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Morphometric and population genetic analyses elucidate the origin, evolutionary significance and conservation implications of *Orchis* ×*angusticruris* (*O. purpurea* × *O. simia*), a hybrid orchid new to Britain

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We report the first confirmed occurrence in Britain of *Orchis* × *angusticruris* Franch. ex Rouy, a hybrid between two closely related orchid species of anthropomorphic *Orchis* (*O. purpurea* Huds. × *O. simia* Lam.) that hybridize frequently in Continental Europe. Seven individual hybrids, most likely F1 plants representing a single interspecific pollination event, first flowered with both parents in May 2006 at a nature reserve in the Chiltern Hills near Goring, Oxfordshire. Univariate and multivariate morphometric analyses (43 characters plus 12 indices), internal transcribed spacer sequencing, plastid microsatellites and amplified fragment length polymorphism (AFLP) analyses together readily separate the parents and confirm that *O. purpurea* was the ovule parent and *O. simia* the pollen parent, presumably reflecting the greater frequency and/or later flowering period of the latter at the site. This study reinforces a more general observation that, in most orchids, the ovule parent contributes substantially more to the hybrid phenotype than does the pollen parent, perhaps reflecting cytoplasmic inheritance. In contrast, the hybrids are placed closer to *O. simia* than to *O. purpurea* in the AFLP tree. Apparently recent arrivals, the few *O. purpurea* plants at Goring contrast genetically with the two other small populations of this species known in the Chilterns, but rather are consistent with relatively uncommon Continental populations. This suggests that the plants may have been deliberately introduced at Goring by man, although transport from the Continent in high-level air currents cannot be ruled out. The Goring population of *O. simia* is likely to have become genetically impoverished through (1) preferential removal of many relatively fit plants to herbaria in the 19th century and/or (2) a catastrophic population crash in the first half of the 20th century. However, both our re-examination of herbarium specimens and our population genetic data indicate past hybridization among anthropomorphic *Orchis* species occurring naturally in the Chilterns. Thus, we tentatively recommend retention of the hybrid plants at Goring, despite their likely anthropogenic origin from Continental material and the partial viability of their pollen and seeds, which offers opportunities for future introgression. Although the Goring hybrids broadly resemble morphologically *O. militaris*, another anthropomorphic *Orchis* still found at two Chiltern localities, sufficient morphological and molecular differences were observed to strongly refute our initial hypothesis that *O. militaris* could have originated through hybridization between ancestors that resembled *O. purpurea* and *O. simia*. The comparatively complex genetic properties evident in both *O. simia* and *O. purpurea* merit further study. *Botanical Journal of the Linnean Society*, 2008, **157**, 687–711.

ADDITIONAL KEYWORDS: amplified fragment length polymorphism (AFLP) – Gower similarity – hybridization – identification – internal transcribed spacer (ITS) – maternal inheritance – Orchidaceae – *Orchis militaris* – plastid microsatellites – principal coordinates – speciation.

INTRODUCTION

Few new taxa have been added to the British orchid flora in recent years. In a very few cases, population genetics and morphometrics have combined to reveal cryptic species, each segregated from within a better-known species that is only subtly morphologically different from the norm (Bateman, 2001, 2006a). One good example is the Hebridean Marsh-orchid, *Dactylorhiza ebudensis*, a recently formed allopolyploid

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endemic to the Hebridean island of North Uist (Bateman, 2006a; O. Paun, pers. comm., 2007). In other, equally rare, cases the novel species is most probably a recently arrived fugitive from mainland Europe, such as the Small-flowered Tongue-orchid, *Serapias parviflora* (Cobbing, 1989; Madge, 1994). Despite the likelihood that such natural northward immigration events will increase in frequency as a result of global warming, such reports inevitably raise accusations from sceptics of assisted transport by some misguided enthusiast (cf. Ettlinger, 1997; Stace, 1997; Preston, Pearman & Dines, 2002; Foley & Clarke, 2005; Harrap & Harrap, 2005; Bateman, 2006a). Thus, further additions to the well-known orchid flora of the British Isles are most likely to reflect not new taxa but rather new combinations of taxa, generated through the formation of novel natural hybrids (e.g. Stace, 1975, 2009). However, both the identification and the origin of such presumed hybrids often spark controversy.

