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Distinguishing between *Epipactis leptochila* (Narrow-lipped Helleborine), *Epipactis helleborine* (Broad-leaved Helleborine) and their putative hybrid, *Epipactis x stephensonii* (Orchidaceae) at the Warburg reserve, Oxfordshire

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Abstract

A drought in May 2020 curtailed snail damage to a population of *Epipactis leptochila* (Godfrey) Godfrey (Narrow-lipped Helleborine) such that in July there were 33 flowering plants in an area well known for this species at the Warburg nature reserve in Oxfordshire (v.c.23). Despite growing in a heavily-shaded area under beech, the typical habitat of *E. leptochila*, some of these plants were actually *E. helleborine* L. (Crantz) (Broad-leaved Helleborine) based on recognized distinguishing characteristics. We took morphological measurements (19 floral characters and 10 vegetative characters) for all the flowering plants present and were able to confirm by statistical analysis our initial species identifications. We were able also to discriminate between the species reasonably accurately using leaf characters alone, such as leaf colour, leaf length to width ratios and the angle of turn of the bottom three leaves. Using the outcomes from this analysis we were able to make predictions about the identity of most of the 82 non-flowering plants present. There were 4 plants that defied clear initial allocation to one or the other species and were suspected to be the hybrid *E. x stephensonii* Godfrey. Statistical analysis confirmed that these plants were indeed distinguishable from either parent, and most characters were intermediate to some degree between the two. The morphology of these putative hybrids is described pending molecular confirmation of their identity.

Keywords: orchid; morphometrics; population studies; taxonomy.

Introduction

This paper describes a morphometric study of two sympatric orchids, *Epipactis leptochila* (Godfrey) Godfrey (Narrow-lipped Helleborine) and *E. helleborine* (L.) Crantz) (Broad-leaved Helleborine) and their putative hybrid *E. x stephensonii* Godfrey at the Warburg reserve (a local Wildlife Trust reserve) in Oxfordshire (v.c.23). *E. leptochila* is a nationally scarce plant; in Oxfordshire, where the typical habitat is heavy shade under beech on thin calcareous soils, with virtually no other plant competition, Erskine *et al.* (2018) describe it as rare. *E. helleborine* can occur in similar habitat, usually where there is more light, but in one area of Warburg the two species are sympatric and grow side by side. Fig. 1 shows a plant of *E. leptochila* in bud in early July.

Figure 1. *Epipactis leptochila* under beech, in bud, early July 2020.

The Warburg reserve holds the largest population of *E. leptochila* in Oxfordshire. Currently all the plants are confined to a zone measuring roughly 150 m by 150 m composed mostly of Beech (*Fagus sylvatica*) but including one small outlier under Hazel (*Corylus avellana*) around 100 m from the main body to the east (taken together these constitute the "the study area").

Following declines in recent years there was an encouraging upsurge in the number of *E. leptochila* present in 2020, almost certainly due to drought conditions in May, which curtailed snail damage to emerging plants. 115 helleborines were identified in the study area, but in a vegetative state there were no clear pointers to whether they were all *E. leptochila* or whether some were *E. helleborine*. After the loss of 26 plants mainly due to snails, there were 89 extant plants at flowering time in mid-July. 33 of these plants flowered (28% of the total number marked), a much higher proportion than in the previous three years and again an outcome of less snail damage. Morphological data were gathered for each plant, though some measurements were missing due to herbivory or other forms of damage. At flowering, some plants were clearly assignable to *E. leptochila* or *E. helleborine* but identifications were complicated by potential introgression and the presence of *E. x stephensonii*.

Methodology

Plants were marked with canes when they first emerged from early May 2020, and their fate followed thereafter through to flowering and beyond. Seven surveys were undertaken between early June and mid-July and on each occasion the status of each plant was noted (e.g. whether in leaf, in bud, chewed, withered, or gone), sometimes including photographs of each plant. Each plant was assigned a simple sequentially numbered marker and many were caged to exclude deer grazing.

The positions of newly emerged plants were determined by triangulation to reference points, 35 posts or recognisable trees, which in turn were triangulated to a known, accurate, grid reference derived from Google Earth (https://earth.google.com/). The accuracy was probably around ±0.5 m and it would be challenging to improve it, because of inaccuracies in triangulating the reference points. A hand-held GPS device (Garmin 64CPS) proved less accurate because of heavy shading, although it had to be used for a small number of plants away from the main population in an outlier.

Leaf measurements

On 14 July when several plants were in flower and others in bud, measurements were made on all plants still extant, whether flowering or not:

- the number of leaves
- the length:width ratio of each of the bottom 3 leaves
- the clockwise turn (in degrees) between one leaf and the next moving up the stem for the second from bottom, third from bottom and fourth from bottom leaves.

Leaf colour is also known as a distinguishing feature though we are not aware of any systematic work to quantify any such difference. Therefore, except for those plants that had succumbed to snail predation, in June pictures of all the plants in leaf were taken alongside a white scale, which was used to correct the white balance to a constant, so that the colour rendition of each picture was equivalent as far as possible. Using photo editing software (Affinity Photo ver. 1.8.5.703, https://affinity.serif.com/en-gb/) each photograph was calibrated to the same RGB value for the scale, and then an area of most of each of the visible leaves was selected. Next an average blur filter was applied to that selected area and the RGB value read off. If there was more than one visible leaf an average was calculated for all the visible leaves (Fig. 2). The R:G, R:B and G:B ratios were calculated.

Figure 2. Determination of Leaf Colour Components

Flower measurements

When plants were in bud or flowering, the number of those buds or flowers was counted, and the plant height, the length of the inflorescence as well as the leaf width and length of all leaves were measured, not just the bottom three.

When fully in flower a close-up photograph (e.g. Fig. 3) was taken of at least one flower on each plant alongside a scale so that the flower width (lateral sepal tip to lateral sepal tip), lateral sepal width and length (visible portion), dorsal sepal width (visible portion), epichile width and length, and the hypochile width could be determined. If the epichile is not perpendicular to the camera then the length measurement will be understated and for this reason such measurements were avoided. Epichile and hypochile widths however are more reliable, being in the same plane (save for a small parallax error). The width of the viscidium if present was measured (zero if not).

