Implications of next-generation sequencing for the systematics and evolution of the terrestrial orchid genus *Epipactis*, with particular reference to the British Isles

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Summary. Recent application of next-generation sequencing technology to Eurasian taxa of the primitive epidendroid orchid clade *Epipactis* section *Epipactis* has further advanced our knowledge of what has become a model system for studying the origins of numerous autogamous taxa from within arguably only one allogamous ancestral species, *E. helleborine* s.s. Current understanding of evolutionary pattern, species re-circumscription and speciation process within *Epipactis* is critically reviewed, emphasising the relevance of recent research based on next-generation sequencing and aiming to better understand taxa native to the British Isles. Conclusions include recognition that (1) the majority of named taxa currently widely viewed as species do not pass rigorous tests of species status, (2) two of the six unequivocal species native to Britain extend further east in Eurasia than was previously supposed, and none is endemic, (3) all scientifically defensible species of *Epipactis* have high frequencies of self-pollination (although autogamy is significantly less frequent in the ancestral species *E. helleborine* than in any of the remaining species derived from it), and (4) fully understanding the speciation process (including substantial increases in autogamy) will require us to further investigate not only pollinator behaviour and the diverse factors determining gynostemium morphology but also the equally diverse factors (including mycorrhizal specificity) likely to influence the invasion of novel, challenging habitat types.

Key Words. allogamy, ancestor-descendant relationships, autogamy, evolutionary radiation, fluctuating asymmetry, geitonogamy, speciation; species circumscription; species concept.

Introduction

The orchid family encompasses more than its fair share of 'critical groups' that present serious challenges to those of us attempting to better circumscribe species and to anyone attempting field identifications within those groups. And if the species themselves are at best difficult to identify and at worst biologically indefensible, what hope exists of confidently identifying putative hybrids between them? In southern Europe, it is bee orchids of the genus *Ophrys* that most commonly furrow the brows of field botanists. But within the British Isles, the orchid groups that strike most fear into the heart are *Dactylorhiza* (especially the tetraploid *majalis* aggregate), its sister genus *Gymnadenia,* and the *helleborine* aggregate within *Epipactis* (i.e. section *Epipactis,* which contains all British species other than the distinctive, phylogenetically distant *E. palustris*: taxonomic authors are given in Table 1). It is perhaps no coincidence that only these three 'critical' genera each include one or two

species currently thought to be endemic to the British Isles, but that all four putative endemic orchids remain taxonomically contentious and difficult to identify, irrespective of whether classical morphology or DNA data are being employed as identification tools.

The latest weapons in the systematist's armoury are a set of DNA-based techniques, collectively termed next-generation sequencing (NGS: this and other technical terms associated with evolutionary genetics are defined in Box 1), that generate vast amounts of genetic data from each plant analysed, encompassing a significant proportion of their genomes (e.g. Harrison & Kidner 2011; Olson *et al.* 2016). At present, these approaches remain technically challenging in terms of data generation, filtration and analysis, at least for a non-expert such as myself. Having already applied one such technique, RAD-seq (Box 1), to *Ophrys* (Bateman *et al.* 2018) and *Dactylorhiza* (Brandrud *et al.* 2019), my colleagues and I then

Table 1. Revised classification of British and Irish *Epipactis* taxa to subspecies level. The downgraded status of '*sancta*' is provisional, pending future whole-genome sequencing and morphometric comparison. Asterisked taxa contain one or more varietal epithets still in occasional use (excluding the chlorophyll-deficient individuals that occur rarely in all species and are better viewed as formae).

Genus *Epipactis* Zinn
  Section *Arthrochilum*
    E. palustris (L.) Crantz

  Section *Epipactis*
    E. atrorubens (Hoffm.) Besser
    E. phyllanthes G.E.Sm.*
    E. purpurata Sm.
    E. helleborine (L.) Crantz*
        subsp. *neerlandica* (Verm.) Buttler
    E. leptochila (Godfery) Godfery*
    E. dunensis (T.Stephenson & T.A.Stephenson) Godfery*
        subsp. *sancta* (P.Delforge) Kreutz

turned our attention to *Epipactis* (Sramkó *et al.* 2019). In doing so we sought to improve the poor phylogenetic resolution achieved by previous studies that were able to target only small numbers of genes (e.g. Bateman *et al.* 2005; Tranchida-Lombardo *et al.* 2011). Although this latest, NGS-based molecular study of *Epipactis* was funded in Hungary, and hence emphasised plants from eastern Europe, its results nonetheless have several intriguing implications for our understanding of the genus in Britain and Ireland, where much previous research has been performed on the genus. It is fortunate that our native populations of *Epipactis* encompass a representative cross-section of the overall morphological and molecular variation found in the genus (Fig. 1).

What has NGS ever done for us?
Sramkó *et al.* (2019) analysed NGS data for 108 plants of 29 named taxa, 27 of which belonged to the *helleborine* group. Geographical sampling extended from Portugal to Russia, and from Denmark to Greece. Detailed analysis and careful interpretation based on an explicit set of taxonomic rules formulated by Bateman (e.g. 2009) resolved the 27 names into just 11 genuine species, plus a further three well-founded subspecies. Determining whether the remaining formal names represented in the analysis are most appropriately viewed as subspecies or, more likely, varieties or formae requires further, more precisely targeted research generating morphometric as well as molecular data. Moreover, a further 38 named taxa recognised by Delforge (2016) within section *Epipactis* remain to be investigated using NGS approaches.

Despite the key historical role played by British botanists in *Epipactis* taxonomy, only nine of the 108 samples analysed by Sramkó *et al.* (2019) were derived from Britain: two plants of *E. dunensis* from the Lancashire

coast plus one from inland Cumbria (var. *tynensis*), single plants of *E. phyllanthes* from the Lancashire coast and an Oxfordshire beechwood, more beechwood plants in the form of *E. leptochila* from its *locus classicus* in Surrey plus a controversial population in Buckinghamshire attributed by some observers to '*E. neglecta*', and a pair of samples of *E. helleborine* s.l. from Kenfig dunes in Glamorgan — one sample from *Salix repens* scrub purporting to be subsp. *neerlandica* and the other from back-dune woods purporting to be subsp. *helleborine*. Sadly, no room was found in the sample set for British material of *E. atrorubens*, *E. purpurata* or the controversial UK endemic from Lindisfarne, '*E. sancta*', despite the fact that both *E. purpurata* and *E. 'sancta'* have their type localities in England. We can nonetheless make some useful observations on their current status in the light of improved understanding of other closely related taxa.

Viewed from a purely methodological perspective, the British plants offer a valuable indication of the impressive accuracy of RAD-seq via Illumina sequencing — the next-generation sequencing techniques used by Sramkó *et al.* to generate their data and resulting trees (e.g. Fig. 2). The two samples of *Epipactis dunensis* collected c. 5 km apart in the Lancashire dunes are perceived as virtually genetically identical. More remarkably, so are the two samples of *E. phyllanthes*, despite the fact that the Lancashire dunes sample was collected 280 km distant from its Oxfordshire counterpart. Less happily, the Welsh duneland sample of *E. helleborine* subsp. *neerlandica* is also strikingly molecularly similar to the sample originally attributed to *E. helleborine* subsp. *helleborine* that was collected in back-dune woodland just 500 m distant. Only the two UK samples of *E. leptochila* deviate from this pattern of close similarity between British pairs; that from the Surrey *locus classicus* of this species lies (appropriately) at the phylogenetic centre of the *leptochila* clade in Fig. 2, whereas the plant from the more controversial Buckinghamshire population is placed as sister to the remaining samples of *E. leptochila* s.l. — an intriguing observation that is discussed at greater length below.

A chequered taxonomic history
Viewed with the benefit of hindsight, the taxonomic history of the *Epipactis helleborine* aggregate reads rather like a masked ball, many of the protagonists catching occasional glimpses of truth but failing to discern the bigger picture. Authors who followed the traditional taxonomic pathway by addressing locally taxonomic problems that actually required a global solution reliably reached contrasting solutions that led to ever greater nomenclatural confusion.