One of the most fertile hunting grounds for hybrids novel to Britain has been the anthropomorphic group of species within the genus *Orchis* (a group that includes *O. militaris*, the type species of the type genus of the orchid family). In recent years, molecular phylogenetic studies and ensuing morphological re-investigations have led to the disassembly of this formerly polyphyletic genus, resulting in approximately half of its constituent species being reassigned to expand the previously monotypic genera *Anacamptis* and *Neotinea* (Bateman, Pridgeon & Chase, 1997; Pridgeon *et al*., 1997; Bateman *et al*., 2003; Kretzschmar, Eccarius & Dietrich, 2006; Bateman, 2007). Species remaining in the re-circumscribed genus *Orchis* fall into two broad categories, each widely distributed across Europe and Asia Minor, which appear reproductively isolated. The two groups are increasingly frequently separated formally, as either sections (e.g. Bournérias & Prat, 2005) or subgenera (e.g. Kretzschmar *et al*., 2006). Members of the clade epitomized by *O. mascula* (*c.* 23 species) have spreading lateral sepals and most have large flowers bearing long spurs. In the more reliably calcicolous anthropomorphic group (*c.* eight species) the lateral sepals are incorporated into the hood, the flowers are in most cases smaller and shorter-spurred and the four-lobed labellum is more deeply divided, conferring on the flower a strikingly anthropomorphic outline (Figs 1, 2). Molecular phylogenies based on the nuclear internal transcribed spacer (ITS) region suggest that the relatively early divergences of *O. anthropophora* and/or of the exclusively Mediterranean *O. italica* render this group paraphyletic (Bateman *et al*., 2003) (Fig. 3). However, plastid-based phylogenies suggest that the anthropomorphic *Orchis* group is monophyletic, albeit still showing *O. anthropophora* and *O. italica* as strongly and apparently basally divergent (Bateman, 2007; R. Bateman & P. Hollingsworth, unpublished data; A. Kocyan, unpublished data). Hybridization occurs frequently within the anthropomorphic *Orchis* group (Godfery, 1933; Summerhayes, 1968; Clapham, 1962; Peitz, 1970; Hunt 1975a, b; Soó, 1980; Bateman & Farrington, 1987; Bateman, 2006b; Kretzschmar *et al.*, 2006).

In Britain, the anthropomorphic *Orchis* species are considered especially charismatic. This partly reflects their extraordinary appearance (Fig. 1) and inferred aspects of their evolution, such as the apparent reduction to a vestigial condition of the spur in *O. anthropophora* that until recently led to its taxonomic segregation as a monotypic genus, *Aceras* (Bateman *et al*., 1997; Pridgeon *et al*., 1997; Bateman *et al*., 2003; Bateman, 2007). However, the increased attention paid to the group has been prompted mainly by strong and increasing conservation interest. Of the four anthropomorphic species occurring in England (none is present in Scotland, Wales or Ireland), *O. simia* and *O. militaris* are now confined to two or three natural localities each and so appear on Schedule 8 of the Wildlife and Countryside Act (Sumpter *et al*., 2004; Cheffings & Farrell, 2005), while *O. purpurea* and *O. anthropophora* are listed among the most rapidly declining of all our native vascular plants (Rose, 1994; Wells, 1994; Preston *et al*., 2002; Braithwaite, Ellis & Preston, 2006), being geographically restricted to southeast England (Rose, 1949; Stewart *et al.*, 1994; Preston *et al*., 2002). Within this region, the anthropomorphic *Orchis* group exhibits diversity hotspots in the North Downs of Kent and Chiltern Hills of Oxfordshire and Buckinghamshire.

The comparative rarity of these species in England has not prevented them from combining to generate several high-profile hybrids in recent years. A single putatively F1 hybrid between *O. simia* and *O. anthropophora*, found in Kent in 1985 and immediately subjected to detailed morphometric examination (Bateman & Farrington, 1987), persisted at the site for several years (Ettlinger, 1997). Although it was suggested that this hybrid might have been produced mischievously by one of the conservation volunteers who were recruited at the time to bulk up the population of *O. simia* by artificially transferring pollinia among inflorescences using a paintbrush (e.g. Foley & Clarke, 2005), individuals of the parental species occurred in close juxtaposition, thereby maximizing the risk of natural cross-pollination (Bateman & Farrington, 1987). In 1998, another Kentish site yielded two hybrid plants identified as *O. purpurea* × *O. anthropophora*, although, unfortunately, issues of site confidentiality precluded more detailed scientific investigation (cf. Rose, 1998; Bateman & Farrington, 1999).

