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Marsh-orchids of Canada: long-standing mysteries partially solved

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Summary. Between 1959 and 1988, three populations of purple-flowered terrestrial orchids attributable to *Dactylorhiza* subgenus *Dactylorhiza* were discovered in Canada. The populations at Timmins, Ontario, and St John's, Newfoundland were strongly marked on both flowers and leaves, in contrast with the anthocyanin-deficient population at Tilt Cove, Newfoundland. All three populations have since experienced a wide range of taxonomic assignments; debates are also ongoing regarding their origin and most appropriate conservation status. Here, we address these questions by combining detailed in situ morphometric analyses based on 52 characters with allozyme profiles and data from nrITS, 15 plastid microsatellites and seven nuclear microsatellites. The allozyme data alone are sufficient to both confirm allopolyploidy and categorically refute past assignments of these populations to *D. incarnata*, *D. maculata*, *D. fuchsii*, *D. majalis* or *D. purpurella*. Several morphometric characters, nuclear microsatellites and nrITS all reliably distinguish each of the three study populations, whereas the two sampled subpopulations from St John's proved near-identical morphologically. In contrast, morphological variation within each of the three populations is strikingly low, particularly in characters other than those influenced by plant vigour. Similarly, compared with 14 European populations, the three Canadian populations proved genetically impoverished (two were near-invariant) and likely experienced recent, extreme genetic bottlenecks during establishment. The three populations differ substantially, both morphologically and molecularly, therefore probably representing independent immigration events. Although clearly attributable to *D. praetermissa*, all three populations deviate significantly in morphology and DNA data from comparable populations sampled across Europe, preventing identification of their precise geographic origins. Any attempt to determine their mode or origin — through natural long-distance transport, or accidental or deliberate introduction by humans – is challenged to explain why three lineages of a single European Marsh-orchid species, each in different ways atypical of that species, arrived independently in North America whereas no other European dactylorchid species has become established there.

Key Words. allopolyploidy, allozymes, Canada, *Dactylorhiza*, Europe, evolutionary mechanisms, Internal Transcribed Spacer, genetic bottleneck, in situ morphometrics, microsatellites, species circumscription, taxonomy.

Introduction

Background

For many years it was believed that only two species of the terrestrial orchid genus *Dactylorhiza* Neck. ex Nevski occurred on the North American continent: the circumboreal *D. viridis* (syn. *Coeloglossum viride*) is widespread throughout much of Canada, whereas *D. aristata* has barely reached Alaska after island-hopping across the Aleutians from Siberia (e.g. Luer 1975). However, in 1959, a distinctly different population of *Dactylorhiza* was found at Timmins, Ontario. This discovery prompted a form of taxonomic Russian roulette that soon encompassed an impressive range of taxonomic assignments to various northwest European species: *D.* (as *Orchis*) *purpurella* (Andrews 1961), *D. maculata* (Luer 1975; Case 1987; Hay *et al.* 1990; Catling & Catling 1991), *D. fuchsii* (Luer 1975; Catling & Sheviak 1993), *D. majalis* (Sheviak *et al.* 2002) and — based on early interpretations of the present data — *D. praetermissa* (R. M. Bateman, pers. comm. 1996; Meades 1999) (Figs 1A, B, 2B). In 1996, a broadly similar population of dactylorchids, also bearing dense purple leaf markings, was discovered 2150 km further east at St John's, Newfoundland (Clase & Meades 1996) (Figs 1C, D, 2C). In 1988, a group of three Canadian botanists encountered another broadly similar, but on this occasion unspotted, population of dactylorchids 340 km north-west of St John's at Tilt Cove, Newfoundland (Figs 1E, F, 2A). Later, it became clear that the presence of these orchids had been known to local inhabitants since about 1920 (Meades 1994). These plants

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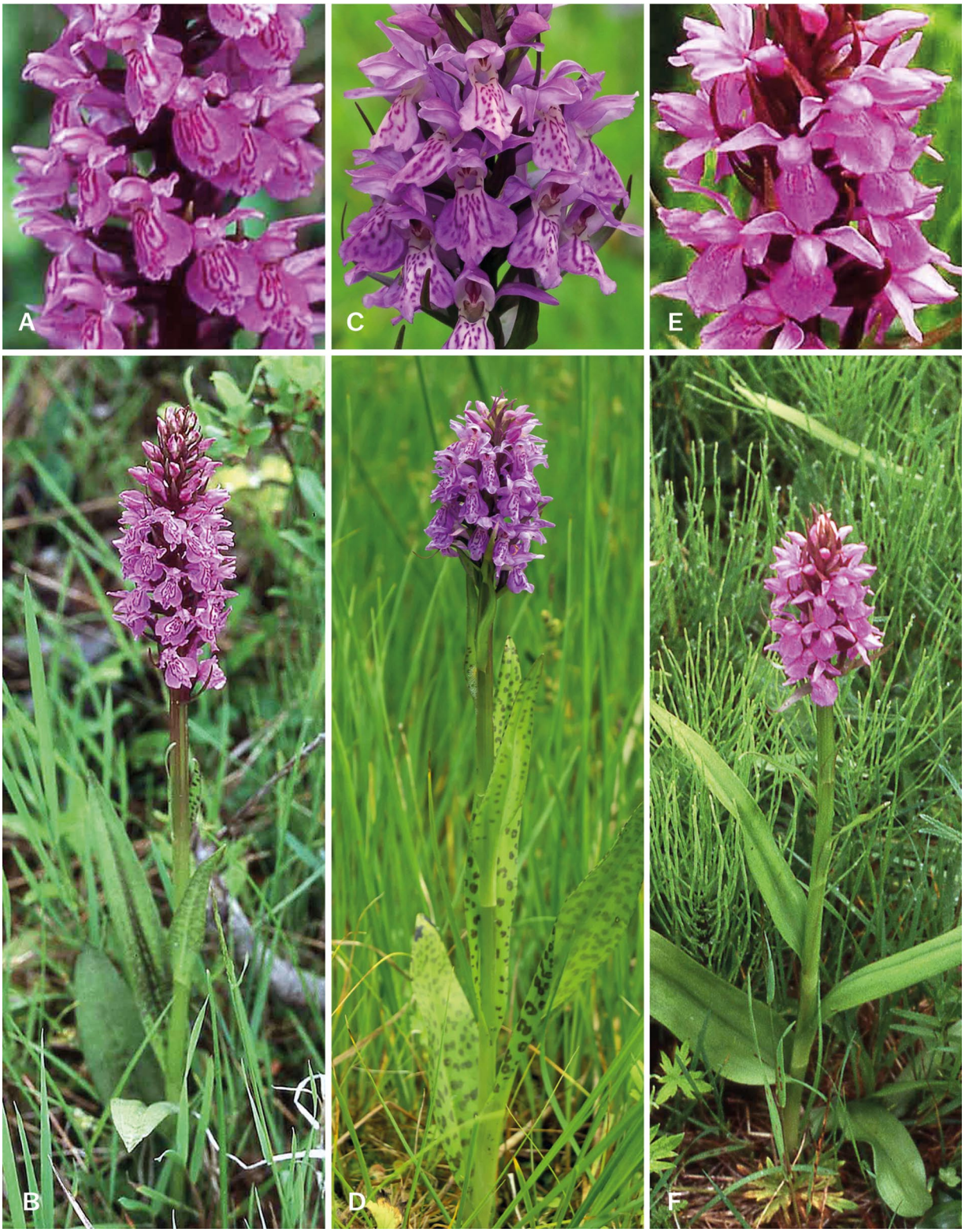

Fig. 1. Representative plants of the Canadian marsh-orchid taxa analysed in the present study. **A**, **B** *Dactylorhiza praetermisssa* var. *junialis*, Timmins, Ontario; 1999. **C**, **D** *D. praetermisssa* var. *junialis*, Nagles Hill, St John's, Newfoundland. **E**, **F** *D.* cf. *praetermissa* var. *praetermissa*, Tilt Cove, Newfoundland; 1994. PHOTOS: **A**, **B**, **E**, **F** SUE MEADES; **C**, **D** GENE HERZBERG.

Fig. 2. Representative habitats of the Canadian marsh-orchid populations analysed in the present study. **A** *Dactylorhiza* cf. *praetermissa* var. *praetermissa*, Tilt Cove, Newfoundland; 1995. **B** *D. praetermisssa* var. *junialis*, Timmins, Ontario; 1999. **C** *D. praetermisssa* var. *junialis*, Nagles Hill, St John's, Newfoundland. PHOTOS: **A**, **B** SUE MEADES; **C** GENE HERZBERG.

were provisionally assigned to *D. incarnata* (Hay *et al.* 1990; Meades 1994), but in 1996, R. M. Bateman suggested that they too were closer morphologically to *D. praetermissa* (Meades 1999; Sheviak *et al.* 2002).

Once expressed, this taxonomic opinion placed the focus of Canadian dactylorchid identity firmly on the Southern Marsh-orchid, *D. praetermissa* — a tetraploid European species (e.g. Bateman & Denholm 2012) or subspecies (e.g. Pedersen 2010) demonstrated to have an allopolyploid origin involving a *D. fuchsii*-like ovule-parent and *D. incarnata* as pollen-parent (e.g. Heslop-Harrison 1954; Hedrén 1996a, 1996c; Paun *et al.* 2011; Balao *et al.* 2016; Brandrud *et al.* 2020). Originally described from southern England (Druce 1914), *praetermissa* is now acknowledged as being frequent in northern France and the adjacent Low Countries, and is rapidly migrating northwards within Britain in response to climate change (Bateman 2022a). The Canadian populations are widely considered to be more likely naturalised than native.

The present project was conceived in 1996 by RB and SM, aiming to clarify the identity of each of these Canadian populations. We employed a combination of detailed in situ multivariate morphometrics, following the methodology established by Bateman & Denholm (1983), and allozyme data, to be analysed within the framework established by Hedrén (1996a). Samples from the project were later forwarded to MH for analyses of major ITS types and both nuclear and plastid microsatellites. Although the existence of early results from this project has received brief mention in earlier publications (Clase & Meades 1996; Meades 1999; Sheviak *et al.* 2002), the data have not until now been presented or their intriguing implications rigorously explored. Additional questions addressed here include determining whether the populations are closely related, whether they show phenotypic and/or genotypic evidence of having passed through recent bottlenecks, identifying their most likely geographic origin(s) and mode of migration. We conclude by assessing the current conservation requirements of this species within Canada and predicting its future behaviour.

Taxonomic note

Even in cases where substantial field sampling has allowed data to be gathered on both morphometrics and genetics (i.e. phenotype and genotype), opinions often differ regarding which category of data should be prioritised, particularly if the two sets of results are not wholly congruent. Even when solid data in both categories are both available and congruent, as with the European dactylorchids discussed here, contrasting valid species concepts can still emerge, and this has definitely occurred in the case of the allotetraploid Marsh-orchids and the Spotted-orchids.

