Contrast in levels of morphological versus molecular divergence between closely related Eurasian species of *Platanthera* (Orchidaceae) suggests recent evolution with a strong allometric component

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We consider the conceptual relationship between pollinator specfificity, genetic isolation and species delimitation, critiquing Darwin's (1877) hypothesis that differential placement of pollinia on the probosces and eyes of sphingid moths explained the divergence of the Eurasians *Platanthera bifolia* and *P. chlorantha* — species that have similar geographic distributions, habitat preferences and flowering times. Subsequent workers have developed Darwin's initial hypothesis into an oft-cited model system for co-evolutionary speciation via a prezygotic barrier. The four datasets gathered from the two species were: (a) morphometric data from 139 individuals of 21 populations in southern England, supported by SEM study of representative flowers; (b) spur-length measurements gathered by many fieldworkers from 3070 plants sampled across western Europe; (c) DNA sequences from two representative individuals of each species from nuclear ribosomal ITS and eight plastid regions; and (d) nrITS sequence data for 50 individuals of the two species and their close relatives in Eurasia. *Platanthera bifolia* and *P. chlorantha* show a strong allometric relationship approximating a 2:3 ratio in mean values for most measurements. The few characters showing greater deviations collectively reflect some but not all aspects of the classical adaptive scenario; moreover, they may be genetically linked. Those characters are sufficient to reliably distinguish between the two species and occasional hybrids. Spur length of both species shows a latitudinal gradient, strongest within the British Isles, that decreases northward by an average of ca. 2% per 100 km. In contrast with this substantial morphological divergence, only one base-pair difference was detected in ca. 9kb of rapidly evolving nuclear and plastid DNA, though inclusion of closely related taxa from the Mediterranean region, Macaronesian islands and eastern Asia increased to seven the number of subtly distinct ITS alleles recovered from the aggregate. Most of the phenotypic variability observed, both within and between *P. bifolia* and *P. chlorantha*, is encompassed within a single allometric cline that conflates — in order of decreasing influence — ontogenetic factors, environmental (epigenetic) influences, and taxonomic distinction. Phenotypic divergence between the two species is less than is generally supposed, and is hypothesised to reflect a simple genetic control that radically increases stigma size in *P. chlorantha*. Our failure to detect species-specific genetic differences between the two allogamous putative species indicates (but cannot conclusively demonstrate) that extensive gene-flow occurs between them, suggesting that previous assertions of pollinator specificity have been greatly exaggerated. This homogeneity could represent hybridisation through secondary contact of formerly distinct species, but this theory is contradicted by the low allelic diversity of the putative species, together with their similar geographic distributions and habitat preferences. Rather, we suspect that these and other closely related taxa represent a relatively early stage of speciation, when phenotypic divergence (which typically reflects minute changes in the regulation of phenotypically expressed genes) inevitably precedes more widespread divergence in the various genic regions that are routinely used in molecular phylogenetics. This phenotypically overt but genotypically cryptic phase of speciation, here termed the genetic divergence lag (GDL), renders such incipient species immune to DNA barcoding. Many incipient species may never achieve a level of genetic isolation sufficient to escape the GDL. In the present case, the

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incipient speciation may have occurred sympatrically and may (thus far) have led only to stabilised polymorphism.

Keywords: Adaptation, Allometry, Co-evolution, DNA barcoding, Geographic cline, Internal transcribed spacer, Morphometrics, Nectar spur, Orchid, *Platanthera chlorantha*, *Platanthera bifolia*, Plastid sequences, Pollinator specificity, Speciation, Species delimitation

Introduction

The genus Platanthera *as a model evolutionary system*

Recent molecular phylogenetic studies, based primarily on the nuclear ribosomal ITS region (Hapeman & Inoue, 1997; Bateman *et al.*, 2003, 2009), have resolved the Butterfly Orchids (genus *Platanthera* Rich.) into six putatively monophyletic sections (reviewed by Bateman *et al.*, 2009). Since Darwin (1877) first conceived a fundamentally co-adaptive scenario to explain the separation of *Platanthera chlorantha* (Custer) Rchb. (an epithet recently the subject of a successful bid for nomenclatural priority over *Orchis montana* F.W. Schmidt: Govaerts, 2009; Brummitt, 2011) from *P. bifolia* (L.) Rich. within section *Platanthera*, several species of *Platanthera* have increasingly been used as models of evolution in general and of presumed adaptation in particular. In North America, hybridisation, allopolyploid speciation, and facultative autogamy have been documented in the *P. dilatata–P. aquilonis* aggregate (Section *Limnorchis*: Wallace, 2004, 2006; Bateman *et al.*, 2009). Also, in Section *Lacera*, inbreeding depression and mutational processes were described in *P. leucophaea* (Wallace, 2003; Holzinger & Wallace, 2004), and pollination frequency, outcrossing-to-inbreeding ratio and selection for spur length were explored in *P. lacera* (Little *et al.*, 2005).

The remaining evolutionary studies have focused on two widespread Eurasian sister species (or, arguably, species complexes) of Section *Platanthera*. *Platanthera bifolia* was used to investigate variable selection for male and female function (Maad, 2000; Maad & Alexandersson, 2004) and reproductive potential (Boberg & Ågren, 2009). Meanwhile, studies of *P. chlorantha* yielded details of nectar secretion and resorption (Stpiczynska, 2003a, b). Naturally occurring mutants have featured in discussions of non-adaptive macroevolution through saltation (a heritable, genetic or epigenetic modification that is expressed as a profound phenotypic change across a single generation and results in a potentially independent evolutionary lineage), notably via modification of the labellum to closely resemble the sepals. This category of transformation has been termed pseudopeloria (Bateman, 1985; Bateman & DiMichele, 2002; Bateman & Rudall, 2006b; Bateman & Sexton, 2008).

The majority of evolutionary studies of the *P. bifolia* aggregate adopted a comparative approach, contrasting particular aspects of *P. bifolia* and *P. chlorantha* that are assumed to have become highly adapted through selection pressures. Nilsson (1978, 1983) used morphometric measurements from both herbarium collections and *in situ* populations in Scandinavia to quantify the features that apparently encouraged placement of the pollinia on the probosces of pollinating moths in *P. bifolia* populations but on the compound eyes of other, closely related moths in *P. chlorantha* populations. Although the phylogenetic study of Hapeman & Inoue (1997) omitted *P. chlorantha*, optimisation of morphological characters across this and subsequent ITS-based phylogenetic trees (Pridgeon *et al.*, 1997; Bateman *et al.*, 2003; Bateman, 2005) suggested that the eye attachment of pollinia in *P. chlorantha* evolved from the more common and conventional proboscis attachment that characterises *P. bifolia*, as predicted previously by Summerhayes (1951). In addition to morphological contrasts, the two species also reputedly differ in fragrance chemotypes (Nilsson, 1983; Tollsten & Berstrom, 1993). Admixed populations of these two species containing phenotypic 'intermediates' (i.e. presumed hybrids) have been studied in southern England (Bateman, 2005; Bateman & Sexton, 2008), the Low Countries (Claessens & Kleynen, 2006; Claessens *et al.*, 2008) and Scandinavia (Nilsson, 1985), where the efficiency of pollinium import and export between the two species was quantitatively estimated (Maad & Nilsson, 2004).

Most of the evolutionary scenarios emerging from these studies paid particular attention to the key role of the morphology of the column (and, to a lesser degree, of the pollinia themselves) in placing the pollinia at appropriate locations on visiting Lepidoptera, and on the function of the nectar-secreting spur in first attracting those pollinators and then persuading them to probe for nectar sufficiently deeply to acquire firmly attached pollinia. Only a minority of terrestrial orchid species in the Northern Hemisphere offer their pollinators a genuine food reward, in most cases nectar; the remainder deceive insects into mistakenly believing that they will be rewarded with food or, less frequently, with copulation (e.g. van der Cingel, 1995; Cozzolino & Widmer, 2005). Although there have been repeated evolutionary transitions between the rewarding and non-rewarding conditions within subtribe Orchidinae (Bateman *et al.*, 2003; Cozzolino & Widmer, 2005), the genus *Platanthera* appears to be uniformly rewarding (Hapeman & Inoue, 1997). Substantial quantities of nectar are secreted by solitary labellar spurs (Stpiczynska, 2003a, b) that differ considerably in mean

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length among the many species in the genus (Bateman & Sexton, 2008; Box *et al.*, 2008).

Rationale of the present study

The main purpose of the present study was to better describe the variation within, and the boundary separating, the two putative sister species, *P. bifolia* and *P. chlorantha* (Fig. 1). We hoped to infer the speciation mechanism most likely to have generated *P. chlorantha* from an ancestor presumed on grounds of phylogenetic placement to have closely resembled *P. bifolia*, thereby testing the classic explanation of their apparent divergence rooted in pollinator specificity (Darwin, 1877; Nilsson, 1983, 1985; Hapeman & Inoue, 1997; see also Bateman *et al.*, 2011; Vereecken *et al.*, 2011). We followed Bateman's (2001) recommendation for species delimitation studies in general of sampling several individuals per study population for both genetic and morphometric analyses. Nuclear ITS and eight regions of the plastid genome were targeted for an initial exploratory round of DNA sequencing. Initial results encouraged us to restrict a subsequent broader survey of the populations to the ITS region, set in the context of the broader phylogenetic survey of the *Platanthera* clade presented by Bateman *et al.* (2009).

Morphometric data were gathered from plants in southern England that yielded ITS data, plus additional individuals from the same populations. Sufficient morphometric characters were chosen to adequately describe all above-ground organs of each plant. In addition, selected flowers were examined under the scanning electron microscope in order to describe in greater detail characters that could potentially influence pollinator specificity.

A much broader geographical survey was then conducted on just one supposedly highly adaptive morphological character, specifically spur length, to determine whether evolutionarily and taxonomically important characters are liable to show substantially greater variation if they are studied more widely across the geographic distributions of the study species (cf. Bateman & Sexton, 2008).

The strongly contrasting levels of morphological versus molecular divergence discerned between *P. bifolia* and *P. chlorantha*, and between other closely related taxa from the Mediterranean (*P. holmboei*, *P. algeriensis*) and the Azores (*P. micrantha*, *P. azorica*), are used as a case-study to address several questions of broader relevance to systematic botany. These include whether: (a) the standard typological approach to classical morphological taxonomy and molecular phylogenetics yields reliable results; (b) any species that has recently diverged from its ancestral lineage will show readily detectable differences in phenotype but not genotype; and (c) such species will inevitably be immune to the taxonomically broad but genetically narrow DNA-based identification approaches collectively temed 'DNA barcoding'.

Materials and Methods

Fieldwork

Fieldwork on the two species of *Platanthera* that occur in the UK was confined to the southernmost parts of England and Wales; specifically, a latitudinally elongate oblong delimited by the counties of Kent, Cornwall, Powys (material supplied by Harold and Jane Lambert) and Suffolk (material supplied by Jonathan Tyler). The decision to restrict the study geographically was taken primarily in order to provide the authors with ready and, if necessary, repeated access to chosen field sites, but it later proved to have been fortuitous. Specifically, a broader survey of spur length in the two species (Bateman & Sexton, 2008: discussed at greater length below) demonstrated a surprisingly strong latitudinal control over the dimensions of at least this particular feature of the plant. In contrast, the present character-rich study spanned a mere 1.5° of latitude, thereby usefully minimising one major influence on the perceived 'mean morphologies' of the two species.

Selection of populations within this limited geographic area aimed to span a representative range of habitats, paying particular attention to baseline soil properties and shade afforded by associated vegetation. Peak flowering period was also estimated. Of the 21 colonies visited by us during May–June 2003 and/or 2004 that contained flowering plants yielding useful data, 11 contained *P. chlorantha* only, eight contained *P. bifolia* only, and two contained both species; in both colonies, small numbers of putative hybrid plants were also found (Appendix 1). For the smaller colonies, all flowering plants in suitable condition were measured (six sites yielded only one measurable plant). In larger colonies, individual plants were selected to adequately represent the range of phenotypic variation and habitat occupancy evident at the locality. In total, measurements were taken from 139 plants: 79 individuals of *P. chlorantha*, 55 individuals of *P. bifolia* and five putative hybrids.

The majority of the 139 plants also yielded samples for prospective DNA sequencing (Appendix 2); two or three flowers (or, less often, leaf tips) excised from each individual were placed in silica gel in the field for rapid DNA extraction and/or longer term storage.

Morphometric analyses

In total, 37 morphometric characters were recorded (Appendix 3). The four characters describing stem plus inflorescence, seven characters describing the leaves, and three characters describing the bracts were recorded in the field on *in situ* plants, measured

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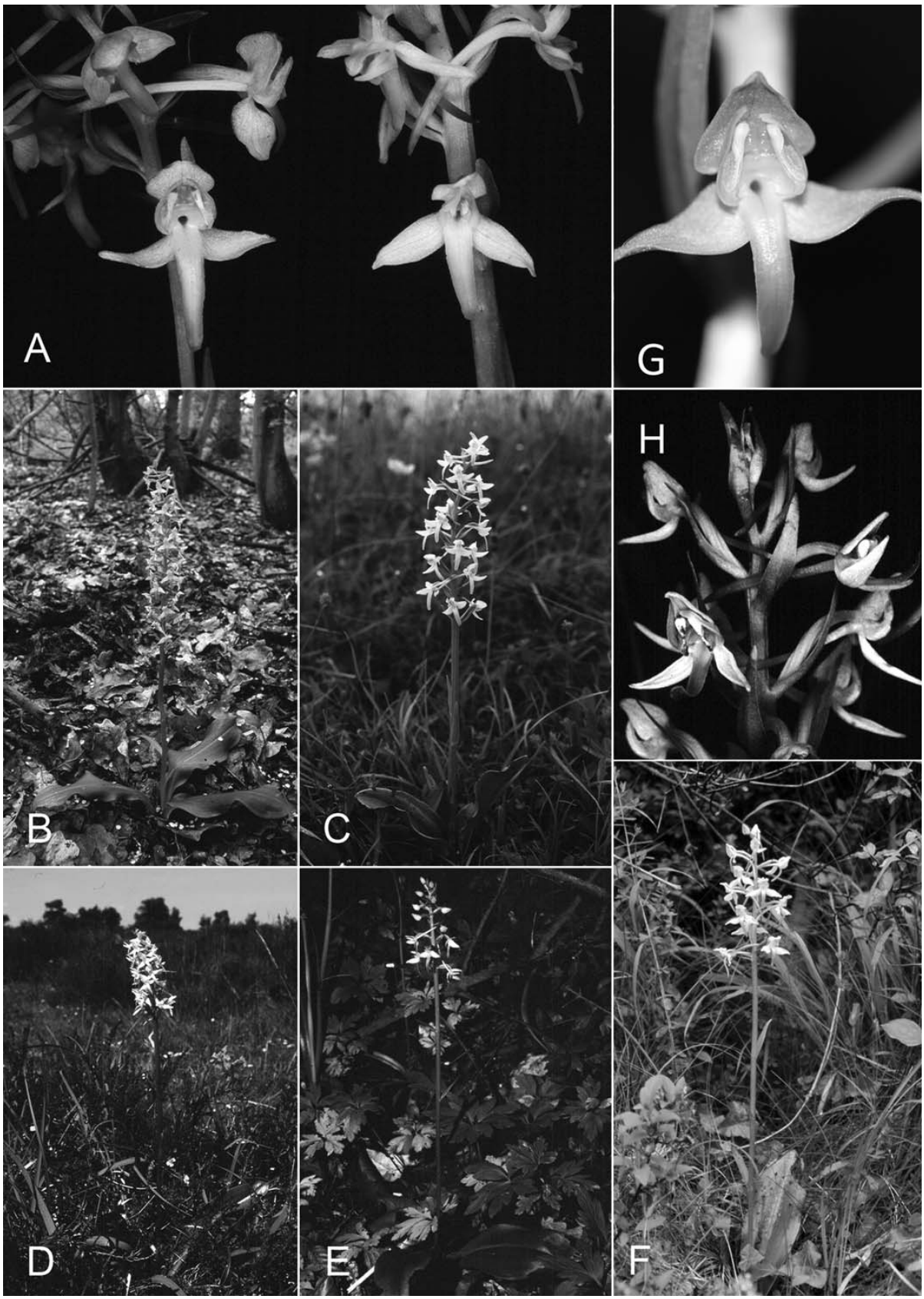

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Figure 1 Habitat races represented by the two study species in the UK. (A) Comparison of inflorescences of *Platanthera chlorantha* (left) and *P. bifolia* (right) co-occurring in the neutral grassland of a cemetery immediately east of Broadford, Skye, Scotland; (B) *P. chlorantha* in beech woodland, East Hoathly, Sussex; (C) *P. bifolia* in calcareous grassland, Morgan's Hill, Wiltshire; (D) *P. bifolia* in acid heathland, Pig Bush, Hampshire; (E) *P. bifolia* in calcareous woodland, Stockbury Hill Wood, Kent; (F) *P. chlorantha* in scrubby grassland, Aston Clinton, Buckinghamshire; (G) Flower of *P. algeriensis* from lowland pinewoods, Marina d'Erba Rossa, Corsica; (H) Flower of mutant green-flowered individual of *P. chlorantha* resembling *P. algeriensis* from mine tailings at Keltney Burn, Perthshire. Images: Richard Bateman, except (B) courtesy of the late Derek Turner Ettlinger.

at a resolution of 1 mm. Destructive measurements of root-tubers were not attempted. A single 'floret' was removed from the middle of the inflorescence for subsequent morphometric characterisation in the laboratory. Organs card-mounted on double-sided adhesive tape for measurement were the labellum (five characters), labellar spur and ovary (together five characters), lateral sepals and lateral petals (together four characters), bracts (three characters) and gynostemium (seven characters). Most floral organs were measured at a resolution of 0.1 mm using a Leitz $\times 8$ graduated ocular. There were two exceptions: gynostemium characters were measured to a resolution of 0.1 mm at $\times 10$ magnification under a Leica MZ8 binocular microscope, whereas floral bract cells (two characters) were recorded in μm at $\times 100$ magnification under a Leica Dialux 20 compound microscope.

The resulting matrix was analysed by multivariate methods using the Genstat v11.2 computer program (Payne *et al*., 2008). All 37 characters were used to compute a symmetrical matrix of indices that quantified the similarities of pairs of individual plants using Gower's (1971) coefficient of similarity. The resulting maximum similarity values were linked to yield a minimum spanning tree expressing their phenetic relationships (Gower & Ross, 1969), and were used to calculate principal coordinates (Gower, 1966, 1985) – compound vectors incorporating positively or negatively correlated characters that are most variable and therefore of potential diagnostic value. The first three principal coordinates (PC1–3) were plotted together in pairwise combinations to assess the degree of morphological separation of individuals, populations and taxa in these dimensions (e.g. Bateman, 2001a), and pseudo-F statistics were obtained to indicate the relative contributions to each coordinate of the original variables.

Spur-length survey

Data obtained for the present study via the morphometric programme formed the core of a much more geographically extensive survey of spur lengths. During 2007, the present authors added data from northwest Scotland and southeast France. Large bodies of data from south-central Scotland were contributed by Roy Sexton (Sexton & McQueen, 2005). Further data from several regions of Britain and the Alps were kindly provided by several members of the UK Hardy Orchid Society, who followed detailed instructions that successfully ensured consistency of measurement among different analysts (Bateman & Sexton, 2007, 2008). Results for the survey up to mid-2007, which encompassed 79 datasets, were discussed in detail by Bateman & Sexton (2008, 2009).

Between late 2007 and late 2012, a further 71 spur-length datasets were added to the matrix. Although only 38 datasets proved usable for latitudinal comparison, these filled critical geographical lacunae in the previous sampling (Appendix 4). On the Continent, sampling in the Alps was extended east into Slovenia (S. and M. Tarrant) and northward into Switzerland (N. Johnson and R. Webb, A. Hughes) and Germany (D. Hughes), samples were obtained from southwest France (R. Bateman and P. Rudall, A. Hughes), and a single population was studied in Normandy (D. Pearce and K. Stott). In the British Isles, new data were gathered in previously unstudied regions of west Ireland (R. Bateman), west Wales (A. Chater), the Welsh Borders (J. Pedlow), and the southern Pennines (S. Cole). Other new samples consolidated previously sampled regions (R. Bateman and P. Rudall, A. Gendle, G. Goodfellow and A. Skinner, L. Harbron and N. Harbron, A. and S. Harrap, K. Stott, N. Henderson and D. Pearce, J. and S. Temporal).

Spur length is a more obviously self-defining parameter than, for example, spur width/diameter. It is most readily measured by placing a 150-mm steel rule against the back of one of the lateral sepals, with the flower still firmly attached to the inflorescence. As flower size had recently been shown to decrease considerably from the base to the apex of the inflorescences of several European orchid species (Bateman & Rudall, 2006a), we specified that the measured flower should be chosen from the middle of the inflorescence and be fully open. We requested that analysts gather a sample of 20 individuals; some samples were substantially larger than this figure, though the limited size of most *Platanthera* populations in the UK meant that many sample sizes were appreciably smaller (cf. appendix 1 of Bateman & Sexton, 2008).

Bateman & Sexton (2008, table 1) pooled spur-length data to enable 21 paired comparisons, using student's t-tests to explore the robustness of the data. These demonstrated that data accumulated by different analysts were reliably comparable, but suggested that the greatest threat to data accuracy lay in continued elongation of the spur following anthesis. Fourteen further comparisons of multiple values for the same population were made during the present study (see below). Then, after culling all unacceptably small and/or duplicate populations, mean spur lengths for the remaining 116 populations were regressed against latitude.