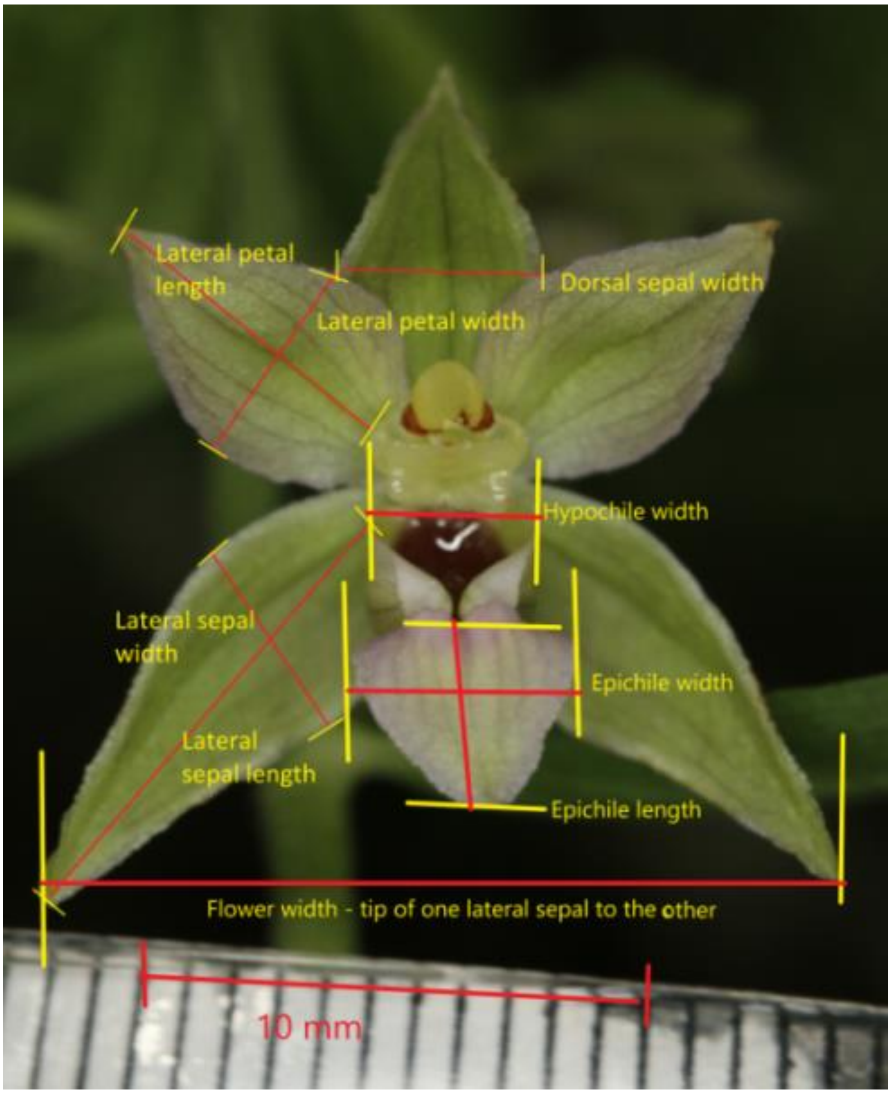


Figure 3. Floral dimensions measured

Fruit set

A count of the number of fruits on each plant was made on 16 August, 2020. The number of seed pods compared to the original number of flowers in July gave the fruit set percentage. Using photographs, and taking only those seed pods flat to the camera, an average of seed pod length and width was calculated, and the angle of attachment to the stem from the upright measured (Fig. 4).

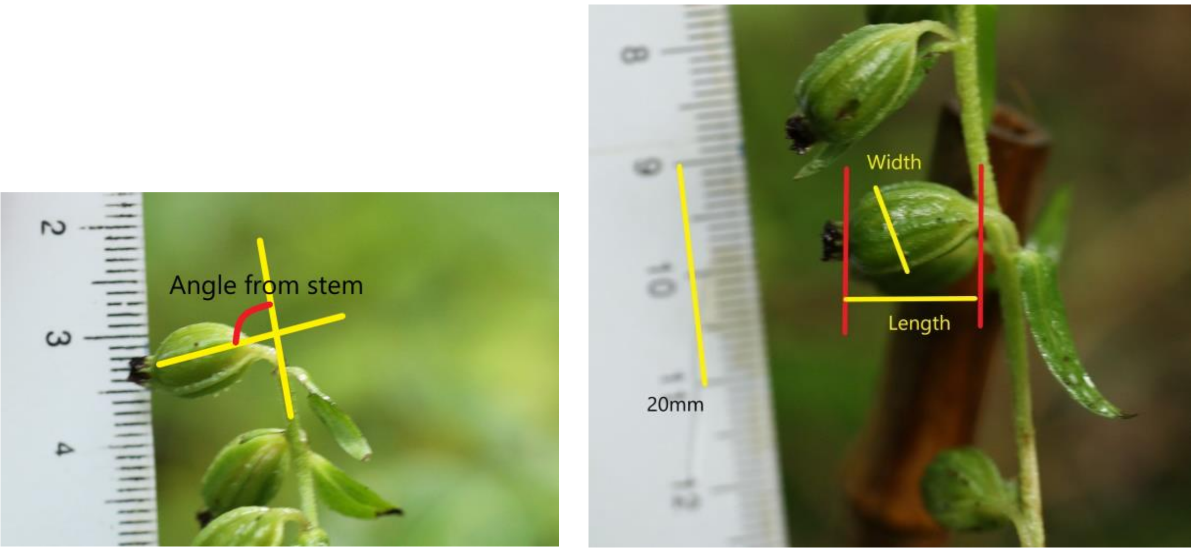


Figure 4. Measurements made on fruiting plants

Data analysis

Statistical analysis was undertaken using R version 4.0.3 (RCore Team, 2020) and in particular the MASS package (Venables & Ripley, 2002). Helpful templates to develop the required coding were the lecture notes provided by Holland (2019).