The 'British School' (e.g. Bateman 2006, 2022a; Bateman & Denholm 2012; Stace 2019; Tyteca & Gathoye 2023) argue that each independent allopolyploidy event generates a novel species, even if the same two species have repeatedly acted as the parents, whereas the Scandinavian School (e.g. Pedersen 1998, 2010; Kühn *et al.* 2019; Hedrén & Tyteca 2020) argue that such taxa are better viewed as subspecies; all of the allopolyploid products derived from the same pair of parental species are then considered conspecific. Thus, for RB, *praetermissa* is a species, whereas for MH (who also questions its monophyly), it is a subspecies of *D. majalis* — a taxon considered to consist of no less than 17 subspecies by Kühn *et al.* (2019) (irritatingly, reducing *D. praetermissa* to a subspecies requires the taxon to then bear the epithet *integrata* rather than the well-established *praetermissa*, due to the law of nomenclatural priority: Pedersen 2010). Similarly, the Scandinavian school treat the dominantly diploid facultative calcicole *D. fuchsii* and the autotetraploid calcifuge *D. maculata* s.s. as subspecies of a single species, *D. maculata* s.l., whereas these taxa are consistently regarded as separate species by the British School. Here, the taxonomy of the British School is given priority (cf. Hedrén *et al.* 2011). Happily, both schools do at least agree that controversial plants with leaves that bear purple spots rather than being unmarked are best attributed to a variety whose most appropriate epithet is *junialis* (Bateman & Denholm 1983; Pedersen 2010).

Materials and Methods

This study has been progressed spasmodically through the last quarter-century, whereas the DNA data were collected by MH periodically between approximately 2013 and 2022. The project was originally constructed around a combination of allozymes and character-rich multivariate morphometrics, sampling at the population level individual plants, each of which provided data for both morphological and molecular analysis.

Fieldwork

Field recording was conducted by SM. Morphometric data on the Canadian plants were collected by RB between 1996 and 1999; ten plants per population were scored at Timmins, Ontario and Pippy Park (St John's, Newfoundland), but only six plants at Nagles Hill (also St John's) and four plants at Tilt Cove, Newfoundland.

The original 1960s lakeside marsh population at the gold-mining town of Timmins, discovered by local educator Fred Cowell, was eventually lost as housing development increased and the area became 'tamed' during conversion into a city park. Cowell therefore

preserved the lineage by transplanting the majority of the remaining orchids from the original Gillies Lake site to the banks of the Mattagami River (Sheviak *et al.* 2002); we sampled this rapidly expanding secondary population in 1999, when it had already exceeded 100 plants (Fig. 2B). The preferred habitat here is seepages over riverine silts and ultramafic rocks, at altitudes approximating 270 m; flowering peaks in late June – early July. Several small populations have since become established to a radius of 8 km from the town.

At St John's, a further five localities now form a transect extending 8 km outwards from the original population on the outskirts of the town (Meades & Brouillet 2019), occupying acidic soils developed on Precambrian sandstones and siltstones. Two subpopulations were sampled for the present study from among a total of approximately 1,000 plants occupying *Sphagnum*-rich seepages, wet meadows and scrubland at c. 70 m asl (Clase & Meades 1996) (Fig. 2C). Adjacent subpopulations were labelled Nagles Hill, sampled in 1996, and Pippy Park, sampled in 1998. Flowering peaks in mid-July in both subpopulations.

Tilt Cove is also a mining area, dominated by copper-rich ultramafic rocks. Here too, populations have over the years waxed and waned in multiple sites encircling the almost deserted lakeside town to a radius of less than 1 km (e.g. Meades 1994, 1995). Of the two extant populations, the smaller upper population occurs among a typical fen flora at c. 65 m asl. The larger lower population is located further west (8 – 20 m asl), occupying a seepage slope that separates the largest mine from Winser Lake, where it is accompanied by a flora typical of a wet meadow (Fig. 2A). We sampled this population in 1995. Plants flower in late July and their numbers are progressively increasing; they now exceed 1,000. The Tilt Cove dactylorchid records remain inexplicably absent from the Global Biodiversity Information Facility (GBIF).

Morphometrics

Character scoring

The 52 characters scored by us, and the methods used to quantify them, were detailed by Bateman & Denholm (1985). Wherever feasible, floral measurements were obtained from flower–bract unit located one third to halfway from the base of the inflorescence, aiming to minimise the effect of the decreasing flower size from the base to the apex of the inflorescence that is evident in most Eurasian orchid species (Bateman & Rudall 2006). Each flower was mounted onto double-sided adhesive tape already attached to a filing card. Following measurement, these cards acted as compact herbarium vouchers. Metric characters for most floral organs were measured at a resolution of 0.1 mm, using a Leitz ×8 graduated ocular.

The colour of the distal half of each labellum was matched to the nearest one or two colour block(s) of the Royal Horticultural Society Colour Chart (RHS 2015). The colours were later quantified through conversion to three CIE (Commission Internationale de l'Eclairage) coordinates. Two of these ('x' and 'y') define a position on a square grid superimposed onto a near-triangular array of colours that pale toward white at the centre of the triangle. The corners correspond with pure blue, pure green and pure red, respectively. Density of pigment was represented by a third coordinate (reflectivity or luminance, 'Y'), which decreases in value outward from the centre of the triangle (see http://hyperphysics.phy-astr.gsu.edu/hbase/vision/cie.html). A compound microscope was used to count bract marginal cells across three fields of view, each 1.5 mm in diameter, before their mean length was calculated and their average angularity was summarised.

The 52 characters scored describe the stem and inflorescence (5), leaves (12), leaf markings (7), bracts and ovary (7), labellum (14), spur (4) and sepals (3). They can alternatively be categorised collectively as metric (21), meristic (3), multistate-scalar (24) and bistate (4).

Data analysis

Our chosen approach to data analysis and interpretation was both detailed and experimental. Morphometric data for individual plants were summarised on an Excel v15.4 spreadsheet. Mean values, plus sample standard deviations and coefficients of variation for all metric and meristic characters, were calculated for every character in each study population. Univariate and bivariate analyses were summarised and presented using Deltagraph v7.1 (SPSS/Red Rock software 2013).

The morphometric matrix for the Canadian plants consisted of 30 individuals × 52 characters and contained 6.5% missing values, primarily because vegetative characters could not be scored for two of the four plants representing Tilt Cove and three of the six plants representing Nagles Hill. Nonetheless, no single character incurred more than 23% missing values. Mean values obtained for the four (sub)populations were then explored in the context of a database of 147 populations that collectively encompasses all dactylorchid taxa known from the British Isles, gathered between 1981 and 2018 by RB and ID (Bateman & Denholm 1983, 1985, 1989, 2012). An initial analysis employed all 147 comparators, but a subsequent analysis was confined to a subset of 72 tetraploid marsh-orchid populations plus the four Canadian data-sets. All three analyses were conducted using multivariate methods within Genstat v14 (Payne *et al.* 2011).

Of the 52 characters scored, four of the seven leaf-marking characters (C46 – C49) were omitted to avoid over-weighting a feature that appears to reflect only a single underlying gene, and the presence or absence of a basal leaf (C36) was omitted because such leaves had proven vulnerable to premature senescence. The remaining 47 characters were used to compute a symmetrical matrix that quantified the similarities of pairs of data sets (i.e. plants) using the Gower Similarity Coefficient (Gower 1971) on unweighted data sets scaled to unit variance. This similarity measure is comparatively effective when presented with a matrix of heterogeneous characters that includes missing values (Gower & Legendre 1986; Lloyd 2016; Bateman 2022b). The resulting matrix was in turn used to construct a minimum spanning tree (Gower & Ross 1969) and subsequently to calculate principal coordinates (Gower 1966, 1985) — compound vectors that incorporate positively or negatively correlated characters that are most variable and therefore potentially diagnostic. Principal coordinates are especially effective for simultaneously analysing heterogeneous suites of morphological characters and have the additional advantage of comfortably accommodating missing values. Such ordinations have proven invaluable for assessing relationships among orchid species and populations throughout the last four decades, employing a consistent analytical approach that was reviewed in detail by Bateman (2001) and Bateman & Rudall (2023).

Three separate multivariate analyses were conducted; the first was based on measurements for individual Canadian plants, whereas the second and third were based on mean values calculated for each analysed variable in each of the four study populations. For each of the three multivariate analyses, the first four principal coordinates (PC1 – PC4) were plotted together in pairwise combinations to assess the degree of morphological separation of individuals (and thereby of populations and taxa) in these dimensions, and pseudo-F statistics were obtained to indicate the relative contributions to each coordinate of the original variables.

Allozymes

Our acquisition of protein data at the University of Edinburgh in 1996 benefited considerably from preliminary allozyme studies already performed on the genus *Dactylorhiza* by MH (published later that year as Hedrén 1996a, 1996b, 1996c). Of seven allozyme loci explored by Hedrén, the three involving phosphogluconate-group substrates (the dimeric *6-pgd* and *pgi* [syn. *gpi*] plus the monomeric *pgm*) had been shown to offer an effective combination of both reliably discriminating between the parents of the western European allotetraploids (*D. fuchsii/maculata* and *D. incarnata*) but also showing some variation among populations of the same putative species of allotetraploids. All three loci had also proven competent to resolve dosage levels (Hedrén 1996b), and hence were selected for the present project.

Chilled leaf tissues were prepared satisfactorily as a crude buffer extract with no elaborate purification or concentration steps; optimisation of pH values proved to be the most crucial methodological challenge (e.g. Wendel & Weeden 1989).

For each individual analysed, approximately 1 cm^2 of leaf was ground in 80 µl of a Tris-HCl extraction buffer (Soltis *et al.* 1983), modified by replacing ß-mercaptoethanol with dithiothreitol. Extracts were absorbed onto paper wicks and proteins were separated on horizontal starch gels at 60 mV for 30 mins until removal of the wicks, after which current was increased to 70 mV for a further 3 – 4 h. *Pgi* bands were resolved on the lithium-borate tris-citrate buffer system of Ashton & Braden (1964), as modified according to Lonn & Prentice (1990), whereas a histidine-citrate buffer system (Wendel & Weeden 1989) was used to separate bands of *pgm* and *6-pgd*. Staining recipes followed Wendel & Weeden (1989) with only minor modifications. Gel patterns were recorded immediately, both graphically and photographically, prior to immersion in a methanol-acetic acid fixative.

Each 20-lane starch gel included extracts from 16 putative allotetraploid individuals, bracketed at either end by extracts from single "standard" plants sampled in Scotland to represent the two diploid parental genomes. The *fuchsii* standard was derived from a small population maintained in cultivation in RBG Edinburgh (1984/1618: tubers originally gathered in 1984 from a coal bing at Gorebridge, Midlothian), whereas the *incarnata* standard was derived directly from a natural population located 21 km east of Edinburgh in extensive dune-slacks at Aberlady Bay, East Lothian. Numbers of individuals analysed per Canadian population were small: four for Nagles Hill and six for Tilt Cove.

When scoring the resultant gels (cf. Weeden & Wendel 1989), bands were designated by lower-case letters, beginning with the most rapidly migrating enzyme. 'Missing letters' denoted alleles found in populations of *Dactylorhiza* outside the present study, but as summarised for European populations by Hedrén (1996a) rather than following later coding employed for a broader spectrum of Eurasian populations by Hedrén (2001). Routine use of the diploid parental 'standards' contributed appreciably to accurate identification of specific bands; nonetheless, the present gel for *pgd* incurred sufficient ambiguity to discourage us from presenting the results for Nagles Hill (denoted by F in Table 3). Enzyme banding patterns were translated into allelic data according to the known tertiary structure of the particular enzyme (Weeden & Wendel 1989) prior to subsequent analysis.

DNA-based data

Materials and data collection

A total of 17 populations attributed to *Dactylorhiza praetermissa* were selected by MH for the present study from among a larger body of data held in his long-term data repository for nuclear and plastid microsatellites. Three Canadian populations were compared with populations from France (1), Belgium (4), the Netherlands (3), Denmark (4), and England (2) (Table 1). Several flowers per plant were field-collected in ziploc bags containing fine-grained silica gel. In the laboratory, DNA was extracted from one to two flowers per plant following the 2× CTAB procedure (Doyle & Doyle 1990), and used to generate data for nrITS, together with both nuclear and plastid microsatellites.