Scanning electron microscopy (SEM)

Selected flowers of both species from the Stockbury area of north-central Kent were stored in 70% ethanol. The spirit collection at RBG Kew yielded

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Table 1 Details of DNA primers used

Locus	Primer	Direction	Reference
ITS	ITS5	F	Baldwin *et al.* (1995)
	ITS4	R	Baldwin *et al.* (1995)
atpB-rbcL	1.1	F	Fofana *et al.* (1997)
	1.2	R	Fofana *et al.* (1997)
petA-psbE	3.5	F	Fofana *et al.* (1997)
	3.6	R	Fofana *et al.* (1997)
rbcL	1F	F	Gastony & Johnson (2001)
	1351R	R	Gastony & Johnson (2001)
rpl16	71F	F	Jordan *et al.* (1996)
	1661R	R	Jordan *et al.* (1996)
rps4	RPS4F	F	Not known
	TRNAS	R	Nadot *et al.* (1995)
rps14-psaB	2.3	F	Fofana *et al.* (1997)
	2.4	R	Fofana *et al.* (1997)
trnL-trnF	TRNC	F	Taberlet *et al.* (1991)
	TRNE	F	Taberlet *et al.* (1991)
	TRNF	R	Taberlet *et al.* (1991)
trnC-rpoB	TRNC-R	F	Ohsako & Ohnishi (2000)
	RPOB-R	R	Ohsako & Ohnishi (2000)

further alcohol-fixed inflorescences of *P. bifolia*, *P. chlorantha* and *P. holmboei*. Selected flowers from each inflorescence were dehydrated through an alcohol series to 100% ethanol. They were then critical-point dried using an Autosamdi 815B CPD, mounted onto stubs using double-sided adhesive tape, coated with platinum using an Emtech K550X sputter-coater and examined under a Hitachi cold-field emission SEM S-4700-II at 2 kV. The resulting images were recorded digitally for subsequent manipulation in Adobe Photoshop. Comparison of fresh and spirit material of *P. bifolia* demonstrated the absence of preservation-related artifacts in the spirit material.

DNA extraction and sequencing

Total genomic DNA was extracted from silica-desiccated floral (or, less often, leaf) material from a total of 50 specimens using the standard CTAB procedure (Doyle & Doyle, 1990) except that extractions were incubated in 500 µl CTAB buffer, 50 µl sarkosyl and 10 µl proteinase-K. The rapidly mutating ITS region of nuclear rDNA (e.g. Baldwin *et al.*, 1995; Hershkovitz *et al.*, 1999) was amplified by polymerase chain reaction using primers ITS4 and ITS5 (Baldwin *et al.*, 1995) and cycling parameters inherited from the initial phylogenetic analysis of subtribe Orchidinae by Pridgeon *et al.* (1997). In addition, eight plastid regions (*atpB-rbcL*, *petA-psbE*, *rbcL*, *rpl16*, *rps4*, *rps14-psaB*, *trnL-trnF*, *trnC-rpoB*) were amplified from two representative specimens each of *P. bifolia* (B798, Warburg; B852, St Anne's) and *P. chlorantha* (B806, Yockletts; B842, Sheepleas). Primers are given in Table 1 and cycling parameters in Table 2. Bidirectional cycle sequencing was carried out on an ABI 3730 capillary DNA sequencer using ABI PRISM BigDye Terminator Cycle Sequencing Ready Reaction Kit (Perkin-Elmer), using the same primers used for amplification. Sequence contigs were assembled in Genotyper and manually aligned in MacClade v4 (Maddison & Maddison, 1992). One exemplar sequence for each of the seven 'alleles' (strictly, ribotypes) identified was deposited in GenBank by Bateman *et al.* (2009).

Tree-building methods

Section *Platanthera* was placed in the broader phylogenetic context of the *Pseudorchis–Neolindleya–Galearis–Platanthera* clade by Bateman *et al.* (2009). Here, we present data for the full ITS1–5.8S–ITS2 assembly for accessions from the *P. bifolia* aggregate only (Appendix 2). Of the 50 accessions reported here, single sequences of both *P. bifolia* (Scotland) and *P. chlorantha* (Italy) were first presented by Pridgeon *et al.* (1997) and another, of *P. holmboei* (Lesvos), by Bateman *et al.* (2003). The remaining 47 sequences were generated specifically for the present study.

Only one complete ITS sequence representing the *P. bifolia* group had previously been deposited in GenBank (AY04975, dated 2004), and this proved to have been misidentified, the sequence being approximately equally and substantially divergent from those of the *Platanthera* and *Galearis* clades. It was therefore omitted from subsequent analyses.

The resulting matrix contained six named members of the Eurasian *P. bifolia* aggregate. Of these, two were represented by only one accession (*P. finetiana* and *P. metabifolia* from China) and three were represented by just two accessions (*P. micrantha*

Table 2 Details of DNA sequencing treatments

Locus	Approximate fragment length	Initial denature	Cycles	Denature	Anneal	Extend	Final extend
ITS	700	94°C, 2 min	25	97°C, 1 min	54°C, 1 min	72°C, 3 min	72°C, 7 min
atpB-rbcL	1550	94°C, 2 min	45	94°C, 30 s	53°C, 30 s	72°C, 2 min	72°C, 7 min
petA-psbE	1450	94°C, 2 min	45	94°C, 30 s	56°C, 30 s	72°C, 2 min	72°C, 7 min
rbcL	1250	94°C, 4 min	35	94°C, 30 s	50°C, 1 min	72°C, 2 min	72°C, 5 min
rpl16	1300	94°C, 3 min	28	94°C, 1 min	50°C, 1 min	72°C, 1 min	72°C, 7 min
rps4	800	94°C, 2 min	35	94°C, 30 s	55°C, 30 s	72°C, 2 min	72°C, 5 min
rps14-psaB	550	94°C, 2 min	45	94°C, 30 s	56°C, 30 s	72°C, 2 min	72°C, 7 min
trnL-trnF	1000	94°C, 2 min	30	94°C, 30 s	55°C, 30 s	72°C, 1 min	72°C, 5 min
trnC-rpoB	1200	94°C, 2 min	30	96°C, 1 min	50°C, 2 min	72°C, 3 min	72°C, 7 min

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and *P. azorica* from the Azores, plus *P. holmboei*, consisting of a *bona fide* plant from Cyprus and a more questionable accession from Lesvos). In contrast, *P. bifolia* and *P. chlorantha* (the primary subjects of this study) were together represented by 42 accessions: three from China, nine from Continental Europe, and the remaining 30 from the British Isles. Three of the UK-sourced DNA samples were putative hybrids; multiple accessions were analysed only from the two sites that yielded putative hybrids (five samples from Bix and three from St Anne's).

Parsimony trees were generated from the matrix using PAUP 4.0b10 (Swofford, 2001); full details of the tree-building procedure were given in Bateman *et al.* (2009).

Results

Molecular phylogenetics

Nuclear ribosomal ITS data

The analysis of Bateman *et al.* (2009) supported that of Hapeman & Inoue (1997) in suggesting that the apparent monophyly of *Platanthera* section *Platanthera* is only weakly supported, and that there is considerable molecular divergence among exclusively Asiatic species of the section (i.e. *P. mandarinorum*, *P. florentia*, *P. bakeriana*). The *P. bifolia* aggregate proved robustly monophyletic but, to our surprise, the 50 accessions of seven putative species yielded only seven ITS alleles (I–VII: Appendix 2, Table 3), and these alleles differed among each other by a maximum of only five base-pair changes (i.e. less than 0.8% divergence: Fig. 2). Also, three of the seven alleles (including the two most common) were found in more than one putative species, and the apparently plesiomorphic allele I was found in no less than five of the seven putative species analysed (Appendix 2). Moreover, the sequence chromatograms of several individual accessions of *P. bifolia* and *P. chlorantha* were consistently observed to contain overlapping traces at specific loci consistent with heterozygosity (absence of funding precluded exploration of these apparent co-occurring alleles via cloning).

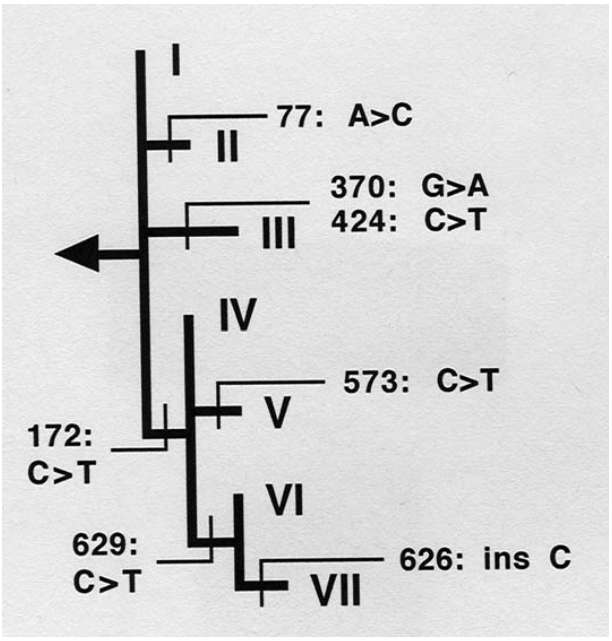

Figure 2 Detail of topology of ITS-based parsimony tree for the *Platanthera bifolia* aggregate, highlighting the position and nature of the small number of base-pair changes (including autapomorphies, and optimised via Acctran). The arrowed branch subtending the entire clade is 18 steps long, with bootstrap support of 100% and a decay index exceeding three. The seven closely similar ITS alleles (I–VII) differ by a maximum of five base-pair changes (for the broader phylogenetic context see Bateman *et al.*, 2009, fig. 1).

Alleles I and II occurred in *P. bifolia* and alleles I, II and III in *P. chlorantha* (Appendix 2, Table 3). Allele III was found only in Chinese accessions of *P. chlorantha* (in two out of three specimens analysed). In Continental Europe and the UK, alleles I and II occurred at equal frequencies (50% : 50%, $n=19$) in *P. chlorantha*, whereas in *P. bifolia* allele I dominated over allele II (78% : 22%, $n=18$), and only one each of the accessions from Continental Europe and the UK contained solely allele I. The 28% difference in frequency of allele I in the two species marginally failed tests of statistical significance. Although individuals carrying both alleles formed 24% of the combined

Table 3 Distribution of ITS alleles I–III among analysed accessions of *P. bifolia*, *P. chlorantha* and their putative hybrids

Geographic region	Allele(s)	*Bifolia*, *n* (%)	*Chlorantha*, n (%)	Hybrids, *n* (%)
China	I	NA	1 (33)	NA
	III	NA	2 (67)	NA
Continental Europe	I	4 (80)	1 (25)	NA
	II	1 (20)	1 (25)	NA
	I+II	0	2 (50)	NA
UK	I	8 (62)	5 (36)	1 (33)
	II	1 (8)	6 (43)	2 (67)
	I+II	4 (31)	3 (21)	0
All regions	I	12 (67)	7 (33)	1 (33)
	II	2 (22)	7 (33)	2 (67)
	I+II	4 (11)	5 (24)	0
	III	0	2 (10)	0

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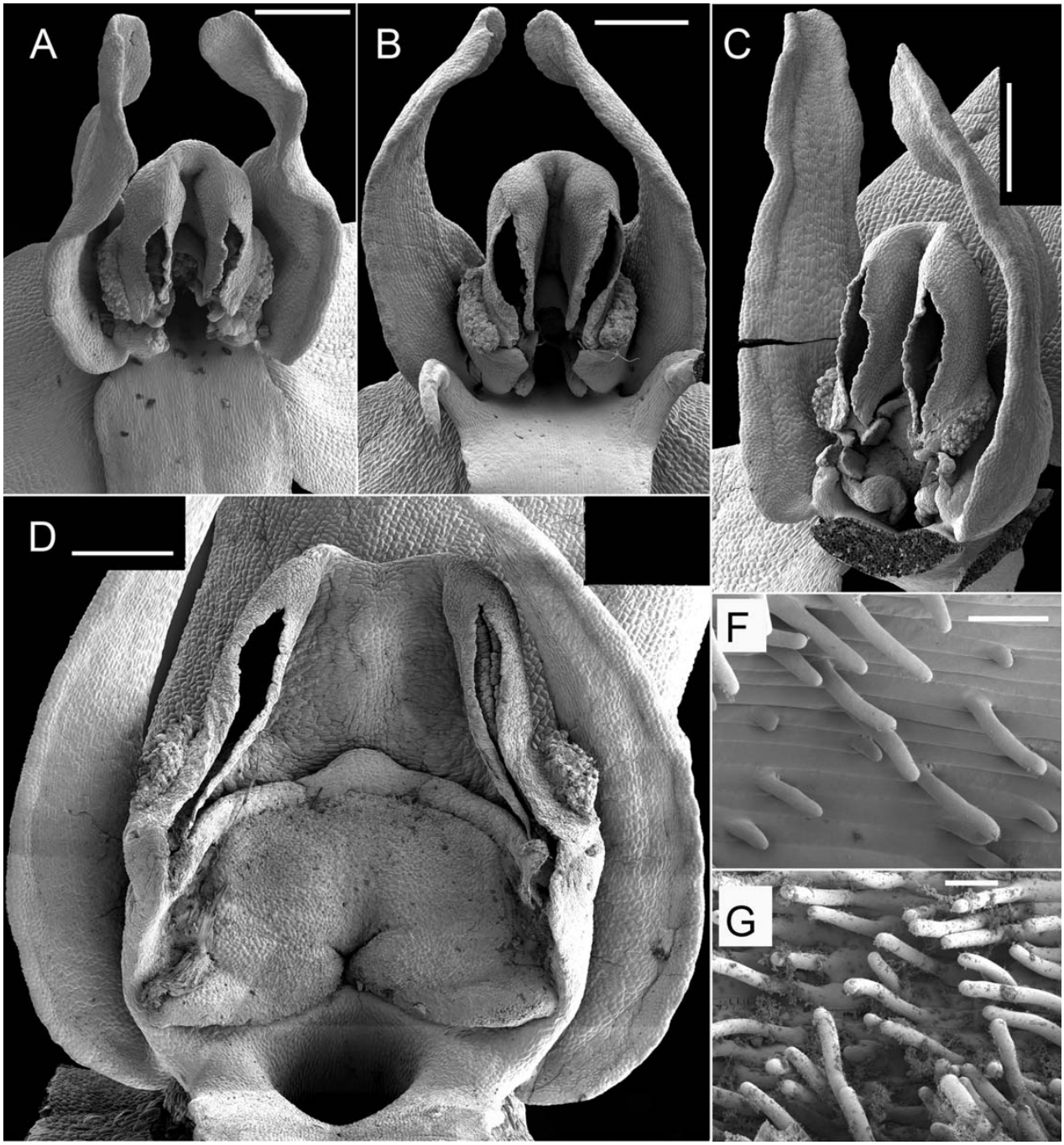

Figure 3 SEM images of the two main study species. *Platanthera bifolia*. (A–C) Three whole flowers from different plants in the same population, showing variation in floral morphology (e.g. note the unusually well-developed lateral horns on the labellum of flower B); (E) enlargement of interior of spur showing well-developed papillae. *Platanthera chlorantha*. (D) Whole flower at similar scale to (A–C), showing much larger stigma, connective and spur entrance; (F) enlargement of interior of spur showing well-developed papillae coated with residue of nectar. Scales: A–D=1 mm; E, F=50 μm. All images: Paula Rudall.

sampling of the two species, surprisingly, none of the three putative hybrids from the UK analysed by us proved to possess both alleles: one contained only allele I and the other two yielded only allele II.

Plastid data

Comparison of two exemplar DNAs each of *P. bifolia* and *P. chlorantha* encompassed two plants containing only ITS allele I (*P. bifolia* from Bix, *P. chlorantha* from Sheepleas), one plant containing only allele II (*P. chlorantha* from Yockletts) and one plant apparently maintaining both allele I and allele II (*P. bifolia* from St Anne's). The most surprising outcome of the plastid sequencing was the failure to detect any difference in the eight moderately to rapidly mutating plastid regions, totalling 9100 bp, that were screened in the present study (*atpB-rbcL*, *petA-psbE*, *rbcL*, *rpl16*, *rps4*, *rps14-psaB*, *trnL-trnF*, *trnC-rpoB*). Even within species, such genetic uniformity is exceptional.

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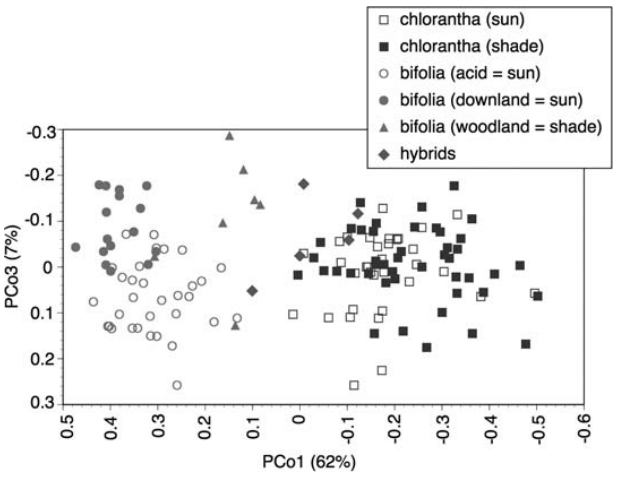

Figure 4 Scanning electron micrographs of mid-bud (A) and late-bud (B) ontogenetic stages of *Platanthera holmboei* flowers. Scale bar=5 mm. All images: Paula Rudall.

SEM of flowers

SEM study of representative flowers of *P. bifolia*, *P. chlorantha* (Fig. 3) and *P. holmboei* (Fig. 4) confirmed and clarified the differences — and similarities — already reported between the two species (Fig. 1).

These reliably anthocyanin-free flowers show the relatively simple perianth that is characteristic of the majority of *Platanthera* species. The six perianth segments are broadly similar in shape (obovate) and size. The dorsal petal and lateral petals form a loose, flexible hood above the gynostemium. The lateral sepals are opposed, spreading and often show slight spiral torsion such that they resemble the wings of a hovering seagull. The labellum is comparatively narrow, parallel-sided and robust, being thicker dorsiventrally; it is typically held vertically, but projects forward in some individuals. Apart from the paired lateral horns that adorn a minority of individuals (Fig. 3B), the only labellar ornamentation is the relatively narrow, filiform spur that projects horizontally backward perpendicular to the labellum. The spur exceeds both the labellum and ovary in length, and so projects beyond, and often touches, the inflorescence axis (Fig. 1A). The interior surface of the spur is rich in papillae (Fig. 3F and G), which presumably are responsible for both secretion and subsequent resorption of substantial quantities of nectar (Stpiczynska, 2003a, b).

The main purpose of the SEM study was to examine in greater detail the morphology of the gynostemium and pollinaria (Fig. 3), which together have long been considered to determine pollinator specificity in the group (Darwin, 1877; Nilsson, 1983, 1992), and reportedly provide the most valuable characters for distinguishing the two species (e.g. Godfery, 1933; Summerhayes, 1951; Clapham *et al.*, 1962; Webb, 1980; Stace, 1987; Harrap & Harrap, 2005; Delforge, 2006).

The gynostemium of *P. bifolia* is relatively narrow (Fig. 3A–C). Only a small connective and rostellum separate the parallel loculi that contain the pollinaria. The viscidia lie immediately above the narrow, circular spur entrance, which is located immediately below the small stigmatic surface; outside the stigma are the short-stalked, tuberculate staminodes that are especially characteristic of *Platanthera*. The pollinia and caudicles that, together with the basal viscidia, constitute the pollinaria are approximately equal in length.

The mature flowers of *P. chlorantha* (Fig. 3D) and *P. holmboei* (Fig. 4B) are typically larger in all their parts than that of *P. bifolia*, though their gynostemia differ from those of *P. bifolia* in shape as well as size (see also Efimov, 2011). The overall effect is as though a flower of *P. bifolia* had been inflated by injecting air into a valve located immediately above the spur entrance. The stigmatic surface is taller and much wider, pushing the viscidia much further apart – that of *P. chlorantha* is reputedly four times the distance separating the viscidia in *P. bifolia*. In contrast, the apices of the thecae are only slightly further apart than in *P. bifolia*; this disparity causes the pollinaria of *P. chlorantha* to converge from base to apex, subtending an angle of approximately 30°. The spur entrance is substantially wider, and the staminodia are significantly longer-stalked, than those of *P. bifolia*. Removal of the pollinaria shows that they are larger and that the caudicles form a greater proportion of their overall length relative to the club-shaped, pollen-coated pollinia. In addition, the dorsal sepal is correspondingly broader, presumably in order to cover the wider gynostemium (Fig. 1A).

The spur of *P. bifolia* appears to be a simple cylinder, whereas that of *P. chlorantha* is expanded towards the apex (Ettlinger, 1997; Bateman & Sexton, 2008). We found spurs of both species to be bilaterally compressed (i.e. oval rather than circular in transverse section), but in *P. chlorantha* – and in the similar *P. holmboei* and *P. algeriensis* – there also appeared to be a significant dorsiventral increase in wall thickness in the distal half to two-thirds of the spur. This often conferred on the spurs of *P. chlorantha* a gently sigmoid shape when viewed laterally (Fig. 1A and F).

Morphometric analyses

The resulting Excel matrix of 139 individuals (Appendix 1) × 37 characters (Appendix 3) yielded an asymmetric matrix of 5,143 cells, of which 733 (14%, a relatively high proportion for a morphometric matrix based on field measurement rather than herbarium sampling) were scored as missing. Missing values were concentrated in characters representing vegetative features and, to a lesser degree, the

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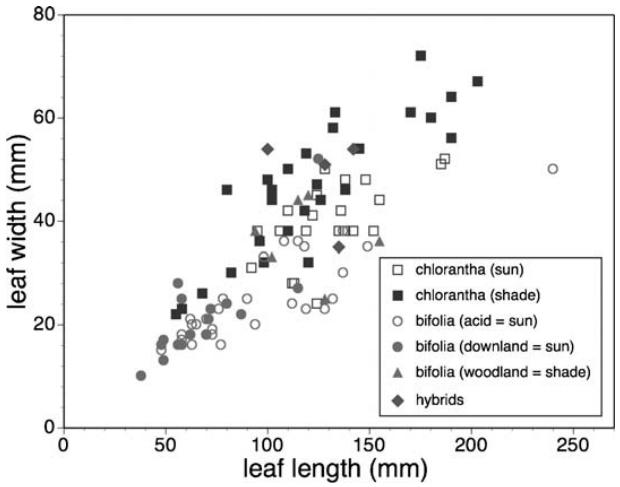

Figure 5 Principal coordinates plot of the first and third axes of combined *Platanthera bifolia* and *P. chlorantha* data matrices representing 21 populations from southern England.