Data were collated for 33 flowering plants, on 17 variables related to flower dimensions and 10 variables related to their vegetative characteristics. The MASS package was used to undertake discriminant analysis on the flower characteristics to confirm species assignment of the initial visual assessment. Where there were gaps in the data for a particular plant they were filled by using average values for the species to which the plant had been attributed. Such amendments applied to 2 *E. helleborine* plants (out of 10), 6 *E. leptochila* plants (out of 19), but none of the putative hybrids. Analysis without the plants where data was incomplete gave very similar results.

With identifications confirmed, discriminant analysis was then applied to the vegetative characteristics. Because there were fewer variables, where there were missing datapoints for a plant, then that plant was discarded from the analysis.

It proved more useful to use ratios between the colour components, RGB, (e.g. R:G) rather than use the colour values themselves. The discriminant results were compared with the initial visual species identification and then used to predict to which species each non-flowering plant belonged from data gathered on the same variables.

Results and Discussion

Population changes

Since the early 1990s the number of plants and number of flowering plants have been counted annually by reserve staff and in the last three years by T. Swainbank.

During 2020 in the study area 115 plants of *E. leptochila*, *E. helleborine* and possible hybrids were marked. Of these 26 plants had succumbed to herbivores by 14 July 2020 when the first flowers were appearing meaning that 89 plants were still extant at flowering time compared to only 14 plants extant in July 2019 and 18 in July 2018. The healthy number of extant plants at flowering time in 2020 was redolent of the situation in the early 1990s.

Previous surveys of the total number of plants may have been ambiguous. It was obvious in 2020 that plants of *E. leptochila* and *E. helleborine* were intermingled to a surprising degree, and that identifying non-flowering plants to species was challenging without the detailed analysis we undertook; surveys of total plants in previous years might therefore be suspect, because they might have included non-flowering plants of *E. helleborine*.

Historic counts of the number of flowering plants are less ambiguous, assuming that the same areas have always been surveyed, and at the appropriate time. These data show an upsurge in 2020 with 23 flowering plants of *E. leptochila* including putative hybrids compared with only 2 plants flowering in 2017, 3 in 2018, and just 1 in 2019. In 2009 there were 15 flowering plants, 16 in 1999, but over 100 in 1990 (Fig. 5). In the same area in 2020 there were 10 flowering plants of *E. helleborine* intermingled with or close to those of *E. leptochila*.

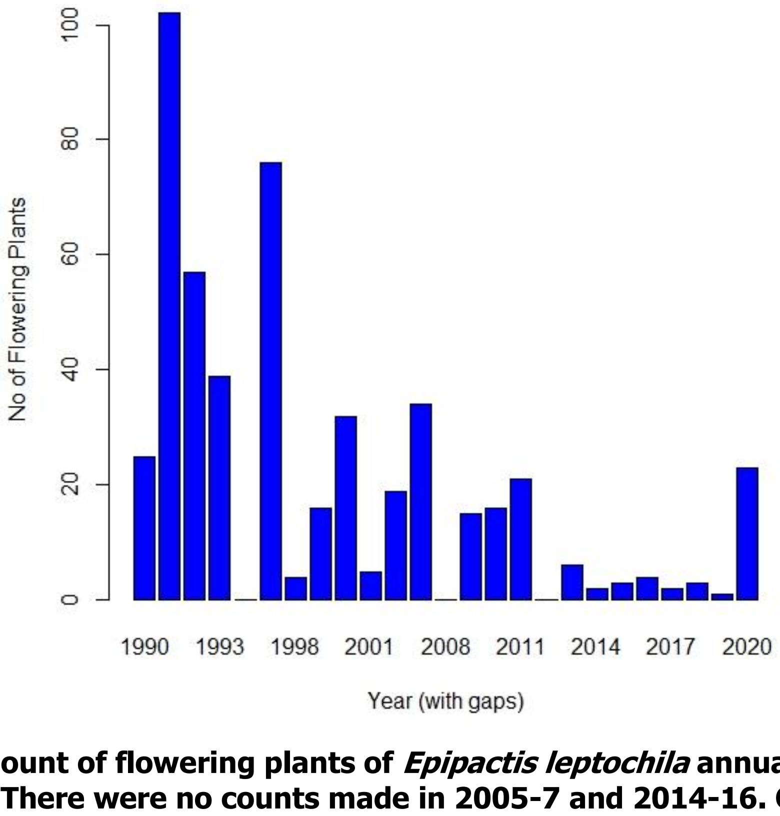


Figure 5. Count of flowering plants of *Epipactis leptochila* annually at Warburg 1990-2020. There were no counts made in 2005-7 and 2014-16. Counts for 2018, 2019 and 2020 were by T. Swainbank, earlier ones were by reserve staff and are included with their permission.

Species Differentiation of Flowering Plants

Molecular methods have become the method of choice to distinguish between plants of the same genera. In the case of *Epipactis* there has been significant recent research to determine species boundaries and the relationships between species using such techniques, with that of Sramko *et al.* (2019) being the most comprehensive, setting out the evolutionary relationships, and noting that the autogamous - allogamous split is less well defined than previously thought.

In the case of *E. leptochila* there still remain unanswered questions about species boundaries. In particular and perhaps of relevance to the plants at Warburg, the debate about a group of plants at Princes Risborough, Buckinghamshire (v.c.24) (Lewis, 2008; Cole, 2008; Harrap, 2009; Kreutz, 2009) continues, with the plants attributed to various taxa including the hybrid *E. x stephensonii*. Kreutz *et al*. (2020) concluded that they represent a variety of a species hitherto unrecognised in Britain, *E. neglecta* (Kumpel) Kumpel *var collina* Kreutz, L.Lewis & G.Giles. This is confusing because *E. neglecta* is described as a subspecies of *E. leptochila* in Kew's database, Plants of the World Online (http://www.plantsoftheworldonline.org/), rather than as a separate species.

