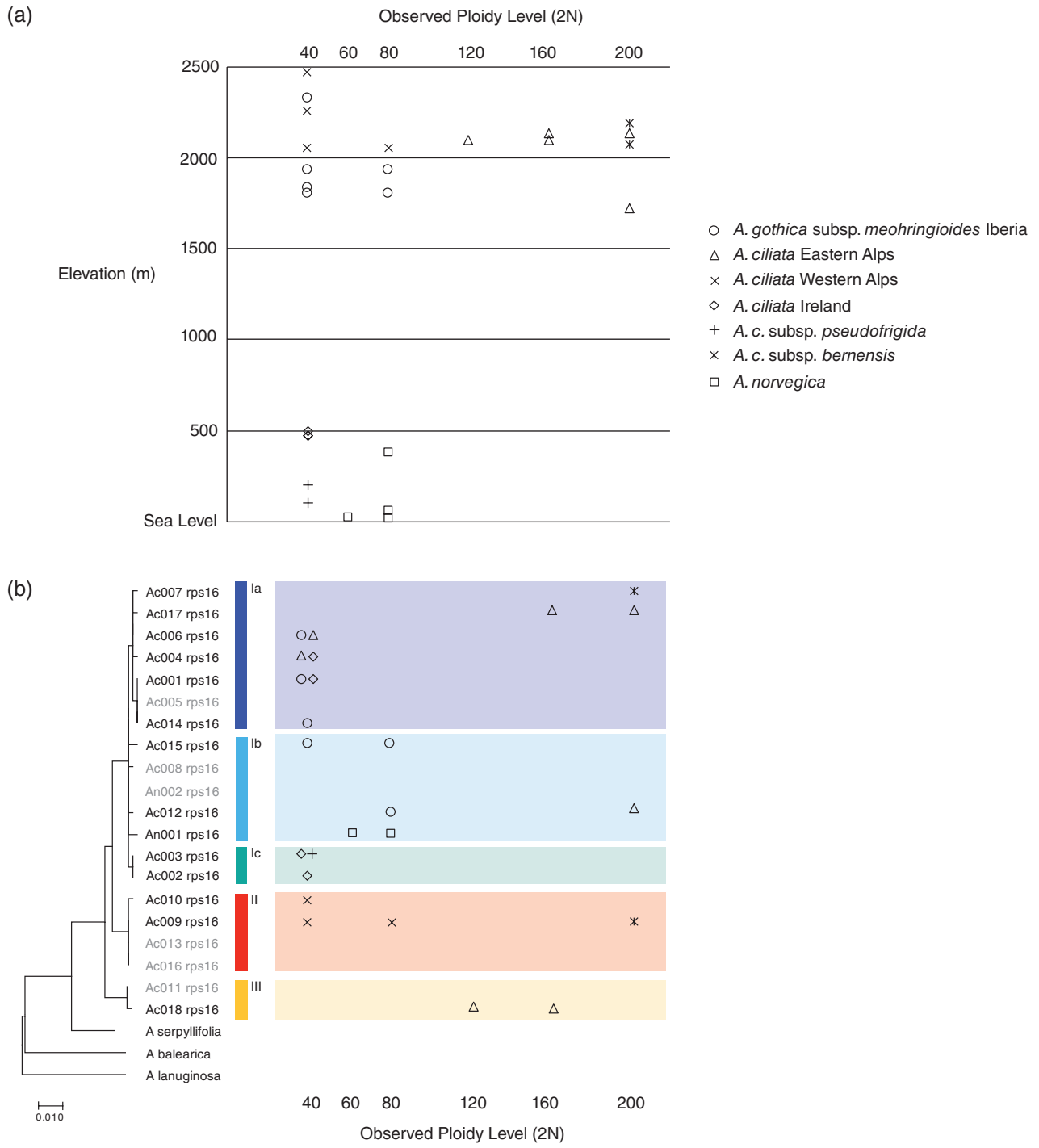

Fig. 3 (a) Recorded ploidy levels in sampled populations and taxa of the *A. ciliata* complex relative to population altitude. (b) Recorded ploidy levels in sample populations and taxa of the *A. ciliata* complex relative to known chloroplast haplotypes for each sample. Maximum likelihood evolutionary history (left) of all haplotypes identified by Dang *et al.* (2012), those included in the present study in bold, was inferred based on the Tamura-Nei model (log likelihood −1380.40) and implemented in MEGA 7.0. Branch lengths measured in the number of substitutions per site. All gaps and missing data were eliminated prior to analysis. Based on 1000 resampling runs, Clades I, II and II have > 95% bootstrap support; Subclades Ia-Ic, > 75% support.