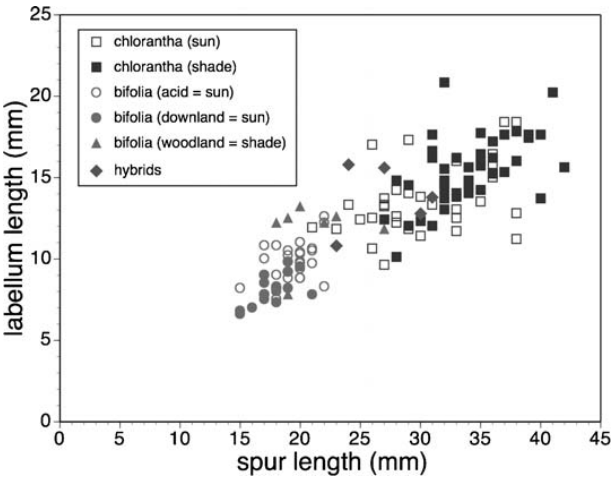

Figure 6 Bivariate scattergram of leaf length versus leaf width (mm) for *Platanthera bifolia* and *P. chlorantha* in southern England.

gynostemium, which was the part of the flower that degraded most rapidly following field sampling. Of the five characters with missing value totals exceeding 25%, two described features of the bract cells, one described the divergence of the viscidia, and two described the gross morphology of the leaves (leaf shape and the angle subtended by the leaf relative to the ground surface).

In the principal coordinates analysis of all accessions (i.e. including both species plus putative hybrids: Fig. 5), the first coordinate reliably separated individuals of *P. chlorantha* (scores <0.02) from those of *P. bifolia* (scores >0.08), though the intervening morphological discontinuity was narrower than expected. This axis also largely separated woodland *P. bifolia* from the remainder, placing them closest to *P. chlorantha*. Of the five putative hybrid plants, four overlapped with that portion of the distribution of *P. chlorantha* closest to *P. bifolia*, whereas the fifth individual overlapped with that portion of the distribution of *P. bifolia* closest to *P. chlorantha*. Compared with other similar morphometric studies of European orchids that employed an identical analytical protocol (e.g. Bateman & Denholm, 1983, 1985, 1989; Bateman & Farrington, 1987, 1989; Bateman *et al.*, 2008; Bateman & Rudall, 2011), the first coordinate encompassed an exceptionally large proportion of the total variance (62%), reflecting the fact that it was strongly influenced by 21 of the 37 measured variables (Table 4). Although floral characters that supposedly distinguish the two species (e.g. viscidial separation, diameter of spur entrance, length of spur) contributed strongly, they operated in conjunction with several other metric characters, both floral and vegetative; all of these characters increased in value from left to right in Fig. 5.

The second coordinate (not figured, but contributing variables given in Table 4) encompassed only 10% of the total variance and proved to be biologically spurious, largely reflecting the suboptimal way in which the intensity of chlorophyll pigment in the lower part of the labellum had been recorded (absent=0, pale green=1, darker green=2). No individual scored absent and, in each species, approximately half of the analysed individuals scored 1 and the other half 2. Such distributions of character states are typical of a character that, statistically if not biologically, is viewed as being highly diagnostic; thus, within each species, the character indicated a false discontinuity between individuals scored as pale green and those scored as darker green, the latter also tending to develop more extensive green pigmentation. We are confident that, had the green suffusion been coded more rigorously — ideally, as a set of continuous variables using a colourimeter or a quantitative colour chart — it would not have appeared discriminatory.

Table 4 Variables contributing to the first three principal coordinates (Fig. 5), listed in order of decreasing contribution. Numbers of variables match character numbers given in Appendix 4. Double slashes separate dominant from subdominant characters. Italicised characters increase in value toward the bottom left of Fig. 5; roman characters increase in value toward the top right

Principal coordinate	Percentage of variance accounted for	Contributing characters
PCo1	62	16,18,19,15,17,7,13,6, 1,21,20,8,12,25,10,2, 14,24,26,33,27
PCo2	10	4//5,*11*,29,37
PCo3	7	11//*37*,*3*,*21*

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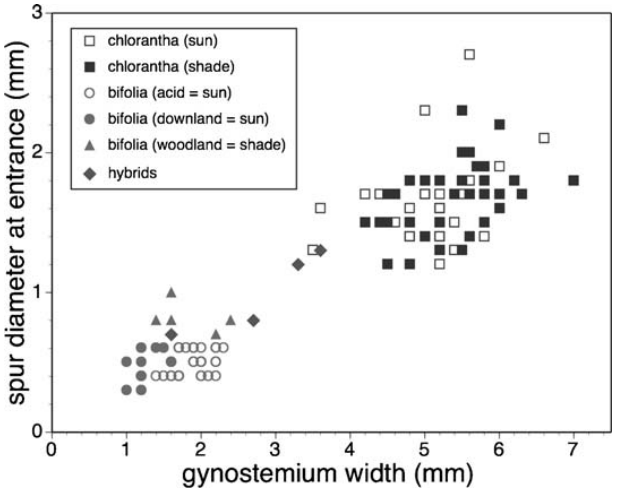

Figure 7 Bivariate scattergram of spur length versus labellum length (mm) for *Platanthera bifolia* and *P. chlorantha* in southern England.

The third coordinate accounted for only 7% of the total variance (Table 4). It was dominated by the position of the lateral outer perianth segments, which projected laterally above the horizontal in the majority of plants but below the horizontal in most plants of *P. bifolia* that occupied acid soils. In addition, plants of *P. bifolia* occurring in open habitats on alkaline soils tended to have leaves adpressed against the soil surface (Fig. 5). In contrast, no internal structure relating to soils or insolation was identified within the *P. chlorantha* cluster. To a lesser degree, this axis also reflected the shorter staminodes of *P. bifolia* relative to *P. chlorantha*.

Bivariate scattergrams were used to explore selected characters that were either previously considered discriminatory between the two species or were identified by the principal coordinates analyses as being of particular interest; three such plots are presented here (Figs. 6–8).

Not surprisingly, the plot of leaf length against leaf width suggested a strong positive correlation between the two parameters (Fig. 6). However, the length: width ratio tends to be greater in *P. chlorantha* from sunny habitats, and in *P. bifolia* from acid soils. Among flowering individuals, small leaves ($<80 \times 30$ mm) dominantly occur in *P. bifolia* and large leaves ($>150 \times 50$ mm) dominantly occur in *P. chlorantha*, but there is a large zone of overlap, demonstrating that these vegetative characters are of limited value in discriminating between the two species.

Both labellum length and spur length offer reasonable levels of discrimination between the two species in southern England (Fig. 7). Discrimination is stronger for spur length, where only two individuals of *P. chlorantha* had spurs less than 23 mm long and only one individual of *P. bifolia* had a spur greater than 23 mm long. Few individuals of *P. chlorantha* had labella less than 11 mm long, whereas, with two exceptions, only *P. bifolia* from woodland habitats exceeded that length.

In contrast with the two previous character pairs, gynostemium width (a proxy for viscidial separation) and the diameter of the spur entrance showed complete discrimination of the two species, which are separated by a broad discontinuity in gynostemium width and a narrow discontinuity in spur diameter (Fig. 8). Three of the four scored hybrids (the fifth could not be measured satisfactorily) occupy intermediate positions (unsurprisingly, given that these characters were used to identify them as hybrids in the field). The fourth hybrid coincides with *P. bifolia*, which appears to show some habitat differentiation in these characters; woodland plants have wider spurs, whereas downland plants have especially narrow gynostemia (Fig. 8). In retrospect, it would have been helpful to measure the pollinaria in greater detail, considering also length of stipe, length of pollen mass and diameter of viscidial disc (cf. Nilsson, 1983).

Spur-length survey

The spur-length survey covered a much broader geographical area than the multivariate analysis. As the early results were examined in detail by Bateman & Sexton (2008), the implications of the present, substantially expanded data matrix are summarised only briefly here.

By the close of the 2012 field season, the database of spur lengths contained 181 datasets (70 for *P. bifolia*) totalling 3,070 individual plants (1,013 for *P. bifolia*), datasets ranging in sample size from a single plant to 148 plants. Of these 181 datasets, 43 were generated by Bateman and Rudall, 30 by Sexton and the remaining 108 by many other recorders. The results presented by Bateman & Sexton (2008) showed clustering of data-points in the Alps, southern England, Cumbria and southern Scotland (interestingly, all three aforementioned regions of the UK are geographical 'hot-spots' for *P. chlorantha* explicitly identified by Foley & Clarke, 2005) (Fig. 9). Subsequent data usefully filled the lacuna in the British Midlands (the new data cover the southern Pennines, Welsh Borders, and west Wales) plus western Ireland and southern France (Appendix 4).

Seventeen datasets consisted of only one or two plants, and so yielded data of severely limited value. Each of the remaining 154 datasets yielded values for the mean spur length and sample standard deviation, omitting a few rare outlying plants wherein spur development had clearly been seriously retarded. A further 39 datasets (nine for *P. bifolia*) duplicated, in

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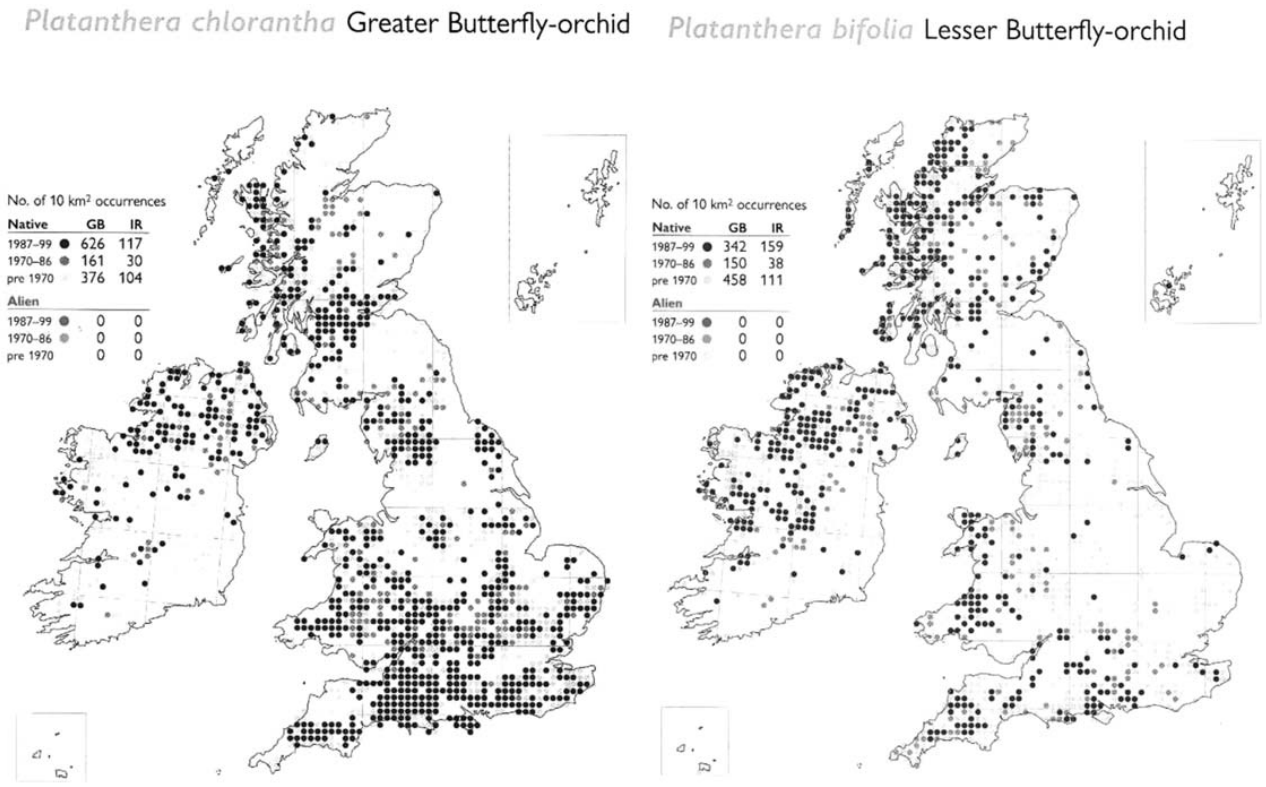

Figure 8 Bivariate scattergram of gynostemium width versus width of spur entrance (mm) for *Platanthera bifolia* and *P. chlorantha* in southern England.

several contrasting ways, other datasets based on the same locality. These duplications usefully allowed Bateman & Sexton (2008) to infer how much of the observed variation in spur length could be attributed to measuring error or to non-genetic (i.e. environmental and/or developmental) influences, rather than to genetic factors. With regard to measuring error, different analysts had demonstrably generated reliable data, and the only serious potential cause of misleading results — rapid increase in spur length close to, and even after, anthesis observed in a population of *P. bifolia* on South Uist — had been precluded by the stringent measuring protocol. The large statistical samples gathered in successive years from populations in south-central Scotland monitored by Sexton & McQueen (2005) and in Sussex by K. Stott, N. Henderson and D. Pearce demonstrated small but, in five out of 11 populations, statistically significant differences in mean spur length between years, suggesting that there exists a modest climatic influence on spur length.

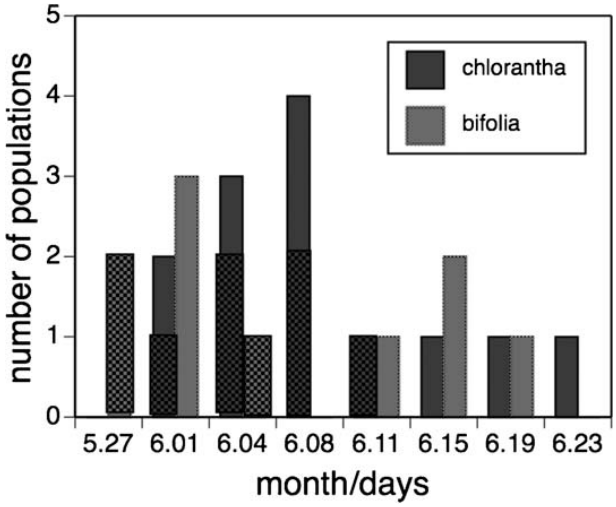

Figure 9 Distributions of *P. chlorantha* (left) and *P. bifolia* (right) in the British Isles (from Preston *et al.*, 2002: 842).

Fourteen further paired comparisons (six involving *P. bifolia*) were made in the present study. Ten of these comparisons involved analysts measuring the same population in successive years, and only one yielded a statistically significant difference: an 8.4% increase in spur length at the Wolstenbury population of *P. chlorantha* between 2007 and 2008 ($P<0.01$: K. Stott, N. Henderson and D. Pearce), though spur length at the site was far more consistent throughout the subsequent years 2008–2012. At Smardale in 2008, L. and N. Harbron observed a 9.1% difference ($P<0.01$) between adjacent woodland and shorter-spurred grassland populations of *P. chlorantha*, supporting our previous assertion that shade significantly influences spur length. However, a similar comparison between adjacent groups of sun and shade plants of *P. chlorantha* in Normandy failed to

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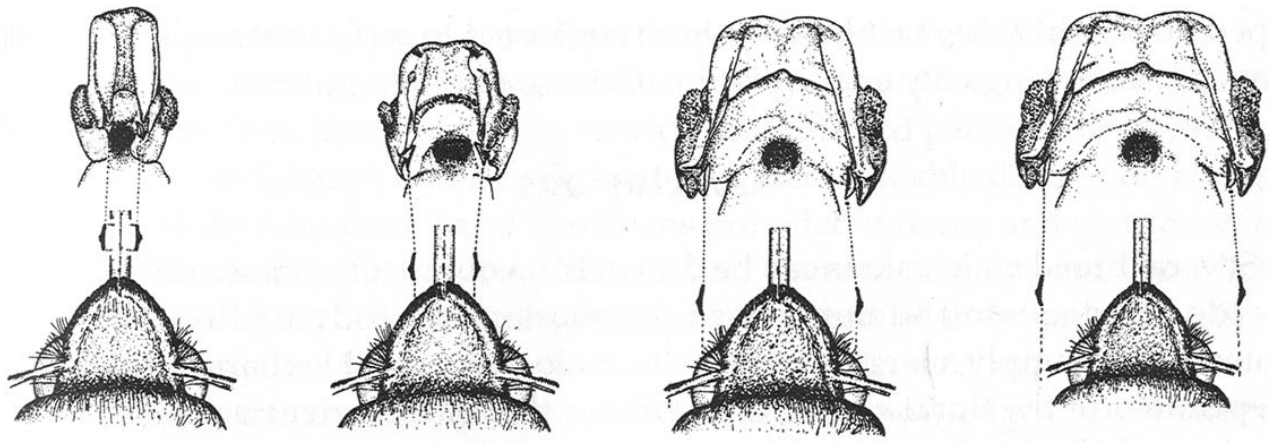

Figure 10 Comparison of the peak flowering periods of study populations of *Platanthera chlorantha* and *P. bifolia*. Cross-hatched populations were located dominantly in shade rather than sun.

yield statistical significance (4.8% difference: K. Stott and D. Pearce). Perhaps the most interesting result was obtained by measuring the Chalford population of *P. bifolia* at the beginning (G. Goodfellow and A. Skinner) and end (R. Bateman and P. Rudall) of its 2008 flowering season, four weeks apart, yielding a substantial 14.7% increase in mean spur length ($P<0.01$). This figure precisely matches the increase in spur length observed by Bateman when comparing spur length of open flowers and buds of *P. bifolia* from the Outer Hebridean island of Benbecula, reinforcing the suggestion of Bateman & Sexton (2008) that spurs of these two species continue to elongate during anthesis.

This hypothesis is currently being tested by us under experimental conditions. Similar observations have been made on other groups within *Platanthera*. For example, 'intermediates and populations [of the *P. dilatata* aggregate] with variable spur lengths are abundant. In some populations in western Canada, in particular, spurs that are very short when the flower is young grow to equal the lip as the flower ages, and in some they may greatly exceed the lip, thereby simulating all three [taxonomic] varieties' (Sheviak, 2002: 557).

Subsequently excluding one of each duplicated pair of datasets (in each case retaining the dataset that was based on the largest sample size) left 116 datasets that could be analysed statistically and so used to seek geographically related patterns in spur length variation for each of the two species.

Discussion

Molecular phylogenetic context of the P. bifolia *aggregate*

The *P. bifolia* aggregate (encompassing all of the taxa listed in Appendix 2) was represented only by a single accession of *P. bifolia* in the study of Hapeman & Inoue (1997) and by single accessions of both *P. bifolia* and *P. chlorantha* in Bateman *et al.* (1997) and Pridgeon *et al.* (1997). A sample representing an eastern Mediterranean segregate of *P. chlorantha*, *P.* cf. *holmboei*, was later added by Bateman *et al.* (2003). Their study surprisingly revealed only a single base-pair separating *P. bifolia* from *P. chlorantha* plus *P.* cf. *holmboei*, a level of divergence lower than that reported for any other pair of *Platanthera* accessions in any previous molecular phylogenetic study.

The present study reports ITS data for 50 accessions of the *P. bifolia* aggregate, together representing seven putative species (including five of the six putative species of section *Platanthera* occurring in Europe: cf. Delforge, 2006). The aggregate is well supported as monophyletic in the ITS tree (Bateman *et al.*, 2009). However, even with this greatly expanded sampling, levels of sequence divergence detected by us were exceptionally low (Fig. 2); a maximum of five base-pairs separated two Chinese accessions attributed to *P. chlorantha* from one of two sequences obtained from the Azorean endemic *P. micrantha*. The only apparent phylogenetic structure that we detected within the *P. bifolia* aggregate was a single-step branch (a single C>T transition at site 172, attracting predictably low statistical support) that separated from the remaining accessions of the *P. bifolia* aggregate the sequences obtained from the Azorean endemics *P. micrantha* and *P. azorica* plus that found in the accession of *P. holmboei* from Cyprus (Fig. 2).

Geographical distribution, habitat preference and phenology of P. bifolia *and* P. chlorantha

P. bifolia and *P. chlorantha* appear to have remarkably similar distributions. Both stretch from the Mediterranean northward to the Arctic Circle, and eastward to the Pacific, though their distributions in central Asia are especially poorly known and complicated by taxonomic uncertainties. Both species occur throughout the British Isles, where their broadly similar distributions (Preston *et al.*, 2002)

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suggest a shared preference for the higher rainfall of the west (Fig. 9). *Platanthera chlorantha* is more frequent on chalk soils in the east than is *P. bifolia*, which is in turn more frequent in northern Scotland. Population numbers are said to be stable for *P. chlorantha* but rapidly declining for *P. bifolia* (Braithwaite *et al.*, 2006), though our field experiences suggest that much of this perceived recent decline to be artefactual.

Both species also show considerable overlap in ecological tolerances (Fig. 1). They can survive levels of insolation from deep shade to full sun, though *P. chlorantha* arguably shows a greater preference for intermediate light levels and less tolerance of exposure. Both species occur in calcareous and neutral soils, but *P. bifolia* has in addition a preference for acid wetlands — a habitat that cannot be successfully colonised by *P. chlorantha*, at least in the British Isles.