Figure 1. A, *Orchis purpurea* × *simia* viewed northward along the Thames. B, *Orchis purpurea* × *simia* inflorescence most closely resembling *O. simia*, Goring. C, *Orchis purpurea* × *simia* inflorescence most closely resembling *O. purpurea*, Goring. D, *Orchis simia* inflorescence, Goring. E, *Orchis purpurea* inflorescence, Goring. F, *Orchis militaris* inflorescence, Marlow. G, *Orchis simia* × *anthropophora* inflorescence, Faversham (1985). H, *Orchis anthropophora* inflorescence, Faversham. All photographs by Richard Bateman.

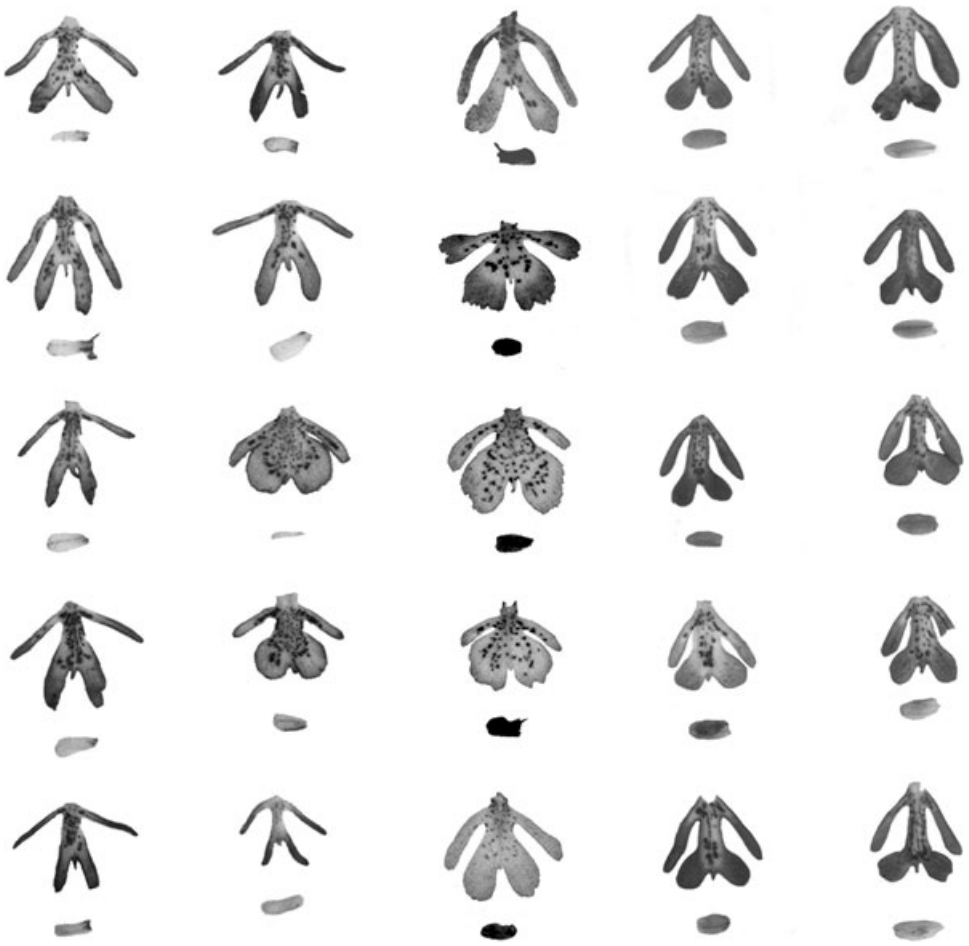

Figure 2. Mounted flowers of the seven hybrids, the two plants of *Orchis purpurea* and a representative flower of *O. simia* (columns 1 and 2) from Goring, five plants of *O. purpurea* from East Kent (column 3), five plants of *O. militaris* from Marlow (column 4) and five plants of *O. militaris* from Turville (column 5). Scale: top-left flower is 18 mm wide.