The UK has consistently played a leading role in progressing the taxonomy of the *Epipactis helleborine* aggregate *per se*. Admittedly, *E. helleborine* itself was first described — presumably from Swedish material — by Linnaeus (1753) and *E. atrorubens* from German material

Box 1. Glossary of evolutionary terms used in this text (in some cases followed in parentheses by more precise definitions specific to the present study of *Epipactis*).

allogamy: The fusion of two gametes derived from two distinct individuals (here, the transfer by insects of pollen from one *Epipactis* stem/clone to the stigma of another)

autogamy: The fusion of two gametes derived from a single individual (here, the transfer of pollen within or between flowers of one *Epipactis* stem/clone)

bootstrap support: A crude but widely used estimate of the strength of particular nodes in a phylogeny that relies on repeatedly constructing sets of trees from subsets of the original data-set and determining the relative frequencies with which particular relationships survive these statistical perturbations

cryptogamy: A specialised form of autogamy in which gametes are transferred within the same reproductive unit without ever being exposed to the external environment (here, the transfer of pollen between fertile stamen and stigma within a single bud that never opens)

evolutionary radiation: A large surplus in the rate of natality over the rate of mortality for species and/or character states within a specified clade [= monophyletic group] through a specified time interval [Bateman 1999, p. 441] (here, simplified to the less rigorous concept of several species apparently originating from within a single ancestral species through a comparatively short period of geological time)

fluctuating asymmetry: Small random deviations from perfect symmetry evident in bilaterally paired structures or within a single bilaterally symmetrical structure (here, a comparison of the dimensions of paired lateral sepals and lateral petals)

geitonogamy: A specialised form of autogamy in which gametes are transferred between different reproductive units of the same stem/clone (here, the transfer of pollen between flowers within the same inflorescence or between different inflorescences of a single genetic clone)

Hardy-Weinberg equilibrium: Core [but simplistic] theory of population genetics stating that in the absence of any evolutionary influences the frequency of particular copies of genes within a population will remain constant across generations

inbreeding coefficient (*F*): The estimated probability that an individual inherited two homologous [comparable] copies of a particular gene from a single ancestor

next-generation sequencing (= high-throughput sequencing): An umbrella term for several analytical methods that allow comparatively rapid sequencing of large numbers of nucleic acids simultaneously for numerous individuals [e.g. Illumina, 454, ion torrent]

monophyletic: A group of taxa or individual organisms encompassing a single hypothesised ancestor and all of its descendants (here, an exclusive group of analysed samples that in a reconstructed evolutionary tree are subtended by a single shared node)

paraphyletic: A group of taxa or individual organisms encompassing a single hypothesised ancestor and some but not all of its descendants (here, a non-exclusive group of analysed samples that in a reconstructed evolutionary tree are subtended by a single shared node)

phylogeny: A diagrammatic hypothesis inferring the evolutionary relationships of a specified group of organisms (here, a rooted dichotomising tree based on RAD-seq data)

polyphyletic: A 'non-group' of taxa or individual organisms encompassing two or more hypothesised ancestors (here, a non-exclusive group of analysed samples that in a reconstructed evolutionary tree are subtended by two or more nodes)

RAD-seq (= restriction site-associated DNA sequencing): A sequencing strategy that uses restriction sites to characterise a significant proportion of an entire genome to identify myriad single nucleotide polymorphisms [SNPs] in next-generation sequence data

reproductive isolation: A degree of evolutionary independence achieved through one or more of a wide spectrum of mechanisms capable of substantially reducing or prohibiting gene flow between co-occurring organisms

speciation: Origin of a novel species from within at least one ancestral species via one or more of a wide spectrum of evolutionary mechanisms

species circumscription: The search for discontinuities in intrinsic properties that distinguish a set of populations and their constituent individuals from all others and imply that they collectively constitute an independent evolutionary lineage (here, primarily reflected in comparatively long internal branches within the RAD-seq-based phylogeny)

species concept: One or more specified fundamental principles considered essential when circumscribing species [many, often highly contradictory, concepts have been proposed in the literature]

by Hoffmann (1804: a taxon quickly elevated to species level by Besser 1809), but the remaining four (or five) species of the aggregate currently viewed as occurring in the British Isles (Fig. 1) have all been based on British types. The next species to be segregated from *E. helleborine* was *E. purpurata*, described as a full species from an atypically anthocyanin-less Worcestershire plant by J. E. Smith (1828). However, even as late as the early 20th Century, both *E. purpurata* and *E. atrorubens* were lumped into *E. helleborine* in the then dominant British Flora of Bentham & Hooker (1920). Another Smith (G. E. Smith 1852) then used Sussex material as the basis for describing *E. phyllanthes*, but with such brevity and in a publication so obscure that his observations were overlooked for several decades (they were even neglected by Godfery [1933] in his otherwise excellent monograph of British orchids).

The collective failure of botanists to correctly circumscribe *E. phyllanthes* festered at the centre of many years of taxonomic confusion. Several taxa occurring in the British Isles were subsequently established that actually belong within *E. phyllanthes*; even Summerhayes' (1951) landmark orchid book discussed not one but two 'species', *E. 'vectensis'* and *E. 'pendula'*. These and other named taxa were subsequently sensibly reduced in rank to varieties of *E. phyllanthes* by Young (1952a, b; see also Young 1962b; Lewis 2017), enabling Summerhayes (1968) to correct his error in the second edition of his book.

Responsibility for the discovery and subsequent advocacy of *Epipactis leptochila* fell to Godfery (1919, 1920, 1921, 1933), based primarily on a morphologically extreme population that still persists today in Surrey (Fig. 1E). It eventually led to a burgeoning of

Fig. 1. Flowers of all widely accepted species of the genus *Epipactis* native to the British Isles, reproduced at a consistent scale. A – C *E. helleborine helleborine*; **D** *E. dunensis* (*locus classicus*); **E** *E. leptochila* (*locus classicus*); **F** *E. phyllanthes*; **G** *E. purpurata*; **H** *E. atrorubens* (all section *Epipactis*); **J** *E. palustris* (section *Arthrochilum*). Horizontal dimension of each image = c. 18 mm. PHOTOS: **A – E, G – J** R. M. BATEMAN; **F** D. M. T. ETTLINGER.

supposed species in Continental Europe that are actually attributable to *E. leptochila* (Sramkó *et al.* 2019). The establishment of *E. dunensis* was more complex. Wheldon & Travis (1913) first drew attention to *dunensis*, which was formally described by T. & T. A. Stephenson (1918) from Lancashire dune material as a forma of *E. helleborine*, but three years later was elevated by them to a variety of *E. leptochila* (Stephenson & Stephenson 1921) — an eyebrow-raising taxonomic status that nonetheless was supported as recently as the 1990s by Allen *et al.* (1993).

Inevitably, *dunensis* was soon raised to species level by Godfery (1926), who had recently first described *E. leptochila* and was therefore well-equipped to identify its morphological distinction from *E. dunensis*. However, all of these treatments were complicated by widespread confusion caused by the ongoing failure of authors to recognise the existence of *E. phyllanthes* as a cohesive and separate species. Northumbrian populations of *E. 'tynensis'* (Fig. 3A, B), a further taxon now clearly best treated infraspecifically within *E. dunensis*, were initially attributed to *E. leptochila* by
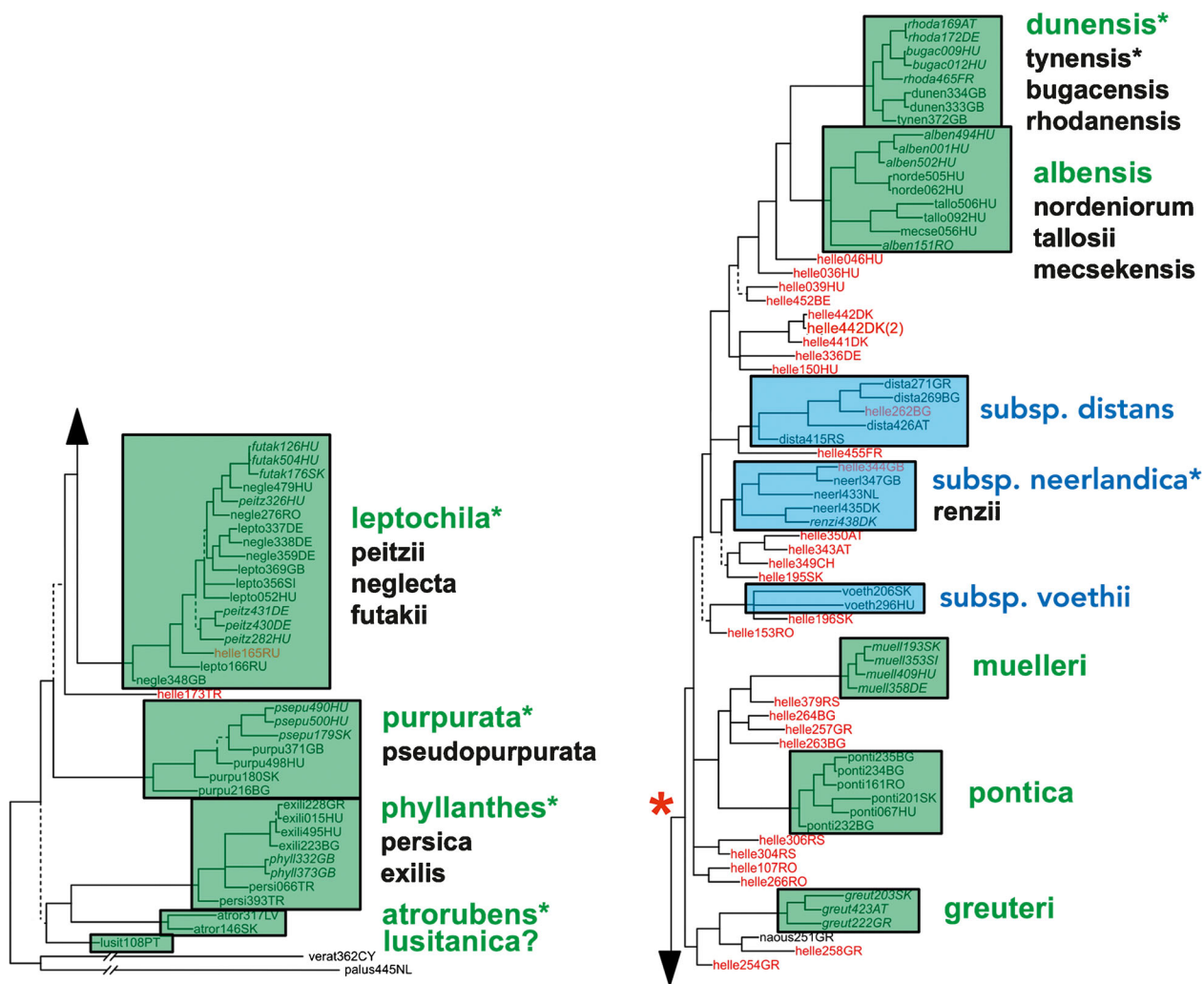
Fig. 2. Maximum Likelihood phylogram depicting the evolutionary relationship of the studied *Epipactis* plants based on RAD-seq data analysed using RAxML. Samples of the ancestral species *E. helleborine* subsp. *helleborine* are highlighted in red. Recircumscribed species are placed in green boxes and subspecies in blue boxes; taxa occurring in the British Isles are asterisked. Collapsed branches attracted approximate likelihood ratio test support values of less than 80% and dashed branches achieved only 80 – 90% support. The red asterisk marks the only branch to have received meaningful support from the previous study of targeted nuclear and plastid genes by Bateman *et al.* (2005). Source country codes: AT, Austria; BE, Belgium; BG, Bulgaria; CH, Switzerland; CY, Cyprus; DE, Germany; DK, Denmark; FR, France; GB, Britain; GR, Greece; HU, Hungary; LV, Latvia; NL, Netherlands; PT, Portugal; RO, Romania; RS, Russia; SK, Slovakia; TR, Turkey. Modified after Fig. 3A of Sramkó *et al.* (2019).