We did not have access to molecular analysis tools to aid our study of the plants at Warburg, but our species assignment used the same approach as that for selecting plants for molecular analysis, i.e. based on conventional morphometric observations, typically influenced by the epichile shape and colouring. The plants at Warburg can be challenging, especially as it is well known that *E. helleborine* can be very variable, and at an early point we thought that there may be a measure of introgression within the population.

Combining two couplets in BSBI's Plant Crib (https://bsbi.org/wp-content/uploads/dlm_uploads/Epipactis_Crib.pdf) distinguishes the species as follows:

E. helleborine: Lowermost leaf blade (much smaller than others) wider than long, cucullate; leaves ribbed, harsh in texture, usually dark green. Flowers usually tinged with dirty purple, rarely pink. Epichile recurved at tip, no longer than wide.

E. leptochila: Lowermost leaf blade longer than wide; leaves scarcely ribbed, silky in texture, often yellowish green. Flowers yellowish green to clear pink. Epichile flat, longer than wide. The tip of the labellum of the recognised subspecies, *E. leptochila* subsp. *neglecta* is however recurved.

A number of other subjective distinguishing characteristics can be found in Stace (2019), Harrap & Harrap (2005) and Cole & Waller (2020):

- Viscidium: well developed in *E. helleborine*; minute or absent in *E. leptochila* as befits an autogamous plant
- Leaves: upper leaves spiral in *E. helleborine*, colour dull to mid green; two-ranked with a fresh green colour in *E. leptochila*. Note though that Jakubska-Busse *et al*. (2017) argue that leaf orientation in the case of *E. purpurata* and probably more generally in the *Epipactis* genus is an unreliable means of discrimination.
- Ovary: usually sparsely hairy, stalk washed purple at base in *E. helleborine*; hairy, flower stalk greenish-yellow in *E. leptochila*.
- Perianth: variable but predominantly green, tinged pink in *E. helleborine*; yellowish green, with a pink-tinged labellum in *E. leptochila*.

- Labellum bosses at base of epichile usually purplish and wrinkled in *E. helleborine*; not as well developed in *E. leptochila*.
- Epichile: heart shaped in *E. helleborine*; shaped like an arrowhead in *E. leptochila*, though Lewis *et al*. (2020) describe a variety with a heart-shaped epichile from Glamorgan, for which they propose the name *E. leptochila* var. *cordata*.
- Hypochile cup: purple to mid brown in *E. helleborine*; wine-red to chocolate brown in *E. leptochila*.

Clearly the appearance of the flower, especially whether the epichile, the bottom part of the lip, is narrow and pointed or broad and curled under respectively is a key distinguishing feature. The 33 flowering plants at Warburg were assigned particularly on the perianth characteristics to either *E. leptochila* or *E. helleborine* (e.g. Fig. 6).

Four plants however defied clear assignment, appearing at first flowering like *E. leptochila* but with age beginning to resemble *E. helleborine*, so that moving up the flower spike the bottom flowers looked like *E. helleborine*, but the top resembled *E. leptochila*. These were considered to be potentially the hybrid *E. x stephensonii*, which has only ever been doubtfully confirmed in Britain. Milecarek (2018) gives a summary of the status and past records for this taxon. Plants found at Whiteleaf near Princes Risborough (v.c.24) have long been considered candidates and one of these is illustrated in Cole & Waller (2020), but there has never been a consensus about their status.

Initially therefore the 33 flowering plants at Warburg were assigned as 19 *E. leptochila*, 10 *E. helleborine* and 4 putative hybrids (illustrated in Fig. 7).

Figure 6. Perianths of 'typical' *Epipactis leptochila* (left) and *E. helleborine* (right)

Figure 7. Perianths of four putative hybrids, *Epipactis* x *stephensonii*

Using these identifications, measurements made on the flowers of the three taxa are summarised in Table 1. Unsurprisingly because the comparative epichile length and width were perhaps the key qualitative determinants of species, they show a marked difference between *E. leptochila* and *E. helleborine* (P=0.01 and 0.02, respectively). Values for the putative hybrids were intermediate, supporting their provisional identification. The presence or absence of a viscidium is an important discriminant between *E. leptochila* and *E. helleborine*; only 2 of the former showed a viscidium (one of which was quite small), as did two of the putative hybrids.

Fruit set in *E. leptochila* was much higher than in *E. helleborine* (P=0.06), an expected result given that one is largely self-fertile, and the other almost entirely dependent on pollination. The seed pods of *E. leptochila* were shorter than those of *E. helleborine* (P=0.02), a useful characteristic were it not for the fact that the low fruit set in the latter makes it of limited application.

Young (1962) reported a difference in the seed lengths of *E. leptochila* (1.15 mm long x 0.27 mm wide) and *E. helleborine* (0.97 mm long x 0.27 mm wide). We examined a small number of seeds of each taxa (n=5) but found no clear differences (Table 2). The hybrid seeds almost always had embryos, so at this most basic consideration, they might be viable.

Table 1. Mean value for floral characters by taxon.