Fifteen size-variable sites were studied in non-coding regions of the plastid genome. Altogether, the sites investigated consisted of five mononucleotide microsatellites, one dinucleotide microsatellite, one combined mononucleotide/dinucleotide repeat, one restriction site, and seven other types of duplications or indels. The combined variation patterns at all marker sites were recognised as haplotypes and numbered according to the annotation system used by Hedrén *et al.* (2008). Haplotypes newly discovered here have been given additional numbers.

Variation in the nuclear genome was estimated using seven nuclear microsatellite loci. Five of these (ms3, ms8, ms11, ms13, ms14) are trinucleotide repeats that were described in Nordström & Hedrén (2007). The remaining two loci were established by Hedrén *et al.* (2018); ms2 was originally found with a dinucleotide repeat motif and all alleles identified here strictly followed the expected pattern of two base-pair differences. Locus D2501 was found to have some alleles that differed by only a single base pair, obliging us to score the entire locus as a single base-pair repeat motif. Microsatellites ms2, ms3 and ms8 are specific for the *incarnata* subgenome, whereas ms13, ms14 and D2501 are specific for the *maculata* s.l. subgenome. ms 11 amplifies well in each of the parental lineages, but in allotetraploid *Dactylorhiza* taxa such as *D. praetermissa*, fragments from the *maculata* s.l. subgenome amplify much more strongly than those of the *incarnata* subgenome, such that only alleles of *maculata* s.l. origins could be scored with certainty. Accordingly, none of the loci produced more than two fragments in any individual, and calculations could therefore be performed as for diploids.

Identification of ITS alleles followed the technique developed by Pillon *et al.* (2007), in which alleles were identified by their combined fragment lengths at two size-variable regions. ITS allele frequencies were estimated from the relative amounts of PCR products detected on the automated sequencer used for separating the size-variable fragments. Details of PCR primers and conditions were given by Nordström & Hedrén (2007) and Hedrén *et al.* (2008). The PCR products from each reaction were mixed with 8.5 µl formamide containing appropriate size markers to enable exact size determination of the amplified fragments. They were then heated at 94°C for approximately 20 minutes before loading onto acrylamide gels and separated by size on an ALFexpress II automated sequencer (Amersham Pharmacia Biotech). Fragment sizes were

Table 1. Details of the three Canadian populations and 14 comparative populations of *Dactylorhiza praetermissa* from Northwest Europe sampled for the present DNA-based analyses (nrITS, plastid haplotypes, and nuclear microsatellites).

Locality	Latitude	Longitude	Taxa[1]	No. inds. sampled	Date sampled
St Johns, Newfoundland, CANADA	47°34′N	52°45′W	jun	10	1998-07-12
Tilt Cove, Newfoundland, CANADA	49°53′11′′N	55°38′36′′W	praet	1	1996-07-24
Timmins, Ontario, CANADA	48°28′N	81°19′W	jun	10	1999-06-26
Neuf Ans, Prouilly, Marne, Champagne, FRANCE	49°17′18′′N	03°41′56′′E	praet/jun	30	2012-06-06
Montagne-**St-Pierre**, Lanaye, Liège, BELGIUM	50°46′44′′N	05°40′60′′E	praet	20	2012-06-26
La **Seigneurerie**, Eynatten, Liège, BELGIUM	50°42′44′′N	06°06′14′′E	praet	26	2012-06-26
Fonteintjes, Zeebrügge, Westvlaanderen, BELGIUM	51°19′23′′N	03°09′32′′E	(praet)/jun	20	2012-06-05
Sashul, Heist, Westvlaanderen, BELGIUM	51°20′10′′N	03°13′50′′E	praet/jun	20	2012-06-05
Voornes Duin, Zuid-Holland, NETHERLANDS	51°53′31′′N	4°02′17′′E	praet	10	2005-06-14
Cronestein, Leiden, Zuid-Holland, NETHERLANDS	52°08′21′′N	4°29′40′′E	praet	10	2005-05-31
Weeribben NR, Overijssel, NETHERLANDS	52°46′55′′N	5°56′48′′E	praet	10	2005-06-23
Køge Søndre Strand, Sjælland, DENMARK	55°27′N	12°12′E	jun	9	2006-06-26
Strøby Egede, Sjælland, DENMARK	55°25′N	12°13′E	jun	7	2007-06-21
Hundige Strand, Sjælland, DENMARK	55°36′N	12°21′E	praet	7	2007-06-23
Brøndby Strand, Sjælland, DENMARK	55°37′N	12°26′E	praet	6	2007-06-23
Chippenham Fen, Cambridgeshire, ENGLAND	52°18′03′′N	00°24′47′′W	praet	14	2005-06-05
Eastleigh, Hampshire, ENGLAND	50°59′53′′N	01°20′26′′W	praet/jun	18	2005-05-30

[1] praet = var. *praetermissa*, jun = var. *junialis*

determined using ALFwin™ fragment analyser 1.03.01 software (Amersham Pharmacia Biotech).

Data analysis: nuclear microsatellites

Data for the seven analysed nuclear microsatellite regions were used to caclculate several population-level measures: number of defined gene copies, number of alleles, effective number of alleles (Nielsen *et al.* 2003), alleleic richness, gene diversity corrected for sample size according to both the Nei (1978) and Pons & Petit (1996) measures, observed heterozygosity, and individual inbreeding coefficient. The data were also used to generate a matrix of Rogers (1991) distances among populations, which was in turn used to calculate principal coordinates (Gower 1966, 1985).

Results

Morphometrics

Table 2 gives mean values and, where relevant, sample standard deviations and coefficients of variation, for all 52 morphological characters analysed in the present study. One character, abaxial leaf markings, predictably proved to be uniformly absent across the three Canadian study populations. Our phenotypic interpretation relies primarily on the following series of three multivariate analyses. Comparative analysis with British and Irish populations relies on data published by Bateman & Denholm (1983, 1985, 1989) and Bateman *et al.* (2023), supplemented with additional study populations not yet published.

Individual plants: Canadian populations only

The single most striking aspect of the principal coordinates plot of individual plants (Fig. 3) is that each of the three Canadian populations forms a discrete and compact group. A typical plot sampling three dactylorchid populations would show substantial overlap among individuals from different populations, whereas here, such overlap is confined to the Pippy Park and Nagles Hill subpopulations of the St John's population. Maximum similarities between individuals in different populations are also remarkably low (dashed lines in Fig. 3). This strong polarisation of phenotypic variation is reflected in the facts that (a) the first two coordinates represent an unusually high proportion of the total variation (47%) and (b) all lower-order coordinates lacked interesting patterns.

The first coordinate separated Tilt Cove from the remaining Canadian populations on the basis of its paucity of discrete anthocyanin markings (particularly rings and loops) on both vegetative and floral organs, and its smaller flowers (Table 2). The second coordinate separated Timmins from St John's on the basis of its annular rather than solid sepal markings, bold looped lip markings, somewhat shorter, spotted bracts with unusually short marginal cells, smaller spurs, shorter leaves and slightly higher ratio of expanded to bracteoidal leaves. In addition, Timmins has a higher frequency of bracts that exhibit diffuse anthocyanins (Table 2).

Population-level analysis: all taxa

Having summarised phenotypic variation among the three Canadian populations, the obvious initial approach to comparative biology was to compare the Canadian populations with all available equivalent European data. Pursued at the population level, the main aim of this analysis was to determine which of the four fundamental groups best accommodated the Canadian populations: diploid spotted-orchids, autotetraploid spotted-orchids, diploid marsh-orchids and allotetraploid marsh-orchids.

The first two coordinates account for 39% of the total variance. The first coordinate separates the spotted-orchids (both diploid and tetraploid) from the diploid marsh-orchids, placing the tetraploid marsh-orchids between the two parental clusters but closer to the diploid marsh-orchids (Fig. 4). The appreciably weaker second coordinate is primarily responsible for separating the diploid and tetraploid marsh-orchids, but interestingly, it also separates Tilt Cove from the remaining Canadian populations. All of the Canadian populations associate with the allotetraploid cluster, but all are also peripheral to that cluster. Tilt Cove is placed on that margin of the allotetraploids that is closest to the diploid marsh-orchids (*D. incarnata* s.l.), whereas the Timmins and near-identical St John's populations occupy the opposite side of the allotetraploid cluster.

Having established that all of the Canadian populations are phenotypically consistent with allotetraploidy, we then sought a tighter focus by repeating the population-level analysis after excluding all non-allotetraploid populations.

Population-level analysis: allotetraploid taxa only

The more focused analysis compares the Canadian populations with numerous populations of the four molecularly cohesive allotetraploid species recognised in the British Isles: the southerly *Dactylorhiza praetermissa*, northerly *D. purpurella* and *D. francis-drucei* (formerly *D. traunsteinerioides*), and westerly Irish endemic *D. kerryensis* (formerly *D. occidentalis*).

This analysis increased the dimensionality of the variation, such that the first four coordinates each achieved a degree of taxonomic separation (Fig. 5). The first coordinate (22.0%) separated *Dactylorhiza francis-drucei* from *D. praetermissa* while simultaneously clearly scoring the Canadian populations as belonging to *D. praetermissa*. Both the second coordinate (13.8%) and third coordinate (9.8%) partially separated *D.*

Table 2. Mean values and coefficients of variation (%) for 52 morphometric characters scored for the four Canadian populations attributed to *Dactylorhiza praetermissa*. NA = not applicable.