The most recent suspected case of hybridization among anthropomorphic *Orchis* species occurred in May 2006 at the Berkshire, Buckinghamshire and Oxfordshire Wildlife Trust reserve at Hartslock, overlooking the River Thames near Goring (Fig. 1A). The reserve has welcomed careful visitors ever since various conservation measures successfully bulked up its once tenuous population of *O. simia* – the final remnant of a once extensive meta-population that stretched along much of the Thames Valley where it cuts through the chalk landscape of the Chiltern Hills (Paul, 1965; Bateman & Farrington, 1989; Harrap & Harrap, 2005, Raper, 2006–2008). The seven problematic plants that are the focus of this paper first flowered in 2006 (Figs 1A–C, 2), immediately attracting much media attention (Anonymous, 2006a, b; Bateman, 2006b; Brown, 2006; Raper, 2006–2008; Walker & Pearman, 2006). The suspected hybrids occurred in a more-or-less linear, downslope array located immediately below two flowering plants of *O. purpurea* (Fig. 1A–C vs 1E), which themselves first mysteriously appeared at the site in 1998. Both these occurrences prompted much speculation regarding their respective origins. Some initial identifications of the plants shown in Figure 1A–C favoured yet another anthropomorphic *Orchis* that is rare and heavily protected in Britain, *O. militaris*, which occurs in increasing numbers at two heavily conserved sites further east in the Chilterns (Figs 1F, 2). However, more careful examination suggested that these striking new arrivals represented yet another case of hybridization among the British anthropomorphic *Orchis*, this time between *O. purpurea* and *O. simia* (Bateman, 2006b; Raper, 2006–2008; Fay *et al.*, 2007), to generate *O.* × *angusticruris* Franch. ex Rouy (= *O.* × *weddellii* E. G. Camus).

The present study explores these plants and their context in considerable detail, building on earlier investigations of the Goring *O. simia* population that used first morphometrics (Bateman & Farrington,

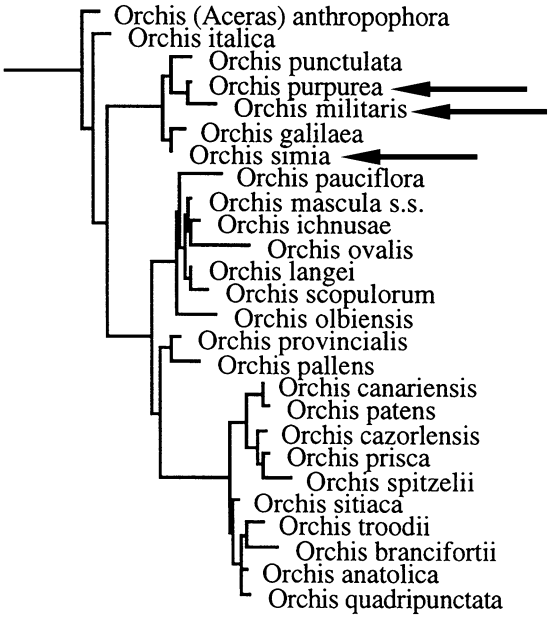

Figure 3. Phylogenetic context of the studied species of anthropomorphic *Orchis* (arrowed), abstracted from the ITS tree of Bateman *et al*. (2003, fig. 2).

1989) and later amplified fragment length polymorphism (AFLP; Vos *et al*., 1995) genetic fingerprinting and plastid microsatellites (Qamaruz-Zaman *et al*., 1998; Qamaruz-Zaman, 2000; Redmond, 2003; Hooper, 2004). Here, we apply a combination of morphometrics and multiple genetic techniques (ITS sequencing, plastid microsatellites, AFLP) to the putative hybrids in order to identify their parentage and maternity, to determine the relative levels of variation present in the hybrids and their parents and to infer inheritance patterns among their phenotypic characters (cf. Bateman & Farrington, 1987; Farrington & Bateman, 1989; Bateman & Hollingsworth, 2004). We then discuss the broader relevance of these observations for (1) determining the provenance of individual plants, (2) inferring speciation mechanisms and (3) formulating conservation recommendations. More generally, we hope that the study will illustrate the benefits than can be derived from adopting a 'forensic' approach to the interpretation of instances of presumed hybridization.