Richards & Swan (1976). These Tyneside populations were later clearly shown to be referable to *E. dunensis* by the molecular studies of Hollingsworth *et al.* (2006; reviewed by Harrap 2009) and latterly Sramkó *et al.* (2019). A taxonomic transfer to *E. dunensis* was formalised at subspecies rank by Kreutz (2007), though Delforge (2016) subsequently argued that *tynensis* should be demoted to a mere variety. A further duneland taxon, *E. helleborine* subsp. *neerlandica*, also occurs in the British Isles (Fig. 3C, D); its significance is highlighted below in a separate section.

Even the most recently described British *Epipactis*, *E. sancta*, rapidly became a taxonomic football. The epithet has been applied only to a single tenuous population endemic to dunes on the small island of Lindisfarne, located just off the northeast coast of England (Fig. 4). It was introduced to the taxonomic community by Delforge (2000) as a variety of the supposedly wholly continental putative species *E. peitzii* — a taxon since rejected as a species by Sramkó *et al.* (2019) and shown to be referable not to *E. dunensis* but rather to *E. leptochila*. *Sancta* was raised to a full species by Delforge in Delforge & Gévaudan (2002), but later placed (more credibly when viewed in the light of recent studies) as a subspecies of *E. dunensis* by Kreutz (2007). Among British authors, the species status of *sancta* was tentatively supported by Squirrell *et al.* (2002), Hollingsworth *et al.* (2006) and Bateman (2006); subse-

Fig. 3. A, B *Epipactis dunensis* var. *tynensis*; **C, D** *E. helleborine* subsp. *neerlandica*; **E** *E. helleborine* subsp. *helleborine* var. *youngiana*, a taxon previously demoted from species level to varietal level based primarily on its possession of a molecular signature indistinguishable from that of var. *helleborine*. PHOTOS: **A – C** D. M. T. ETTLINGER; **D, E** R. M. BATEMAN.

quently also by Harrap & Harrap (2009) and Stace (2010). Supporting arguments rested primarily on the undesirably narrow grounds that *sancta* combined

molecular characteristics of *E. dunensis* (as inferred from nuclear-encoded allozymes) and *E. leptochila* (as inferred from the plastid region *trnL-F*). Given perennial con-

Fig. 4. *Epipactis* 'sancta', a supposed species probably attributable to *E. dunensis* s.l.; this is one of only four putative species of orchids potentially endemic to the British Isles and therefore of considerable conservation interest. **A** duneland habitat on Lindisfarne showing two plants in the centre foreground; **B** typical whole plant; **C** inflorescence; **D** close-up image of individual flower. PHOTOS: **A** – **C** R. M. BATEMAN; **D** D. M. T. ETTLINGER.

cerns regarding the most appropriate status for *sancta* (e.g. Richards & Squirrell 2009), the absence of this taxon from the more data-rich analysis of Sramkó *et al.* (2019) is regrettable; sadly, suitable material was unavailable when the analyses were performed.

Understandably, Sramkó *et al.* (2019) saw no ongoing reason to further explore *'Epipactis youngiana'* (Fig. 3E). This supposed local endemic was first described from a disused lead mine in Northumbria by Richards & Porter (1982), prompting tentative additional reports of the newly described taxon from several other regions of the British Isles (e.g. Ettlinger 1997, 1998) and briefly becoming the subject of a UK Biodiversity Action Plan (Anonymous 2007). The credibility of this taxon as anything more than a variety or forma of *E. helleborine* was seriously wounded by an allozyme study performed by Harris & Abbott (1997) before being unequivocally killed as a putative species by Hollingsworth *et al.* (2006), on the solid grounds that plants of var. *youngiana* always occur among, and freely exchange genes with, substantial numbers of more typical *E. helleborine* plants. A varietal status for *youngiana* was formally established by Kreutz (2004) and was subsequently widely accepted (Lang 2004; Bateman 2006; Hollingsworth *et al.* 2006; Stace 2010; Delforge 2016).

Lastly, there has in recent years been considerable informal discussion regarding a rather dispersed and sporadic 'metapopulation' of *Epipactis leptochila* centred on the Chilterns escarpment east of Princes Risborough, Buckinghamshire. At least three explanations have been offered for its apparently somewhat deviant morphology of certain subpopulations; either attribution to the Continental taxa *E. muelleri* or *E. 'neglecta'* (the latter shown to be synonymous with *E. leptochila* by Sramkó *et al.*: Fig. 2) or gene exchange with nearby plants of *E. helleborine*. Unfortunately, the data gathered by Sramkó *et al.* (2019) are ambiguous with regard to the gene exchange hypothesis; a coalescence analysis presented as a 'heat map' and designed to identify gene exchange between close relatives proved negative, but on the other hand, the analysed plant from Risborough yielded an F measure of inbreeding (see discussion below) that was consistent with the comparatively allogamous (cross-fertilising) *E. helleborine* but radically different from that typical of the more strongly autogamous (self-fertilising) *E. leptochila*. Thus, the jury remains undecided regarding the identity of this particular population.

Kenfig Dunes as a case-study

A remarkable amount of attention has been paid to the members of the *Epipactis helleborine* aggregate occurring in the extensive dune systems of South Wales, particularly that near Kenfig in Glamorgan. This dune system has at various times been accused of harbouring four named taxa in the aggregate: *E. 'cambrensis'*, *E. 'neerlandica'*, *E. 'youngiana'* and *E. helleborine* s.s. (cf. Ettlinger 1997, 1998; Lewis 2003; Lang 2004; Lewis & Spencer 2005; Delforge 2006; Harrap & Harrap 2009; Lewis *et al.* 2009; Clark 2011; Jacquemyn *et al.* 2018; Kapteyn den Boumeester 2018b). *Epipactis 'cambrensis'* of Thomas (1950) falls well within the unusually broad spectrum of varietal-level morphological variation shown by populations of *E. phyllanthes* (Lewis & Spencer 2005; Lewis *et al.* 2009) and so need detain us no longer. However, the plants awarded the epithets *youngiana* and especially *neerlandica* present a more interesting challenge (Fig. 3).

The first written evidence of the apparent presence of what they both regarded as *Epipactis helleborine* var. *neerlandica* in Kenfig is a 1952 letter from British *Epipactis* expert Donald Young to Dutch orchid taxonomist Pieter Vermeulen (Kapteyn den Boumeester 2018b). It is a taxon that had been first described from the Netherlands by Vermeulen (1949) as a variety of *E. helleborine* — elevated to a subspecies by Buttler (1986) and finally — with painful inevitability, to full species by Delforge *et al.* (1991). But it was Ettlinger (1997, 1998) who explicitly drew the attention of British orchid enthusiasts to the possible occurrence of *neerlandica* at Kenfig. Lewis (2003) and Lewis & Spencer (2005) confirmed the presence of plants at Kenfig that resembled the Dutch coastal taxon *neerlandica* but also noted the presence of occasional plants in scrubby back-dune woodland that appeared to be more typical of *E. helleborine helleborine*. Also present was a further morphotype that they considered to more closely resemble the supposed British endemic *youngiana* of Richards & Porter (1982). Lewis (2003) credibly speculated that *youngiana* was a taxon that originated in multiple regions due to heavy metal pollution of soils. Following extensive international consultation, Lewis *et al.* (2009) confirmed the presence at Kenfig of *neerlandica* but decided that this taxon should also include the plants previously attributed by them to *youngiana*.