No.	Character	Timmins		Pippy Park		Nagles Hill		Tilt Cove	
		Mean	*CV*	*Mean*	*CV*	*Mean*	*CV*	*Mean*	*CV*
1	Lip, length to central lobe (A)	8.47	7	8.82	11	8.75	13	7.53	12
2	Lip, presence sinuses	1		0.9		0.67		0	
3	Lip, length to sinus (C)	7.05	6	6.68	10	6.35	27	NA	NA
4	Lip, length to lateral lobe (B)	7.56	6	7.18	10	6.58	26	NA	NA
5	Lip, maximum width (D)	9.94	4	11.57	10	11.32	9	7.70	8
6	Lip, position of width vs length	1.6		1.7		1.67		2	
7	Lip, lateral lobe reflexion	4.7		4.5		4.83		3.5	
8	Lip, CIE colour x	284.4	2	280.8	3	289.0	4	290.5	1
9	Lip, CIE colour y	206.1	15	200.0	14	220.7	14	188.5	8
10	Lip, CIE colour reflectivity (Y)	19	36	20	5	25.2	50	16	25
11	Lip, pattern type	4		3		2.5		1	
12	Lip, pattern distribution	1		1.2		1		1	
13	Lip, pattern contrast	2.9		2.2		2.67		1	
14	Lip, lateral lobe indentation	0.4		0.2		0.67		0	
15	Spur, length	5.56	8	7.54	7	7.07	6	4.93	16
16	Spur, width at mouth	2.88	6	3.76	8	3.63	4	2.35	18
17	Spur, width halfway	2.39	4	3.33	8	3.17	6	2	18
18	Spur, curvature	3.5		4.1		3.83		3.8	
19	Lateral sepals, position	3		3.5		3.33		2.8	
20	Lateral sepals, solid spots	0		1.7		1.33		0	
21	Lateral sepals, ring spots	2		0.1		0		0	
22	Bracts, length basal	24	22	30.7	25	32.67	26	27.5	NA
23	Bracts, length floral	15	15	17.4	12	17.67	11	18	NA
24	Bract, diffuse anthocyanin	0.4		1.5		1		0.5	
25	Bract, spotting	0.8		0.2		0		0	
26	Bract, marginal cell length	56	12	79.5	16	73.83	14	83.8	13
27	Bract, marginal cell shape	2.8		2.3		2.67		3	
28	Stem, total height	32.7	22	39.8	25	38.3	30	27	NA
29	Inflorescence, length	70.3	32	66.7	43	71.3	24	57.5	NA
30	Ovary, length	10.3	8	11.9	7	12.83	6	8.8	14
31	Inflorescence, no. of flowers	32	26	34.6	46	25.33	52	26.5	NA
32	Stem, diameter	5.76	15	7.48	34	6.73	38	4.8	NA
33	Stem, diffuse anthocyanin	0.1		0		0.33		0.5	
34	Leaves, no. of expanded	3		3.7		4.33		3.5	
35	Leaves, no. of bracteoidal	2		1.8		1.33		2	
36	Leaves, basal sheath	NA	NA	NA	NA	NA	NA	NA	NA
37	Leaf, length longest	127.5	18	172.4	14	196.3	31	159.5	NA
38	Leaf, width widest	27.8	15	31.7	31	29	33	26	NA
39	Leaf, posn longest vs widest	1.7		1.7		1.33		1	
40	Leaf, posn longest up stem	6.3	49	7.9	35	12.7	25	5.5	NA
41	Leaf, shape uppermost	2.4		2.6		3		2.5	
42	Leaf, shape longest	3.7		3.7		3.3		3	
43	Leaf, shape lowermost	3.9		3.9		3.7		4	
44	Leaf, apical hooding	0		0		0		0.5	
45	Leaf, shade of green	3		2.2		2.67		1.5	
46	Leaf spots, presence adaxial	1		1		1		0	
47	Leaf spots, percentage cover	68	16	50	21	40	25	0	NA
48	Leaf spots, distribution	2.3		2.3		2		NA	
49	Leaf spots, shape	4.4		3.2		3		NA	
50	Leaf spots, size	4.4		4.3		4.7		NA	
51	Leaf spots, percentage ring	0.8		1		1		NA	
52	Leaf spots, presence abaxial	0		0		0		0	

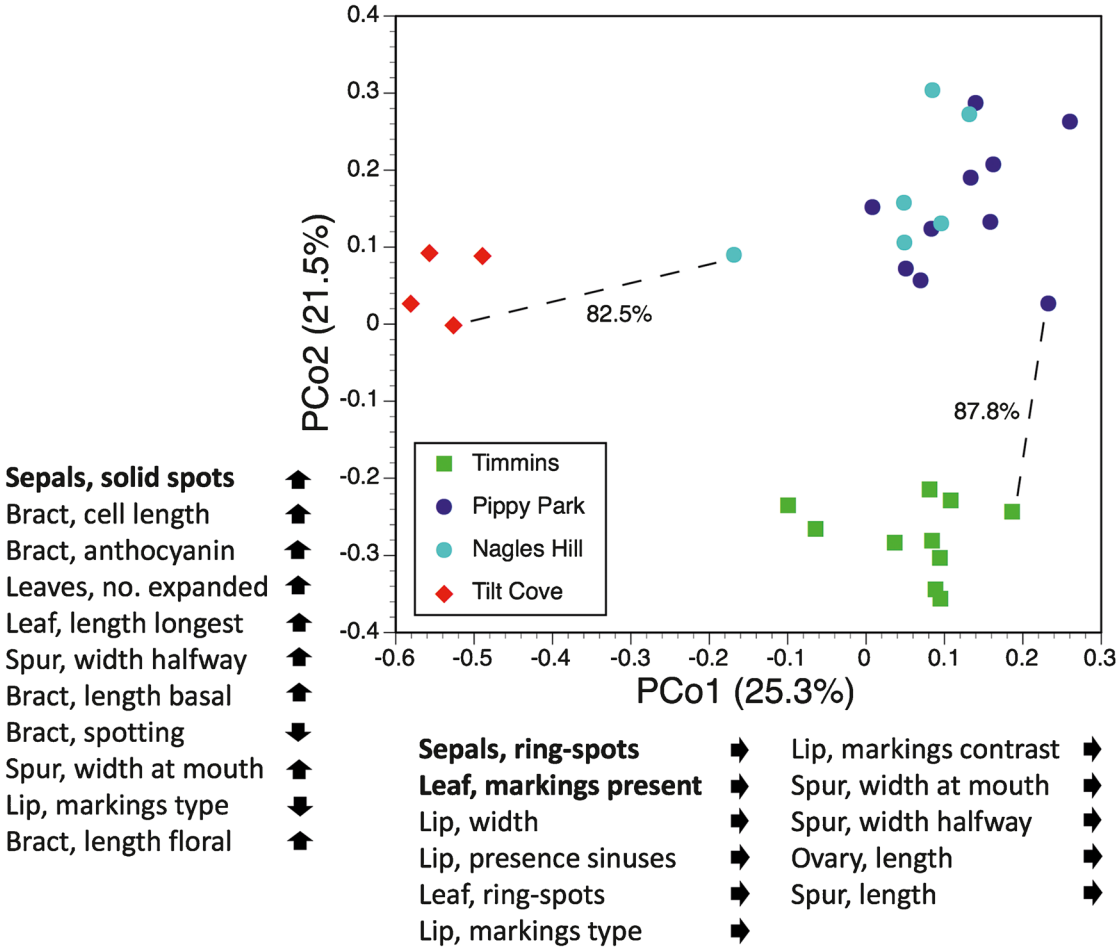

Fig. 3. Plot of the first two principal coordinates for 47 diverse morphological characters measured in a total of 30 plants representing three populations (and two subpopulations) of Canadian marsh-orchids. Parenthetic percentages represent the proportion of the total variance accounted for by each coordinate. Characters contributing significantly to each coordinate are listed in order of decreasing importance, with arrows indicating the direction of increase in value of each character listed; boldface characters are dominant. The three populations form three discrete clusters, linked by comparatively weak Gower Similarities (dashed lines).

francis-drucei and *D. praetermissa* from the remainder, but interestingly, the third coordinate also strongly separated St John's and Timmins from all other populations included in the analysis (Fig. 5B), due primarily to their predominantly annular leaf markings. The fourth coordinate (7.7%, not shown) largely separated *D. kerryensis* from the remaining taxa. Lastly, it is noteworthy that the two subpopulations from St John's scored almost identically on all of these plots, neatly illustrating the effectiveness of this analytical approach for indicating closeness of relationship.

Overall similarities

Further insights were gained through surveying the symmetrical matrix of Gower Similarities for populations of all taxa. Unsurprisingly, the Pippy Park and Nagles Hill subpopulations of the St John's population had by far the strongest similarity with each other (a remarkable 98.1%). Their next strongest similiarities (96.7% and 96.6%, respectively) were with Brambridge, a southern English water-meadow population of *Dactylorhiza praetermissa*. This population included plants of leaf-marked var. *junialis* and also provided the strongest similarity with Timmins, albeit at a lower percentage (93.1%). However, Timmins had almost equally strong similarities with two populations of leaf-marked *D. purpurella* var. *cambrensis* from the north coast of Scotland (e.g. Bateman *et al.* 2023). Predictably, Tilt Cove showed a contrasting pattern, returning a strongest similarity (95.8%) with Braunton, a dune-slack population of *D. praetermissa* var. *praetermissa* from the coast of southwest England. In contrast, the overall similarities of Tilt Cove to the three remaining Canadian populations were unusually low (83.2 – 86.6%).

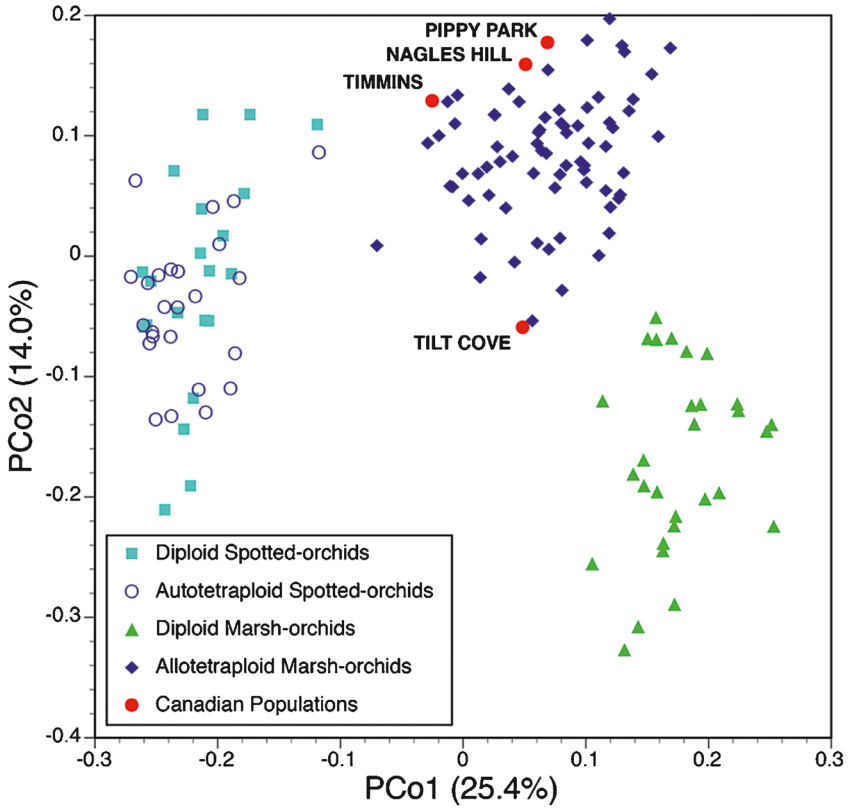

Fig. 4. Plot of the first two principal coordinates for 47 diverse morphological characters measured in four *Dactylorhiza* samples from Canada and 147 populations from the British Isles, analysed as population mean values. Parenthetic percentages represent the proportion of the total variance accounted for by each coordinate. All four Canadian samples are placed peripheral to the cluster of British and Irish populations of tetraploid marsh-orchids.

Restricting comparison of Canadian populations to tetraploid marsh-orchid populations only yielded similar patterns of maximum similarity. The exceptionally strong link between the two subpopulations from St John's (97.0%) persisted, as did the strong similarity of the English Brambridge population to St John's (95.2%) and, at a lower percentage, to Timmins (91.3%). Tilt Cove also retained both its closest allegiance with Braunton (albeit reduced to 93.6%) and its low similarities with the remaining Canadian populations (78.5 – 79.8%).

Character variation

The main lessons to be learned from plotting labellum length versus width (Fig. 6) were that the correlation between these two perpendicular parameters was remarkably strong within all three Canadian populations, and that each population had a distinctive length: width ratio, largely reflecting contrasts in widths (mean values were 7.7 mm for Tilt Cove, 9.9 mm for Timmins and 11.5 mm for St John's: Table 2). In contrast, the plot of spur length versus width (Fig. 7) shows an allometric ratio of approximately 2: 1 that is shared by all three populations, but a discontinuity separates the large St John's spurs from their exceptionally small equivalents at both Timmins and Tilt Cove.