MATERIAL AND METHODS

Field sampling

Each study plant from Goring was vouchered via 1 : 1 ring-flash photographs. Under the supervision of the site warden, four flowers were removed from each of the seven suspected hybrids and both flowering plants of *O. purpurea*. Because of the small number of *O. purpurea* present at Goring, additional plants were measured from a large population at Covert Wood in East Kent, the heartland of the species in the UK. In addition, both of the native populations of *O. militaris* that have survived in the Chiltern Hills were measured, to allow comparison with the superficially similar hybrid plants at Goring. For each sampled plant, two flowers were placed in silica gel for subsequent DNA extraction. The third flower was rapidly examined under the light microscope to obtain a rough estimate of pollen fertility within the pollinia, while the fourth provided morphometric data.

Samples of anthropomorphic *Orchis* species from further afield, for genetic comparison with the Goring plants, were taken from stock collections of silica gel-dried material held by RBG Kew and by R. M. Bateman. These included plants of *O. purpurea* from the two other known localities in the Chiltern Hills (near Pangbourne and near Bix) and plants of *O. militaris* from the two localities that have persisted in the Chilterns (near Marlow and near Turville). We attempted to sample another outlying UK population of *O. purpurea*, the Avon Gorge (Willis, Martin & Taylor, 1991), but it appears to have been extirpated (N. Hudson & S. Parker, pers. comm., 2007); nor were we able to obtain in time material from the recently reported outlier on Porton Down, South Wiltshire (Anonymous, 2007). In order to provide a suitable phylogenetic context, samples of *O. simia*, *O. purpurea* and *O. militaris* were included from localities widely distributed across Europe.

Morphometric analyses

A standard suite of morphometric characters was compiled during previous studies of anthropomorphic *Orchis* species (Bateman & Farrington, 1987, 1989) and is here reproduced with minor modification as Appendix 1. The 43 characters scored described the stem and inflorescence (7), leaves (7), labellum (17), spur (3), lateral petals (2) and lateral sepals (7). They can alternatively be categorized as metric (29), meristic (5), scalar (6) and presence/absence (3). These variables were in turn used to generate 12 ratios that were devised to better describe the shapes of certain structures.

Stem, inflorescence and leaf characters were scored in the field from *in situ* plants. Floral, ovary and bract characters were obtained from a representative flower; this was consistently located 30–40% of the distance from the base to the apex of the inflorescence, in order to minimize the effect of the substantial diminution in flower size along this axis (Bateman & Rudall, 2006). In the case of the study of *O. simia* at Goring in 1986, the flowers were measured *in situ* on the parent plant, thereby incurring