The number and nature of the morphological characters that supposedly distinguish *neerlandica* from *helleborine* s.s. have been much debated and the majority found to be seriously compromised (cf. Vermeulen 1958; Buttler 1986; Kapteyn den Boumeester 1989, 2018a, b; Van den Bussche 2004; Kreutz 2005; Kuiper *et al.* 2009; Lewis *et al.* 2009). After monitoring through several years the morphology of Kenfig plants of both duneland *neerlandica* and woodland *helleborine* s.s., Clarke (2011) advanced an elegant set of qualitative observations that led him to conclude that the *neerlandica* morphotype was merely an environmentally induced ecotype (termed by him an 'ecad') of *helleborine* s.s. and predicted that they would prove to lack genetic distinction. Based on field observations made in 2009, Kapteyn den Boumeester (2018b) also reached the conclusion that the dune-

land and woodland plants represented the same taxon, but instead argued that "the plants growing among the trees were not subsp. *helleborine* but were instead subsp. *neerlandica*, adapted to the shade."

I must confess that, prior to data collection, I viewed with considerable scepticism the idea that the Welsh plants attributed to *neerlandica* would prove to be comparable with those in its core distribution along the North Sea coastline of Belgium, the Netherlands and Denmark (reviewed by Ehlers *et al.* 2002; Jacquemyn *et al.* 2018). It seemed highly unlikely that a taxon with a very recent origin, most likely emerging in these North Sea dune systems (Jacquemyn *et al.* 2018), would have a radically disjunct distribution that included South Wales. However, that is the most likely interpretation that can be placed on the RAD-seq results obtained by Sramkó *et al.* (2019).

Their NGS tree reveals a single cluster of five relevant samples, four attributed to *neerlandica* and one to *helleborine* s.s. (Fig. 2). This shows that the Kenfig plants attributed to *neerlandica* and *helleborine* s.s. are strongly similar genetically, certainly sufficiently similar to be viewed as representing the same subspecies (cf. Figs 1A – C, 3C, D). Their closest relative is a sample of *neerlandica* from the Netherlands coast, and the British and Dutch samples have as their closest relative samples from Danish dune systems of *neerlandica* and its supposedly strictly self-pollinating segregate, the Danish endemic *neerlandica renzii* (the two samples also proving very similar to each other). These relationships suggest that *neerlandica* most likely originated recently and in Denmark, later spreading westwards to the Netherlands and eventually to South Wales. Certainly, these results seriously challenge my initial scepticism regarding the disjunct distribution of *neerlandica*; indeed, they suggest that *neerlandica* should be sought in the intervening extensive dune systems of Norfolk and Lincolnshire.

Unfortunately, other than the supposed sample of *Epipactis helleborine* s.s. from the Kenfig back-dune woodland, Sramkó *et al.* did not include any samples of *E. helleborine* s.s. from the British Isles, so we cannot be absolutely certain that British populations would yield genetic profiles comparable with those of the French, Belgian and Danish populations that were sampled. However, on present evidence it seems probable that Clark (2011) and Kapteyn den Boumeester (2018b) were correct to synonymise at species level the Kenfig *neerlandica* (including the already debunked *'youngiana'*) with *E. helleborine* s.s. Indeed, Kapteyn den Boumeester (2018b) gains the laurels by predicting that (a) the whole Kenfig population should all be attributed to a single taxon, (b) that the relevant taxon is indeed *neerlandica*, and (c) that *neerlandica* is a valid subspecies. Certainly,

these dune populations evidently merit more than the varietal status collectively awarded to them by several colleagues (including Stace 2010); they are rather a stabilised and possibly expanding western European dune ecotype best viewed as *E. helleborine* subsp. *neerlandica* (Jacquemyn *et al.* 2018; Sramkó *et al.* 2019).

Thus, after about 70 years of debate, three 'species' once again become one, albeit a species (*Epipactis helleborine* s.l.) containing a subspecies that might eventually evolve into a *bona fide* species (*neerlandica*) and a variety that most likely will be subsumed back into the mainstream (*youngiana*).

Revised taxonomic circumscriptions

Given recent observations, most notably the newly published NGS data of Sramkó *et al.* (2019), we can now usefully review our current understanding of the circumscription of all of the genuine species (and some infraspecific taxa) occurring in the British Isles (Fig. 2), and produce an updated infrageneric classification at the levels of section, species and subspecies (Table 1).

With regard to British national floras, we can now see that the species-level circumscriptions published by Clapham (1962), Clapham *et al.* (1987) and Stace (2010) were remarkably accurate; only Stace's (2010) admittedly lukewarm recognition of *Epipactis sancta* as a local endemic species deviates from my current views, and was probably prompted by my own arguably premature statements (Bateman 2006). Admittedly, Stace (1991) had earlier treated *E. dunensis* as a mere variety of *E. leptochila*, while Sell & Murrell (1996) treated both *E. leptochila* and *E. dunensis* as varieties of *E. muelleri*. In contrast, the analysis of Sramkó *et al.* (2019) reinforces previous morphological and molecular evidence that *E. leptochila* and *E. dunensis* are relatively distantly related to each other (*contra* the rather quixotic rearguard action fought by Richards & Squirrell 2009) and that the equally evolutionarily distant *E. muelleri* is exclusively Continental. Moreover, Sell & Murrell followed Stace (1991), Allen *et al.* (1993) and Ettlinger (1997, 1998) in recognising *E. helleborine* var. *youngiana* (Fig. 3E) as a full species, presumably because their traditional taxonomic treatment was published immediately prior to publication of the first molecular study of this taxon (Harris & Abbott 1997). These and other fundamental weaknesses in the formal treatment of British and Irish orchids by Sell & Murrell (1996) ably illustrates that further understanding of our flora is unlikely to be achieved through herbarium-based floras.

From a purely nomenclatural perspective, the species-level synonymies identified by Sramkó *et al.* (2019) of supposedly Continental (especially eastern European) species with their British counterparts reliably benefit our native species; the relevant taxa

were formally described (often from British material) prior to any synonymous taxa being established in Continental Europe based on non-British types. Thus, it falls to Continental systematists to decide whether to accept the evidence presented by Sramkó *et al.*, demonstrating the scientifically demonstrated requirement for synonymisation according to the law of nomenclatural priority. Regrettably, nomenclatural issues all too often become the tail that wags the proverbial taxonomic dog. Certainly, the chequered taxonomic history of European *Epipactis* makes abundantly clear the desirability of pursuing a geographically and taxonomically broad, monographic approach to taxonomy, rather than establishing formally named taxa on a piecemeal, *ad hoc* basis over a considerable period of time. Such an approach simply generates confusion that can require centuries to remedy.

Viewed from a British perspective, the Sramkó *et al.* study makes clear that no *bona fide Epipactis* species is endemic to the British Isles. Even the most westerly species, *E. dunensis* and *E. leptochila*, extend as far east as Hungary (cf. Molnár 2011), and the majority of the valid species stretch at least as far as the eastern boundary of Europe. Unfortunately, it is not presently feasible to assess species distributions — certainly not beyond the British Isles — because traditional taxonomy has yet to catch up with current systematic knowledge. Based on past experience, it may never do so.

Certainly, the number of formal names proposed for *Epipactis* taxa still continues its steady expansion, most recently affecting Russia (Fateryga & Fateryga 2018). For example, a supposed new autogamous species endemic to a narrow region of Russia east of the Black Sea, *E. 'euxina'*, was recently segregated from *E. 'persica'* by Fateryga *et al.* (2018) as a result of traditional taxonomic comparison with *E. 'persica'*, *E. 'exilis'*, *E. 'fageticolca'* and *E. leptochila*. It seems extremely unlikely that *E. 'euxina'* is a *bona fide* species, given that the DNA data of Sramkó *et al.* (2019) show that the cases for species status are equally poor for *persica*, *exilis* and most likely *fageticola*; all of these supposed taxa are actually attributable to *E. phyllanthes*. In other words, the taxonomic over-splitting of *E. phyllanthes* that was attempted in Britain during the early part of the 20th century and was later universally rejected (even before molecular data became available) is now being attempted elsewhere in Europe during the early part of the 21st century. Such taxonomic over-splitting is further epitomised by another recent paper (Ardelean *et al.* 2018) that reports the discovery in Romania of four additional 'species' of *Epipactis*, three of which lack the necessary biological credentials of a *bona fide* species; the Sramkó *et al.* tree (Fig. 2) shows that *E. 'nordeniorum'* and *E. 'tallosii'* belong within *E. albensis*, and *E. 'exilis'* within

E. phyllanthes. Clearly, it is time to step back and seriously review current, genuinely scientific knowledge of the genus.

Species concept(s) employed

We should perhaps pause at this point to consider which rules and criteria were employed by Sramkó *et al.* (2019) in order to convert the relationships of sampled plants (summarised here as Fig. 2) into species and subspecies. I had published a set of guidelines on several previous occasions (e.g. Bateman 2009), but tackling section *Epipactis* required the addition of codicils to those rules. "Recognise only monophyletic groups" became "Recognise monophyletic groups except where a paraphyletic ancestral species remains clearly identifiable", while it proved necessary to amend "Preferentially divide the tree at branches that are relatively robust (and usually comparatively long within the context of the tree in question)" to take account of the longer branches caused by increased retention of mutations and/or fixation of alleles in plants that are predominantly autogamous.