Fig. 8 presents histograms of six non-metric characters that help to discriminate among the Canadian populations, each ultimately dictated by discrete or diffuse anthocyanin pigments. Once again, the two subpopulations from St John's are sufficiently similar that they can be discussed as a single entity. Lip markings are reliably loops only and high-contrast in Timmins, but they are spots only and low-contrast in Tilt Cove; St John's is both intermediate and more variable in these characters. Lateral sepal markings discriminate effectively among the three populations: they are absent from Tilt Cove, ubiquitous but uniformly annular in Timmins, and almost wholly solid in St John's, where again they vary somewhat in contrast. Bract spotting characterises a majority of Timmins plants, a minority of St John's plants, and is absent from Tilt Cove plants. Leaf markings are also absent from Tilt Cove but are ubiquitous (and predominantly annular) in both St John's and Timmins.

Allozymes

Table 3 compares the allele frequencies obtained for two of the three studied Canadian dactylorchid populations with populations of all of the well-founded species known to occur in northwest Europe. Letters denoting specific alleles of the three loci follow Hedrén (1996a, 1996b, 1996c).

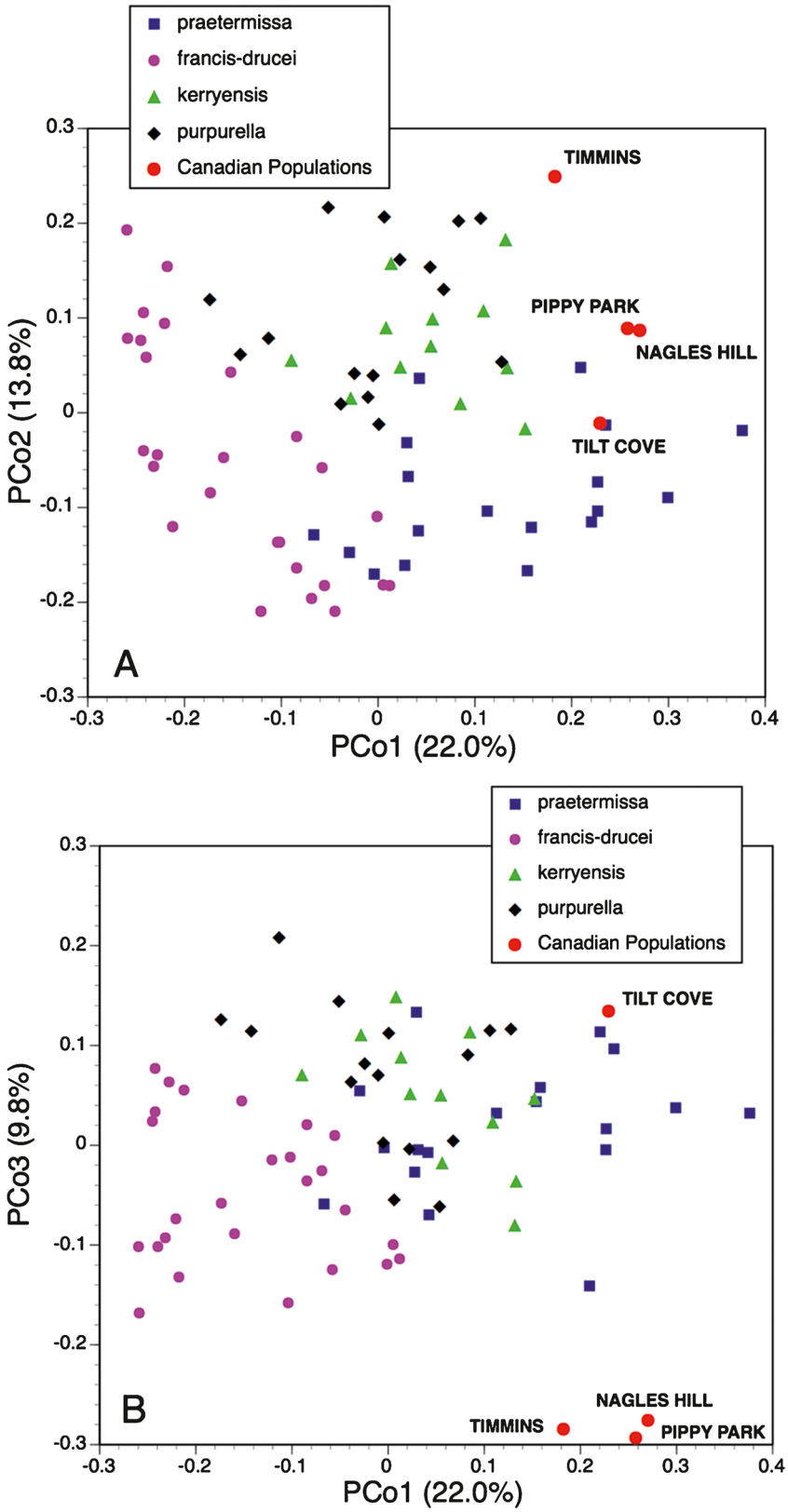

Fig. 5. Plots of the first and second principal coordinates (**A**) and first and third coordinates (**B**) for 47 diverse morphological characters measured in four *Dactylorhiza* samples from Canada and 72 tetraploid marsh-orchid populations from the British Isles, analysed as population mean values. Parenthetic percentages represent the proportion of the total variance accounted for by each coordinate. The Canadian populations are most similar to, but also peripheral to, the 18 British populations of *D. praetermissa*, and Tilt Cove is strongly separated from the other Canadian populations on the third coordinate.

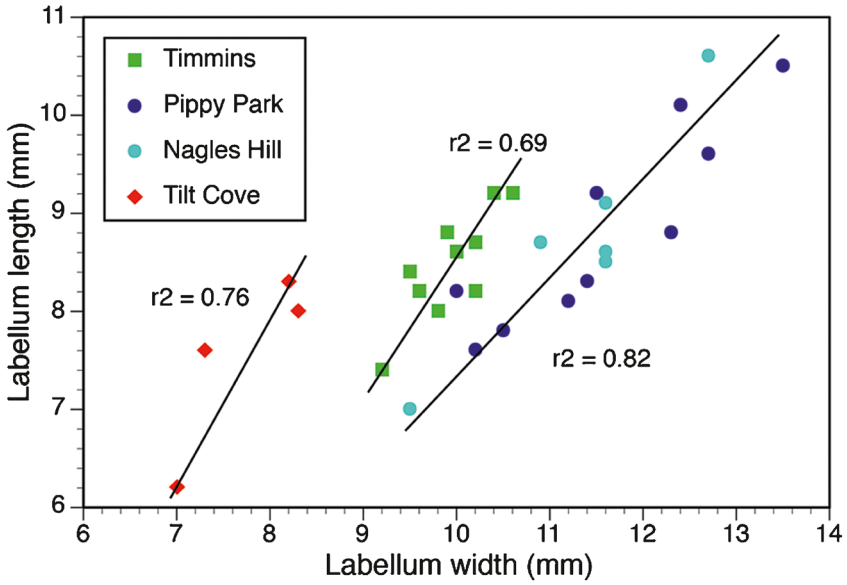

Fig. 6. Plot of maximum length vs maximum width of the labella for individuals sampled from the three Canadian populations of tetraploid marsh-orchids. Each population has a distinctive length: width ratio, and St John's also shows greater internal variation in size.

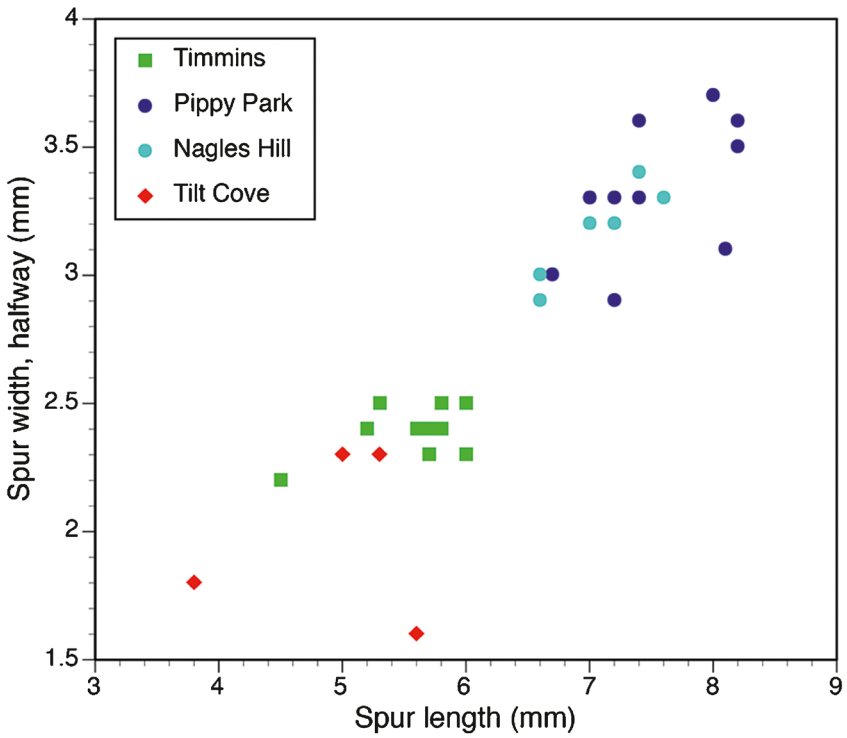

Fig. 7. Plot of maximum length vs width halfway long their length of the spurs for individuals sampled from the three Canadian populations of tetraploid marsh-orchids. All populations have a similar length: width ratio, but St John's spurs are consistently much larger.

No variation was detected either within or between the Nagles Hill (St John's) and Tilt Cove populations. The *pgi* locus reliably yielded the *b* and *e* alleles that typify all allopolyploid *Dactylorhiza* taxa, the *b* allele having been inherited from the *D. fuchsii* parent and the *e* allele having been inherited from the *D. incarnata* parent. The *pgm* locus consistently bore the *b* allele inherited from Continental rather than British populations of *D. incarnata*, together with the *e* allele that is the most common allele in *D. fuchsii*; this combination of alleles rules out *D. purpurella* as a possible identification for the Canadian populations (Bateman *et al.* 2023). The *pgd* locus yielded tentative data only from Tilt Cove; the resulting *ab* genotype is characteristic only of *D. praetermissa* and *D. majalis* s.s. (Table 3). Thus, all three allozyme loci yielded results consistent with the Canadian dactylorchids being assignable to *D. praetermissa*. Our allozyme results concur with