The majority of authors claim that, in comparable situations, flowering peaks in *P. bifolia* 7–10 days ahead of that in *P. chlorantha*. However, this assertion receives relatively little support from our estimates of peak flowering in our study populations from southern England (Fig. 10). During the mid-2000s we estimated mean flowering periods of June 6th for *P. bifolia* (apparently obscuring a bimodal distribution of population phenology) and June 9th for *P. chlorantha* (a figure that coincides with the single mode). In both species, UK populations in shade tended to flower earlier than those in more open situations (Fig. 10). Nilsson (1983) suggested that flowering of *P. bifolia* marginally precedes that of *P. chlorantha* in Sweden but that the converse occurs in Denmark and Germany. However, yet further south, Mediterranean segregates commonly considered to be more closely related to *P. chlorantha* (*P. holmboei*, *P. algeriensis*) flower substantially later than co-occurring *P. bifolia*.

Thus, the most striking feature exhibited by these putative sister species is substantial overlap — in geographical distribution, habitat preference and phenology. This has led one of us to speculate that *P. chlorantha* could have originated sympatrically, from within *P. bifolia* (Bateman *et al.*, 2004; Bateman, 2005). However, it is surprising that this overlap in extrinsic properties has not given rise to more records of hybrids, or of locations supporting substantial numbers of both species where hybridisation would be predicted to occur.

Divergent speciation between P. bifolia *and* P. chlorantha*: summary of a classic adaptive scenario*

At this point, it should prove helpful to briefly summarise the often-repeated adaptive scenario that has been developed to explain the species boundary that is universally considered to separate *P. chlorantha* from *P. bifolia* — a scenario that was first conceived by Darwin (1877) and later refined in an exceptional series of papers by Nilsson (1978, 1983, 1992), based on observations made primarily in southeast Sweden. Both morphological inference and direct ecological observation show that the dominant pollinators of both species are night-flying sphingid and noctulid moths. Surprisingly, few authors have considered whether the moths typically alight on the flower (Claessens *et al.*, 2008) or hover in front of it, using their long probosces to probe the correspondingly long labellar spur in order to exploit the abundant nectar that it contains (Stpiczynska, 2003a, b). Moths are covered in loose waxy scales and trichomes that are unlikely to permit firm and reliable attachment of the viscid discs of the pollinaria. Thus, the only stable and resilient surfaces in the anterior region of the moth are the proboscis and the pair of compound eyes (Fig. 11).

Assuming that all erstwhile pollinators probe into the spur via its entrance, the distance separating the viscid discs that terminate the pair of pollinaria will be crucial in dictating which potential pollinator will be most efficient in transferring pollen masses among flowers. The closely spaced viscidia of *P. bifolia*, located immediately adjacent to the spur entrance, are assumed to be well adapted for attachment to the proboscis. In contrast, the much greater distance separating the viscidia of *P. chlorantha* is consistent with the amount of eye separation shown by at least some medium-sized sphingids and noctulids, suggesting ocular rather than proboscoid placement of pollinia. However, ocular attachment will occur only if the moth presses its head firmly against the gynostemium. The insect must therefore be coerced to fully extend its proboscis into the spur in order to access the nectar, suggesting that the average spur of *P. chlorantha* should be optimised to be marginally longer than the average proboscis of the preferred pollinating insect(s) (Nilsson, 1978).

These inferences led naturally to the assumption that any primary hybrids between *P. bifolia* and *P. chlorantha* would have intermediate placements of viscidia. If a flower of *P. chlorantha* was visited by a moth whose eyes were separated by a distance significantly shorter than that separating the viscidia of the orchid, the viscidia would contact waxy plates on the 'cheeks' or maxillary palps of the moth rather than its eyes, precluding effective transport to another flower for cross-pollination. Contrasting individuals of *P. chlorantha* that exhibited unusually large viscidial separation would fail to contact the head of the moth, and hence the pollinia would not be removed. In contrast, we do not need to consider the consequences of viscidial placements closer than

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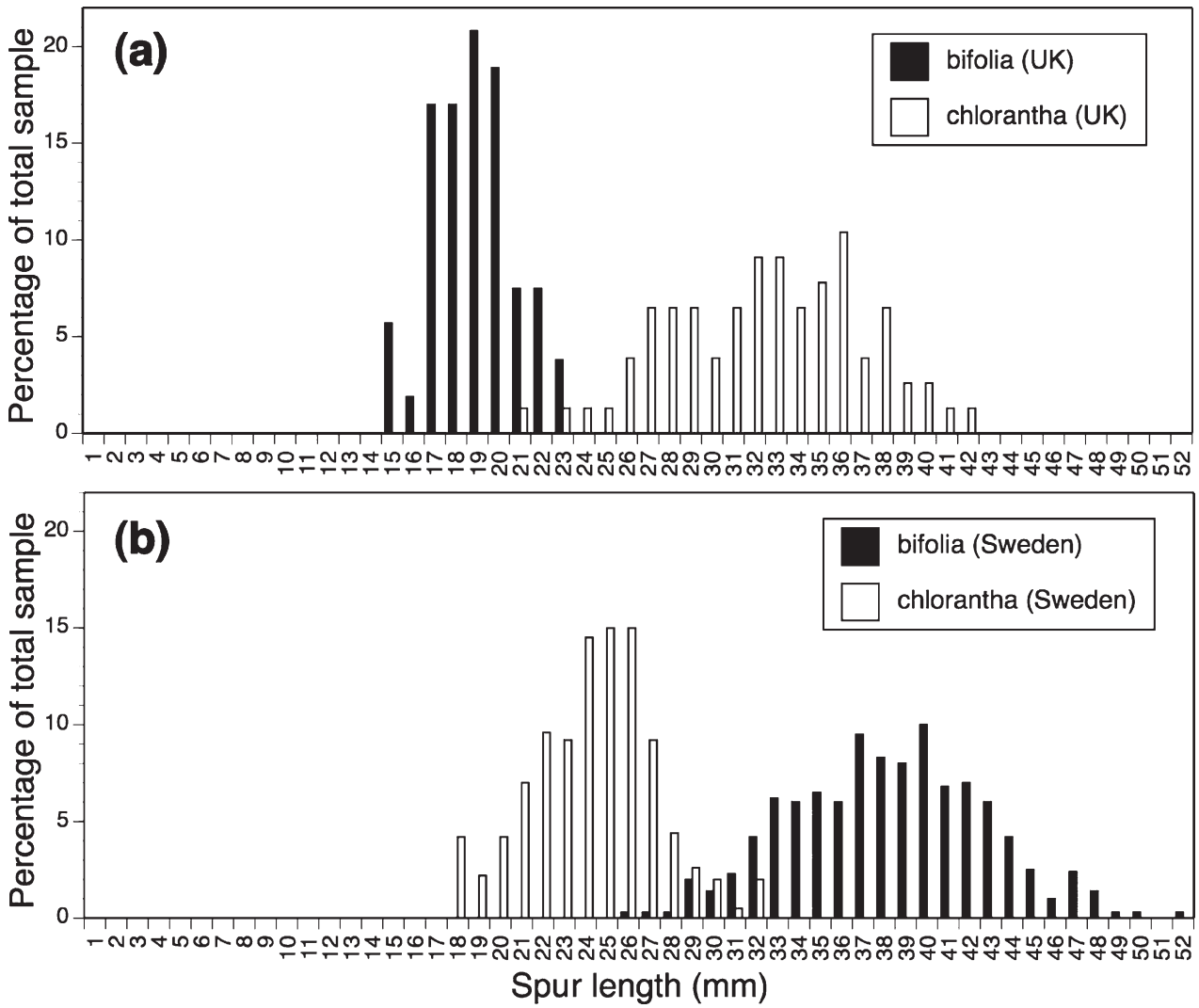

Figure 11 Evolutionary scenario previously advanced as an adaptive explanation for the contrasting pollinium placements in *P. bifolia* and *P. chlorantha* (fig. 15.8 of Hapeman & Inoue, 1997, based largely on fig. 5 of Nilsson, 1983). Gynostemia represent, from left to right, *P. bifolia*, *P. bifolia* × *chlorantha*, normal *P. chlorantha*, exceptionally wide *P. chlorantha* (see text for further explanation).

that observed in *P. bifolia*, because closer placements are precluded by a structural constraint, specifically the presence of the intervening spur entrance.

The classic adaptive scenario described above is attractive because it is both elegant and internally consistent. If correct, it allows additional predictions. Firstly, eye attachment of pollinaria is more likely to yield a narrow range of pollinator species than is proboscis attachment, as attachment should be possible to the probosces of a wide range of moth species, whereas eye attachment requires a more precise spatial match between the relevant structures of flower and pollinator. In addition, pollination should be possible irrespective of where the pollinaria are placed along the length of the proboscis, since the pollen masses should at some point contact the stigmatic surfaces as the proboscis penetrates progressively more deeply into the spur. Thus, one would predict that spur length would be placed under stronger selection pressure in orchids preferring eye attachment, where successful attachment requires the moth to probe deeply into the spur, than in orchids with proboscis attachment, where the mere presence of a nectariferous spur may be sufficient to encourage pollinarium attachment. Lastly, the classic theory states that primary hybrids will be incapable of reproduction, since their intermediate viscidial placement will be too narrow to be attached to the eyes of the pollinator of *P. chlorantha* but too wide to become attached to the proboscis of any potential pollinator; any F1 hybrids should therefore be immune to successful pollination.

Morphometric analysis appears broadly consistent with the adaptive scenario, but potentially adaptive characters appear subordinate to a strong allometric overprint

Analysis of individual morphometric characters revealed few surprises. Characters that we predicted

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would discriminate poorly between *P. bifolia* and *P. chlorantha*, such as leaf dimensions, did indeed perform relatively poorly (Fig. 6). Other characters, such as labellum and spur length, were helpful but not wholly reliable (Fig. 7). Nonetheless, the small number of reproductive characters that are supposedly reliably discriminatory, such as gynostemium width and spur diameter, largely fulfilled their promise (Fig. 8).

However, the multivariate analysis encompassing all characters scored Fig. 5, Table 4) suggests a more interesting pattern. In some of our previous morphometric studies of other groups of European orchids, the first principal coordinate has discriminated between two or more taxa, whereas the second coordinate has primarily represented differences among plants in vigour — in other words, it has reflected dominantly ontogenetic variation in a substantial number of (mostly metric or meristic) characters (Bateman & Farrington, 1987; Bateman, 2001a). In studies encompassing a smaller amount of taxonomic diversity, the vigour coordinate can encompass more of the total variation than does the taxonomic coordinate (Bateman & Denholm, 1989; Bateman & Farrington, 1989; Bateman & Rudall, 2011). However, in the majority of cases, taxonomically and ontogenetically important characters become intermixed in the first two or three coordinates (Bateman & Denholm, 1983, 1985; Bateman *et al.*, 2008), thereby substantially complicating interpretation of the plots. But in the present case, taxonomic and ontogenetic sources of variation have become combined in the first coordinate — a novel outcome that accounts for an unprecedented 62% of the total variance.

In an attempt to better understand this phenomenon, we compared the mean values in the two species for a range of biologically significant floral and vegetative characters, and ranked them in order of decreasing ratio of *P. bifolia* relative to *P. chlorantha* (Table 5). The only character that showed a significantly greater mean value in *P. bifolia* than in *P. chlorantha* was flower number per inflorescence. Because flowers of *P. bifolia* are significantly smaller than those of *P. chlorantha*, more flowers can be accommodated in the same length of cylindrical, racemose inflorescence (Bateman & Rudall, 2006a). Also, orchid inflorescences tend to be more lax in shaded habitats, and a larger proportion of our sample of *P. chlorantha* plants occurred in woodland and scrub compared with *P. bifolia* (Appendix 3). Two further characters measured by us are statistically identical in the two species: total number of leaves and bract cell length. The latter presumably reflects the identical chromosome numbers ($2n=42$) in the two species (Afzelius, 1922; Cauwet-Marc & Balayer, 1986).

At the opposite end of the spectrum occur a small number of potentially developmentally correlated gynostemial characters that show mean values in *P. bifolia* that are 50% or less of the equivalent mean values in *P. chlorantha*: stigma width, gynostemium width, spur diameter, pollinium length and

Table 5 Rank order ratios of mean values for selected vegetative and reproductive characters between *P. bifolia* and *P. chlorantha* in southern England

Bifolia/chlorantha	Character	Mean±SD *bifolia* ($n \leqslant 57$)	Mean±SD *chlorantha* ($n \leqslant 79$)
Vegetative characters			
124%	Flower number	15.6±5.9	12.5±4.1
103%	Bract marginal cell length (μm)	52.4±7.6	50.7±8.5
99%	Leaf number	5.4±0.7	5.4±1.1
82%	Stem diameter (mm)	2.7±0.7	3.3±0.7
78%	Inflorescence length (mm)	67±28	87±31
72%	Longest leaf length (mm)	90±37	126±34
68%	Floral bract length (mm)	9.9±2.2	14.6±2.1
65%	Floral bract width (mm)	2.7±0.6	4.1±0.7
64%	Stature (cm)	23.5±7.6	36.7±9.1
59%	Longest leaf width (mm)	25.5±10.2	43.3±11.7
Floral characters			
73%	Lateral sepal length (mm)	8.2±1.5	11.3±1.3
65%	Ovary length (mm)	11.0±1.9	16.9±2.2
65%	Labellum length (mm)	9.6±1.8	14.7±2.3
64%	Labellum width (mm)	2.2±0.3	3.5±0.5
59%	Spur length (mm)	19.2±2.3	32.5±4.5
58%	Lateral sepal width (mm)	3.1±0.6	5.3±0.7
49%	Column length (mm)	2.2±0.5	4.5±0.6
44%	Pollinarium length (mm)	1.7±0.4	3.9±0.4
34%	Spur width at mouth (mm)	0.53±0.14	1.66±0.26
33%	Column width (mm)	1.7±0.4	5.3±0.6
23%	Stigma width (mm)	0.9±0.2	4.1±0.8

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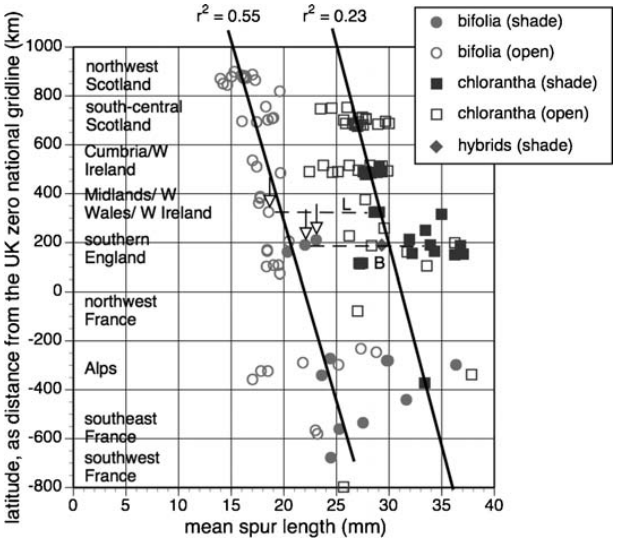

Figure 12 Spur lengths (mm) of *Platanthera bifolia* and *P. chlorantha* from southern England (top, present study) compared with data for these species from southeast Sweden (bottom, Swedish data abstracted from Nilsson, 1983).

gynostemium length. It is these characters that allow the two species to be distinguished with an acceptable degree of confidence.

It is striking that ratios for the remaining characters — the majority of both the vegetative and floral categories — approximate two-thirds, falling between 59% and 82% (Table 5). This suggests that there is an unusually strong allometric relationship between the two *Platanthera* species. With the exception of very few characters, one can generate a typical *P. chlorantha* plant simply by adding ca. 50% to the metric dimensions of a typical *P. bifolia* plant. Rather than indicating the differential expansion and contraction of metric characters that is the hallmark of selection-mediated adaptation, the entire plant appears to have been 'inflated' to a similar degree across its various organs. In other words, evolutionary changes are being conflated with (or possibly confused with) ontogenetic changes. The most obvious explanation of this phenomenon would be increased resourcing in *P. chlorantha* relative to *P. bifolia*.

In order to consider this hypothesis further, we explored in greater detail one character that is: (a) supposedly under strong selection pressure in *Platanthera* due to co-evolution with pollinators; (b) potentially responsive to allometric/ontogenetic shifts; and (c) readily and consistently measured in the field by multiple operators — specifically, the length of the nectariferous labellar spur.

Spur length within both Platanthera species appears controlled primarily by a latitudinal gradient and secondarily by local environment

Given the reputedly strong influence of nectar spurs on pollination efficiency, it is not surprising that evolutionary biologists have long paid considerable attention to the spurs of orchids in general (Darwin, 1877; Rudall & Bateman, 2002; Box *et al.*, 2008, 2012; Bell *et al.*, 2009) and of *Platanthera* in particular (Nilsson, 1978, 1983, 1985; Wood & Neiland, 2001; Maad & Nilsson, 2004; Little *et al.*, 2005; Claessens & Kleynen, 2006, 2011; Bateman & Sexton, 2008; Claessens *et al.*, 2008; Boberg & Ågren, 2009; Buttler, 2011; Lorenz *et al.*, 2012).

Our first indication that the pattern of spur lengths in the *P. bifolia* aggregate may be more complex than was previously supposed occurred when we used histograms to compare the spur-length data that emerged from our morphometric study in southern England with spur-length measurements taken from populations in southern and eastern Sweden by Nilsson (1983). Both datasets revealed classic bell-curve distributions that appeared consistent with a polygenic feature under selection, and showed limited overlap between the two species (Fig. 12). Superficially, there appeared to be only small offsets in the peaks of the two curves between southern England and southern Sweden, but we soon realised that the two bell-curves were in fact transposed between the two counties: *P. bifolia* actually has substantially shorter spurs than *P. chlorantha* in southern England but substantially longer spurs than *P. chlorantha* in southern Sweden (Bateman *et al.*, 2004; Bateman, 2005).

It was this startling insight that encouraged initiation of our Europe-wide survey of spur lengths in *P. bifolia* and *P. chlorantha* (first reported in detail by Bateman & Sexton, 2008). That matrix is here supplemented with valuable additional data that fill previous geographical lacunae, but that further complicate the already intriguing patterns and inferences reported by Bateman & Sexton.

Regression of mean spur length against latitude (Fig. 13) demonstrated that spur length distinguishes the two species only if latitude is taken into account; for example, populations of *P. bifolia* from the Vercors in southeast France show mean spur lengths similar to those of populations of *P. chlorantha* occurring approximately 1300 km to the north in southern Scotland (ca. 26 mm) (for a critical account of previous, inaccurate representations of spur length in these species see Bateman & Sexton, 2008). More importantly, the regressions also revealed strong correlation coefficients between spur length and latitude. The additional data did not affect the correlation for *P. bifolia* (r^2 decreased slightly from 0.56 to 0.55), whereas that for *P. chlorantha* was considerably weakened (r^2 decreased from 0.42 to 0.23), due largely to inclusion of a single exceptionally short-spurred population from Andorra (Fig. 13). Nonetheless, similar trends still characterise both species; each shows a reduction in

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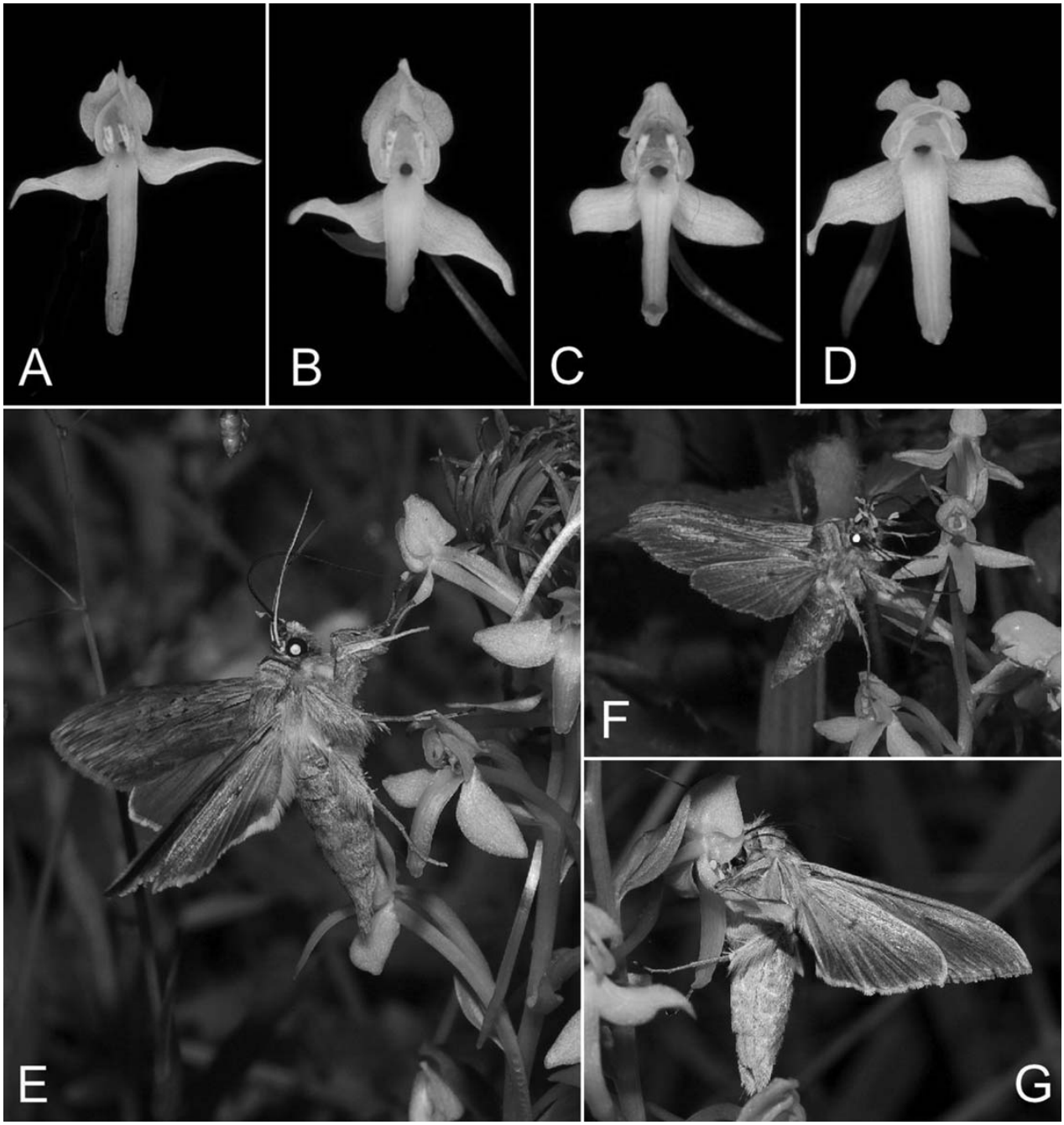

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Figure 13 Mean spur length (mm) plotted against latitude for 54 populations of *Platanthera bifolia* (left, r^2=0.55) and 61 populations of *P. chlorantha* (right, r^2=0.23). Shade and open habitats are also distinguished. The dashed lines connect mean values for both parents and putative hybrids at the Bix Bottom (B) and Lynclys (L) localities; the three arrowed populations of *P. bifolia* show evidence of introgression. The hybrids were omitted from the linear regression analysis (Matrix expanded from Bateman & Sexton, 2008, fig. 3; study sites additional to that study are listed in Appendix 4).

mean spur length of approximately 2% per 100 km of increasing latitude.