The requirement for monophyly recognises inclusive groups where there is no need to qualify any group by making exceptions (for example, in Fig. 2, if we were to decide to recognise *Epipactis 'pseudopurpurata'*, it would be necessary to circumscribe *E. purpurata* as "all plants in the *E. purpurata* group except those attributed to *E. pseudopurpurata*"). But in Fig. 2, recognising 13 monophyletic groups as 10 species and three subspecies has left us with a "paraphyletic rump" of several clusters of samples of *E. helleborine* s.s. — the ancestral species that apparently gave rise to all the remaining taxa. This is exactly what one would expect to see in a molecular phylogeny in genuine cases of ancestor–descendant relationships; I suspect that the reason such phylogenetic patterns are rarely encountered is because that relationship rapidly becomes masked by further mutations and extinction of intermediate lineages, such that for non-recent speciation events ancestor and descendant species are more likely to be represented as sisters. Thus, in this particular case, recognising 13 monophyletic species and subspecies in Fig. 2 obliges us to accept one non-monophyletic species, as the only other option would be to divide *E. helleborine* into several supposed species that would not be distinguishable on morphological grounds.

In this context, it is important to note in passing that some hard-core phylogeneticists and population geneticists deny the applicability to genetic data of the concept of ancestor–descendant relationships. Part of the inspiration for such a puritanical position can perhaps be traced back to the famous pronouncement of Greek philosopher Heraclitus (c. 570 B.C.) that roughly translates as "No man ever steps in the same

river twice, for it's not the same river and he's not the same man." In this case, allele frequencies observed in the putative ancestor and descendant species today would differ from those pertaining at the time when genuine *Epipactis* species first became (largely) genetically independent from each other. This statement may be true for neutral genetic markers effectively acting as mutational Geiger counters, but for those markers that provide ongoing cohesion of the two species and allow their morphological discrimination, it cannot be true because those discriminating differences would not then persist. Species circumscription is, at its heart, a search for morphological discontinuities that have genetic underpinnings that in turn provide a high degree of reproductive isolation (Bateman 2001, 2012, 2016). In this particular case, the emergence of several *bona fide* species from within *E. helleborine*, thus rendering *E. helleborine* profoundly paraphyletic, is taken here as offering strong if circumstantial evidence for its role as a (very) common ancestor. Although this statement constitutes an admission that *E. helleborine* as circumscribed here has not remained fully coalesced from a genetic perspective, I find acknowledging this fact a small price to pay for then being able to explore a far more comprehensive evolutionary scenario for section *Epipactis*.

Returning to the core thread of using evolutionary trees to circumscribe taxa, branch length becomes exceedingly important for differentiating between monophyletic groups that should be treated as species, subspecies and groups worthy of varietal rank at best. Monophyly alone is an inadequate basis for circumscribing taxa. Species are expected to be subtended by a comparatively long branch, subspecies to be subtended by a shorter one (although in the present case, subspecies that are presumed autogams are also identified by longer terminal branches subtending individual plants (Fig. 2). Congruence with traditional taxonomy is also considered when dividing up a tree, although congruence with morphological phylogenies or morphometric ordinations would offer more powerful and informative comparisons. Sadly, such analyses are not currently available across section *Epipactis*.

The application of monophyly as a criterion for species circumscription is more controversial (and certainly more problematic) than applying it to circumscribe supraspecific taxa. Clearly, it alone cannot circumscribe species; a family (human, not taxonomic) of parents and children is likely to be resolved as monophyletic if they are genetically tested and the resulting data are imported into mathematical algorithms designed to generate dichotomous trees, but that property alone does not make the family a distinct species. The length (usually broadly proportional to the statistical robustness) of branches in trees is an essential companion to monophyly.

So what does this simple set of rules (perhaps better described as guidelines) and underlying principles mean in terms of species concepts applied? Although some ancillary information employed in the taxonomic decision-making of Sramkó *et al.* (2019) was derived from other studies presenting traditional morphological descriptions, together with crossing experiments and ecological observations broadly linked to so-called biological species concepts, the main thrust of their arguments was based on their own genetic data. The data were explored within a conceptual framework that is lineage-based and largely corresponds with both the cohesion species concept of Templeton (1989) and the evolutionary species concept of Wiley & Mayden (2000: 73): "an evolutionary species is an entity composed of organisms that maintains its identity from other such entities through time and over space and that has its own independent evolutionary fate and historical tendencies."

Evolutionary implications

The questions
The *Epipactis helleborine* complex (Figs 1, 3, 4) has become a textbook case of plant evolution. Initial interest focused on the realisation that flowers of the majority of the species segregated from *E. helleborine* s.l. had various morphological features that collectively rendered them more likely to experience (or, to put it another way, less able to prevent) self-pollination (e.g. Young 1948, 1952a, b, 1962a, b; Proctor & Yeo 1973; Richards 1982, 1986a, b; Robatsch 1995; Proctor *et al.* 1996; Claessens *et al.* 1998; Pedersen & Ehlers 2000; Squirrell *et al.* 2002; Hollingsworth *et al.* 2006; Claessens & Kleynen 2011).

Observers therefore began to ask the question of whether this phenomenon followed a gradual trend or underwent a more radical evolutionary transition? And if a genuine transition, was it responsible for the speciation event or simply a downstream consequence of it? As the theoretical background to plant reproductive biology became more sophisticated, later authors raised additional questions, such as whether dominant or even complete autogamy represented an evolutionary dead-end or whether such species could alternatively have given rise to further species (e.g. Takebayashi & Morrell 2001; Igic & Busch 2013; Wright *et al.* 2013; Barrett *et al.* 2014), either themselves also dominantly autogamous or possibly even reverting to a considerably greater frequency of cross-pollination? If the transition to autogamy is indeed an evolutionary dead end then all of the species that appear dominantly autogamous must by definition have evolved independently. If this is the case, then which species acted as the ancestor of which autogamous species?

At this point, more systematics-oriented questions come into play. Could the centuries of taxonomic confusion between *Epipactis helleborine* and the remaining taxa in the *helleborine* complex actually reflect an evolutionary cause, specifically multiple origins of new species in different geographic locations across Eurasia from within a single ancestor, namely *E. helleborine helleborine*? If so, approximately when and where did each speciation event occur? And if most or all of these speciation events do indeed prove to have occurred over a short time-scale from within a single geographically widespread species (i.e. *E. helleborine* s.s.), can we legitimately refer to the *E. helleborine* complex as having undergone a genuine evolutionary radiation, using the criteria established by Bateman (1999; definition in Box 1)? Last, but by no means least, if these speciation events occurred recently when measured against a geological time-scale, can we justifiably refer to that radiation as still being active? Such bold assertions have rarely been made for specific taxonomic groups within the plant kingdom, as they are extremely difficult to demonstrate with any great conviction.

What the *F*?

Their NGS matrix allowed Sramkó *et al.* (2019) to address each of these questions. Ironically, I believe that the first question — the nature of the evolutionary transition leading to speciation — remains the most difficult to answer with confidence. Sramkó *et al.* chose to seek evidence of allogamy versus autogamy in their NGS using two contrasting statistical approaches. *F* statistics offer a direct estimate of inbreeding (strictly, of heterozygosity relative to Hardy-Weinberg equilibrium) in each plant, which should provide a good approximation of the level of self-fertilisation that has occurred in the lineage averaged backwards through numerous generations. The co-ancestry approach is also designed to expose examples of recent gene exchange between comparatively closely related plants. No obvious examples of recent hybridisation were exposed by Sramkó *et al.*'s (2019) co-ancestry analysis. In contrast, the *F* statistics revealed six individuals of diverse taxonomic attribution that possessed low *F* values comparable with those of *Epipactis helleborine*. Two of these were plants identified upon collection as being respectively *E. lusitanica* (a taxon morphologically similar to, and arguably synonymous with, *E. helleborine*) and *E. 'naousaensis'*, a reputedly rare and poorly understood local endemic from Greece that proved genetically indistinguishable from a plant of *E. helleborine* collected nearby. The four remaining samples showing low *F* values were all single individuals of taxa supposed to be either facultative or obligate autogams: an *E. phyllanthes exilis* from Bulgaria, an *E. phyllanthes persica* from Turkey, an *E. helleborine distans* from Russia, and an *E. leptochila*

from a Buckinghamshire population that is considered by some visiting orchid enthusiasts to be of hybrid origin. I find it difficult to explain these anomalously low *F* values, so obviously atypical of all other individuals assigned to the same taxon, in any way other than through hybridisation occurring within the last few generations of the 'family history' of each of these plants.