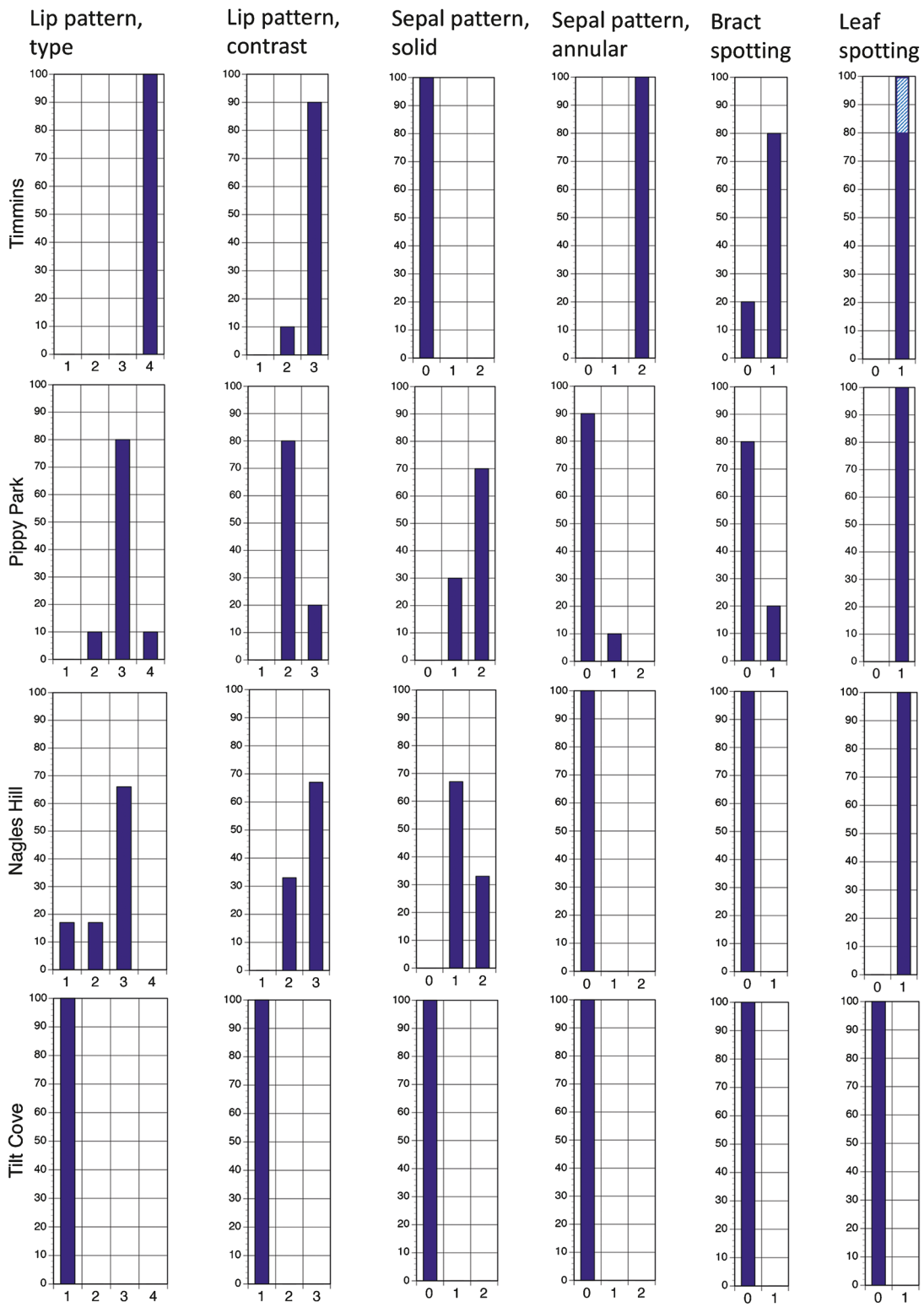

Fig. 8. Frequencies within the Canadian populations of competing states for six characters that represent discrete anthocyanin markings on flowers, bracts or leaves. Lip pattern, type: 1, dots plus dashes; 2, dashes only; 3, dashed plus loops; 4, loops only. Lip pattern, contrast (relative to background pigmentation): 1, low; 2, medium, 3, high. Sepal pattern, solid spots: 0, absent; 1, pale; 2, strong. Sepal pattern, annular markings: 0, absent; 1, pale; 2, strong. Floral bract, markings: 0, absent; 1, present. Leaf, markings on upper surface, solid plus annular: 0, absent; 1, present (cross-hatching indicates markings uniformly solid rather than dominantly annular).

those of Pedersen (2010) in failing to find any distinction between *D. praetermissa* var. *praetermissa* and var. *junialis*.

DNA-based data

Data representing the Canadian population at Tilt Cove should be treated with caution, as only a single plant was available for analysis (Table 1).

Internal Transcribed Spacer (ITS) alleles

Populations of *Dactylorhiza praetermissa* are most commonly dominated by any two of three nrITS alleles: III, V and/or X (Pillon *et al.* 2007; Nordström & Hedrén 2009; Hedrén *et al.* 2011). This pattern is followed by the St John's population, which is dominated by allele V (86%) with subordinate allele X. Among the 14 comparative populations analysed here, the most similar were from Sashul in Belgium and Køge in Denmark. However, in contrast, the Tilt Cove and Timmins populations were fixed for allele VI — an allele that was found in only two of the comparative populations, both Danish, and was fixed in only one: Brøndby. Allele VI is rare to absent in all dactylorchid taxa, but is most frequently reported from *D. fuchsii* (Pillon *et al.* 2007; Ståhlberg & Hedrén 2008).

Plastid haplotypes

All three Canadian populations proved to be fixed for plastid haplotype C (87), which was present in 11 of the 14 European populations of *Dactylorhiza praetermissa* and dominated nine of them. Previous studies of the species also showed C to be the most frequent haplotype in this species, A being widespread but subordinate to C (Pillon *et al.* 2007; Hedrén *et al.* 2008, 2011; Nordström & Hedrén 2009). The minority of populations lacking haplotype A (59) are widely geographically scattered. Both haplotypes A and C are indicative of maternal inheritance from *D. fuchsii* or its close relatives.

Nuclear microsatellites

The nuclear microsatellites offered no surprises for four of the five well-established microsatellite loci; ms8, ms11, ms13 and ms14, all of which yielded results from the three Canadian populations that were typical of European *Dactylorhiza praetermissa*. For ms8, all three Canadian populations were fixed for the 206 allele, which is frequent in all European countries. For ms11 and ms13, the three Canadian populations were dominated by the 159 and 83 alleles, respectively, both of which were fixed in Timmins and Tilt Cove. These alleles are common across much of Europe, though our limited sampling suggests that ms11-159 is comparatively rare in England and France. Timmins and Tilt Cove were also fixed for ms14 allele 287, which interestingly otherwise dominated only the Dutch Weeribben population. In contrast, St John's was fixed for the 290 allele that dominated all other European populations. A more surprising and incongruent pattern was observed in ms3, where it was Tilt Cove and St John's that appeared fixed for the near-ubiquitous European 162 allele, whereas Timmins was deviant, being fixed for a 153 allele that in Europe inhabited only a mere 2% of plants in one population — Seigneurerie in Belgium.

Moving on to consider the two novel nuclear microsatellite regions analysed here, all three Canadian populations diverged greatly in the hypervariable ms2 region. Although ms2 alleles ranged from 147 to 221, those most common in European populations spanned the narrower range 179 – 187. St John's mixed the 183 allele, most frequent in the Netherlands and the UK, with the 187 allele, most frequent in the Netherlands and Belgium. The single analysed Tilt Cove plant yielded a unique mix of allele 179, a minority component of single populations from each of the Netherlands, Denmark and England, with allele 159, otherwise found only in the English Chippenham population, where it achieved a mere 7% frequency. Lastly, fixation also characterised microsatellite region msD2501. Timmins and St John's were fixed for the 78 allele, which was found in all 14 European populations but had its lowest frequencies in the two English populations (29% and 39%). In contrast, Tilt Cove was fixed for the less frequent 76 allele, which otherwise was found in only five of the European populations, reaching its highest frequency (39%) in the English Eastleigh population.

Five European populations yielded private alleles (they were especially frequent at Neuf Ans), whereas no private alleles were detected in the Canadian populations.

The multivariate analysis of aggregated nuclear microsatellite data (Fig. 9) yielded a first coordinate, accounting for 39% of the total variance, served primarily to separate the Timmins and Tilt Cove populations from the remainder. However, these two populations were widely separated on the second coordinate (24%), which also separated all seven English, French and Belgian populations from five of the seven Dutch and Danish populations. The third coordinate (13%, not shown) separated the Belgian St Pierre population from the remainder but brought together the Timmins and Tilt Cove populations.

Summary statistics for the Canadian and European populations (Table 4) confirmed that Timmins and apparently also Tilt Cove present negligible genetic diversity, both within and between individuals. All parameters are consistent with recent establishment of each population via a single genotype. In contrast, some genotypic variation is evident at St John's, but all

Table 3. Allozyme allele frequencies for the Canadian Nagles Hill (St Johns) and Tilt Cove populations, compared with those presented in previous studies of western European dactylorhizas in Scandinavia (Sc) and the British Isles (BI). Sources: Bateman *et al.* (2023) for the British and Irish data other than *Dactylorhiza praetermissa*, Hedrén (1996b) for Scandinavian *D. purpurella*, Hedrén (1996a) for the remaining taxa. A few minor alleles have been omitted.

Locus	**6-pgd**			**pgi**		**pgm**				
Allele	**a**	**b**	**c**	**b**	**e**	**a**	**b**	**c**	**d**	**e**
D. fuchsii (Sc)	0	47	52	99	0	0	0	3	24	61
D. incarnata (Sc)	100	0	0	0	100	0	100	0	0	0
D. incarnata (BI)	3	97	0	0	100	0	4	96	0	0
D. purpurella (Sc)	14	76	10	50	50	0	6	44	3	47
D. purpurella (BI)	1	99	0	50	50	0	0	50	0	50
D. traunsteineri (Sc)	51	2	36	48	50	0	50	3	36	10
D. lapponica (Sc)	50	0	23	38	50	0	50	9	41	0
D. francis-drucei (BI)	44	8	48	51	49	0	47	3	50	0
D. praetermissa (BI)	49	43	7	47	49	2	45	0	23	15
D. majalis s.s. (Sc)	51	38	9	48	51	4	46	0	40	10
D. sphagnicola (Sc)	49	1	26	56	38	1	50	0	43	7
D. maculata (Sc)	0	27	70	85	0	0	0	6	51	38
Nagles Hill (n = 4)	F	F	0	50	50	0	50	0	0	50
Tilt Cove (n = 6)	50	50	0	50	50	0	50	0	0	50

measures indicate considerably lower variation than was detected in most of the 14 European populations: average number of alleles 1.43 vs 2.49, effective number of alleles 1.24 vs 1.80, allelic richness 1.41 vs 2.05, corrected gene diversity 0.15 vs 0.28, and observed heterozygosity 0.10 vs 0.22. Inbreeding coefficient is predictably somewhat higher at St. John's: 0.33 vs 0.26 (Table 4).

Four of the 14 European populations also showed evidence of genetic impoverishment, presumably indicating recent founding: the Belgian population from St Pierre plus three of the four Danish populations (Brøndby, together with the identical closely juxtaposed populations from Køge and Strøby). The Danish populations approach (and arguably constitute) the present northern limit of naturally occurring *Dactylorhiza praetermissa* populations in Europe, and hence may be the relatively young products of northward migration, whereas St Pierre was documented as being recently established on waste ground.

Discussion

Taxonomic challenges

The marsh-orchids and spotted-orchids constitute a taxonomic 'critical group', within which it is notoriously difficult to circumscribe, diagnose and identify taxa. Inherent sterility barrers are at best weak, permitting extensive hybridisation among species and at least some introgression (e.g. De hert *et al.* 2012; Balao *et al.* 2016; Stace *et al.* 2016). Further problems are caused by the predisposition of the clade to undergo polyploidy, a situation that is exacerbated by repeated allopolyploidy events between different strains of the same parental pair of spotted-orchid seed-parent and marsh-orchid pollen-parent (e.g. Pillon *et al.* 2007; Hedrén *et al.* 2008; Balao *et al.* 2016; Brandrud *et al.* 2020; Wolfe *et al.* 2023). The most frequent pairing successfully generating allopolyploids is that between *Dactylorhiza fuchsii* and *D. incarnata* (or their immediate predecessors), which is responsible for the origins of not only *D. praetermissa* but also those of several other widely recognised taxa (e.g. Hedrén 1996a, 2008; Pillon *et al.* 2007; Brandrud *et al.* 2020).