Character	***E. leptochila***		***E. helleborine***		**?*E x stephensonii***	
	Average	**Std Dev**	**Average**	**Std Dev**	**Average**	**Std Dev**
Epichile length mm **(EL)**	3.58	0.83	2.67	0.41	3.32	0.49
Epichile width mm **(EW)**	3.87	0.71	5.06	0.52	4.21	0.51
Hypochile width mm **(HW)**	4.30	0.68	3.94	0.46	3.82	0.41
Flower width mm **(FW)**	12.89	3.26	14.62	1.37	14.08	2.37
Viscidium width **(Visc)**	0.08	0.21	0.90	0.13	0.37 **	
Lateral sepal length (visible part) **(LSL)**	6.60	1.43	7.56	1.06	7.47	1.35
Lateral sepal width **(LSW)**	4.70	0.88	4.45	0.75	4.10	0.53
Dorsal sepal width mm **(DSL)**	4.81	0.98	4.24	0.5	5.07	1.1
Lateral petal length (visible part) **(LPL)**	4.46	0.89	5.39	1.58	5.07	1.15
Lateral petal width **(LPW)**	3.90	1.14	4.34	0.79	4.12	0.3
Height cm **(H)**	32.7	12.0	37.6	8.6	27.3	6.8
Inflorescence cm **(I)**	7.8	3.8	9.9	4.7	7.5	1.8
Number of flowers **(F)**	10.8	7.7	12.6	7.1	7.5	2.3
Calculated flower density	1.35	0.75	1.74	1.14	0.99	0.09
Fruit set expressed as a decimal (eg 0.5 =50%) **(FRS)**	0.62	0.38	0.25	0.30	0.77	0.35
Seed pod length mm	9.8	1.2	11.6	0.8	10.6	1.5
Seed pod width mm	6.2	0.9	6.4	1.0	6.1	1.1
Seed point attachment to stem °	111.4		101.3		125.6	
Number	10		19		4	

** 2 plants had a viscidium, the other 2 did not

Table 2 Seed dimensions of selected plants from each taxon

	Mean length (mm)	Mean width (mm)
E. leptochila	1.07	0.21
E. helleborine	1.09	0.195
?*E. x stephensonii* plant 1	1.125	0.195
?*E. x stephensonii* plant 2	1.295	0.21

The density of flowers on the spike was higher for *E. helleborine* compared to *E. leptochila* but the difference was not statistically significant.

A more rigorous quantitative discriminant analysis was undertaken using the four characters used for the initial visual assignment, namely epichile width and length, hypochile width and width of the viscidium (zero if absent). The predictive accuracy was 87.9% with one of the 19 *E. leptochila* plants assigned to *E.*

helleborine and a second plant to *E. x stephensonii*. One of the four putative hybrids was assigned to *E. leptochila* and another to *E. helleborine*, but nevertheless two remained 'different' to either full species.

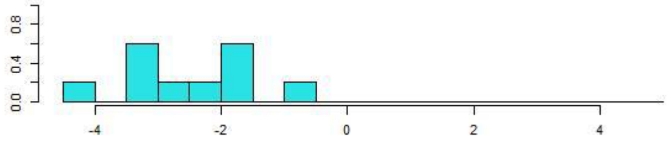
Using all the variables in the table except for seed pod dimensions and attachment angle (because not all plants went on to develop fruits), gave a clean separation on the first linear discriminant function (which described 89.3% of the total variation). The predictive accuracy was 100%, with all *E. helleborine* and *E. leptochila* correctly assigned as were the putative hybrids.

Repeating this analysis but excluding the viscidium width the predictive accuracy of the model surprisingly remained unchanged at 100%, with all plants placed in their preassigned groups. A possible explanation is that two of the *E. leptochila* plants had at least a partially developed viscidia, which is not uncommon. Therefore the presence or absence of a viscidia needs to be treated with caution and indeed it is known that some *E. leptochila* have a viscidium which falls off early. In contrast the viscidium of *E. helleborine* goes when the flower is pollinated and the anthers removed.

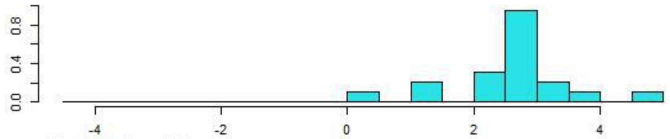
The equation for the powerful first discriminant function is as follows (see Table 1 for character identifiers):

LD1 = 0.12***F**-0.13***I** - 1.58***EW** + 1.53***EL** - 0.07***HW** - 0.16***FW** - 0.64***LSL** + 1.39***LSL** + 0.03***DSW** - 0.33***LPL** - 1.01***LPW** + 1.19***FRS** + 2.59

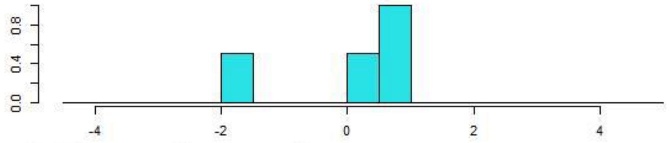
If LD1 is greater than 1 then the plant is almost certainly *E. leptochila*. If between 0 and 1, then it is probably a hybrid, and if negative then it is probably *E. helleborine* (although there is some overlap with the hybrid). The separation achieved is shown in Figure 8.



E.helleborine



E. leptochila



? E.x stephensonii

Figure 8. Grouping of taxa on LD1 based on floral characters

To test how important the labellum and viscidium measurements are to discrimination, a repeat analysis was undertaken with all the measurements of the flowers, floral dimensions and fruit set but excluding the labellum measurements, the epichile width and length, the viscidium width and the hypochile width. These labellum measurements when taken subjectively lie at the heart of the initial qualitative discrimination between the two species, namely the relatively thin and pointed epichile vs. the wider recurved epichile on one hand, and the presence or otherwise of a viscidium on the other. Of course there were other variables which were not measured because of the difficulty of objectively quantifying them such as such as the colour of the epichile bosses, and the colour of the ovary to stem attachment. Nevertheless the remaining 10 variables produced a predictive accuracy of 87%, the same as that for the labellum variables alone. One initially assigned *E. leptochila* plant was placed in *E. helleborine* and another in ?*E* x *stephensonii*, whilst the putative hybrids were again split as one *E. leptochila*, one *E. helleborine*, and two ?*E.* x *stephensonii*. It is surprising that the species (and at least some of the putative hybrids can be separated out fairly accurately on the length and width of the dorsal sepal, the lateral sepal width and length (visible part) and fruit set. Further, predictive accuracy is maintained even excluding fruit set, but the separation gap between species is much lower.

Species Differentiation Using Vegetative Characteristics
For all plants in the study area, whether flowering or not, several measurements were made on the vegetative characteristics including leaf length and width, orientation around the stem, and colour. These vegetative data were not used in the assignment of plants to species by discriminant analysis, because the flower characteristics alone gave a fully adequate separation. However, the discriminant analysis was repeated using just the vegetative characteristics of the flowering plants to assist with predicting species identity of non-flowering plants. Vegetative measurements are summarised in Table 3.