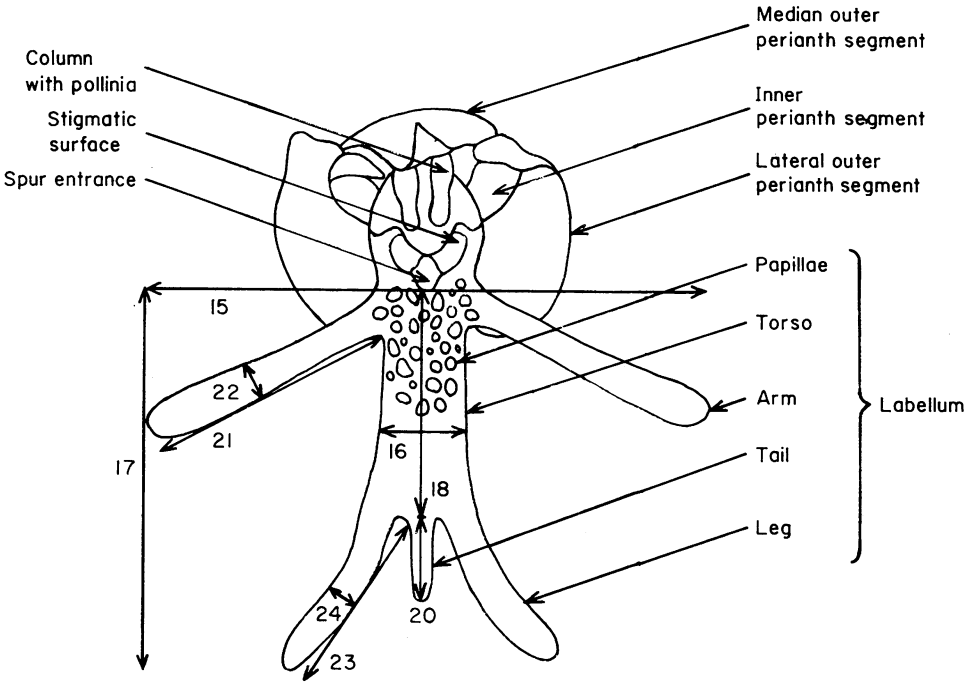

Figure 4. Terminology of floral parts and locations of labellum dimensions measured. Numbers refer to characters listed in Appendix 1 (after Bateman & Farrington, 1989, fig. 1).

undesirably large errors of measurement in some characters (notably widths of the exceptionally narrow 'legs'). Hence, during the 2006 sampling, the flowers of *O. purpurea* and the putative hybrids were removed and mounted on double-sided adhesive tape, thereby allowing both more accurate measurement and permanent vouchering (Fig. 2).

Figure 4 summarizes nine linear measurements taken from each labellum and explains the anthropomorphic terminology adopted here for simplicity to describe parts of the labellum. The colours of the 'limbs' and (for some accessions) the 'torso' of each labellum, and of the reverse surfaces of the outer perianth segments, were matched to the nearest colour block(s) of the Royal Horticultural Society Colour Chart (Anonymous, 1966) and converted to three Commission Internationale de l'Eclairage (CIE) coordinates. Two of these ('x' and 'y') define a position on a square grid superimposed onto a triangular array of colours that pale towards white at the centre of the triangle. The corners correspond to pure blue, pure green and pure red. Density of pigment is measured by a third coordinate (reflectivity, 'Y'), which decreases in value outward from the centre of the triangle (Bateman & Denholm, 1985).

Data for individual plants were summarized in an Excel spreadsheet. Means, sample standard deviations and coefficients of variation were calculated for each character and each ratio in the samples of the hybrids and of both putative parents (although the regrettably small number of flowering individuals – specifically, two – precluded statistical analysis of *O. purpurea* from Goring).

The morphometric matrix of individuals × characters was unusual among such matrices in being complete; there were no missing values. However, for a few individuals (particularly of *O. simia*), this completeness was achieved by extrapolating the length of leaves that had suffered burnt apices, and by estimating from photographs the bract lengths of the two measured individuals of *O. purpurea*. The assembled data were analysed by multivariate methods using Genstat v9.3 (Payne *et al.*, 2006). Character 12 was omitted from further analyses as it largely duplicated character 13 and character 19 was discarded as it partially duplicated character 20. Character 43 was omitted as it scored zero for all of the plants included in the present analyses (it serves primarily to distinguish the related species, *O. anthropophora*). Ratios 'a' to 'l' were omitted as they, by definition, duplicated their constituent characters.

The remaining 40 characters were used to compute a symmetrical matrix that quantified the similarities of pairs of data sets (i.e. plants) using the Gower Similarity Coefficient (Gower, 1971) on unweighted data sets scaled to unit variance. This was in turn used to construct a minimum spanning tree (Gower & Ross, 1969) and subsequently to calculate principal coordinates (Gower, 1966, 1985) – compound vectors that incorporate positively or negatively correlated

characters which are most variable and therefore potentially diagnostic. Principal coordinates are especially effective for simultaneously analysing heterogeneous suites of morphological characters and can comfortably accommodate missing values. As such, they have proven invaluable for assessing relationships among orchid species (reviewed by Bateman, 2001) and populations (Bateman & Farrington, 1989), and between putative hybrids and their presumed parents (Bateman & Farrington, 1987; Farrington & Bateman, 1989; see also Adams, 1982; Bateman & Hollingsworth, 2004).