Greatly improved knowledge of evolutionary mechanisms operating within the group has clarified but in no way eliminated taxonomic disagreements. The great majority of taxonomic publications by-pass the cycle of targeted sampling, data collection, data analysis and inference that is central to genuine science, and therefore fall within the unsatisfactory realm of authoritarian opinion. Where data have been collected they tend to represent either genotype or phenotype; studies collecting both categories of data remain rare. And even in cases where both categories of data have been gathered, opinions differ regarding which category of data should be prioritised if the results are not wholly congruent. Lastly, as discussed in the 'Taxonomic Note' that concludes our Introduction, even when rigorous data-sets for both morphometrics and DNA-based techniques have not only been acquired but have also proved acceptably congruent, contrasting species concepts can still fuel ongoing taxonomic controversies (cf. Pedersen 2010; Bateman & Denholm 2012). *Dactylorhiza praetermissa* can arguably be distinguished from all other Western European

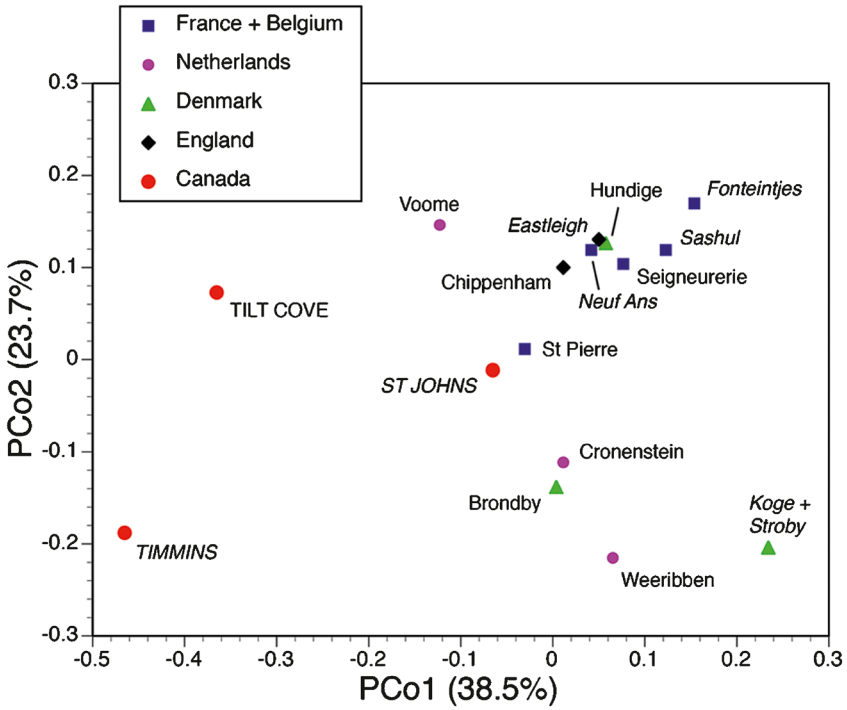

Fig. 9. Plot of the first two principal coordinates for Rogers distances derived from seven nuclear microsatellite loci obtained from three *Dactylorhiza* populations from Canada and 14 comparative populations of *D. praetermissa* from Northwest Europe. Populations containing at least some leaf-marked individuals attributable to var. *junialis* are italicised. Køge and Strøby were fixed for the same alleles at all analysed loci and hence received identical scores for the first two coordinates. Parenthetic percentages represent the proportion of the total variance accounted for by each coordinate.

allopolyploids by nuclear sequencing (microsatellites and RAD-seq) and from most others by its ITS sequences, plastid haplotypes and allozyme profiles, particularly when they are employed in combination. However, in no case are the molecular contrasts large — unsurprisingly, given that (a) the allopolyploids are descended from different lineages of the same pair of parental species, and (b) gene-flow remains frequent among the allotetraploids and their putative diploid ancestors (e.g. Hedrén & Tyteca 2020). Consequently, the observed degree of morphological overlap among the resultant allopolyploids is predictable from first principles.

A further factor complicating taxonomic circumscription and assignment on the basis of morphology is pleiotropy, particularly that determining the distribution of anthocyanin pigments across the various organs that constitute a dactylorchid. It has only gradually become apparent that most dactylorchid species maintain, albeit at different frequencies, anthocyanin-high and anthocyanin-low phenotypes (and presumably equivalent underlying genotypes). Anthocyanins are expressed in both discrete and diffuse modes. Diffuse anthocyanins provide the ground colour to the flowers but also often occur at varying densities throughout the bracts and/or upper stem. Discrete

Table 4. Summary statistics for seven concatenated nuclear microsatellite regions, comparing the three Canadian populations with aggregate values for 14 Northwest European populations of *Dactylorhiza praetermissa*.

Population	St Johns	Tilt Cove[4]	Timmins	NW Europe	
				Mean	SSD
Sample size (n)	10	1	10	14.8	7.6
No. of defined gene copies	20	2	20	29.38	15.12
No. alleles (NA)	1.43	1.14	1	2.49	1.15
Effective no. alleles (NAe)[1]	1.24	1.00	1	1.80	0.62
Allelic richness (AR: k=12)[2]	1.41	NA	1	2.05	0.74
Corrected gene diversity (He, = h)[3]	0.147	0.143	0	0.281	0.174
Observed heterozygosity (Ho)	0.100	0.143	0	0.218	0.144
Individual inbreeding coefficient (Fi)	0.330	NA	NA	0.214	0.186

[1] After Nielsen *et al.* (2003); [2] Expected number of alleles among 12 gene copies; [3] Figures were identical for Nei (1978) correction for sample size, He, and Pons & Petit (1996) measure based on unordered alleles, h; [4] Only one individual was analysed, weakening statistical credibility.

anthocyanins form dot, dash and/or loop markings on the labellum and/or lateral sepals of the flower, but also form solid or annular markings on the adaxial surfaces of the leaves and (less often) on the abaxial leaf surface, bracts and/or stem. The diffuse and discrete categories of anthocyanin expression are not always positively correlated, but within each of these two categories, positive correlation is stronger among different characters and pleiotropy is therefore suspected. Consequently, anthocyanin characters have often been over-weighted when making taxonomic judgements (Bateman 2006, 2011; Hedrén *et al.* 2011; Bateman & Denholm 2012), particularly when they ignore the considerable polymorphism exhibited by many dactylorchid populations.

One such case is *Dactylorhiza praetermissa* var. *junialis* (syn. *pardalina*). This anthocyanin-rich mode of *D. praetermissa*, colloquially named the Leopard Marsh-orchid (and lavishly illustrated by Kreutz 2019), bears bold floral markings and also possesses leaf markings; both of these sets of pigmentation characters are usually strongly expressed and often present as annular rather than solid markings. The visual impression is sufficiently striking that some taxonomists have suggested that *junialis*/*pardalina* merits recognition as a subspecies (e.g. Soó 1960; Nelson 1976; Kreutz 2004) or even as a full species (Pugsley 1935) or nothospecies (Averyanov 1986). Other workers have accused *junialis* of most likely being the product of introgression with *D. fuchsii*, arguing that genuine *D. praetermissa* consistently lacks leaf markings (discussion in Summerhayes 1951), or have explored finer morphometric partitioning of *junialis* (Tyteca & Gathoye 1993). However, *D. praetermissa* has now been subjected to sufficiently intense analysis, both genotypic and phenotypic, to be confident that it is a *bona fide* cohesive taxon that supports both anthocyanin-poor and anthocyanin-rich modes (Bateman & Denholm 1983, 2012, unpublished; Pedersen 2010; Pedersen & Hedrén 2010; Zonneveld 2019; Brandrud *et al.* 2020). Morphometric analysis conducted by Pedersen (2010) yielded a dendrogram that suggested conspecificity of *praetermissa* s.s. and *junialis* but a principal components ordination that largely separated individuals of the two taxa. However, analyses by Bateman & Denholm (1983, unpublished) show that only the much-discussed leaf markings suggest possible hybridity; once these are omitted from a multivariate analysis, plants of *junialis* adopt their correct placement as possessing phenotypes typical of *praetermissa*, rather than of hybrids between *praetermissa* and species of spotted-orchid.

Taxonomic identity

Having outlined what is, in truth, an even more complex taxonomic framework than is implied above, we can now focus on conclusively identifying the ambiguous Canadian populations. From a genotypic perspective, the present allozyme data alone are sufficient to (a) confirm allopolyploidy of the Timmins and Tilt Cove populations and (b) reject past assignments of these populations to *Dactylorhiza incarnata*, *D. fuchsii*, *D. maculata* or *D. purpurella* (cf. Hedrén 1996a, 1996b, 2001; Bateman *et al.* 2023); indeed, they also rule out *D. sphagnicola* and members of the *D. traunsteineri* group such as *D. francis-drucei* (Table 2). However, the allozyme data cannot discriminate between *D. praetermissa* and the closely related but exclusively continental allotetraploid *D. majalis* s.s.

Moving on to consider DNA data, plastid microsatellites can discriminate among most of the British and Irish allotetraploids other than *Dactylorhiza praetermissa* versus *D. francis-drucei*, and a similar situation is evident in nrITS; both species typically share allele III with either V or X (Pillon *et al.* 2007; Hedrén *et al.* 2011). However, plastid haplotypes can discriminate between *D. praetermissa* and *D. majalis* s.s. in continental populations (Hedrén & Tyteca 2020), and nuclear microsatellites proved to be completely discriminatory among the British and Irish allotetraploids (Hedrén *et al.* 2011). The most effective discrimination among the allotetraploids, which helpfully indicated their mutual monophyly, was achieved using RAD-seq data, though more extensive sampling is desirable than that conducted by Brandrud *et al.* (2020). Unfortunately, such data are not available for the Canadian populations.

Among those DNA-based data that *are* available for the Canadian populations, both the plastid and nuclear microsatellites are most consistent with assignment to *Dactylorhiza praetermissa*, though intriguingly, the Timmins data for nuclear microsatellite region ms3 are most consistent with *D. praetermissa* subsp. *schoenophila* sensu Bateman & Denholm (2012; see also Bateman 2020). The most surprising result was provided by ITS data for the Canadian populations; although St John's yielded the expected combination of alleles V and X, Timmins and Tilt Cove proved to be fixed for the unusual allele VI. Rare to absent in all allotetraploids, allele VI is more often found in the diploid Spotted-orchids — occasionally in *D. fuchsii* but more frequently in its southeastern European equivalent, *D. saccifera* s.l. (cf. Devos *et al.* 2006; Stahlberg & Hedrén 2008; Brandrud *et al.* 2020; Bateman 2021). This troublesome ITS variant was found in only two of the 14 comparative populations of *D. praetermissa*, both Danish, though allele VI was previously identified in about half of the samples of this species that were analysed from Britain by Pillon *et al.* (2007).

Our multivariate morphometric analyses offer a little more comfort to those observers who have in the past hazarded a range of taxonomic identifications of the Canadian populations. Although the multivariate plots concur with our allozyme and sequencing data in

suggesting that all three analysed populations should be assigned to *Dactylorhiza praetermissa*, they also show that all three Canadian populations are distinct from each other in multiple characters and demonstrate that all are morphologically atypical of the species as characterised through 18 British populations. Tilt Cove is the *praetermissa* population that most closely resembles its paternal ancestor, *D. incarnata*, whereas characters such as the dense leaf markings of the St John's and especially Timmins populations place them on that margin of the *praetermissa* cluster that is located closest to the Spotted-orchids: the diploid *D. fuchsii* and autotetraploid *D. maculata* (Fig. 4). Timmins also showed fairly strong overall morphometric similarities to var. *cambrensis*, the anthocyanin-rich mode of *D. purpurella* (Bateman & Denholm 2012; Bateman *et al.* 2023).