Sramkó *et al.* (2019) chose to analyse their *F* statistics primarily in the context of four prior categories: obligate allogams, facultative allogams, facultative autogams and obligate autogams. Attribution of each plant to one of the four categories was achieved largely on reproductive biology as inferred from features of the gynostemium, a few anecdotal observations of its morphology commonly being generalised across each named taxon. No direct quantitative examination was made of the gynostemia of individual study plants *in vivo* at the time of sample collection for genetic analysis. In the absence of such data, it seems to me more logical to re-analyse the *F* statistics on the basis of membership of monophyletic groups as re-circumscribed within the rooted molecular tree published by Sramkó *et al.* (Fig. 2). It also seems reasonable to omit from the calculations the six anomalous plants discussed above. The results of the revised analysis are presented here as Fig. 5. It should be emphasised that sample sizes per re-circumscribed species are small (2 – 27, mean 7.7) and that error bars
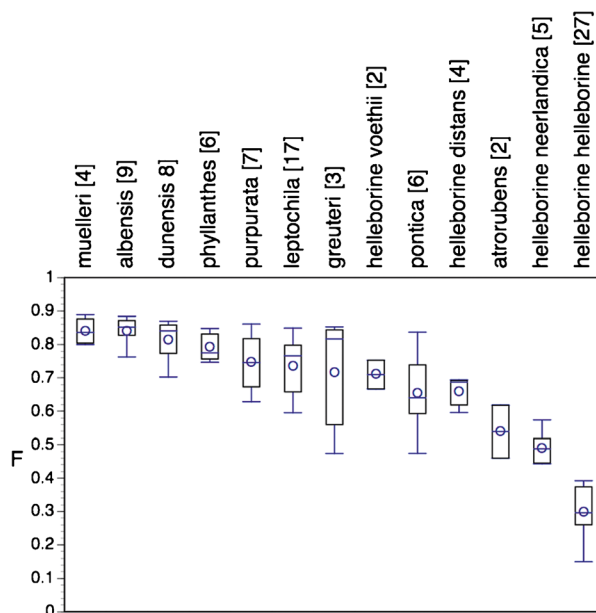

Fig. 5. Box plot showing means and sample standard deviations of the inbreeding coefficient (*F*) per re-circumscribed taxon within the *Epipactis helleborine* group, based on figures calculated by Sramkó *et al.* (2019) and produced for the present study using Deltagraph v.7.1.2. The blue circles denote mean values. The black box is terminated by the 25th and 75th percentiles; the horizontal blue line within the box denotes the 50th percentile, and the extended blue 'whiskers' mark the 10th and 90th percentiles. Sample sizes are given adjacent to the taxon names.

for the less well-sampled among the clades are therefore comparatively large.

Three reputedly dominantly cleistogamous taxa, shown by Sramkó *et al.* to be best viewed as subspecies or varieties, yielded remarkably consistent mean *F* values: +0.83 for *Epipactis purpurata pseudopurpurata*, +0.82 for *E. phyllanthes exilis* and +0.80 for *E. leptochila futakii*. Given that the gynostemium of cleistogamous plants is (at least in theory) inaccessible to potential pollinators, it seems logical to assume that these are the *Epipactis* plants that are least likely to experience cross-pollination. If so, they provide us with a useful indication of *F* values that are likely to represent near-obligate autogamy. In fact, it is clear from Fig. 5 that these mean values are matched by the largely non-cleistogamous species *E. muelleri*, *E. albensis*, *E. dunensis* and *E. phyllanthes* s.l., and only slightly exceed values obtained for *E. purpurata* s.l., *E. leptochila* s.l. and possibly *E. greuteri* (which here has a mean value that is distorted by one deviant sample). Values for *E. pontica* (comparatively variable), *E. helleborine voethii* and *E. helleborine distans* are somewhat lower (c. +0.65), as are those for the two outgroups selected from within *Epipactis* section *Arthrochilum* (*E. palustris* and *E. veratrifolia*). Values for *E. atrorubens* and *E. helleborine neerlandica* approximate +0.5, the supposedly obligately autogamous var. *renzii* of *neerlandica* actually achieving a far from obligate *F* value of just +0.45. Only at this point in the gradation of *F* values do we encounter a genuine jump in values; the species ancestral to this plexus, *E. helleborine helleborine*, has a substantially lower mean of c. +0.3 ± 0.1. Sramkó *et al.* (2019, fig. 3) argued that three of the four transitions between their prior groups were statistically significant; only the supposed transition separating their bins for facultative versus obligate autogamy failed a Mann-Whitney test. But re-assessing the data on the basis of re-circumscribed species, as in the present Fig. 5, gives a substantially different result. It serves primarily to distinguish the ancestral plexus of *E. helleborine helleborine* (and perhaps also *lusitanica*) from the remaining, evolutionarily derived species and subspecies, which do not fall comfortably into a series of convenient reproductive bins. All taxa other than *E. helleborine helleborine* apparently grade from frequent to near-complete autogamy.

At this point, it is important to note that the theoretical range of the *F* statistic is not from zero to plus one, as might be inferred from Fig. 5, but rather from minus one (theoretical maximum outbreeding) to plus one (theoretical maximum inbreeding). Several complicating factors mean that it would be over-simplistic to interpret the results summarised in Fig. 5 as a strictly arithmetic scale and thus view a mean of zero as a perfect balance between inbreeding and outbreeding. Nonetheless, it is clear from these figures that even *Epipactis helleborine* s.s. is actually far removed from an obligate allogam. Sramkó *et al.* (2019) listed several factors that could, either individually or collectively, push all *Epipactis* species toward frequent autogamy. They included the much-discussed structural variation in morphological features competent to increase cohesion of pollen tetrads within pollinia or to preclude pollen massulae from reaching the stigma of the same flower. It is important to remember that any genetic control of such features is inevitably overprinted with ecophenotypic phenomena such as rapid desiccation of the viscidia that attach pollinaria to visiting insects.

However, on the basis of field observations in particular, I suspect that the relatively high frequency of autogamy is caused primarily by geitonogamy — the tendency for insects to visit multiple flowers on the same inflorescence, thereby transferring pollen between genetically identical flowers (e.g. Kropf & Renner 2008). This phenomenon is especially prevalent in species of flowering plants that offer a substantial nectar reward, as do all *Epipactis*, because the reward extends the residency of an insect on the inflorescence (e.g. Claessens & Kleynen 2016). Insect residency is also increased if inflorescences bear large numbers of large flowers, as do all *Epipactis* taxa, and if rhizomatous growth allows a single plant to generate multiple inflorescences — a particularly strong trend in members of the section *Arthrochilum* outgroup but also frequently observed in most members of section *Epipactis*. It therefore seems likely to me that, even in *E. helleborine* s.s., only a minority of successful pollination events are cross-pollinations. And in the experimentally demonstrated weakness of any intrinsic sterility barriers (Tałalaj & Brzosko 2008; Brys & Jacquemyn 2016), self-pollination is highly likely to lead to self-fertilisation. Given that experimental crossing experiments yielded very similar results for *E. helleborine* s.s. and *E. atrorubens*, the contrast in their mean *F* values evident in Fig. 5 is unlikely to reflect any intrinsic factors and hence is more likely to reflect a higher average frequency of geitonogamy in *E. atrorubens*. These are readily testable hypotheses.

Another F: fluctuating asymmetry

Further evidence of routine autogamy (albeit circumstantial evidence) can be gained from the morphology of the affected organisms. Theory states that protracted autogamy and the resulting decrease in population-level genetic diversity should lead to increased frequencies of developmental anomalies (e.g. Graham *et al.* 2010; Klingenberg 2010). And certainly, in the case of the *Epipactis helleborine* complex, the tendency in several species for the labellum to become simplified and more sepal-like (e.g. Young 1952b; Bateman & Rudall 2006) supports such an inference. The simplest way to explore the phenotypic effects of

prolonged autogamy is to study the amount of deviation from perfect bilateral symmetry in specific organs — a phenomenon termed fluctuating asymmetry. I therefore mounted and measured the widths (and, in the case of the lateral tepals, lengths) of all six tepals of flowers in order to measure any deviation between left and right tepals (lateral tepals) or left and right half-tepals (median tepals). The study was based on two flowers removed from each of ten plants of *E. helleborine* (Fig. 1A) and ten plants of *E. leptochila* (Fig. 1E), both species being sampled at the Surrey *locus classicus* of *E. leptochila* in August 2016.

Positive correlation of asymmetry values proved to be poor between the pairs of flowers taken from the same plant, indicating that the resulting data were noisy and probably incorporated a degree of experimental error. This was particularly true when attempting to define a midline in order to measure half-widths of the median tepals; these data were therefore discarded from further analysis. Averaged out over all four lateral tepal measurements for both flowers per plant from all ten plants, the degree of asymmetry was estimated at $3.5 \pm 1.8\%$ for *Epipactis helleborine* and $4.0 \pm 1.9\%$ for *E. leptochila* (set against a background of c. 1% measuring error for both species). Thus, although mean right–left asymmetry was, as expected, assessed as being greater in the more strongly autogamous *E. leptochila*, the quantitative difference between the two species was slight and not statistically significant. It would appear that although functional constraints over the floral phenotypes of the more strongly autogamous *Epipactis* species may have diminished, the genetic consequences have not (yet) become sufficiently entrenched to adversely affect developmental controls on their floral symmetry.

The (provisional) answers

We now return to the rhetorical questions posed at the beginning of this 'Evolutionary Implications' section. Based on current evidence, the re-circumscribed species within the *Epipactis helleborine* group are best simply apportioned among two reproductive categories rather than four: moderately autogamous s.l., mainly reflecting geitonogamy (primarily *E. helleborine helleborine*), and strongly to almost obligately autogamous (the remainder), reflecting genetically and/or environmentally induced self-pollination of individual flowers, supplemented with geitonogamy in all autogams other than the few genuinely cleistogamous populations. Provided that populations currently attributed to *E. helleborine* in Iberia (e.g. Tyteca & Dufrene 1994), Russia (e.g. Efimov 2004) and Asia Minor can in the future be more clearly confirmed as correctly identified — an outcome that is by no means certain — the NGS tree of Sramkó *et al.* (2019) implies that all of the remaining, more strongly autogamous species (highlighted in green on Fig. 2) and at least

three valid subspecies (highlighted in blue) arose independently from within this single ancestral plexus.