Admittedly, the assertion of a broadly linear relationship between spur length and latitude could be challenged. For example, the spur-length data for *P. bifolia* (Fig. 13) could be interpreted as showing a constant size of approximately 18 mm for most of the latitudinal range, deviating only at the two extreme ends of the latitudinal gradient (i.e. downwards in northwest Scotland and upwards in France and parts of the Alps). Similarly, data for *P. chlorantha* could be considered to be constant for most of its latitudinal range, plateauing in much of the UK and France at approximately 28 mm — a similar figure also characterises this species in the Low Countries (Claessens and Kleynen, 2006). However, the figure deviated upward to values of 32–38 mm in the majority of populations occurring in southern England and 38 mm in both populations measured in the Alps (Fig. 13). The lower r^2 value for *P.*

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chlorantha relative to that for *P. bifolia* primarily reflects a relatively large spread of mean spur lengths in southern England (most of the data being derived from the present morphometric study) and especially in Continental Europe, culminating in a mean value in the Andorra population of 26 mm, 11 mm shorter than the length predicted at that latitude by the regression line. Nonetheless, applying to the spur-length data (Fig. 13) several curve-fitting algorithms more complex than simple linear regression failed to improve the statistical fit of line to data points for either species of *Platanthera*.

The obvious temptation is to interpret these latitudinal trends as reflecting adaptation of the spur to proboscis length in the respective moth pollinators attributed to these two orchid species, at least within the British Isles, where the relationship between spur length and latitude is strongest. However, as noted in the previous section, the present morphometric data, gathered in southern England from all major features of the plants of both species, suggest that most features of *P. bifolia* — not just spur length — are on average only two-thirds of the dimensions of equivalent features in *P. chlorantha* (Table 5). A similar ratio was evident among a more limited range of floral dimensions recorded in south-central Scotland by Roy Sexton (unpublished). If this ratio is repeated across most of the (largely coincident) ranges of the two *Platanthera* species, the latitudinal gradient in spur length observed in this study (Fig. 13) could simply represent an allometric ratio that characterises most organs of the plant and so is merely reflected in spur length.

The observation that plants of both species become on average larger in all their organs further south led Bateman & Sexton (2008, 2009) to speculate that this variation could have a dominantly environmental cause. Specifically, they hypothesised that more southerly plants could be better resourced as a result of greater photosynthetic activity — the presumed consequence of a greater relative degree of insolation at lower latitudes impacting on the expanded leaves of plants, which are typically paired (1% of flowering plants of *P. bifolia* and 6% of flowering plants of *P. chlorantha* have only one expanded leaf: Bateman & Sexton, 2009).

However, three pieces of evidence challenge this resource-based hypothesis. Firstly, during the annual growth cycle of these species, leaf expansion and inflorescence elongation occur during a period (April–May) when the inclination of the axis of the Earth relative to the position of the sun minimises the difference in insolation levels between the Mediterranean region and the much higher latitudes of northern Scotland. Secondly, our data for both species suggest that, at a particular latitude, habitat exerts a significant influence on spur length; in particular, plants of both species occupying more shaded habitats have on average longer, rather than shorter, spurs (Fig. 13). This pattern is similarly difficult to explain; it may simply reflect etiolation of the spur as a result of the lower light intensity. Thirdly, a 5-year (2008–2012) observational dataset obtained from the large Wolstonbury population of *P. chlorantha* by K. Stott, N. Henderson and D. Pearce showed simultaneous modest reductions in mean values for spur length, width of largest leaf and plant height that distinguished the warm, dry years of 2008 and 2009 from the comparatively cold, wet summers of 2010–2012; only flower number appeared unaffected by this transient regional climatic shift (Table 6). This pattern refutes the hypothesis that larger leaves formed in one year would presage more flowering plants of greater vigour in the following year (cf. Bateman & Sexton, 2009).

Aspects of the habitat most likely to influence spur length include soil parameters, notably pH and moisture content, and the degree of shade experienced by the orchids. Our data on soils are inadequate (admittedly, there exists much anecdotal evidence that *P. bifolia* is more tolerant than *P. chlorantha* of wet acidic soils), but for both species we were able to compare large numbers of populations occupying closed and open habitats.

Among our sampled populations, those that are shaded do not extend northward beyond southern England for *P. bifolia* and beyond northern England for *P. chlorantha* (Fig. 13). In the case of both species, within specific latitudinal zones, most populations occurring in shade have longer spurs than those occurring in the open (i.e. the majority are placed on, or to the positive side of, the regression line in Fig. 13). Similar but more substantial differences between short 'sun' spurs and longer 'shade' spurs have been reported from central Europe (Lorenz *et al.*, 2012) and Scandinavia (Boberg & Ågren, 2009), but this pattern of divergence appears to break down in Poland (P. Baraniecki, pers. comm., 2008).

We have gathered anecdotal evidence that, at least in Mediterranean regions such the Vercors area of southeast France, exposed plants suffer greater water stress in spring than their woodland counterparts;

Table 6 Mean values for four phenotypic parameters made from large samples of individuals from the *Platanthera chlorantha* population at Wolstonbury Hill, Sussex (all dimensions in mm; raw data courtesy of Kathy Stott, Neville Henderson and David Pearce)

Year	*n*	Spur length	Leaf width	Flower number	Plant height
2008	56	30.0	47.5	13.8	391
2009	45	29.5	50.1	14.2	420
2010	60	28.6	41.9	14.4	334
2011	60	27.6	40.1	13.5	338
2012	60	28.0	43.3	13.7	366

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this could result in less vigorous growth. However, studies of populations of *P. bifolia* on the Baltic island of Öland (Nilsson, 1983; J. Maad, pers. comm., 2007) similarly revealed substantial divergence between short-spurred populations of *P. bifolia* in grassland (means of 19–23 mm) and those occupying deciduous woodland (means 28–40 mm). The considerable elongation of the *Platanthera* spur observed during anthesis raises the possbility that at least some of the difference between sun and shade plants reflects a plastic response to the local environment. We would be especially interested to learn the relative contributions to spur elongation of cell expansion versus cell division.

Returning to potential genetic influences on phenotype, we note that although some of our study populations of both *Platanthera* species encompassed several hundred flowering plants, the majority were small, many typically yielding less than 10 flowering plants in any one year. Where such small effective population sizes are maintained, deviation from previous distributions of spur length can readily be achieved via genetic drift, whereas in contrast the potential for long-term directional or disruptive selection is greatly reduced in small populations (e.g. Tremblay *et al.*, 2005). Genetic drift merits consideration in any study of spur-length variation in *Platanthera*, though its random effects would have been expected to obscure the apparent correlation between habitat preference and mean spur length if that correlation reflected genetic factors, irrespective of whether the differences actually represent selection or drift.

In the interests of balance, we should close this section by noting that data published by other authors show that positive deviations in the spur length of *P. bifolia* (mean values as high as 27–34 mm) characterise much of Scandinavia, differing substantially from our Scottish data. However, we also note that spur length in *P. bifolia* is radically shorter in the birch forests of northern Scandinavia (Nilsson, 1983, 1985; Tollsten & Berstrom, 1993; Maad, 2000), yielding values comparable to those observed in Scotland (means of 18–21 mm). In other, albeit smaller-scale, morphometric surveys, Buttler (2011a) reported spur lengths from individual plants of *P. bifolia s.l.* as ranging from 12 to 41 mm, and a similar range of 13–40 mm was reported by Lorenz *et al.* (2012), while seven populations from Poland averaged 22–33 mm (P. Baraniecki, K. Ciesielski, L. Dudek, W. Hanak, L. Krajewski and M. Scelina, pers. comm., 2008).

Much more research is required to untangle the Gordian Knot of factors that together determine spur length in the *P. bifolia* group. Nonetheless, we believe that our data constitute strong (albeit circumstantial) evidence that spur length is under at most relatively weak, sporadic and/or local selection pressure in these populations (a conclusion reached, on the basis of more sophisticated data, for the North American *P. lacera* by Little *et al.*, 2005). Within the observed ranges of spur size, both the large-scale latitudinal gradient and the smaller-scale habitat differences appear to influence spur length more strongly — and certainly at a larger scale — than does pollinator-mediated selection. In addition, our data suggest that substantial spur elongation occurs not only during but also long after anthesis. This ontogenetic influence renders spur length a constantly moving target for selection, most likely precluding precise optimisation to particular pollinator species.

DNA sequence data fail to discriminate among the Eurasian Platanthera species, suggesting substantial gene flow between taxa

Anyone surveying the literature on the comparatively impoverished orchid flora of the British Isles would soon realise that it has always been characterised by ongoing debates regarding the taxonomic status of many (perhaps the majority) of the erstwhile species and by a great enthusiasm for recording hybrids, usually on the basis of undesirably limited evidence (Bateman & Haggar, unpublished). Given these generalisations, the long-term taxonomic stability awarded to *P. bifolia* and *P. chlorantha* appears remarkably atypical.

Firstly, despite their obvious morphological similarity, the status of *bifolia* and *chlorantha* as distinct and separate species has rarely been challenged during the last century (Bateman & Sexton, 2008). One has to delve backward through time as far as the Victorian era in order to find direct conflict, specifically to a heavyweight contest between no lesser figures than the authors of the first comprehensive flora of the British Isles, Bentham & Hooker (e.g. 1886), and Darwin (1877). Darwin took considerable exception to the willingness of Bentham & Hooker to treat the two British *Platanthera* taxa as mere varieties of a single species — a decision taken on the grounds that 'intermediate' forms occurred between them (cf. Summerhayes, 1951). Comparison with our data shows that Darwin was driven to exaggeration in his determination to acquire widespread acceptance of species status for the two taxa, stating that 'the two forms differ in a large number of characters, not to mention general aspect and the stations inhabited', and, even more startlingly, that 'these two forms certainly differ from one another more than do most species belonging to the same genus'. Having noted that loose waxy scales on the heads of visiting moths most likely confine placement of pollinia to the proboscis (*P. bifolia*) or eyes (*P. chlorantha*), Darwin (1877: 73–4) concluded that there is no 'doubt that the Larger and Lesser Butterfly Orchids are distinct

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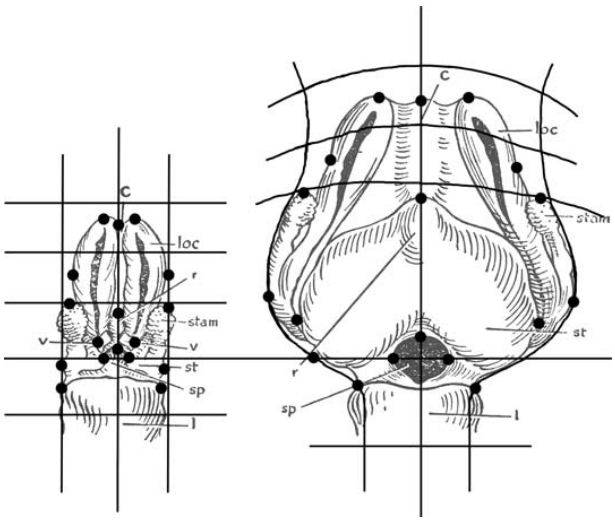

Figure 14 Flowers of (A) *Platanthera bifolia*, (D) *P. chlorantha* and (B, C) two putative hybrids from the Warburg Reserve, Bix Bottom, Oxfordshire. Scale bar=5 mm. (E–G) *Cucullia umbratica* moths using their probosces to remove pollinia (arrowed) from flowers of (E, F) the natural hybrid *P. chlorantha* × *bifolia* and (G) *P. chlorantha* at Wylre, southern Limburg, Netherlands. Images: A–D=Richard Bateman, E–G=Jean Claessens (Claessens *et al.*, 2008, figs. 1, 5, 6).

species, masked by close external similarity'. Thus, Darwin appealed to both reproductive isolation (via contrasting pollinator specificities) and morphological distinction to support his strongly held taxonomic beliefs. However, the data underpinning his assertions of pollinator specificity were confined to a small number of observations made in the vicinity of his Kentish home, Down House, and his morphological arguments are weakened by internal contradiction: the two species supposedly show differences that are greater than those of most congeneric species but are simultaneously 'masked by close external similarity'. One might be forgiven for supposing that Darwin had access to DNA sequence data.

Secondly, there has been an extraordinary paucity of confident records of hybrids between the two species (=*P.* × *hybrida* Bruegger). In a rare show of unity rooted in long-term scepticism, successive generations of authors have argued that evidence of hybridisation between the British *Platanthera* species is inconclusive, and that most if not all putative hybrids should be regarded as aberrant individuals of one of the supposed parents (cf. Godfery, 1933; Summerhayes, 1951; Hunt, 1975; Sell & Murrell, 1996; Stace, 1997; Foley & Clarke, 2005; Harrap & Harrap, 2005; Bateman & Haggar, unpublished). Further questionable arguments frequently deployed against the existence of hybrids included apparent differences in habitat preference and/or peak flowering period. And if any hybrids did miraculously appear, their fitness would presumably be substantially reduced by their suboptimal presentation of the viscidia to pollinators — in other words, by evolutionary constraints imposed by the pollinator species.

Several recent investigations, including the present study, have appreciably weakened each of these arguments. Firstly, we failed to detect substantial differences in flowering periods in our study populations (Fig. 10). The two taxa also clearly show substantial overlap in habitat preferences, and their respective geographical distributions within the British Isles are also broadly similar (Fig. 9). Not surprisingly, the two taxa co-existed at a significant minority of our study sites. Hence, five of the 137 plants (<4%) included in our morphometric survey were identified as hybrids, and similar frequencies of hybridisation have been reported on mainland Europe, from both Scandinavia (Nilsson, 1985) and the Netherlands (Claessens & Kleynen, 2006; Claessens *et al.*, 2008). We identified hybrids primarily on the basis of their intermediate pollinarium positions; recognising four such plants from Bix Bottom, Oxfordshire (Fig. 14), plus one less certain identification from St Anne's Chapel, Cornwall (regrettably, no data could be obtained from its rapidly decaying gynostemium). In addition, our renewed spur-length survey (Appendix 4) prompted recognition of convincing hybrids at Bulls Cross, Gloucestershire (Hughes, 2007), Llynclys Common, Shropshire (Whild & Lockton, 2007; J. Pedlow, pers. comm., 2008) and Cors Caron, Cardiganshire (Chater, 2010). One of the putative Bix hybrids was identified in the field as *P. bifolia*, but its identification was re-assessed in the wake of the morphometric multivariate analysis (Fig. 5), which encouraged us to examine more carefully the scores of this plant for the most taxonomically diagnostic characters.

Basically, the mean ratio of 2 : 3 identified in most features of *P. bifolia* versus *P. chlorantha* provides insufficient discrimination to reliably distinguish the two species. Consequently, characters showing greater contrasts in value are needed to even tentatively identify hybrids. In practice, this constraint leaves as discriminatory only spur diameter plus certain gynostemial features such as column width, stigma width and pollinium length (Table 5), together with characters not directly measured in the present study such as the separation and diameter of the viscidia.

Given the evident difficulty of morphologically identifying putative F1 hybrids between *P. bifolia* and *P. chlorantha*, there is little likelihood of confidently identifying F2 hybrids or backcrosses with the parents using phenotypic characters. Bateman & Sexton (2008) tentatively inferred the occurrence of introgression in mixed populations of *Platanthera* through the circumstantial evidence provided by their spur-length data. Specifically, although the putative hybrids from Bix (and elsewhere) predictably had

mean spur lengths intermediate between those of the co-existing parental species, spur-length measurements for supposedly pure plants of *P. bifolia* in this population are the longest of any in southern England (Fig. 13), suggesting that there has been some genetic input from co-occurring plants of *P. chlorantha*.

Similar observations of spur intermediacy in hybrids were made on larger numbers of putative hybrids of *P. bifolia* × *chlorantha* occupying the Baltic margins of Sweden by Nilsson (1985), though along this coast *P. bifolia* typically has spurs that are substantially longer than those of *P. chlorantha* — an extraordinary character reversal relative to the more typical condition (Bateman, 2005; Bateman & Sexton, 2008). Indeed, intermediacy of spur dimensions has characterised all of the many quantitative case-studies of hybridisation among spurred European orchids known to us (reviewed by Bateman & Haggar, unpublished). This reliable intermediacy renders extraordinary the report of extensive hybridisation in two mixed populations of *Platanthera* in the Netherlands, where the putative hybrids show longer average spur lengths (32 mm) than either of the co-existing putative parents (*P. chlorantha*=26 mm; *P. bifolia*=23 mm: Claessens & Kleynen, 2006, pers. comm., 2012; B. Gravendeel, pers. comms., 2007, 2012). It seems likely that the evolutionary processes operating in these ambiguous Dutch populations are more complex than simple introgression.

Much has been learned about inheritance patterns of morphological characters in other taxonomically controversial genera of Eurasian Orchidinae such as *Dactylorhiza* (Bateman & Haggar, unpublished) and *Ophrys* (Malmgren, 1992, 2008; Bateman *et al*., 2010, 2011), by conducting reciprocal crosses of known taxa in controlled conditions and raising substantial numbers of progeny from single crossed or selfed capsules. Other experiments have demonstrated the relative weakness of postzygotic isolation (Scopece *et al.*, 2007, 2010; Cozzolino & Scopece, 2008). Unfortunately, *Platanthera* has proved to be relatively recalcitrant to both cultivation and rearing of seeds in controlled conditions. We would be especially interested to see how the crucial metric characters that distinguish *P. bifolia* and *P. chlorantha* (Figs. 5 and 8), plus more subtle (and presumably polygenic: Box *et al*., 2008, 2012) variables such as spur length (Figs. 7 and 13), would segregate in the F1 generation and in backcrosses.

We naturally anticipated that our broad programme of DNA sequencing would allow us to conclusively separate the two parents and thus, by definition, identify their hybrids, hopefully also distinguishing among any F1, F2 and backcrossed plants. We were therefore astonished to discover that 9.8 kb of supposedly rapidly-changing nuclear and plastid DNA regions yielded only one variable site — and to learn that even that one variable site ultimately proved not to be species specific. These data strongly suggested not only that there remains considerable ongoing gene flow between the two species but also that it occurs at a greater rate than is consistent with our present, phylogenetically based estimate of the frequency of hybrid individuals among these populations. However, ironically, our failure to detect *any* species-specific genetic difference and our discovery of only one non-species-specific difference meant that we could not test rates of gene flow, or indeed conclusively demonstrate that it was in fact occurring — a frustrating manifestation of the 'Catch 22' principle.

More recently, screening of 20 plastid regions in Italian *Platanthera* plants by Pavarese *et al.* (2011) yelded minor variations in the *psbk–trns* and *psaA–ycfex3* spacers, which allowed some discrimination among populations and taxa. Italian *Platanthera chlorantha* formed a single group with *P. algeriensis* from Tunisia and Sardinia, but in contrast, populations of *P. bifolia* were divided into three molecularly disparate groups, originating respectively from the Alps, southern Italy and central Sardinia plus Tunisia — the latter relatively tall plants attributed to var. *kuenkelii* sensu Baumann (1981). Although these plastid regions offer some promise for improved assessment of gene-flow patterns among *Platanthera* species, confident interpretations of the results of Pavarese *et al.* will require much broader geographic sampling (cf. Bateman *et al*., unpublished).

The classic adaptive scenario revisited

Past assertions that strong selection pressure routinely acts on spur length have focused on morphological and ecological data for the two *Platanthera* species in Scandinavia (Nilsson, 1978, 1983, 1985; Maad, 2000; Maad & Nilsson, 2004), where mean spur length of *P. bifolia* in particular appears to show greater regional variation than is evident elsewhere (admittedly, we have documented comparatively broad spreads of mean spur lengths in southern England for *P. chlorantha* and especially in the Alps *s.l.* for *P. bifolia*: Fig. 13). The Swedish data suggest that strong pollinator specificity among different species of moth provides a meaningful level of reproductive isolation separating the two species and different geographic/habitat races within *P. bifolia*. Indeed, the mechanism of contrasting pollinarium placements originally inferred by Darwin (1877) has become a popular model system for illustrating evolutionary relationships between plant and pollinator (e.g. Hapeman & Inoue, 1997).

The classic model states that *P. bifolia* places its pollinaria anywhere along the length of the proboscis of the moth, whereas *P. chlorantha* requires the moth to

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press its head against the column in order to attach the viscidia to the surface of its eyes. This contrast in the mechanism of pollinarium placement would suggest that spur length should be more critical to the reproductive success of *P. chlorantha* than to that of *P. bifolia*. Setting aside sites that yielded very small sample sizes and those showing evidence of introgression, coefficients of variation for spur length within our study populations range 2–18% for *P. bifolia* (typically ca. 11%) and 6–14% for *P. chlorantha* (typically ca. 9%). Thus, there is no obvious contrast between the two species in presumed selective pressure affecting spur length; their respective bell-curves exhibit similar dispersions about the mean (e.g. Fig. 12).