affirm a number of key patterns. Although there is a considerable difference in elevational range between Iberian *A. gothica* populations and those of *A. norvegica* far to the north, the two groups share a common ploidy and phylogenetic identity that suggests a possible common origin. Similarly, *A. ciliata* subsp. *ciliata* in Ireland and

subsp. *pseudofrigida* in Svalbard share the same ploidy and haplotype identity, indicating a close ancestral relationship. *Arenaria ciliata* in the western Alps is distinguished from populations in the eastern Alps both by ploidy and haplotype identity, suggesting the two biogeographic groupings are distinct. In broad terms, these data indicate at least four genetic groupings within the species complex, each with a discrete distribution pattern.

Ploidy variation in this dataset is not clearly correlated with altitude, but two patterns are nonetheless clear: high-ploidy individuals have only been found at high elevation, and at low elevations only low-ploidy individuals are recorded (Fig. 3a). The majority of haplotype lineages are represented within high-elevation high-ploidy populations, with a slightly narrower range of identities represented in the lower-ploidy populations at high elevation, and the least range is evident in low-ploidy individuals that occur at low elevation (Fig. 3b).

This complex biogeographic structuring is not uncommon in arctic-alpine species. The impact of positive and negative selection during recolonization, combined with frequent population fragmentation and coalescence cycles, has facilitated a distinctive multilayered genetic structure in many cold-habitat species (Brochmann *et al*. 2004; Birks 2008). Within this dynamic, changes in ploidy arise in the first instance locally, and over shorter time intervals remain associated with localized genetic identities and populations. Over longer intervals these ploidy changes can introduce barriers to mating that trigger more significant divergence among related population lineages (Levin 1983; Soltis & Soltis 2009; Wood *et al*. 2009).

In the data presented here there is no clear inference of genetic divergence as a result of ploidy-induced reproductive isolation. Such a scenario could be supported if the observed lower ploidy identities in alpine populations (for example evident for haplotypes Ac009 and Ac012, Fig. 3) had emerged *via* haploidization, and there is some evidence of ploidy reduction among *A. norvegica* populations that have immigrated to Iceland in the postglacial period (Table 2, Fig. 2e). An alternative (and more parsimonious) scenario is that all populations arose from a reduced ploidy ancestral stock, with a base count, for example, of 2N = 40 (which is exhibited by 12 of 20 populations, and 9 of 14 haplotype lineages). Based on preliminary consideration of the haplotype phylogeographic signal, this ancestral stock would most likely have been associated with the Alps region during the mid- to late-Pleistocene (Dang 2012; Dang *et al*. 2012; Howard-Williams 2013), which after spatial and altitudinal migration gave rise to several region-specific polyploidization events, which were most pronounced in the east. Importantly, this scenario of more recent localized polyploidization in the *Arenaria ciliata* complex, especially within isolated mountain populations, is not unusual among arctic-alpine species, where it is generally associated with climate oscillations and range shifts during the late Pleistocene and early Holocene (Brochmann *et al*. 2003; Brochmann *et al*. 2004). More specifically, postglacial colonization of higher-altitude habitats by alpine plant species is correlated both with polyploidization and an increased frequency of apomictic reproduction, most likely in response to elevated UV radiation levels, greater stress extremes and the reduced mating possibilities that exist across sparsely vegetated and exposed slopes (Guggisberg *et al*. 2006; Schinkel *et al*. 2016).

The data presented here confirm a complex polyploid structure within the *A. ciliata* group, one that is not readily compatible with the population genetic structure inferred from haplotype data. Resolving the timing and biogeographic context of the inferred polyploidization events relative to chloroplast and nuclear lineages within the *A. ciliata* complex will involve consideration of a broader range of genomic data (C. Meade *et al*., in preparation).

In terms of data acquisition, the deployed method yielded accurate ploidy data from an archival collection, quickly adding an additional layer of genomic information for an ongoing biogeographic study. With the gradual increase in archival material collections across botanical research, this resource is readily amenable to ploidy analysis.

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