Given this comparatively rare example of the recent origin of several taxa from within just one ancestral taxon, via one or more processes that have generated species in some cases but only subspecies in others, it is difficult to view the *Epipactis helleborine* aggregate as having undergone anything other than an evolutionary radiation as defined in Box 1 (albeit lacking the desirable comparative yardstick of a fossil record of *Epipactis* — necessary if we were to establish the background rates of speciation and extinction required by the conservative definition proposed by Bateman 1999). Moreover, that radiation would appear to remain active today. This conclusion highlights the value of the *helleborine* aggregate as a model group for the study of plant evolution.

The most obvious possible exception to the general statement that all of the more recent species were derived from *Epipactis helleborine* s.s. is the highly autogamous sister pairing of the predominantly western European *E. dunensis* and the predominantly central European, later-flowering *E. albensis* (Fig. 2). Their relationship as mutually monophyletic sisters means that one species probably (though not definitely) gave rise to the other, although the tree does not help us to identify which of the two species is the more likely to be ancestral to the other. However, this relationship does usefully imply that, although evolutionary trajectories favouring increased autogamy do generally result in species that are evolutionary dead ends, this popular assumption may actually constitute an evolutionary guideline rather than an unbreakable rule.

Each of the genuine *Epipactis* species that occurs in Britain and Ireland (Table 1) has as its sister an individual of *E. helleborine* from central or eastern Europe. This is not entirely surprising, given that western European samples were in a minority among the samples analysed. Nonetheless, the results are consistent with all of the species occurring in Britain and Ireland having originated in eastern Europe or possibly, in the case of *E. phyllanthes*, even in Asia Minor. Only the western European *E. helleborine* subsp. *distans* appears to have actually originated *in* western Europe, given that its sister sample of *E. helleborine* subsp. *helleborine* was collected in France. Even the western European *E. helleborine* subsp. *neerlandica*, a taxon characteristic of the western seaboard of Europe, has as its sister-group four individuals of *E. helleborine* subsp. *helleborine* sampled in Switzerland, Austria and Slovakia (Fig. 2). It appears that *Epipactis*, a genus of the orchid subfamily Epidendroideae, shares with other Eurasian orchid genera assigned to subfamily Orchidoideae a pattern of successive speciation events occurring

during an overall westward migration of the lineage.

It is clear that speciation in the *Epipactis helleborine* complex is reliably positively correlated with increased inbreeding frequency, which interestingly is routinely manifested as an increased rate of retained mutations or allele fixation and thus generates longer subtending branches on molecular trees (Fig. 2). However, at this point I feel it essential to deploy the age-old maxim of science that correlation does not necessarily reveal causation. It is noteworthy that the ancestral species *E. helleborine* is even more widespread and frequent than are its descendant species, presumably because they reliably show narrower ecological tolerances. Having said that, some of the derived species do possess the ability to invade habitats shunned by *E. helleborine helleborine*. Examples include on the one hand *E. dunensis* and *E. helleborine neerlandica* occupying poorly stabilised dune 'soils' exposed to intense sunshine but on the other hand *E. leptochila* surviving in darkened calcareous beechwoods devoid of other ground vegetation. Indeed, in the woodlands of the Chiltern Hills and North Downs of southern England it has proven possible to use the respective distributions of *E. helleborine* and *E. leptochila* versus that of *E. purpurata* to map the boundary between thin calcareous chalk soils and the thicker mildly acid clay-rich soils developed in the overlying chalk residue that is informally termed clay-with-flints.

These observations lead me to wonder whether our long-term obsession with the reproductive biology of these plants has to some degree blinded us to potentially important events occurring at the opposite end of the plant from any pollinator visitation, specifically the roots. Ample evidence has accumulated to show that these species rely heavily on mycorrhizae for carbon nutrition (Bidartondo *et al.* 2004; Jacquemyn *et al.* 2016; Schiebold *et al.* 2017), and a study of fungi on the roots of *Epipactis microphylla* suggested a comparatively high degree of mycorrhizal specificity (Selosse *et al.* 2004). Is it possible that transitions in mycorrhizal communities could have permitted occupation of these more austere, insect-deficient habitats, and that the increased frequency of autogamy in these species was a later consequence, rather than a cause, of speciation?

Certainly, there is an intriguing symmetry in the relationship between the moderately autogamous *Epipactis helleborine* s.s. and the remaining, more strongly autogamous taxa within the *helleborine* group. *Epipactis helleborine* s.s. is undeniably the more widespread and locally frequent among the species, but its more strongly autogamous derivatives are also more geographically widespread than conventional wisdom suggested; I suspect that, collectively, they sequester as much biomass

as their shared parental species, suggesting the existence of some form of emergent trade-off between the number of novel lineages generated and degree of ecological success. In summary, it would be overly simplistic to view from first principles these strongly autogamous taxa as being ecologically inferior to their more allogamous brethren.

As a postscript to this section of the paper, I would emphasise that section *Epipactis* offers excellent opportunities to explore experimentally feedback loops between evolutionary lineage and environment. In terms of pollination biology, both of the main drivers toward autogamy — gynostemium phenotypes permitting self-pollination of a single flower and geitonogamy permitting cross-pollination of genetically identical flowers on a single ramet — are vulnerable to ecophenotypy. A comparatively desiccating environment soon renders the viscidium dysfunctional and the pollinium friable, thereby encouraging self-pollination, and even partial or more rarely complete cleistogamy also tends to occur more frequently in dry soils. Meanwhile, factors such as exposure to wind and degree of shade inevitably affect the frequencies (both relative and absolute) of visits from various species of potential pollinating insects and the behaviour that they typically exhibit once they have elected to alight on an inflorescence. Lastly, I note that these environmental influences would impact more strongly, and have a more rapid and direct effect on evolution, if it can be demonstrated that they can lead to heritable change through epigenetic mechanisms (e.g. Paun *et al.* 2010).

UK conservation status of section *Epipactis* taxa requires radical revision

In the case of section *Epipactis*, the comparatively recent Red List for England (Stroh *et al.* 2014) simply echoes comments and decisions made a decade earlier in the Red List for Great Britain (Cheffings *et al.* 2005). This treats section *Epipactis* under the rather prejudicial banner of "apomicts and other complex groups", arguing that *"Epipactis phyllanthes, E. leptochila, E. sancta, E. dunensis* and *E. youngiana* form a problematic complex of self-pollinated plants in which species limits are the subject of [?ongoing] research. Speciation is considered to be still taking place. Conservation for this group may be best to be 'process-based'." (Cheffings *et al.* 2005: 6).

Understandably, *youngiana* was placed on the 'Parking List' of rejected taxa, presumably on the grounds that it "shows incontrovertible evidence that it should be placed at a lower rank than subspecies" (Cheffings *et al.* 2005: 98), although the main evidence justifying that statement was not published until the following year (Hollingsworth *et al.* 2006). Remarkably, all of the remaining species of section *Epipactis*,

including the widespread *E. helleborine*, appear on the Red Lists. Most are categorised as Least Concern, although *E. leptochila* and *E. dunensis* are listed as Data Deficient on the spurious grounds that data for these two species are confounded by the fact that they were formerly recorded as conspecific. In fact, the period of taxonomically confused mapping was brief and the mutually exclusive distributions of the two species have actually long been well documented. In contrast, the taxon for which data deficiency is genuinely most problematic, *E. 'sancta'*, is categorised as Endangered, primarily on the grounds of an endemic population size estimated in 2013 at just 70 plants and despite currently being seriously 'data deficient'. *'Sancta'* was also labelled as an "international responsibility", as was *E. phyllanthes*. This status is defined as requiring us to be "sure that the UK holds more than 25% of European populations." In the case of *E. phyllanthes*, this designation is unlikely to survive Sramkó *et al.*'s (2019) demonstration that *E. phyllanthes* is conspecific with *E. exilis* and *E. persica* (and probably with a further four exclusively mainland Eurasian *Epipactis* 'species' recognised by Delforge 2016) (Fig. 2).

The statements made in the Red Lists that self-pollination is frequent and speciation is still taking place within section *Epipactis* are amply justified by the results of recent research. However, arguing that all putative species of section *Epipactis* should appear on the Red List because they as a group are being actively researched seems somewhat perverse. Is knowledge of taxonomic groups that are *not* being actively researched truly more concrete? Indeed, what proportion of the British and Irish flora has ever been the subject of more than superficial scientific research? Moreover, the concept that cases of active speciation require "process-based" conservation strategies is simply unsustainable. Fig. 2 and Table 1 clearly illustrate the substantial number of species and subspecies that have evolved in recent times from within *E. helleborine*. In order to preserve the full evolutionary potential of section *Epipactis* it would indeed be necessary to conserve the species that is demonstrably the cradle of multiple dominantly self-fertilising lineages, namely *E. helleborine*, but this species is widespread, locally common and even occasionally accused of being invasive (reviewed by Squirrell *et al.* 2001). Conserving the whole of section *Epipactis* would hardly be a practical prospect when set against the many other factors and species that should be considered when making conservation decisions. Given that these speciation (and subspeciation) events are likely to represent the few successes among myriad failed attempts, how can we possibly predict which populations will eventually lead to speciation and so prioritise them? In the increasingly man-made world in which we now live, we have little choice but to leave evolution in general, and incipient speciation in

particular, to take care of itself. 'Process-based conservation' is literally for the birds.