There were some clear differences between the two species. The red, green and blue ("R,G,B") components of leaf colour differed significantly using Welch's 2 sample T Test (P=0.02, 0.02 and 0.03 respectively). Note however that R and G are correlated (correlation coefficient 96%). There were also statistically significant differences in the leaf turn between the second and third leaf (P=0.005) and the average length to width ratio of all the leaves (P=0.06). Differences in other vegetative characters were not statistically significant.

The putative hybrids showed a significant difference in the R:G ratio compared to *E. leptochila* (P=0.03) but not to *E. helleborine*, but differed significantly in the average length to width ratio for the bottom three leaves compared to *E. helleborine* (P=0.05). It seems therefore that the putative hybrids resemble each parent in different vegetative characters.

The colour differences are subtle, and challenging to show one plant against another under the same light conditions. We were unable to achieve this at Warburg but Fig.9 shows a plant of *E. leptochila* next to one of *E. helleborine* at Sheepleas in Surrey (v.c.17) in 2019. The former is a rather more yellowish green, compared to the bluish green of the latter.

Table 3 Measurements of leaf variables for each taxon.

Variable	***E. leptochila***		***E. helleborine***		**?*E.* x *stephensonii***	
	Average	**Std Dev**	**Average**		**Average**	**Std Dev**
No of leaves (**LNo**)	5.75		7.40		5.00	
Leaf colour – red **(R)**	92.9	17.6	108.2	14.3	103.0	8.9
Leaf colour - green **(G)**	120.0	17.0	134.2	13.2	127.2	11.4
Leaf colour - blue **(B)**	42.6	12.1	59.9	22.2	47.2	10.8
Leaf colour red to green ratio **(R:G)**	0.77	0.05	0.80	0.04	0.81	0.02
Leaf colour red to blue ratio **(R:B)**	2.33	0.82	1.99	0.61	2.26	0.39
Leaf colour green to blue ratio **(G:B)**	3.03	1.00	2.49	0.80	2.78	0.43
Leaf 1, the bottom leaf, length to width ratio **(LtoW1)**	1.70	0.39	1.48	0.21	1.75	0.08
Leaf 2, the second leaf up length to width ratio **(LtoW2)**	2.23	1.25	1.62	0.22	2.29	0.36
Leaf 3, the third leaf up, length to width ratio **(LtoW3)**	2.19	0.45	1.90	0.35	2.66	0.64
Clockwise turn between leaves 1 and 2 ° **(Turn2)**	185		187		172	
Clockwise turn between leaves 2 and 3 ° **(Turn3)**	177		203		165	
Clockwise turn between leaves 3 and 4 ° **(Turn4)**	182		200		217	
Average clockwise turn for all the leaves ° (**AveTurn**)	167		182		180	
Average length to width ratio for the first 3 leaves **(LtoWAve)**	1.97		1.66		2.42	

For some plants the data were incomplete either because leaves had been chewed or because of incomplete fieldwork; after taking these out, 21 plants remained, of which nine were *E. helleborine*, eight were *E. leptochila* and four were ?*E. x stephensonii* (confirmed by the discriminant analysis on flower characteristics described above). Discriminant analysis on the leaf variables, taking the ratios R:G and R:B to represent the colour variables gave good separation between the species with a prediction accuracy of 90.5%. The fit was better than an analysis using the three individual colours that gave a fit of 80.9%, improving to 85.7% if G, which is correlated with R is omitted, and compares favourably with the fit achieved in an analysis based on labellum and viscidium variables described earlier. Based on vegetative characters, one of the *E. leptochila* plants was placed in *E. helleborine*, and one of the putative hybrids was placed in *E. leptochila.*

Figure 9. *E. leptochila* (right) next to *E. helleborine* (left) at Sheepleas in Surrey in 2019

The equation for the powerful first discriminant function using vegetative characters is as follows (see Table 3 for character identifiers):

LD1 = -8.0304* **R:G** + 0.3484***R:B** - 0.0041***Turn2** - 0.0305***Turn3** +0.007***Turn4** +1.2535***LtoW1** - 0.3133***LtoW2** + 0.9925***LtoW3** + 6.9538

If LD1 is negative then the plant is almost certainly *E. helleborine*. A positive value of LD1 identifies *E. leptochila* or ?*E.* x *stephensonii*. The separation achieved is shown in Fig. 10.

The second discriminant function is:

LD2 = 6.9115* **R:G** – 0.5185***R:B** - 0.0099***Turn2** - 0.0006***Turn3** + 0.0181***Turn4** - 0.3253***LtoW1** - 1.2852***LtoW2** + 2.4197***LtoW3** **-** 8.0504

It has some value for distinguishing *E. leptochila* from ?*E.* x *stephensonii* but only if the possibility of *E. helleborine* has been excluded.

Omitting the R:B ratio leaving only the R:G ratio to represent leaf colour does not change the discrimination though the coefficients change. Using the R:B ratio but not the R:G ratio reduces the prediction accuracy to 81%. Looking at redundancy among the remaining variables the analysis was repeated omitting one of the variables in turn and comparing the revised predictive accuracy with that with all the variables (Table 4).