Relevant in this context is the experimental work of Boberg & Ågren (2009), who one summer artificially shortened by one third the lengths of the spurs borne by some plants in a relatively long-spurred population of *P. bifolia* growing in woodland on the Swedish island of Öland. Although shortening the spurs reduced fruit set by ca. 15%, it is a moot point whether, in a species that routinely generates such a vast surplus of seed over that necessary to replace the parental generation, this modest decrease is of any relevance to the actual reproductive performance of the plants in question.

If we now consider the proboscis versus eye placement scenario in greater detail we see that it constitutes an argument against introgression between *P. bifolia* and *P. chlorantha*, rather than against hybridisation *per se*. The key assumption is that the requirement for precision in attaching pollinaria to moths means that the prospective pollinator would not be able to remove the pollinaria; thus, primary hybrids could only act as pollen recipients, not as pollen donors (Nilsson, 1983). This assumption was overturned in most dramatic fashion by Claessens *et al.* (2008), who succeeded in capturing images of *Cucullia* moths in the act of removing pollinia via their probosces from presumed F1 hybrids in the Netherlands (one such image is reproduced here as Fig. 14E). Other images showed moths removing pollinia from flowers of *P. chlorantha* via probosces rather than adhering to the prescribed eye attachment (Fig. 14F). Clearly, both hybridisation and subsequent introgression are feasible.

Darwin (1877) and subsequent commentators (e.g. Summerhayes, 1951; Nilsson, 1983) argued adamantly that only long-tongued moths effect pollination in European *Platanthera*. However, Darwin also persistently rejected observations of other natural historians, notably Müller (1868), that many European orchids did not actually reward their pollinators, whereas subsequent studies have proven Müller right; many orchid species succeed by deceiving rather than rewarding pollinators (e.g. van der Cingel, 1995; Neiland & Wilcock, 1998; Cozzolino & Widmer, 2005; Schlüter & Schiestl, 2008; Claessens & Kleynen, 2011). Many authors have noted that species offering genuine rewards to pollinators generally achieve substantially higher frequencies of pollination than do co-existing food-deceptive species (Neiland & Wilcock, 1998; Cozzolino & Widmer, 2005). Moreover, that success reflects in part the large number and diversity of insects capable of transferring pollinaria among flowers of rewarding orchids; for example, Nilsson (1979) reported 12 insect species carrying pollinaria within a single population of *Herminium monorchis* during a single flowering season, while Patt *et al.* (1989) observed at least six species removing pollinaria from a population of *Platanthera stricta* in the Pacific Northwest of North America, the pollinators spanning not only Lepidoptera but also Diptera and Coleoptera.

However, fewer authors have noted that this comparatively successful pollination rate has not translated into greater numbers of rewarding than non-rewarding species or to greater average size or number of populations per rewarding species. And even fewer authors have noted that there is no obligation on pollinators to accept any reward offered. Surely, an insect that can demonstrably be repeatedly duped into visiting and pollinating an unrewarding orchid will not baulk at the prospect of pollinating a species that offers a reward, irrespective of whether the insect in question can satisfactorily exploit that reward?

Having thus hypothesised from first principles that at least some occasional pollinators of *Platanthera* would show behaviour patterns that are unlikely to be influenced by spur length, we were not surprised when more recent surveys of insects carrying pollinia of *Platanthera* species in southern Scotland revealed the existence of spectra of pollinators (Sexton & McQueen, 2005; Bateman & Sexton, 2008), rather than following the archetypal (but arguably mythical) 'one orchid is co-adapted with one pollinator' model. Scottish catches included three moth species that are known pollinators of *P. chlorantha* in Sweden (cf. Nilsson, 1978, 1983; J. Knowler, pers. comm., 2006). More recent and extensive studies of pollinators of *P. bifolia* across Scandinavia (Boberg *et al.*, 2007) have similarly identified contrasting spectra of pollinators at different latitudes and in different habitats, seriously complicating any attempt to correlate spur length (and other features of the flower) with preferred pollinator(s).

Recent combined population genetic and morphological studies of gene flow in other groups of European orchids also counsel caution when assuming strong pollinator specificity. Conventional wisdom states that most *Gymnadenia* species have pink

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flowers with long nectariferous spurs and so must be pollinated by butterflies, whereas most *Dactylorhiza* species have pink flowers with shorter, wider, nectarless spurs and so must be pollinated by bees (e.g. van der Cingel, 1995; Neiland & Wilcock, 1998). Yet these genera produce the greatest frequency of *bona fide* intergeneric hybrids of any European orchid genera (Farrington & Bateman, 1989; Bateman & Haggar, unpublished), showing that their pollinator spectra must overlap. Even in the notionally most pollinator-specific orchid genus, *Ophrys*, natural hybrids occur among all major species groups, demonstrating both their interfertility and the fact that pollinators frequently transfer pollinia between species (cf. Paulus & Gack, 1990; S. Cozzolino, pers. comm., 2007; Devey *et al.*, 2008, 2009; Bateman *et al.*, 2010, 2011). In addition, the ability to identify pollinia through DNA sequencing (e.g. Soliva & Widmer, 2003) means that we can assess pollinator error rates, which even in *Ophrys* are proving to be relatively high. Also, artificial crosses among *Ophrys* species have demonstrated fertility in F1s, F2s and back-crossed progeny (S. Cozzolino, pers. comm., 2007; Scopece *et al.*, 2007, 2010; Malmgren, 2008; Cozzolino & Scopece, 2008; Bateman *et al.*, 2010, 2011).

Thus, current (albeit limited) evidence suggests that *P. bifolia* and *P. chlorantha* similarly introgress whenever they co-exist in substantial numbers in the same habitat or in immediately adjacent habitats. We will continue to seek, and apply, molecular markers that would allow us to better quantify levels of gene flow (cf. Pavarese *et al.*, 2011). The jury is still out regarding the degree of reproductive isolation enjoyed by the two European *Platanthera* species; at present, we cannot even exclude the possibility that gene flow is sufficiently high that all European *Platanthera* taxa should best be viewed as conspecific.

We suspect that some past observers have preferred to prioritise speculative assertions of pollinator fidelity above field observations recording intermediate plant morphologies. Current evidence suggests that there are major contrasts in pollinator spectra for each *Platanthera* species in different geographical locations. It would be interesting to see whether there is also habitat-related variation, and whether spectra in any particular region differ substantially between years. Clearly, it is desirable to gather data on the range and frequency of success of pollinators from other regions of Europe to test the degree of pollinator specificity enjoyed by each of the two species. Nilsson (1983) reported substantial differences in fragrance compounds between the two taxa in southeast Sweden; these too need to be explored across the full geographic range of the species.

Nonetheless, current evidence suggests that it would be difficult to develop a co-adaptive scenario to explain the maintenance of the *P. bifolia* and *P. chlorantha* phenotypes that would be applicable across the range of either species.

The P. bifolia *aggregate outside northern Europe*

Thus far, our account has focused on the relatively well-researched relationship between *P. bifolia* and *P. chlorantha* in northern Europe. However, our DNA-based analyses (Appendix 2, Fig. 2) included a few representatives of other named taxa that are assigned to the *P. bifolia* aggregate but occur beyond its northern European heartland. To the east in Asia lies a poorly understood mélange of subtly varying morphology that includes *P. metabifolia*. To the south occurs the green-flowered but otherwise *P. chlorantha*-like pairing of *P. algeriensis* (not yet sequenced) in the western Mediterranean region and *P. holmboei* further east. And the only *Platanthera* species to occur in the Macaronesian Islands are another green-flowered pairing, *P. micrantha* and *P. azorica*, confined to the Azores (Rückbrodt & Rückbrodt, 1994; Delforge, 2003; Bateman *et al.*, unpublished). These two taxonomically controversial pairs of putative species provide interesting comparisons with the pairing of *P. bifolia* and *P. chlorantha*.

Taxa from Asia

Beginning with eastern Asian taxa, we suspect that populations occur in the region that are morphologically indistinguishable from northern European *P. chlorantha* (e.g. Chen *et al.,* 1999) and *P. bifolia* (Inoue, 1983; Lee, 1998: 1116). In the case of *P. bifolia*, the Oriental populations are usually assigned to *P. metabifolia*, but we are unconvinced by the morphological characters that supposedly distinguish *P. 'metabifolia'* from bona fide *P. bifolia* (larger viscidia, longer caudicles). Indeed, Nilsson (1985) suggested that these populations represented either subtle selectively-driven deviations from *P. bifolia* or a hybridogenic origin between *P. bifolia* and *P. chlorantha*. Certainly, our single accession of *P. 'metabifolia'* revealed the presence of ITS allele I, which dominates *P. bifolia* elsewhere (Appendix 2, Table 3). One of our three accessions of *P. chlorantha* from China also yielded allele I, but the two remaining accessions were the only samples to yield allele III, which differs from the apparently plesiomorphic allele I in two base substitutions (Fig. 2). This suggests that cryptic species may occur within *P. chlorantha s.l.* in eastern Asia.

Taxa with large green flowers

The majority of recent authors have recognised two further species that closely resemble, and supposedly largely replace, *P. chlorantha* in the Mediterranean region: *P. holmboei* occurs in shaded, typically

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montane woodlands in the eastern Mediterranean (Cyprus, Turkey, Palestine, and possibly the Aegean), whereas *P. algeriensis* occurs in open, wet, typically montane habitats from Morocco and Spain in the west to Corsica, Sardinia and mainland Italy in the east. Taxonomic treatment of these taxa has varied considerably. They were ignored by Webb (1980) in *Flora Europaea* and treated as subspecies of *P. chlorantha* by Davies *et al.* (1983). Sundermann (1980) and Baumann & Künkele (1982) only recognised *P. algeriensis* (as a subspecies and a full species, respectively). Buttler (1991) and Delforge (2006) listed both taxa as species, though Buttler stated that each taxon deserved, at best, subspecies status (a view echoed by Kreutz, 2005), and Delforge similarly noted their close morphological similarity.

Gölz & Reinhard (1990) measured 39 morphometric characters from single populations each of *P. algeriensis* (Sardinia), *P. holmboei* (Cyprus), *P. bifolia* (Switzerland) and two populations of *P. chlorantha* — one from Switzerland and the other from the Aegean island of Lesvos. Their study suggested strong similarity between the two populations of *P. chlorantha* (despite their geographical disparity), moderate similarity between *P. holmboei* and *P. algeriensis*, but weaker and approximately equal similarities between the three main entities (*P. bifolia*, *P. chlorantha*, *P. holmboei* plus *P. algeriensis*). Re-examination of Gölz & Reinhard's (1990) data reveals that most dimensions of the gynostemium and labellum are intermediate between those typical of *P. bifolia* and *P. chlorantha*, though somewhat closer to the latter, especially in the case of *P. holmboei*, with its smaller flowers and more subdued expansion of the distal half of the spur. Our SEM studies of *P. holmboei* flowers reinforced this perception (cf. Figs. 3 and 4). The available data are consistent with an origin of *P. algeriensis* and *P. holmboei* by hybridisation between *P. bifolia* and *P. chlorantha*, or with divergence of *P. bifolia* and *P. chlorantha* from a morphologically intermediate ancestor resembling *P. algeriensis–holmboei* (all these taxa reputedly have a chromosome number of $2n=42$: e.g. Afzelius, 1922; Scrugli, 1980; Cauwet-Marc & Balayer, 1986). Sceptics might argue that *P. algeriensis* and *P. holmboei* are distinguished from both *P. bifolia* and *P. chlorantha* by having green flowers that often bear brown pollinia and strongly recurved labella, but we have found mutant plants among populations of *P. chlorantha* in northern Europe (Fig. 1H) that closely resemble the *P. algeriensis–holmboei* phenotype (Fig. 1G); clearly, this phenotypic transition is clearly easily achieved and hence most likely reflects simple genetic controls.

We also note that most features of *P. holmboei* are on average about two-thirds the size of *P. algeriensis*, thus echoing the allometric ratio documented in the present study between *P. bifolia* and *P. chlorantha*. The interesting exception is mean spur length, which would be predicted to exceed 25 mm based on the latitudinal gradients observed in *P. bifolia* and *P. chlorantha* (Fig. 4), but which in practice does not exceed 20 mm (Gölz & Reinhard, 1990). However, in the absence of morphometric data from the potential zone of geographical overlap (Italy and the Balkans), we cannot presently reject the possibility that *P. algeriensis* and *P. holmboei* together constitute a geographically correlated morphological continuum. On the other hand, our recent field observations suggest that populations assigned to *P. algeriensis* in Tunisia, Sardinia and Corsica may differ significantly from supposedly conspecific populations found in Morocco and Iberia.

Similarly, we currently lack potentially valuable ITS data from *P. algeriensis*. Plastid microsatellites failed to distinguish Sardinian populations of *P. algeriensis* from populations of *P. chlorantha* in mainland Italy (Pavarese *et al.*, 2011). In contrast, both of the accessions attributed to *P. holmboei* that we analysed for ITS yielded interesting results. The accession from Lesvos contained the plesiomorphic allele I, typical of *P. bifolia* and *P. chlorantha* (Appendix 2, Fig. 2), tending to support assignment of the controversial *Platanthera* populations from Lesvos to *P. chlorantha* rather than to *P. holmboei* (as per Gölz & Reinhard, 1990). However, the plant from Cyprus (the type region of *P. holmboei*: Lindberg, 1942) yielded the rare allele IV, which differs from the plesiomorphic allele I in just one substitution (Fig. 2). Possession of this genotype offers a tentative indication that bona fide *P. holmboei* did not originate recently from *P. bifolia* or *P. chlorantha*, or via hybridisation between these taxa.

European taxa segregated from P. bifolia s.s.

A more recent and more energetic debate has surrounded the possibility of dividing the *P. bifolia* aggregate into species, subspecies and/or varieties. Three decades ago, Baumann (1981) described from Tunisia a segregate of *P. bifolia* characterised only by relatively tall stems, large basal leaves and long, lax inflorescences. Named *P. kuenkelii*, this vegetatively delimited taxon was understandably downgraded to a subspecies by Kreutz (2005) and to a variety by Delforge (2000, 2006).

Surprisingly, the recent plastid microsatellite study of Pavarese *et al.* (2011) suggested not only that an outlying population of *kuenkelii* in Sardinia matched those from Tunisia but also that this taxon appears to be molecularly distinct from other forms of *P. bifolia* found in northern and central/southern Italy, respectively. This limited body of molecular data

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encouraged Lorenz *et al.* (2012) to confirm *P. kuenkelii* as a full species. Furthermore, they assigned to *P. kuenkelii* a supposed Caucasian endemic previously named *P. bifolia* ssp. *atropatanica*, which on morphometric evidence appeared intermediate between *P. kuenkelii* and *P. bifolia s.s*. Their analysis of central European populations of the *P. bifolia* aggregate suggested incongruence between vegetative characters, which primarily distinguish *P. kuenkelii* ssp. *kuenkelii* from the remainder, and floral characters, which more readily distngush *P. chlorantha* from *P. bifolia sensu latissimo*. These data were used by Lorenz *et al.* (2012) to challenge the recent clasification of Buttler (2011), who used a more restricted morphological data-set to argue that floral characters allow separation of a full species, *P. fornicata*, from *P. bifolia* on the basis of the typically greater lengths of its labellum, spur and pollinaria. Buttler also perceived *P. fornicata* ssp. *atropatantica* as synonymous with *P. kuenkelii*, this subspecies being perceived as morphologically intermediate between *P. fornicata* ssp. *fornicata* and *P. bifolia*.

Given present evidence, it remains possible that these and other comparable studies simply constitute attempts to find elusive biologically meanigful boundaries in what are actually continua of morphological variation that, depending on the prior preference of the observer for particular evolutionary mechanisms, could reflect either local adaptation or local drift. The DNA-based study of Pavarese *et al.* (2011) hinted at the possibility that biologically meaningful structure may indeed exist within the *P. bifolia* aggregate, but we strongly suspect that all of the presently competing classifications of the group remain far from optimal. Answering such challenging questions requires Europe-wide surveys that combine motrphological and molecular approaches.

Taxa with small green flowers

The two putative *Platanthera* species endemic to the Azores, *P. micrantha* and the rarer *P. azorica* (Schlechter, 1920; Rückbrodt & Rückbrodt, 1994; Delforge, 2003; Carine & Schaefer, 2010), are of particular interest as they constitute the only potential example of divergent speciation among orchids on the Macaronesian islands; all of the other Macaronesian orchid species can be explained by immigration of mainland European species followed by founder effect and/or anagenetic change in phenotype (Bateman, 2001b; Bateman *et al*., unpublished). Morphologically, the plants diverge considerably from other members of *Platanthera* section *Platanthera*, apparently more closely resembling boreal members of section *Limnorchis*, which is represented by *P. hyperborea* in Iceland, and the former section *Lysiella* (transferred to section *Platanthera* by Hapeman & Inoue, 1997, but diverging substantially in ITS sequences from the *P. bifolia* group), which is represented by *P. oligantha* (?=*P. obtusata*) in Scandinavia. The Azorean species differ from *P. bifolia* and *P. chlorantha* in showing gradation from basal to cauline leaves rather than a sharp discontinuity. Their flowers are smaller and dominantly green, their gynostemia are minute, and their spurs are short (<10 mm), never exceeding the ovary.

Most observers formally (if often tentatively) recognised both of the Azorean *Platanthera* species (Hansen, 1972; Rasbach, 1974; Sundermann, 1980; Buttler, 1991; Rückbrodt & Rückbrodt, 1994; Delforge, 2003; Kreutz, 2005), though Webb (1980) and Davies *et al.* (1983) treated *azorica* as a comparatively rare variety of *P. micrantha*, arguing that the two taxa have overlapping geographic distributions and habitat preferences (sun or shade, in damp acidic — and typically volcanigenic — soils of upland regions). Startlingly, the recent IUCN Red-Listing revision also treated all Azorean *Platanthera* as a single species, albeit an officially Endangered one (Rankou *et al*., 2011). The most striking morphological differences between the two taxa are the even shorter (arguably near-vestigial) spur of *P. micrantha* and the fact that its labellum is vertical or more often recurved, whereas the more elongate labellum of *P. azorica* is strongly decurved, sometimes to the degree where its forward orientation obscures from view the gynostemium and spur entrance. But *P. azorica* is also routinely reported as being noticeably larger and more robust in all its parts than *P. micrantha*; we speculate that the 2: 3 averaged size ratio evident in the pairings of *P. bifolia* and *P. chlorantha*, and of *P. holmboei* and *P. algeriensis*, might also characterise the relationship between *P. micrantha* and *P. azorica*.

We anticipated substantial divergence in ITS sequences between the Azorean *Platanthera* species and their mainland counterparts, matching the substantial ITS divergence found in all other comparisons made to date between orchid sister species in Macaronesia and their sister-species in mainland Europe (e.g. *Himantoglossum* [*Barlia*] *metlesicsianum* vs *H. robertianum*, *Orchis canariensis* vs *O. patens*, *Dactylorhiza foliosa* versus *D. fuchsii*/*D. maculata*: Bateman *et al*., 2003). However, this was not the case. Analyses of our two DNA samples of *P. micrantha* yielded two sequences that differed by only a single substitution. One of these sequences was allele IV, interestingly otherwise found only in the one unequivocal accession of *P. holmboei* (Appendix 2). The second allele, V, simply differed by a single additional substitution (Fig. 2). The two accessions of *P. azorica* appeared derived relative to *P. micrantha* due to the putative synapomorphy of an additional substitution in alleles VI and VII; allele VII also exhibited a one

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base-pair insertion relative to Allele VI (Fig. 2). Thus, despite their substantial morphological divergence from other members of the *P. bifolia* aggregate, the Azorean species appear to have originated from within that group only recently. The only member of the aggregate that currently occurs in northwest Africa and Iberia is *P. algeriensis*, which therefore constitutes the most likely ancestor of the Azorean species. This knowledge has encouraged us to renew our search for DNA samples of these little-known taxa, the results of which will be published elsewhere (Bateman *et al.*, unpublished).

The perils of typology

Classical morphological taxonomy, morphological phylogenetics and molecular phylogenetics all tend to be pursued as essentially typological ventures. In classical taxonomy, the holotype is paramount. In morphological phylogenetics, each species is represented by generalised codings that typically ignore polymorphism within characters. In molecular taxonomy, at least until very recently, a species was usually represented by a single DNA sample extracted from a single individual. Our morphometric and molecular studies of the last decade usefully illustrate the dangers inherent in the typological approach.

With regard to morphology, our morphometric data have clearly shown that interspecific differences between *P. bifolia* and *P. chlorantha* conflate characteristic ontogenetic variation in organ size with a weaker species-specific overprint. Both inferred causes have similar outcomes, specifically allometric variation in quantitative properties of all of the organs that constitute the individual plant. That two factors are compounded in influencing these characters could never have been inferred other than by large-scale sampling and detailed measurement of populations across the range of both putative species (e.g. Bateman, 2001a, 2011).

This point is particularly well-illustrated by the geographically broad data on spur length in *P. bifolia* and *P. chlorantha*. These data demonstrate conclusively that spur length is discriminatory between the two species (Fig. 13), but only if: (a) statistically valid samples are measured in each population; and (b) latitude is taken into account (Bateman & Sexton, 2008). When latitude is ignored, the majority of the ranges of spur length ascribed to these species by previous authors either fail to accommodate a substantial proportion of *Platanthera* populations (if they are too narrow) or fail to suggest any taxonomically diagnostic potential (if they are too broad). We suspect that similar patterns are commonly reflected in quantitative characters presented in diagnoses of herbarium-based floras. There is no adequate substitute for access to geographically and ecologically extensive field data.