Ongoing challenges

Despite recent progress, the systematic challenges posed by the *Epipactis helleborine* group are far from settled. History has taught me that the predilection shown by the European orchid community for piecemeal formal description of new taxa in the absence of meaningful scientific data will continue unabated, along with subsequent games of snakes-and-ladders (or perhaps tit-for-tat?) as formally named taxa migrate between taxonomic ranks with little or no underlying justification, either theoretical or empirical (reviewed by Bateman 2012, 2016, 2018). Indeed, my own previous, tentative advocacy of *E. 'sancta'* as a full species based primarily on a single small difference in the plastid *trnL-F* gene (Bateman 2006) may eventually prove to fall within this particular category of sin.

One obvious weakness of even present knowledge is that resource limitations meant that Sramkó *et al.* analysed only 27 of the 65 putative species recognised by Delforge (2016), although I suspect that the putative species carefully selected for their NGS analyses may eventually prove to have encompassed all of the *bona fide* species other than *Epipactis microphylla*. It is already clear that the majority of the 'species' within the *helleborine* group that are currently recognised in European orchid monographs fail the crucial test summarised by Bateman (2009) of monophyly plus adequate levels of genetic divergence (it should probably be noted once again at this point that Fig. 2 makes clear that *E. helleborine* s.s. is itself clearly strongly paraphyletic rather than monophyletic, as a direct consequence of its impressive ability to generate other, more strongly autogamous, species). However, greatly increased population sampling is desirable to strengthen these early attempts to re-circumscribe species and infraspecific taxa using population-level NGS data (Bateman 2012, 2016).

But by far the greater problem in progressing species circumscription in the genus *Epipactis* is the consistent unwillingness of researchers (not least, of myself and my colleagues) to commit the extensive periods of time necessary to gather large, geographically and taxonomically broad morphometric data-sets. These are an essential pre-requisite for the multivariate analyses needed to integrate morphological studies with the increasingly sophisticated molecular investigations. Compared with other taxonomically problematic genera such as *Dactylorhiza* and *Gymnadenia*, *Epipactis* has been greatly under-researched morphologically. Richards (1982) scored just ten metric characters in British populations of several *Epipactis* species, primarily to compare levels of morphological variation rather than to circumscribe taxa. The studies of *E. helleborine* in Sweden by Ehlers *et al.* (2002) and of *E. leptochila* in the

Alps by Gévaudan (1999) adopted an approach broadly similar to that of Richards. Tyteca & Dufrene (1994) studied a wider range of morphological characters in populations of several members of the *E. helleborine* group located along the Atlantic seaboard of Continental Europe. However, their study was also somewhat weakened by confining their matrix to characters that were both continuously variable (metric and meristic *sensu* Bateman 2001) and macroscopic, omitting less readily coded characters such as flower colours or cell shape and size (perhaps for this reason, the credible pairing of *E. atrorubens* and *E. microphylla* was placed improbably close to *E. helleborine* in the similarity-based comparative tree of Tyteca & Dufrene 1994). A similar range of characters was studied by Jacquemyn *et al.* (2018) when comparing two populations each of *E. helleborine helleborine* and *E. helleborine neerlandica* in Belgium. Forty years of experience have taught me that at least 40 morphometric characters need to be measured in order to give an adequate overall assessment of the morphology of an orchid (or indeed of any other vascular plant); in practice, this means that scalar and presence/absence characters must be scored in addition to metric/meristic measurements (Bateman 2001). And *Epipactis* plants are sufficiently morphologically complex to permit recognition of considerably more than 40 characters.

Within the British Isles, several questions regarding *Epipactis* remain to be answered with the necessary confidence. The most obvious of these is the need to conduct NGS and morphometric studies to determine the most appropriate status for *E. 'sancta'* (Fig. 4). At present, several pieces of circumstantial evidence encourage me to believe that *sancta* is most appropriately treated as a subspecies of *E. dunensis*; if this ultimately proved to be the case it would return this population full circle to the species assignment originally determined by Young (1962a). Firstly, although *sancta* is only now being subject to rigorous morphometric analysis, and usable morphometric data have not yet been gathered for comparable taxa, the morphology of *sancta* appears on present inadequate evidence to differ only very subtly from that of *E. dunensis* (primarily the absence from the pedicel of pink anthocyanin pigments — a characteristic unlikely to have any functional, and thus selective, value). Secondly, the limited DNA data currently available suggest that *sancta* more closely resembles *E. dunensis* in nuclear genes but *E. leptochila* in plastid genes (although very few genes of either category have been sequenced thus far). Of relevance here is the fact that subsequent NGS techniques have suggested that, for closely related orchid taxa, nuclear genes are on average more accurate than plastid genes (Bateman *et al.*, unpublished). Thirdly, the NGS study of Sramkó *et al.* (2019) effectively challenged the credibility of species status awarded by others to any of the

supposed local endemic species that they sampled across Europe; all 11 of the *bona fide* species that survived species-level re-circumscription are geographically widespread. This outcome makes the rare, extremely local endemic *sancta* (Fig. 4) appear much more anomalous than previously.

The NGS-prompted recognition that some *Epipactis* species extend much further east in Europe than was previously widely believed also encourages further consideration of the converse question of whether one or more of the species currently thought to be confined to mainland Europe may actually be present in the British Isles. For example, *E. muelleri* — long recognised as occurring immediately beyond the English Channel in the Low Countries — has long been sought in the UK but has yet to be convincingly found here (cf. Young 1962a; Delforge 1995; Lang 2004). Of course, current limitations on morphological knowledge mean that even if *E. muelleri* does occur in the British Isles it would be challenging to record with confidence, thus making yet another strong case for developing an NGS-based portable device suitable for field-based sequencing of DNA (e.g. Bateman 2016). In the case of other taxa, notably *E. helleborine* subsp. *neerlandica* (Fig. 3C, D), improved knowledge means that determining the distribution of the subspecies within the British Isles is now the primary issue, rather than simply confirming its presence. Surprisingly, the work of Sramkó *et al.* (2019) suggests that *neerlandica* might have a single evolutionary origin, most probably in the Dutch dunes. If so, *neerlandica* subsequently spread to South Wales, rather than arising independently there as I previously believed. The single-origin hypothesis requires a migratory jump westward that is exceptionally long-distance, even by the standards of the dust-seed-equipped orchid family. Also, post-glacial sea level fluctuations mean that *neerlandica* most likely arrived in the South Wales dunes within the last 3,000 years. Like *E. muelleri*, *neerlandica* also would be most readily identified using a portable device capable of DNA-based analysis. The apparent absence from Ireland of *E. leptochila* (admittedly, there have been occasional rumours that it occurs in Co. Antrim: Curtis & Thompson 2009) and especially of *E. dunensis* also appears surprising, given the availability of apparently suitable habitats; further, carefully targeted exploration in Ireland appears desirable.

Primary hybrids have long been sporadically reported in Britain between *Epipactis helleborine* and each of *E. atrorubens*, *E. purpurata* and *E. leptochila* (Godfery 1933; Stace 2010; Richards *in* Stace *et al.* 2015). I suspect that, through the last four decades, I have during fieldwork seen rare examples of each of these three combinations, but again genetic data are necessary for confident identification. Despite the fact that both species are dominantly autogamous, it seems

likely that *E. dunensis* and *E. phyllanthes* also hybridise at least rarely; at some localities they occur together in large numbers, most notably along the Lancashire dunes. Lastly, hybrids between *E. leptochila* and *E. dunensis* were listed as occurring in Britain by Lang (2004), despite the mutually exclusive distributions in Britain of the supposed parents. Overall, there is a need to study gene flow within multi-species populations of *Epipactis*, if only to further explore the apparent incongruence between the inbreeding coefficients (*F* statistics) and co-ancestry heat map presented by Sramkó *et al.* (2019, their figs. 3B and 5).

Much progress has been made in recent years in better understanding speciation patterns and processes within section *Epipactis*. If we continue to operate on the long-held assumption that gynostemium morphology has at least some influence on the likelihood of self-pollination, it is essential that we determine the relative contributions of genetic, epigenetic and ecophenotypic (i.e. environmental) factors in determining that morphology. We also need much more ambitious pollinator observations — studies that are designed specifically to track the course of pollen flow among flowers rather than simply identifying the pollen vectors to species level (while accepting that making sufficient numbers of observations will not be easy for the species with the lowest frequencies of cross-pollination). And we need to pay far more attention to 'housekeeping' aspects of *Epipactis* plants, such as the effectiveness of their photosynthetic machinery under contrasting light levels, the nutrient-absorbing ability of their rootstocks, and the identity and nature of the mycorrhizae that both inhabit the roots and assist germination of the dust-seeds (Jacquemyn *et al.* 2016). Determining the sequence in which key events occur during their evolution — change of floral morphology, increased autogamy, altered habitat preference — will be crucial to finally unravelling speciation process(es) operating in these provocative yet intriguing plants.

Acknowledgements

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