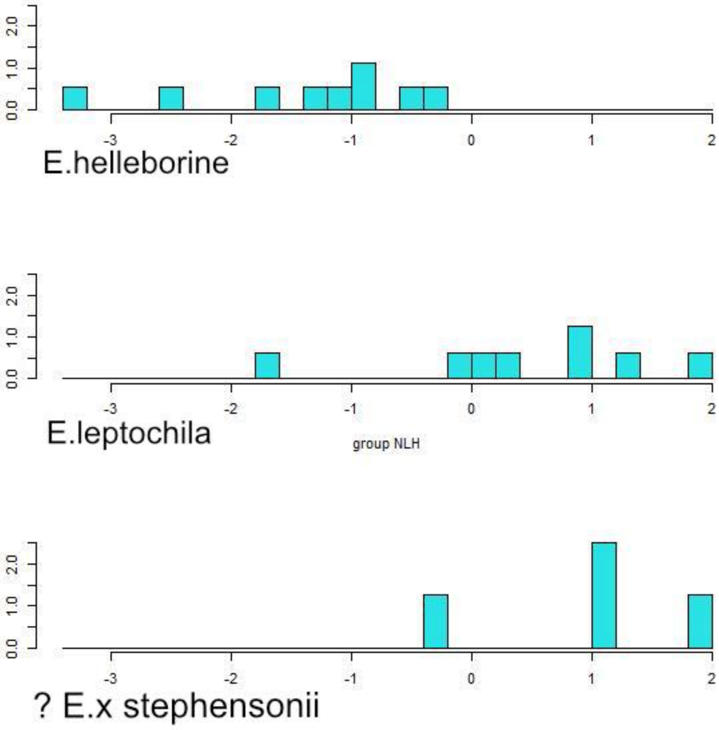


Figure 10. Grouping of taxa on LD1 based on vegetative characters

Table 4. Effect of eliminating variables one by one on predictive accuracy.

	Predictive accuracy
R:G ratio plus all the leaf turn and L:W ratios	90.5% (2 out of 21 plants incorrect)
Without Leaf Turn2	90.5%
Without Leaf Turn3	76.2 (5 out of 21 plants incorrect)
Without Leaf Turn4	80.9
Without L:W1	85.7
Without L:W2	76.2% (5 out of 21 plants incorrect)
Without L:W3	80.9

Clearly leaf width is important and perhaps best is an average measure for the first three leaves, especially say where one or two have been damaged by snails. Surprisingly the length to width ratio of the second leaf up is more important than the base leaf, perhaps because in many situations the base leaf, being small and often bract-like, is easily overlooked, or often chewed away, with the second leaf being mistaken for the base leaf. Leaf turn at the base of the stem is unimportant but above that, well-recognised differences come into play with *E. leptochila* being more two-ranked than *E. helleborine*.

These outcomes were then used to predict the ID of all the vegetative plants, for which we had sufficient comparative data on the relevant characters. Starting with 82 plants we had to exclude 29 plants from a full prediction because of insufficient data, often because the whole plant or leaves were too badly damaged. However, we did though have leaf colour data for all but eight plants. A discriminant analysis on just the leaf colour variables for the previously-assigned flowering plants met with only modest success – an accuracy of only 57%. Given this constraint, our analysis of non-flowering plants for which we had only leaf colour data, suggested that quite a number of these were in fact hybrids, and that hybridisation at Warburg is unexpectedly frequent. It has always been a challenge to assign non-flowering plants at this location to one or other species and this might be the explanation.

We also considered other characteristics of non-flowering plants that might help to distinguish between the two species. In particular we looked at leaf edges and the degree of crenulation and whether or not the main leaf veins had papillae. Poland and Clement (2009) suggest there are differences in the leaves of the two parents: *E. leptochila* has distinctly papillate leaf veins and undulate papillate leaf edges whereas for *E. helleborine* the leaf veins are only obscurely papillate, and there are irregular longish papillae on the leaf edges. Otherwise leaf edges and veins have received limited attention. Cole & Waller (2020) provide some information for British taxa. Jakubska-Busse & Gola (2010) gave details of the leaf morphology of *E. helleborine* while Bernardos et al (2004) report differences in leaf crenulation between *E. helleborine* and two Iberian species, *E. tremolsii* and *E. lusitanica*. We did note some differences in the leaf margins of *E. leptochila* and *E. helleborine* but intra-specific variation, within the latter especially, questions their diagnostic value.

Leaf margins at x100 magnification are shown in Fig. 11 of typical examples of the two species plus a putative hybrid. Curiously the leaf edge of the hybrid was more regular than either of the parents; the papillae of *E. helleborine* plant were longer than those of *E. leptochila*, but we had examples of the former where the papillae were quite short. There were examples of leaf vein papillation in *E. leptochila* but it proved difficult to use this characteristic objectively and consistently.

Putative Hybrids

There appear to be no reliable records for the hybrid, *E. x stephensonii* from Britain. Mielcarek (2018) provided a summary of the status of this enigmatic taxon, and was surprised at the lack of photographs in the published literature or more casually in on-line fora. In addition to the two specimens mentioned by Mielcarek (2018) there is a single record on the BSBI Distribution Database (https://database.bsbi.org/), dating from 1984 and from near Marlow, Bucks (v.c.24) recorded by D.M. Turner Ettlinger, though there is no mention of this in his monograph (Ettlinger, 1997). Instead he notes only that it has been found in Southern England. There are two specimens in the Natural History Museum Herbarium (https://www.nhm.ac.uk/our-science/collections/botany-collections/british-irish-herbarium.html) (one from Coopers Hill Wood, and the other from Painswick, both in East Gloucestershire (v.c.33).

Cautiously therefore we identified four of the 33 flowering plants in our study area with ambiguous morphology as probable hybrids and the discriminant analysis confirmed that from flower morphology that these could be grouped as distinct from either parent. Using flower morphology but excluding labellum variables and the viscidium width (essentially whether or not there was a viscidium) then two of the four remained distinct but one plant was reassigned to *E. leptochila* and another to