This observation is reinforced when we consider the evolution of our own interpretations of available spur-length data through the last few years. Our detailed morphometric survey of both species in southern England was completed in summer 2004. When spur-length data were abstracted from the matrix they showed two classic bell-curves with limited overlap between the two species (Fig. 12). Any idea that these curves might be diagnostic of the two species was rapidly abandoned when we realised that the two curves were transposed relative to the Swedish spur-length data of Nilsson (1983). Our detailed, but UK-dominated, spur-length survey of 2007 suggested that latitude was the single most important factor controlling spur length in *Platanthera*, but the subsequent addition of more populations has complicated this picture (Fig. 13). Most notably, it revealed comparatively wide variation among Continental populations of both species, and reduced from 0.42 to 0.23 the r^2 value of spur length regressed against latitude for *P. chlorantha*. Thus, even our current matrix of 116 populations appears to be a suboptimal sample.

Moving on to ITS sequences, this paper presents analyses of 42 specimens attributed to *P. bifolia*, *P. chlorantha* or putative hybrids (Appendix 2). With the exception of two Chinese specimens identified as *P. chlorantha*, all of these accessions yielded allele I, allele II, or in a few cases both allele I and allele II (the two alleles differ by only one base-pair: Fig. 2).

Completed in 1996, the first papers to present ITS data for these species (Bateman *et al.*, 1997; Pridgeon *et al.*, 1997) were typological, each species being represented by just one individual. The Scottish sample of *P. bifolia* yielded allele I and the Italian sample of *P. chlorantha* yielded allele II, leading those authors to assume that this one base-pair difference was species-specific.

By 2003, we had accumulated ITS sequences for a further 12 plants; all were derived from English populations, and were chosen to represent a wide range of habitats (Bateman *et al.*, 2004). Of the six individuals of *P. chlorantha* sampled, five (83%) had exclusively allele II and only one (17%) had exclusively allele I. In contrast, of seven individuals of *P. bifolia* sampled, four (57%) had allele I and the remaining three proved to be polymorphic for alleles I and II. Ironically, the one putative hybrid analysed, which would have been predicted to be polymorphic if particular ITS alleles had proven to be species-specific, appeared to be monomorphic for allele II. Thus, although exceptions had been detected and frequent polymorphism recognised, in 2003 allele I

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still appeared to be a reasonably utilitarian (albeit imperfect) indicator of *P. bifolia* and allele II of *P. chlorantha*.

The present dataset (Table 3, Appendix 2), which was completed in 2004, contains data for 39 accessions of the two species and their putative hybrids. None of the three putative hybrids was polymorphic for ITS: one yielded only allele I and the other two yielded only allele II. The 19 samples of *P. chlorantha* analysed yielded equal numbers of plants dominated by allele I and allele II (seven each=37%), together with five plants (26%) that clearly contained both alleles. The 17 samples of *P. bifolia* included 11 plants (65%) that apparently contained only allele I but only two plants (12%) that contained only allele II, plus four plants (24%) that yielded both alleles.

Thus, the percentage of plants of *P. chlorantha* that we perceived as containing only allele II fell progressively from 100% in 1996 to 83% in 2003 and 37% in 2004. Crucially, what appeared from the typological evidence of 1996 to be a species-specific diagnostic character was shown by the population-level data of 2004 to lack any statistically significant discriminatory power to separate the two putative species, even when most of the samples had been drawn from a narrow geographical range to limit geographical variation.

Should morphologically distinguishable, molecularly indistinguishable entities be recognised as species?

Species distinction or stabilised polymorphism?

As we have seen, *P. bifolia* is readily morphologically distinguishable from *P. chlorantha*, albeit using a small number of metric characters of the gynostemium that potentially reflect simple genetic control. In contrast, our exploration of both nuclear and especially plastid genomes identified only one variable site; moreover, that one site in the ITS region was not a species-specific difference capable of distinguishing between *P. bifolia* and *P. chlorantha* (Table 3). Contemporaneous attempts in other laboratories to develop tools to study genetic variation at the population level in the *P. bifolia* aggregate, such as plastid microsatellites and AFLPs, were also reportedly undermined as a result of encountering only genetic uniformity (e.g. M. Hedrén, pers. comm., 2006). Eventually, Pavarese *et al.* (2011) detected in two plastid spacers slight variations among Italian populations of the two species, but these differences similarly proved not to be species-specific. Thus, the *P. bifolia* aggregate poses a fundamental question of considerable relevance across the plant kingdom. In particular, the phenotypic–genotypic disparity between *P. bifolia* and *P. chlorantha* forces us to decide that either (1) bona fide species need not differ consistently and reliably in DNA sequences of rapidly changing nuclear and plastid regions, or (2) there is no valid species distinction between them.

If the second statement were true, how could we explain the morphological distinction evident between the two widely accepted species? The inference of a simple genetic control dictating the two contrasting gynostemium morphologies raises the possibility that the two morphs could simply represent a stabilised genetic polymorphism, analogous to that observed in, for example, red versus yellow flower colour in the *Dactylorhiza sambucina* and *D. romana* aggregates (e.g. Gigord *et al.*, 2001). This hypothesis challenges us to seek any evidence that could indicate that the two morphologies diverged under directional or disruptive selection. At present, the remarkable paucity of any divergence in routinely sequenced regions of the genomes precludes a direct answer to this question. Indirect evidence of selectively mediated divergence could be sought in the form of contrasting geographical distributions or phenologies, but the two putative species appear remarkably similar on both criteria (Figs. 9 and 10). However, in northwest Europe, *P. bifolia* does seem to have a broader soil pH tolerance than *P. chlorantha*, most notably including populations that show a predilection for acidic flushes rich in insectivorous plants (Fig. 1D), thereby hinting at the possibility of as-yet undetected differences in ecologically significant genes (notably, acid soil tolerance is also apparent in the Mediterranean and Azorean taxa). On the other hand, given the considerable overlap in ecological preferences of the two species, it is surprising that they do not co-occur more often, and it is notable that where they do co-occur, one putative species is usually far less frequent than the other. Again, two possible explanations offer themselves. Firstly, the less frequent morph could have migrated into a population of contrasting morphology and ben rapidly assimilated through hybridisation. Alternatively, given the small size (and very small effective size, N*e*) of many *Platanthera* populations, the polymorphic population could rapidly reach monomorphism of one randomly-favoured morph simply through genetic drift (e.g. Tremblay *et al.*, 2005).

We will end by considering the possibility that Darwin (1877) and almost all subsequent authors were correct in asserting that *P. chlorantha* really is a species distinct from *P. bifolia*. We have one advantage over Darwin in being able to use molecular phylogenetic evidence to infer that the expanded gynostemium of *P. chlorantha* is derived relative to the much narrower gynostemium of *P. bifolia* (cf. Hapeman & Inoue, 1997; see also Efimov, 2011). Given their apparently identical genotypes, it is not

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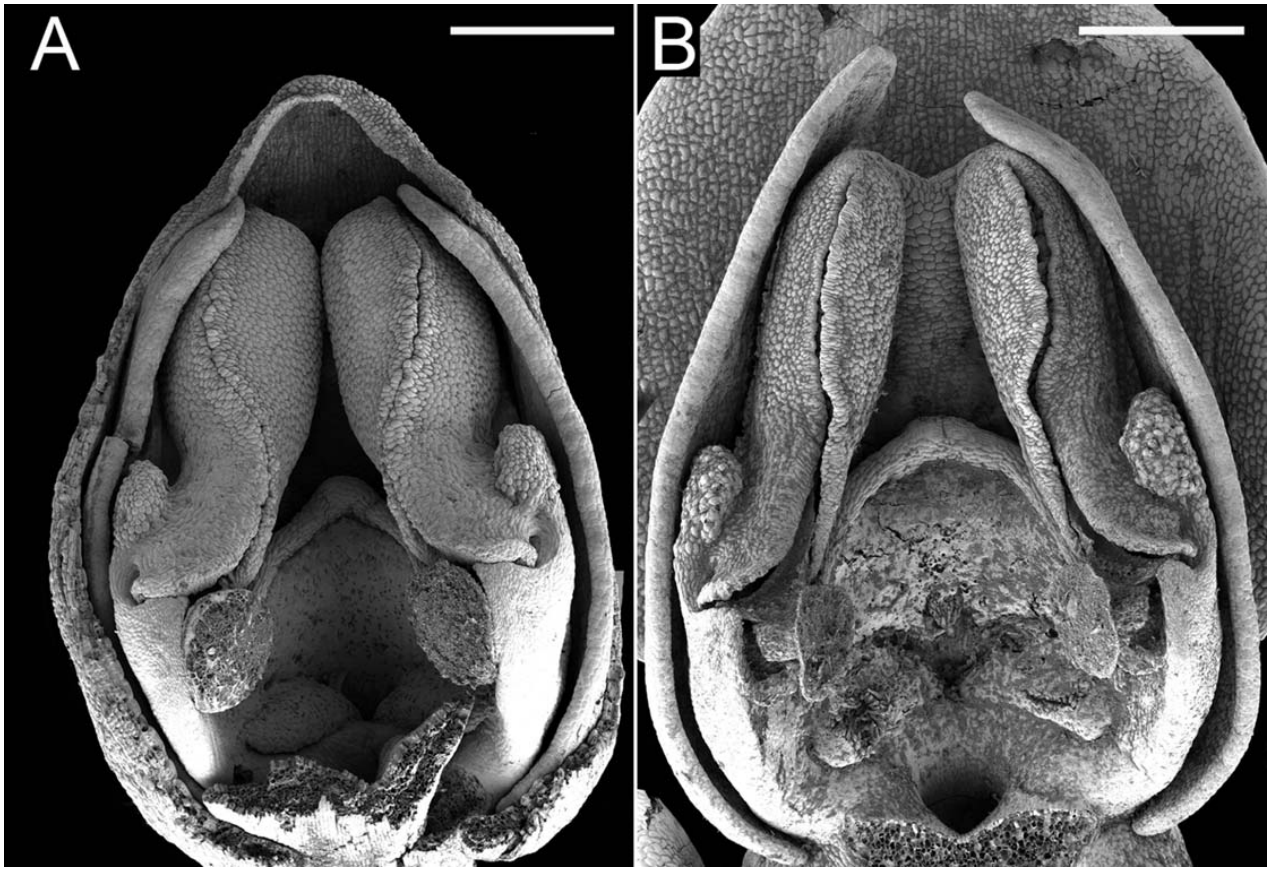

Figure 15 Thompson nets demonstrate how the inferred phenotypic transition from *P. bifolia* (left) to *P. chlorantha* (right) is estimated to have entailed an approximately two-fold increase in gynostemium size and spur diameter but a three-fold increase in stigma size. The net is superimposed on a base diagram adapted from Summerhayes (1951, fig. 17); dots indicate putatively homologous points.

outrageous to reconfigure this hypothesis in terms of ancestor–descendant relationships and thus assume that *P. chlorantha* originated from within *P. bifolia*. This thought experiment encouraged us to consider how the divergence of *P. chlorantha* from *P. bifolia* would have been represented through time in: (a) the phenotypic shift that marked the speciation event; (b) the few genes directly responsible for the phenotypic shift; and (c) the remainder of the genome that is not directly responsible for the phenotypic shift (but is presently the source of almost all molecular phylogenetic data).

This discussion is predicated on the assertion of Bateman (1999: 446) that 'if morphological evolution follows a punctuational pattern (dictated by long periods of stabilising selection that are only occasionally broken by temporary release from selection and consequent speciation) and thus there is no morphological clock, but if in contrast genomic mutation is broadly clock-like [and thus gradualistic], then in phylogenetic terms the vast majority of morphological character-state transitions occur *during* speciation events and the vast majority of molecular character-state transitions occur *between* them' [author's italics]. In addition, the morphological shift typically affects only one of the two daughter lineages (in this case, *P. chlorantha* diverging from a *P. bifolia*-like ancestor), whereas molecular changes are likely to occur with roughly equal frequency in both daughter lineages. Obviously, the morphological shift that is considered to be coincident with the speciation event must have been prompted by at least one change in one or more key phenotypically-expressed genes, but those genetic (or epigenetic) changes are typically minute in scale and can only be detected through careful pinpointing using painstaking evolutionary-developmental genetic approaches; they will not be encountered during a routine molecular phylogenetic study, as the regions routinely sequenced are not relevant.

A critical role for stigmatic peramorphosis?

Reviewing the genus *Platanthera*, Efimov (2011, fig. 1) noted exceptional variation in gynostemium morphology among species but also acknowledged the existence of high morphological platicity within those species. We gained some insight regarding how the transition from *P. bifolia* to *P. chlorantha* phenotypes could have occurred by superimposing a Thompson net (Thompson, 1917) onto Summerhayes' (1951) classic rendition of the *P. bifolia* gynostemium and then

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transferring that net to the putatively derived flower of *P. chlorantha* (Fig. 15). This exercise revealed that the transition from ancestral to derived gynostemium required approximately equal estimated expansions of 225% in spur diameter, 180% in gynostemium height, and 210% in the distal width of the gynostemium, but that the gynostemium expanded by 300% at its widest — that is, proximal — point. The Thompson net strongly suggests that the crucial character separating the two putative species is stigma size, and that accommodating the larger stigma forced the viscidia of *P. chlorantha* much further apart. This could be achieved most simply by an extended phase of development (hypermorphosis) or an increased rate of development (acceleration) of the stigma — modes of peramorphosis within the broader category of heterochrony (e.g. Alberch *et al.*, 1979). The converse phenomenon of truncated development (paedomorphosis) has previously been used to explain speciation via transitions in floral phenotype in some other European orchid groups (e.g. Rudall & Bateman, 2002; Bateman & Rudall, 2006b; Box *et al.*, 2008; Bell *et al.*, 2009; Rudall, Perl & Bateman, unpublished).

This hypothesis would best be tested by carefully documenting ontogenetic time series for flowers of both species. We here preview future, more detailed, investigations by comparing mid-stage and late-stage buds of *P. holmboei*, a taxon that closely resembles *P. chlorantha* (Fig. 4). The more elongated and expanded lateral petals and dorsal sepal of the right-hand bud demonstrate its more advanced ontogenetic state. It also shows greater vertical and especially lateral expansion that separates the paired viscidia and loculi, which open along their somewhat sigmoid sutures. This gynostemial expansion also pushes the stigmatic region forward, rendering it less concave.

Developmental-genetic evidence from *Arabidopsis*, summarised by Balanzá *et al.* (2006), suggests that development of its gynoecium is crucially controlled by an auxin gradient that declines from apex (stigma) to base (gynophore) (Nemhauser *et al.*, 2000). It is not yet clear how confidently we can apply conclusions based on *Arabidopsis* to the far more strongly dorsiventrally polarised, fused andro-gynoecium that characterises orchids (e.g. Rudall & Bateman, 2002; Mondragón & Theissen, 2009). Nonetheless, it is tempting to speculate that the peramorphosis inferred in the stigmatic region of *P. chlorantha* (and *P. holmboei*) could reflect mutation of one of several genes implicated in controlling gynoecium development. *STYLISH1* (*STY1*, SHI gene family: e.g. Fridborg *et al.*, 1999; Ecklund *et al.*, 2010) is expressed in the apical region of the developing gynoecium of *Arabidopsis*, the resulting DNA-binding transcriptional activities regulating genes such as *YUCCA4*, which encodes a key enzyme in the auxin biosynthetic pathway (Sohlberg *et al.*, 2006; Jain & Khurana, 2009). The auxin gradient could be mediated by *ETTIN* (*ETT*, a member of the Auxin Response Factor family [ARF] family: Pekker *et al.*, 2005) toward the base of the gynoecium, where it represses the widely expressed *SPATULA* (*SPT*, a bHLH gene: Heisler *et al.*, 2001; Groszmann *et al.*, 2010) in order to ensure that ovaries are formed, rather than an excess of stigmatic and/or gynophoric tissue. We suspect that even a subtle change in the inferred auxin gradient(s) would be sufficient to expand the stigmatic region by the amount observed to separate *P. chlorantha* from *P. bifolia* (Fig. 15).

The potential significance of the genetic divergence lag (GDL)

Returning to our synthetic model, we consider the 'punctuated phenotype plus gradualist genotype' model of evolution to apply equally to selection, drift or saltation as the cause(s) underlying any speciation event that involves the 'attempted' divergence of lineages. The model predicts that there will be a substantial lag time between the (typically very brief) period of phenotypic divergence that marks a particular speciation event and the accumulation of detectable mutations in those regions of the nuclear, plastid and mitochondrial genomes that are routinely used to reconstruct phylogenies (e.g. Bateman, 2011). We herewith term this period when the existence of the novel lineage is phenotypically overt but genotypically cryptic as the GDL. The length of the GDL for a particular lineage (and indeed whether the lineage ever successfully breaks out of the GDL; most incipient lineages are probably assimilated back into the ancestral lineage without ever achieving breakout velocity) will, in allogamous systems, depend largely upon: (a) the mutation rates in the relevant genic regions; and (b) the frequency of gene flow between the ancestral lineage and the descendant lineage. The mutations are the raw materials essential for molecular lineage recognition, and genetic isolation is essential to allow those potentially ubiquitous genetic markers to reach fixation (classical population-genetic theory shows that even low levels of gene-flow reliably preclude fixation of even highly beneficial alleles in populations).

The GDL is of considerable importance because novel lineages residing in the GDL are, by definition, immune to DNA-barcoding approaches to plant identification (cf. Tautz *et al.*, 2003; Savolainen *et al.*, 2005; Lahaye *et al.*, 2008; CBoL, 2009). In an analysis of six plastid regions in 71 specimens representing 48 species of Costa Rican orchids (12 represented by multiple accessions), Lahaye *et al.*

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(2008) reported genetic distances that ranged from 0.0022±0.003 to 0.0163±0.0211 for interspecific comparisons and from 0.0014±0.002 to 0.0077±0.0146 intraspecific comparisons. They therefore drew positive inferences regarding the discriminatory power of such sequences among orchid species, implying that the genetic uniformity recovered here is exceptional, and that between rather than within putative species, it is extraordinary.

Admittedly, the present example of molecular uniformity concerns only two putative species (arguably six, if the Mediterranean and Azorean taxa are also considered), but exceptionally low ITS and plastid divergence has also been recorded in several other European orchid clades, some of which encompass far greater phenotypic diversity of supposedly dominantly allogamous species: these include *Gymnadenia* subgenus *Nigritella* (Hedrén *et al.*, 2000; Bateman *et al.*, 2003, unpublished), *Serapias* (Bateman *et al.*, 2003; but see Pellegrino *et al.*, 2005) and *Ophrys* (cf. Schlüter *et al.*, 2007; Devey *et al.*, 2008, 2009; Bateman *et al.*, 2010, 2011; Vereecken *et al.*, 2011). Similarly, the *Dactylorhiza incarnata* aggregate is rich in phenotypic diversity (Bateman & Denholm, 1983) but shows little or no variation in allozymes (Hedrén, 1996), ITS sequences, plastid haplotypes (Pillon *et al.*, 2007; Hedrén *et al.*, 2011) or even AFLPs (Hedrén *et al.*, 2001). Even lineages that successfully surpass the GDL phase can resist molecular delimitation. For example, several closely related species of anthropomorphic *Orchis* (Fay *et al.*, 2007; Bateman *et al.*, 2008) and most *Dactylorhiza* (Hedrén *et al.*, 2001, 2011; Pillon *et al.*, 2007; Bateman, 2011b) appear to have successfully developed their own distinctive ITS signatures, only to subsequently suffer widespread hybridisation involving levels of gene flow sufficient to generate complex patterns of ITS alleles and plastid haplotypes that transgress species boundaries. Moreover, these detailed studies overruled earlier, typological ITS studies (Pridgeon *et al.*, 1997; Bateman *et al.*, 2003) that had optimistically suggested that reliable molecular identification would be possible.

Clearly, the genetic divergence lag — epitomised here by the much-discussed 'model' relationship between *P. bifolia* and *P. chlorantha* — seriously compromises both species delimitation and species identification by molecular methods — one observer's stabilised polymorphism is another observer's incipient species. On a more positive note, the shorter the period of time (and degree of genetic change) that has elapsed since the initial phenotypic divergence occurred, the easier it should be to pinpoint the few genetic (or epigenetic) change(s) that actually initiated the speciation event. In other words, the molecular phylogeneticist's loss is likely to be the evolutionary-developmental geneticist's gain, offering us a tantalising potential window into the innermost machinery of speciation.

Acknowledgements

Harold and Jane Lambert, and Jonathan Tyler, generously provided sets of flowers for morphometric analysis from Powys and Suffolk, respectively, while the Azorean accessions were kindly collected by Monicá Moura. We thank Jean Claessens and Barbara Gravendeel for loaning the images of pollinium removal (Fig. 14E–G). We are grateful to Lavinia Robba for laboratory assistance during the latter stages of the sequencing project. We also thank the following members of the Hardy Orchid Society and the Botanical Society of the British Isles for donating additional data to the spur-length survey: Arthur Chater, Sean Cole, Peter Daltry, Ian Denholm, Jonathan Fenton, Alan Gendle, Graham Goodfellow and Ann Skinner, Lois and Nigel Harbron, Anne and Simon Harrap, David and Christine Hughes, Tony Hughes, David Johnston, Nigel Johnson and Rosemary Webb, Sarah Longrigg, Jackie Pedlow, Roy Sexton, Kathy Stott, Neville Henderson and David Pearce, Simon and Moira Tarrant, John and Shelagh Temporal, and Iain and Christine Wright, together with a Poland-based group consisting of P. Baraniecki, K. Ciesielski, L. Dudek, W. Hanak, L. Krajewski and M. Scelina.