Molecular analyses

Sampling and DNA extraction

Samples were collected and dried using silica gel (Chase & Hills, 1991) or were sourced from the DNA bank of the Jodrell Laboratory, Royal Botanic Gardens, Kew (http://data.kew.org/dnabank/homepage.html). Total genomic DNA was isolated using a 2 × cetyltrimethylammonium bromide (CTAB) extraction method modified from Doyle & Doyle (1987) by washing the DNA pellet in 70% ethanol and suspending in TE buffer (10 mM Tris-HCl at pH 8.0, 1 mM EDTA). The suspended DNA solution was purified and concentrated using Nucleospin® Extract II DNA purification columns, following the manufacturer's protocol (Machery-Nagel). Each DNA was subsequently quantified using an Eppendorf biophotometer.

Sequencing analysis

Amplification of the ITS region of nuclear DNA was carried out in 25 μL reactions, containing 22 μL of 2.5 mM Mg polymerase chain reaction (PCR) master mix (Abgene Ltd, Epsom, UK), 1 μL bovine serum albumin (BSA; 0.04%), 0.5 μL each of forward and reverse primers (at a concentration of 100 ng/μL) and approximately 50 ng DNA template. The primers used to amplify the region were AB101 and AB102, as specified by Douzery *et al*. (1999).

The PCR profile consisted of initial denaturation of samples at 94 °C for 2 min, followed by 28 cycles of denaturation at 94 °C for 1 min, annealing at 52 °C for 1 min and extension at 72 °C for 3 min, followed by a final extension phase of 7 min at 72 °C. PCR products were purified with Macherey Nagel Nucleospin® Extract II DNA purification columns using the manufacturer's protocol (GmbH and Co., Düren, Germany). Dideoxy cycle sequencing was then performed with the chain termination method using ABI (Applied Biosystems Inc., Warrington, UK) Prism Big Dye version 3.1 reaction kit, following the manufacturer's protocols. The products were run on an ABI 3100 Genetic Analyzer.

For samples that were found to contain multiple ITS sequences, constituent single copies were isolated by cloning into a vector (pGem-T Easy Vector, catalogue no. A1360; Promega Ltd, Madison, WI, USA). The transformed bacterial colonies were then used as DNA template in a further round of ITS amplification and sequencing, using the M13 primers in the Promega kit.

Sequence editing and assembly were performed using Sequencher v.4.5 (Genecodes Corporation, Ann Arbor, MI, USA). Sequences were aligned by eye using the phylogenetic software package PAUP v4.0b2A (Swofford, 2001), in accordance with the guidelines of Kelchner (2000). Many of the comparative data were provided by Hooper (2004).

Aligned sequence data from the ITS1–5.8S–ITS2 assembly were analysed using the Fitch parsimony algorithm (Fitch, 1971) in PAUP v4.0b2A. Heuristic searches were performed with 1000 replicates of random taxon entry using tree bisection–reconnection (TBR) and limiting the number trees held at each step to 20, to reduce the amount of time spent swapping on large numbers of suboptimal trees. Support for the tree branches was obtained by performing bootstrap analysis with 1000 replicates of simple taxon addition, with TBR swapping and retaining 20 trees per replicate.

Plastid microsatellite analysis

Five microsatellite markers, previously identified for *Orchis* by Redmond (2003) and Hooper (2004), were used to determine the haplotypes of each of the samples: Omarps, Ospprpl16A, Ospprpl16B, OsppA and Orch1. Amplification of the regions was carried out in 25-μL reactions containing 1 μL DNA template (*c.* 50 ng), 0.2 μL of each of the forward and reverse primers (at 20 μM), 0.8 μL BSA and 18 μL PCR master mix with a $MgCl_2$ concentration of 2.5 mM (ABGene, Surrey, UK). For OsppA, Omarps16 and OsppBrpl16, the PCR conditions consisted of initial denaturation at 94 °C, followed by 30 cycles of denaturation at 93 °C for 30 s, annealing at 50 °C for 1 min and extension at 72 °C for 1 min. The final extension phase was 72 °C for 8 min. A lower annealing temperature of 48 °C was used for OsppArpl16 and Orch1. The fluorescently labelled microsatellite fragments were detected via an automated sequencer (ABI Prism 3100 Genetic Analyzer), using a ROX internal size standard (DNA of known sequence labelled on one strand with ROX NHS-ester dye). Raw data were collected and sized using ABI Prism 3100 Collection and Genescan® 3.7 software. The sizes of the fragments for each region were then recorded and the combination of sizes for each microsatellite region was used to determine a haplotype for the sample, thereby allowing comparison of individual plants.