E. helleborine. Using just leaf characteristics, three of the four were distinct, but one conformed to *E. helleborine*.

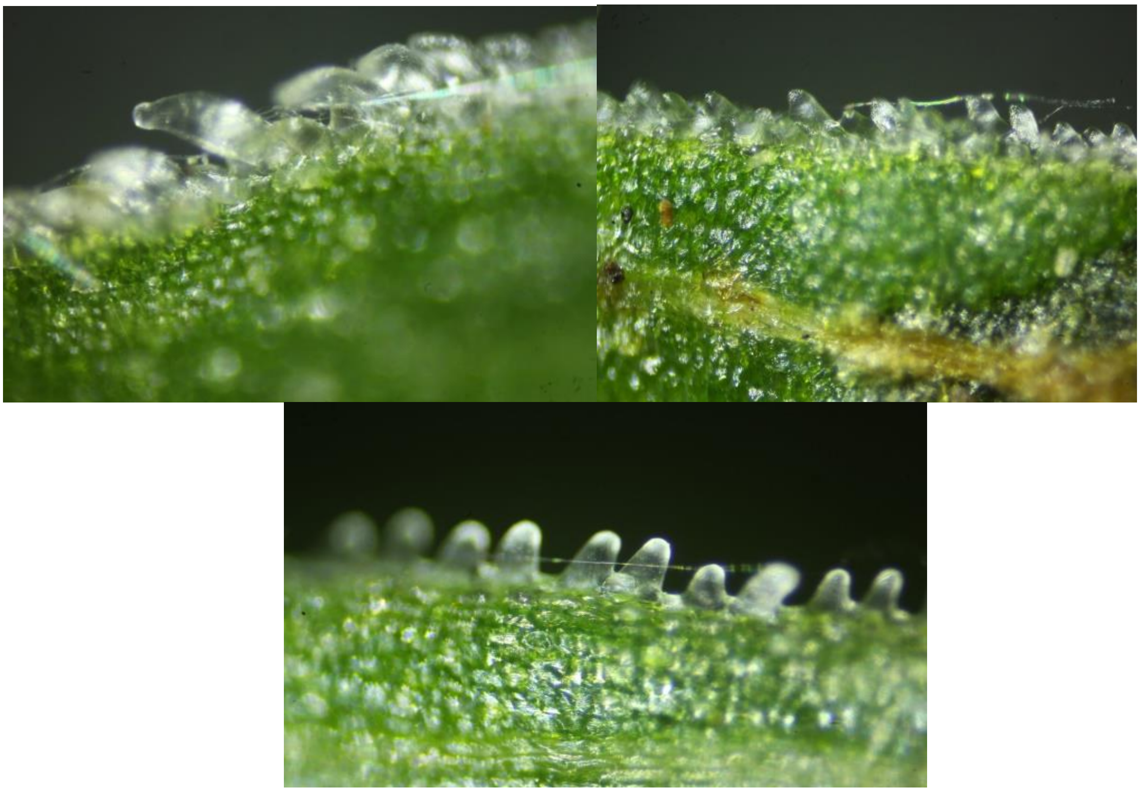


Figure 11. Leaf margins of representative plants of *Epipactis helleborine* (above left), *E. leptochila* (above right) and ?*E.* x *stephensoni* (below).

We consulted BSBI's experts and supplied photographs of two of the plants we thought were hybrids. One of the BSBI Orchid referees, Richard Bateman, thought that one plant was possibly a hybrid but that the other was probably *E. leptochila*. John Richards, BSBI's *Epipactis* referee, thought they might be *E. leptochila* subsp. *neglecta*. Given the continuing taxonomic uncertainties, and the fact that they were dealing with photographs rather than seeing the plants in context, these equivocal responses and difference of view are understandable.

Mean values for the four putative hybrids lay between the means for the parents for viscidium width (two out of four plants had a viscidium, but the other two did not), flower width (i.e. tip of one lateral sepal to the other), epichile width, epichile length, lateral petal length, lateral petal width, seed pod length, leaf colour red component, leaf colour blue component and leaf colour green component. In terms of lateral sepal length the mean was closer to *E. helleborine*. The mean values were closer to *E. leptochila* for four characters: fruit set, leaf turns and length to width ratio of bottom leaf. Anomalous results were found for hypochile width, lateral sepal width, dorsal sepal width, and the length to width ratio of the second and third leaf, where the mean for the putative hybrids was either greater or smaller than that for either parent.

One plant always assigned as putative *E.* x *stephensonii* throughout all the discriminant analyses. A second, confirmed in all the analyses using flower and vegetative characteristics except when the inner perianth measures were excluded, was studied in detail to document its development from mid-June to flowering in mid-July and fruit set at the end of August (Fig.12).

Figure 12. Stages in the development of a putative *Epipactis* x *stephensonii* at Warburg in 2020. Photos taken (from left to right) on 12th June, 7th July, 19th July and 28th August.

Flowers when they first opened, resembled *E. leptochila*, but later as they aged began to resemble *E. helleborine* but without the typical recurved labellum tip (Fig. 13).

Conclusions

Epipactis helleborine and *E. leptochila* coexist at Warburg in habitat more suited to the latter than the former. Using discriminant analysis we were able to distinguish reliably between the two species on the regularly used labellum characters and other perianth characteristics, but also using vegetative measures, especially leaf colour allied with the length to width ratio of the bottom three leaves. Using these criteria identified from flowering plants, we were able to assign most non–flowering plants to one of the two species.

There were four anomalous plants whose flowers looked different to either *E. helleborine* or *E. leptochila* and we propose that these are the hybrid *E.* x *stephensonii.* Our statistical analyses confirmed that these plants showed a level of distinctiveness from both parents on flower characteristics and, with a lower degree of accuracy, on leaf variables. Final confirmation of their hybrid status requires confirmatory molecular analysis, but nevertheless from our morphological evidence we believe this to be the first reliable record of *E.* x *stephensonii* in Britain.

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Colin Williams and Kelly Hedges of the Berks, Bucks and Oxon Wildlife Trust (BBOWT) provided valuable support and assistance to our study and provided access to BBOWT's datasets. John Richards and Richard Bateman were especially helpful in providing opinions on the plant photographs which we shared with them. Ian Denholm provided very significant assistance and advice in editing the paper pre-publication. John Hunnex, curator of the Natural History Museum Herbarium, provided access and support to review the collection.

Figure 13. Flowers of a putative plant of *E.* x *stephensonii* at Warburg in 2020. Top left: lowermost flower on 7th July; Top right: second flower up inflorescence on 14th July; Bottom left: uppermost flower on 19th July; Bottom right: uppermost flower on 26th July.

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