Thus, there was at least some morphological justification for each of the previous near-miss attempts at formal identification. Nonetheless, we regard the five categories of data available to us as being sufficient to demonstrate that the St John's and Timmins populations are assignable with a reasonable level of confidence to *Dactylorhiza praetermissa* var. *junialis* and the Tilt Cove population to *D. praetermissa* var. *praetermissa*.

Origins

Given the paucity of known sites for *Dactylorhiza praetermissa* in North America, and a confirmed native distribution of the species that is confined to England, northern France and the Low Countries, its official status in Canada as naturalised is understandable. However, the routine production of vast quantities of "dust-seeds" by orchids confers upon them the potential for long-distance dispersal across the Atlantic Ocean in high-level air currents, either in isolation or through the vector of migrating birds. Moreover, *Dactylorhiza* species have thus far been shown to be mycorrhizal generalists (e.g. Jacquemyn *et al.* 2012), suggesting that there is a reasonable probability that a European seed alighting in North America could by chance encounter suitable mycorrhizal partners. Similarly, their reputedly dominantly allogamous pollination through food-deception means that a wide range of insects, especially bees (most commonly of the genus *Bombus*: Claessens & Kleynen 2011) have been observed removing pollinaria from various Marsh-orchids in Europe. Given that an estimated 856 bee species are native to Canada (Sheffield *et al.* 2017), including at least 46 *Bombus* species, it should not have proven difficult for a newly-arrived *D. praetermissa* (or indeed, for any closely related taxa) to find suitable partners to ensure pollination. If there exists a natural constraint that would filter out all immigrant dactylorchid taxa other than *D. praetermissa* (with an apparent preference for the rarer var. *junialis* over var. *praetermissa*) we have yet to envisage it. Nonetheless, in theory at least, natural migration into Canada remains a credible hypothesis.

Alternative explanations for the arrival of *Dactylorhiza praetermissa* in North America require human assistance, either accidentally and unknowingly (e.g. through the transport of mining equipment shipped from the UK with biological packing materials such as hay: Meades & Clase 1996) or through deliberate transport of tubers of these attractive plants from the Old World to the New, presumably as erstwhile garden plants. Can we discriminate among these three intriguing hypotheses?

Remarkably, the three Canadian populations of *Dactylorhiza praetermissa* analysed by us collectively span almost the entire morphometric and genetic ranges reported from the many populations of this species that we sampled in western Europe. In contrast, within each Canadian population we encountered almost no genetic variation, and the morphological variation that was documented is judged sufficiently low to be consistent with our direct observations of genetic uniformity. Populations of *D. praetermissa* showing similarly low variance have been reported from the UK (e.g. the Baldock population of var. *praetermissa*) and Belgium (e.g. the Louvain-la-Neuve population of var. *junialis*: Tyteca & Gathoye 1993). The near-constancy in anthocyanin-related characters within each of the Canadian populations is especially striking (admittedly, St John's appears a little more variable in these characters than Timmins and Tilt Cove, just as it is a little more variable in nuclear microsatellites: Table 4); greater variation would be predicted in metric and meristic characters simply in response to non-genetic contrasts in vigour among individuals (e.g. Bateman & Denholm 1989; Bateman & Rudall 2011). One possible exception is an intriguing white-flowered plant reported from Tilt Cove (Meades 1994; Clase & Meades 1996); albinos occur occasionally in diploid and autotetraploid species of *Dactylorhiza*, but they are rarely encountered in any allotetraploid taxon (e.g. Eccarius 2016). Suspected of being fixed for non-functional alleles in the anthocyanin-assembly pathway, albino individuals are most likely to occur in small inbred populations. All of our data sets support initial suggestions (Meades 1994; Clase & Meades 1996) that it is highly likely that each of the Canadian populations was established relatively recently by a single demonstrably self-compatible individual, either seed or tuber. If so, each population has by definition passed through the ultimate genetic bottleneck.

Moreover, each of the Canadian populations is sufficiently distinct from the remainder, both phenotypically and genotypically, that it is extremely unlikely that any of these populations gave rise to any other. Although all three Canadian populations are identical in allozyme profiles and plastid haplotypes, they differ greatly in ITS alleles and nuclear microsatellites

(Fig. 8). ITS alleles and microsatellite ms14 link Timmins with Tilt Cove, whereas microsatellite ms3 links St John's with Tilt Cove, and microsatellite msD2501 links St John's with Timmins. Morphometric results (Figs 3, 4) predictably suggest greater similarity between the two populations of var. *junialis* — Timmins and St John's — but nonetheless, similarities among all three populations are comparatively weak other than that between the two subpopulations sampled in St John's (Figs 4, 5). Viewed in aggregate, our results reject hypotheses of ancestor–descendant relationships between any of these populations; each population almost certainly represents an independent colonisation event into Canada.

This startling conclusion only serves to deepen the mystery of the origin of the Canadian populations. The association of the orchids at both Timmins and Tilt Cove with substantial historical mining activities led Meades (1994, 1999) to argue that colonisation most likely took place through transport from Europe of mining equipment, which was often packed in meadow hay, or alternatively as European hay exported as livestock feed in the case of the St John's population (Clase & Meades 1996). Certainly, deliberate spreading of the seed of *Dactylorhiza praetermissa* mixed with hay recently led to the successful establishment of a population of this orchid in Southern Scotland, almost 200 km north of the species' current natural northern limit in the British Isles (cf. Bateman 2022a; Trudgill 2022).

However, if the Canadian localities were colonised through seeds, either through natural means or accidental introduction by humans, they should randomly sample their three independent source populations in Europe. If so, the probability would be extremely low that all three immigrant colonies would deviate so strongly from both the ITS compositions and especially the phenotypes that are typical of the species in Europe. Compared with 147 plants from 18 English populations of *Dactylorhiza praetermissa*, both Timmins and St John's have average densities of leaf markings exceeding all of them and a greater frequency of annular markings. Also, spurs of Timmins plants are on average shorter than 98% of English plants. Tilt Cove plants have spurs shorter than 99% of English comparators and narrower than 96%, together with a labellum length: width ratio that is greater than 97%. These observations suggest to RB that the phenotypes of the Canadian colonies were most likely selected by people as being unusually visually attractive. The chosen plants, presumably transplanted as tubers, were exceptionally boldly (but differently) marked in the case of St John's and Timmins, but exceptionally pale in the case of Tilt Cove — features making them likely to stand out from the crowd. Militating against these arguments is the absence of clear evidence of historical garden cultivation of dactylorchids in Canada (which seems especially improbable in spartan mining towns), and once again the question of why only *D. praetermissa* would consistently be preferred above all other European dactylorchid species. Admittedly, *D. praetermissa* is arguably the most ecologically tolerant among the western European allotetraploid taxa (e.g. Bateman & Denholm 2023).

We should also revisit previous understandable assumptions that the Canadian populations originated in Britain (Meades 1994; Clase & Meades 1996; Sheviak *et al.* 2002). Only a minority of British populations of *Dactylorhiza praetermissa* contain leaf-marked individuals assignable to var. *junialis* (Bateman & Denholm 1983). Within most of those populations, only a minority of plants bear leaf markings. And among leaf-marked plants, few show the density of leaf markings that characterises both Timmins and St John's, where markings typically cover at least 50% of the upper leaf surface. Such visually striking plants are actually encountered more frequently in the Netherlands (Kreutz & Dekker 2000; Kreutz 2019) and Belgium (Tyteca & Gathoye 1993). ITS profiles might be taken to tentatively indicate Danish sources, though the Danish populations are similarly suspected of recent establishment, and ITS compositions can vary considerably within allotetraploid dactylorchids (e.g. Hedrén *et al.* 2018). Nuclear microsatellites offer contradictory hints; ms2 and ms3 vaguely suggest British origin, whereas results for ms11 imply that a British or French origin is least likely for St John's and Timmins. Given next-generation sequencing data, it might eventually prove possible to determine genetically whether the Canadian plants originated from the British Isles or continental Europe, but given present data we can only continue to speculate.

Moreover, there is one final mystery that remains even more difficult to explain: Whatever the means by which *Dactylorhiza praetermissa* travelled repeatedly to Canada, why has it not been joined there by any of its European congeners? Among the allotetraploid dactylorchid taxa, *D. praetermissa* is comparatively frequent, often forms large populations, and has a relatively broad ecological tolerance (e.g. Summerhayes 1951; Harrap & Harrap 2009). Nonetheless, European species such as *D. fuchsii*, *D. maculata* and *D. majalis* s.s. are rapid and flexible colonists; compared with *D. praetermissa*, they are even more common and geographically widespread, at least as ecologically tolerant, and arguably are equally attractive and garden-worthy. And other allotetraploids with northwestern European distributions, such as *D. purpurella* and *D. lapponica*, are judged especially well-adapted to both cool northerly climates and the formerly glaciated terrains that characterise Canada (Nordström & Hedrén 2008, 2009; Bateman 2011). Why then would either nature or mankind repeatedly sample only one European species of

Dactylorhiza while so determinedly ignoring all of the others?

Potential future

Metapopulations of *Dactylorhiza praetermissa* in all three of our study areas now exceed (perhaps greatly) 1000 plants, and each has demonstrated the ability to migrate at least locally once some specific localities were rendered uninhabitable. Thus, they present no immediate conservation concerns.

The three populations of *Dactylorhiza praetermissa* analysed here were the only known localities for the species in North America when our sampling took place in the late 1990s. However, consultation of the Global Biodiversity Information Facility in March 2022 revealed recent records encompassing three additional regions of Canada. The species has been deliberately introduced (H. Mann, pers. comm. 2015) into several localities in the area surrounding Pasadena and Corner Brook, on the west coast of insular Newfoundland, while in the centre of the island, multiple populations have reputedly appeared around the dam complex west of Meelpaeg Reservoir. The third new population is well-separated from the others; it was found near the village of Chartierville, east of Montreal and close to the border separating Quebec from the US state of Maine.

Thus, if *Dactylorhiza praetermissa* was indeed brought by humans to Newfoundland at least a century ago, either deliberately or accidentally, it has already spread to four centres on the island, each establishing local metapopulations spanning a radius of (1 –) 5 – 8 km. It would be interesting to discover whether any of the three new areas resemble genotypically and/or phenotypically any of the three earlier-discovered localities. For example, a distance of 850 km separates the Chartierville locality from the nearest previously known locality, at Timmins.

Given that *Dactylorhiza praetermissa* occupies a fairly broad spectrum of habitats, tolerates soils that extend from calcareous to neutral pH and encompass a wide range of soil moisture (e.g. Summerhayes 1951; Pikner 2014; Kreutz 2019), and readily invades recently abandoned industrial sites, it would not be surprising to see it spread rapidly within North America. Although all six known Canadian localities occupy a fairly narrow latitudinal zone (47.5 – 50° N), and most occur at low altitudes (Chartierville is the highest, reaching c. 500 m), expansion northward and upward appears likely in response to global warming. In Britain — a small land area rich in field botanists — detailed long-term monitoring suggests that the northern margin of *D. praetermissa*'s distribution is presently migrating northwards along the east and west coasts at an estimated rate of 20 km per decade (Bateman 2022a; Bateman *et al.* 2023), and saltational jumps of at least 35 km have been inferred (admittedly, this performance is exceeded by that of the Bee Orchid, *Ophrys apifera*, which has apparently achieved a northward migration rate of 65 km per decade and saltational jumps of at least 75 km). These UK-derived figures may well prove comparable with the future behaviour of *D. praetermissa* in North America.

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Declarations

Conflicts of Interest The authors declare that they have never entertained even the slightest hint of any actual or potential conflict of interest of any kind.

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