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Appendix 1: Details of morphometric localities. Flowers from Lanbedr were supplied by Harold and Jane Lambert, those from Lavenham by Jonathan Tyler

Locality	Habitat	Shaded	Year(s)	Number		
				Chlorantha	*Bifolia*	Hybrids
Sylvia's Meadow, ST ANNE'S Chapel, Tavistock, Cornwall	neutral coarse grassland	N	2003	1	10	1
Elkham's Grave, MEAD END, Rhinefield, S Hants	Acid wet heath/bog	N	2003		8(+2)	
BADBURY Rings, Shapwick, NW Wimborne, Dorset	Calcareous coarse grassland/scrub	N	2004	10		
PIG BUSH, Denny Lodge, S Hants	Acid heath	N	2003		10	
STEPHILL Bottom, Denny Lodge, S Hants	Acid heath	N	2003		4(+6)	
Broad Down, WYE Downs, SE Wye, E Kent	Calcareous coarse grassland	N	2004	1		
PARK GATE Down, Elham, E Kent	Calcareous grassland	N	2004	1		
YOCKLETTS Bank, Waltham, E Kent	Calcareous open woodland	Y	2003/4	5(+4)		
SHEEPLEAS Wood, West Horsley, Surrey	Calcareous coarse grassland/scrub	N	2003	1(+9)		
Great CHEVERELL Hill, SW Lavington, Wilts	Calcareous coarse grassland	N	2004		1	
Coppice NW SHELDWICH, S Faversham, E Kent	Neutral woodland	Y	2004		1	
Traffic island, LONGREACH Wood, Stockbury, W Kent	Calcareous coarse grassland/scrub	N	2004	10		
STOCKBURY Hill Wood, Elham, W Kent	Calcareous open woodland	Y	2003/4		3(+5)	
Walkers Hill, PEWSEY Downs, N Alton Barnes, Wilts	Calcareous grassland	N	2004		5	
MORGAN'S HILL, Calstove Wellington, SE Calne, Wilts	Calcareous grassland	N	2004		10	
HOMEFIELD Wood, Medmenham, Bucks	Calcareous woodland/scrub	Y	2003/4	8		
Warburg Reserve, BIX Bottom, Oxon	Calcareous woodland/scrub/grassland	Y	2003/4	11	3(+1)	4
DANCERS END, Aston Clinton, Bucks	Calcareous scrub/open woodland	Y	2004	1		
ASTON CLINTON Ragpits, Bucks	Calcareous scrub/coarse grassland	Y	2004	10		
Ty-Commins, LLANBEDR, Crickhowell, Powys	Neutral coarse grassland	N	2004	10		
Lineage Wood, E Bridge Street, W LAVENHAM, W Suffolk	Neutral woodland	Y	2004	10		
Total				79	55	5

Appendix 2: Samples of the *P. bifolia* aggregate yielding ITS sequences. Sequences sources from previous publications (in square brackets): 1=Pridgeon *et al.* (1997), Bateman *et al.* (1997); 2=Bateman *et al.* (2003). *=sequence representing that particular allele in the parsimony analysis summarised in Fig. 2

Number	Taxon	Locality	Collector(s)	Date	ITS allele(s)
Southeast Asia (5)					
K13038	*Platanthera finetiana*	China	YB Luo		I
K13073	*Platanthera metabifolia*	China	YB Luo		I
*K13071	*Platanthera chlorantha*	China	YB Luo		III
K13001	*Platanthera chlorantha*	China	YB Luo		III
K13072	*Platanthera chlorantha*	China	YB Luo		I
Continental Europe (16)					
B2195	*Platanthera azorica* 1	Lagoa di Fogo, San Miguel, Azores	M Moura	25.06.08	VI
B2196	*Platanthera azorica* 2	Lagoa di Fogo, San Miguel, Azores	M Moura	25.06.08	VII
B2193	*Platanthera micrantha* 1	Lagoa di Canario, San Miguel, Azores	M Moura	24.06.08	IV
B2194	*Platanthera micrantha* 2	Lagoa di Canario, San Miguel, Azores	M Moura	24.06.08	V
*Bsn	*Platanthera micrantha*	Azores	J Vogel	?	IV or V
*B1398	*Platanthera holmboei*	Copse N crossroads, Mandria, Troodos, C Cyprus	R Bateman	07.03.06	IV

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Appendix 2: Continued

Number	Taxon	Locality	Collector(s)	Date	ITS allele(s)
B443 [2]	*Platanthera* cf. *holmboei*	SW Agiasos, Lesvos, Aegean Greece	M Lowe	15.05.99	I
B1152	*Platanthera* cf. *chlorantha*	Artemisia–Sparti Road, SC Peloponnese, S Greece	R Bateman	18.04.05	(I+)II
B565	*Platanthera* cf. *bifolia*	S Munciaratti, ESE Collesano, Palermo, Sicily	R Bateman	24.04.00	I
B707	*Platanthera chlorantha*	SE Mass. Orimini, W Martina Franca, SC Italy	R Bateman	19.04.02	I
Ksn [1,2]	*Platanthera chlorantha*	Italy	W Rossi		II
B1078	*Platanthera bifolia*	Neuhaus, nr Marinzell, Nieder-Osterreich, Austria	M Fischer	19.06.04	I
B141	*Platanthera bifolia*	Meadow NE Scharnitz, NW Innsbruck, Austria	R Bateman	14.07.97	II
B140	*Platanthera chlorantha*	Woods NE Scharnitz, NW Innsbruck, Austria	R Bateman	14.07.97	I+II
B737	*Platanthera bifolia*	Flush W Farnigen, SW Altdorf, Switzerland	R Bateman	11.07.02	I
B971	*Platanthera bifolia*	Surbaix, W Lyon, France	R Manuel	04.06.04*	I
Southern UK (27)					
B1064	*Platanthera chlorantha*	Broad Downs, Wye, Kent	R Bateman	18.06.04	I
B1065	*Platanthera chlorantha*	Park Gate Down, Elham, Kent	R Bateman	18.06.04	I+II
B806	*Platanthera chlorantha*	Yockletts Bank S, Kent	R Bateman	07.06.03	II
B1007	*Platanthera bifolia*	Coppice NW Sheldwich, S Faversham, Kent	R Bateman	30.05.04	I
B799	*Platanthera bifolia*	Stockbury Hill Wood, Kent	R Bateman	07.06.03	I
B1009	*Platanthera chlorantha*	Longreach Wood, Stockbury, Kent	R Bateman	30.05.04	II
B842	*Platanthera chlorantha*	Sheepleas, West Horsley, Surrey	R Bateman	20.06.03	I
B975	*Platanthera chlorantha*	Lineage Wood, E Bridge St, W Lavenham, W Suffolk	J Tyler	03.06.04	I+II
B1062	*Platanthera chlorantha*	Dancers End, Aston Clinton, Bucks	R Bateman	16.06.04	I
B1052	*Platanthera chlorantha*	Aston Clinton Ragpits, Bucks	R Bateman	16.06.04	I+II
*B787	*Platanthera chlorantha*	Homefield Wood, Medmenham, Bucks	R Bateman	01.06.03	II (type)
B798	*Platanthera bifolia*	Site 1A, Warburg Reserve, Bix, Oxon	R Bateman	01.06.03	I
B797	*Platanthera bifolia* (x *chlorantha*?)	Site 1B, Warburg Reserve, Bix, Oxon	R Bateman	01.06.03	II
B992	*Platanthera chlorantha* x *bifolia*	Site 3, Warburg Reserve, Bix, Oxon	R Bateman	05.06.04	II
B997	*Platanthera* cf. *chlorantha*	Site 5, Warburg Reserve, Bix, Oxon	R Bateman	05.06.04	I
B794	*Platanthera chlorantha*	Site 2, Warburg Reserve, Bix, Oxon	R Bateman	01.06.03	II
B832	*Platanthera bifolia*	Elkhams Grave, Mead End, Rhinefield, Hants	R Bateman	15.06.03	I
B812	*Platanthera bifolia*	Stephill Bottom, Denny Lodge, Hants	R Bateman	15.06.03	(I+)II
B822	*Platanthera bifolia*	Pig Bush, Denny Lodge, Hants	R Bateman	15.06.03	I(+II?)
B1043	*Platanthera bifolia*	Walkers Hill, Pewsey Downs, N Alton Barnes, Wilts	R Bateman	12.06.04	II
*B1033	*Platanthera bifolia*	Morgans Hill, SE Calne, Wilts	R Bateman	12.06.04	I (type)
B1030	*Platanthera bifolia*	S slope Great Cheverell Hill, SW Lavington, Wilts	R Bateman	12.06.04	I
B1020	*Platanthera chlorantha*	Badbury Rings, Shapwick, NW Wimborne, Dorset	R Bateman	11.06.04	II
B1081	*Platanthera chlorantha*	Ty-Commins, nr Llanbedr, Crickhowell, Powys	H Lambert	27.06.04	I
B852	*Platanthera bifolia*	Sylvia's Meadow, St Anne's Chapel, Tavistock, Cornwall	R Bateman	27.06.03	I(+II?)
B862	*Platanthera bifolia* (x *chlorantha*?)	Sylvia's Meadow, St Anne's Chapel, Tavistock, Cornwall	R Bateman	27.06.03	I
B863	*Platanthera chlorantha*	Sylvia's Meadow, St Anne's Chapel, Tavistock, Cornwall	R Bateman	27.06.03	II
Northern UK (3)					
B717	*Platanthera bifolia*	Rhos-y-Gad, Pentraeth, Anglesey, N Wales	R Bateman	03.06.02	I+II
B61	*Platanthera bifolia*	Ardnish Peninsula, Broadford, Skye, W Scotland	R Bateman	19.06.96	I
B62 [1,2]	*Platanthera bifolia*	Loch a Mhuilinn, Applecross, Wester Ross, W Scotland	R Bateman	21.06.96	I

Appendix 3: Morphometric characters measured for *Platanthera bifolia* aggregate

Categories F and G were measured in the field, categories A–E in the laboratory from excised flowers (category D under a binocular microscope, category E characters 22 and 23 under a compound microscope).

A. Labellum (5 characters)

1. Length (0.1 mm)

2. Maximum width (excluding basal teeth, if present) (0.1 mm)

3. Reflexion, on a scale 1–4 (1=slightly decurved, 2=vertical, 3=slightly recurved, 4=strongly decurved)

4. Depth of green pigmentation, on a scale 0–2 (0=white [state not recorded among sampled individuals], 1=pale green, 2=dark green)

5. Maximum extent of green pigmentation (% of distance from apex to base)

B. Spur/ovary (5 characters)

6. Spur length (0.1 mm)

7. Spur width/mouth (0.1 mm)

8. Spur width halfway (0.1 mm)

9. Spur curvature, on a scale 1–5) (1=strongly recurved, through to 5=strongly decurved)

10. Ovary length (mm)

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C. Sepals and lateral petals (4 characters)

11. Lateral sepal position, on a scale 1–3 (1=substantially below horizontal, 2=more-or-less horizontal, 3=substantially above horizontal)
12. Lateral sepal length (0.1 mm)
13. Lateral sepal width (0.1 mm)
14. Lateral petal length (0.1 mm)

D. Gynostemium (7 characters)

15. Maximum length of column (0.1 mm)
16. Maximum width of column (0.1 mm)
17. Maximum width of stigma (0.1 mm)
18. Length of pollinarium (0.1 mm)
19. Separation of viscidia (0.1 mm)
20. Separation of pollinaria apices (0.1 mm)
21. Length of staminode (0.1 mm)

E. Bracts (5 characters)

22. Mean cell diameter (μm)
23. Mean cell shape, on a scale 1–3 (1=barrel-shaped, 2=subangular, 3=angular)
24. Width floral bracts (0.1 mm)
25. Length floral bracts (mm)
26. Length basal bracts (mm)

F. Stem and inflorescence (4 characters)

27. Stem height, above ground level (including inflorescence) (cm)
28. Inflorescence length (mm)
29. Number of flowers/buds
30. Stem diameter (0.1 mm)

G. Leaves (7 characters)

31. Number of bracteoidal (cauline) leaves
32. Number of expanded (basal) leaves
33. Maximum width of longest leaf (mm)
34. Length of longest leaf (mm)
35. Position of maximum width relative to maximum length, as measured from the point of attachment to the stem (mm)
36. Degree of 'petiole' development, on a scale 0–2 (0=no basal contraction, leaf lanceolate, 1=obscure basal contraction, leaf obtuse, 2=clear basal contraction, leaf obovate)
37. Angle of expanded leaf relative to soil surface, on a scale 1–3 (1=0–30°, 2=31–60°, 3=61–90°)

Appendix 4: Spur-length data additional to that documented by Bateman & Sexton (2008, appendix 1)

Species	Recorder(s), Year	Locality	Habitat	Shade	N	Mean	SSD	CV (%)
Chlorantha	A Hughes, 2009	Port d'ENVALIRA, Pyrenees, Andorra	Grassland	N	19	25.68	2.79	10.9
Bifolia	R Bateman/P Rudall, 2009	GUILHAUMARD, Cevennes, SE France	Limestone scrub	Y	10	24.46	3.13	12.8
Bifolia	S+M Tarrant, 2008	RAKOV Skocjan, Slovenia	Woodland	Y	6	31.67	2.94	9.3
Chlorantha	S+M Tarrant, 2008	SLIVNICA, Slovenia	Woodland	Y	1	27.00	NA	NA
Chlorantha	A Hughes, 2009	LAUTERBRUNNEN, Switzerland	Meadow	N	14	33.36	2.59	7.8
Bifolia	A Hughes, 2009	WIXI, Bernese Oberland, Switzerland	Coniferous woodland	Y	2	23.50	NA	NA
Bifolia	N Johnson/R Webb, 2008	GASTERNTAL, Kandersteg, Switzerland	Scrubby roadside bank	Y?	21	23.62	2.63	11.1
Chlorantha	N Johnson/R Webb, 2008	Road to UESCHINENTAL, Kandersteg, Switzerland	Sunny roadside bank	N	20	37.80	3.21	8.5
Bifolia	D Hughes, 2008	Woods above HUNGERBURG, Innsbruck, Austria	Spruce woods	Y	13	29.92	2.93	9.8
Bifolia	D Hughes, 2008	IGLS, Austria	Spruce woods	Y	10	29.80	4.37	14.7
Bifolia	D Hughes, 2008	Open woodland, HALLTHAL, Austria	Open woodland	Y	10	24.40	2.32	9.5
Bifolia	D Hughes, 2008	MITTENWALD, SC Germany	Grassy banks	N	5	28.80	1.64	5.7
Bifolia	D Hughes, 2008	BENEDIKTBEUREN, SC Germany	Damp meadow	N	3	27.33	0.58	2.1
Bifolia	D Hughes, 2008	HUEFINGEN Woods, E Freiburg, SW Germany	Spruce woods	Y	1	25.00	NA	NA
Chlorantha	K Stott *et al.*, 2008	JUMIEGE, nr Rouen, Normandy, France	Chalk grassland/quarry	N?	27	26.98	2.38	8.8
Chlorantha	K Stott *et al.*, 2008	WOLSTONBURY Hill, Sussex	Grassland and scrub	(N)	56	29.97	3.02	10.1
Chlorantha	K Stott *et al.*, 2009	WOLSTONBURY Hill, Sussex	Grassland and scrub	(N)	45	29.48	2.70	9.2
Chlorantha	K Stott *et al.*, 2010	WOLSTONBURY Hill, Sussex	Grassland and scrub	(N)	60	28.56	2.66	9.3
Chlorantha	K Stott *et al.*, 2011	WOLSTONBURY Hill, Sussex	Grassland and scrub	(N)	60	27.58	2.77	10.0
Chlorantha	K Stott *et al.*, 2012	WOLSTONBURY Hill, Sussex	Grassland and scrub	(N)	60	27.97	3.60	12.9
Chlorantha	R Bateman/P Rudall, 2008	Orchid paddock, Isle of Wight Woods, PORTON Down, Hants	Grassland	N	1	37.00	NA	NA
Chlorantha	R Bateman/P Rudall, 2011	Juniper Bottom, BOX HILL, Surrey	Chalk scrub	Y	10	32.20	2.66	8.3
Bifolia	R Bateman/P Rudall, 2008	MORGAN'S HILL, Calstove Wellington, SE Calne, Wilts	Grassland	N	20	18.43	2.30	12.5
Chlorantha	G Goodfellow/A Skinner, 2008	SOMERFORD Common, Wilts	?Limestone grassland	N?	26	28.35	2.14	7.6

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Appendix 4: Continued

Species	Recorder(s), Year	Locality	Habitat	Shade	N	Mean	SSD	CV (%)
Chlorantha	R Bateman/P Rudall, 2009	ASTON ROWANT, Oxon	Chalk grassland	N	7	36.21	2.38	6.6
Bifolia	G Goodfellow/A Skinner, 2008	Strawberry Banks, E CHALFORD, E Stroud, Gloucs	Limestone grassland	N	148	20.48	2.62	12.8
Bifolia	R Bateman/P Rudall, 2008	Strawberry Banks, E CHALFORD, E Stroud, Gloucs	Limestone grassland	N	20	24.00	2.50	10.4
Bifolia × ?chlor.	R Bateman/P Rudall, 2008	Strawberry Banks, E CHALFORD, E Stroud, Gloucs	Limestone grassland	N	1	22.50	NA	NA
Chlorantha	R Bateman/P Rudall, 2008	Strawberry Banks, E CHALFORD, E Stroud, Gloucs	Limestone grassland	N	1	34.00	NA	NA
Chlorantha	A Chater, 2008	Meadow, Winllan, TALSARN, Cardigan	Hay meadow	N	44	29.52	1.75	5.9
?Hybrids	A Chater, 2008	Pasture, W bank Afon Teifi, S end CORS CARON, Cardigan	Floodplain pasture	N	30	21.70	2.02	9.3
Chlorantha	A+S Harrap, 2009	HONEYPOT Wood, W Dereham, Norfolk	Woodland	Y	20	34.98	2.38	6.8
Chlorantha	S Cole, 2007	Cloud Wood, BREEDON on the Hill, Leics	Damp willow woods	Y	12	28.58	2.84	9.9
Chlorantha	J Pedlow, 2007	'Firwood', LYNCLYS, S Oswestry, Shropshire	Woodland, meadow, lawns	(N)	20	29.45	2.39	8.1
Bifolia	J Pedlow, 2007	LLYNCLYS Common, S Oswestry, Shropshire	?Open heathland	N	20	18.55	2.44	13.2
Bifolia × chlor.	J Pedlow, 2007	LLYNCLYS Common, S Oswestry, Shropshire	?Open heathland	N	1	25.00	NA	NA
Bifolia	S Cole, 2007	Swallow Moss, ENE LEEK, Staffs	Damp acid moorland	N	21	17.60	2.48	14.1
Chlorantha	S Cole, 2007	CALVER, SE Macclesfield, Derbs	Scrubby limestone hillside	N	8	27.75	3.57	12.9
Bifolia	R Bateman/P Rudall, 2009	Lough GELAIN, W Corrofin, Co. Clare, Ireland	Calcareous wetland	N	8	17.75	2.54	14.3
Bifolia	R Bateman/P Rudall, 2009	Lough BAILIE, W Corrofin, Co. Clare, Ireland	Rough pasture	N	4	17.75	0.96	5.4
Bifolia	A Gendle, 2008	OUTLY Moss	Valley mire	N	20	18.95	1.95	10.3
Chlorantha	A Gendle, 2008	Piers Gill, DENTDALE	Hay meadow	N	8	26.88	3.19	11.9
Chlorantha	A Gendle, 2008	Railway, DENTDALE	Railway cutting, grassland	N	15	22.40	2.71	12.1
Chlorantha	A Gendle, 2008	WAKEBARROW	Ancient woodland	Y	6	29.33	2.73	9.3
Chlorantha	A Gendle, 2008	Township plantation, WHITBARROW	Ancient woodland	Y	7	27.79	0.99	3.6
Chlorantha	A Gendle, 2008	A6/A591 junction, S Kendal, SE Cumbria	Coarse grassland	N	19	29.45	3.48	11.8
Chlorantha	A Gendle, 2008	FIRBANK churchyard		N?	10	27.35	1.33	4.9
Chlorantha	L+N Harbron, 2008	SMARDALE NNR, W Kirkby Stephen, SE Cumbria	Woodland	Y	32	28.73	2.46	8.6
Chlorantha	L+N Harbron, 2008	SMARDALE NNR, W Kirkby Stephen, SE Cumbria	Woodland	N	19	26.11	3.03	11.6
Bifolia	A Gendle, 2008	WAITBY Greenriggs, W Kirkby Stephen, SE Cumbria	Grassland	N	20	18.23	2.28	12.5
Bifolia	A Gendle, 2008	Railway near WAITBY Greenriggs, SE Cumbria	Railway cutting, grassland	N	3	18.00	2.65	14.7
Chlorantha	A Gendle, 2008	CROSBY GARRETT	Open railway cutting	N	6	29.33	1.83	6.2
Chlorantha	L+N Harbron, 2008	ARGILL Woods+Pastures, ?W Kirkby Stephen, SE Cumbria	Steep grassland	N	17	26.21	3.22	12.3
Chlorantha	A Gendle, 2008	AUGILL, N Stainmore	Hill pasture	N	6	23.75	2.38	10.0
Bifolia	R Bateman, 2012	S MULLAGHMORE, N Sligo, Co. Sligo, Ireland	Maritime meadow	N	20	17.05	1.36	8.0
Chlorantha	R Sexton, 2008	BOMAINS Meadow LNR, Bo'ness, Falkirk, ?Fife	Coarse grassland	N	16	27.19	1.90	7.0
Bifolia	R Sexton, 2008	Wester BALGAIR, Stirling	Wet grazed meadow	N	6	19.32	2.03	10.5
Bifolia	R Sexton, 2008	BALLANGREW Meadow, Stirling	Wet grazed meadow	N	7	17.87	1.65	9.3
Bifolia	R Sexton, 2008	QUOIGS Meadow, Perth	Coarse grassland	N	9	20.64	1.62	7.9
Bifolia	J+S Temporal, 2009	LUSKENTYRE, South Harris, Outer Hebrides	Grassy roadside verge	N	20	15.30	2.14	14.0

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