AFLP analysis

Approximately 250 ng of total genomic DNA was digested using *Eco*RI and *Mse*I enzymes, and ligated to double-stranded adaptors in a single reaction with the samples incubated at 37 °C for 2 h. Restriction–ligation, pre-amplification and selective amplification of fragments used PE Applied Biosystems Inc. (ABI) AFLP® kits, following Applied Biosystems plant mapping protocols with half-volume reactions. All thermal cycling was carried out on an Applied Biosystems Geneamp® PCR System 9700. The fluorescently labelled AFLP fragments were detected with the use of an automated sequencer (ABI Prism 3100 Genetic Analyzer), using a ROX internal size standard. Raw data were collected and sized with the use of ABI Prism 3100 Collection and Genescan® 3.7 software.

Primer testing was carried out on an anonymous subsample of eight individuals; 12 large genome primer pairs (3 + 4 selective bases) were tested. Two primer–pair combinations (*Eco*RI ACA–*Mse*I CAGC and *Eco*RI ACA–*Mse*I CTAG) were selected on the basis of the quality and quantity of bands produced and these were subsequently used in AFLP reactions on all samples. Positive controls, consisting of reference DNA samples from Applied Biosystems AFLP® kits and negative controls of water were used in all reactions.

The Genescan files were imported into Genotyper® 3.7, where the electropherograms were viewed and the presence and absence of bands were scored by eye. The presence/absence matrix obtained from the scoring of bands was subjected to Principal Coordinates Analysis (Gower, 1966) using the software package le Progiciel R v4·0d (Casgrain, 1999) and the Dice similarity measure (Dice, 1945), subtracting all values in the matrix from 1 to obtain genetic distances.

RESULTS

Variation in single morphometric characters

Characters separating the parents

Bateman & Farrington (1987; see also Bateman & Farrington, 1989; Bateman & Hollingsworth, 2004) defined three categories of character significance when comparing mean scores for two populations or meta-populations:

1. *Taxonomically diagnostic*. No overlap of twice the sample standard deviations of the populations (for continuous metric/meristic characters and ratios) or mutual exclusion (for scalar characters). Such characters are usually available if the populations being compared represent different bona fide species.
2. *Taxonomically informative (non-diagnostic)*. No overlap of the sample standard deviations or less than 33% class-sharing for scalar characters.
3. *Taxonomically uninformative*. Overlap of the sample standard deviations or more than 33% class-sharing for scalar characters.

Their original study (Bateman & Farrington, 1987), which compared single, coexisting populations of two anthropomorphic species, *O. simia* and *O. anthropophora*, revealed 20 diagnostic characters, nine taxonomically useful characters and 11 taxonomically uninformative characters. These figures contrasted strongly with a subsequent comparison of two geographically disparate populations of *O. simia* (Bateman & Farrington, 1989), which unsurprisingly revealed no taxonomically diagnostic characters, six taxonomically informative characters (all but one vegetative) and 33 taxonomically uninformative characters. The present comparison of *O. simia* and *O. purpurea* yielded a result remarkably similar to that comparing *O. simia* and *O. anthropophora*; 19 diagnostic characters (plus five ratios), seven taxonomically informative characters (plus two ratios) and 12 taxonomically uninformative characters (plus five ratios), together with three characters considered invariant or inapplicable.

The taxonomically diagnostic and informative characters are distributed fairly evenly across the various organs of the plant. *Orchis purpurea* is reliably larger than *O. simia* in all vegetative characters other than bract length; it also significantly exceeds *O. simia* in labellum and spur dimensions other than the lengths of the labellum per se, the arms and the tail (Table 1). The contrast in widths is especially strong; *O. purpurea* averages double the torso width, treble the arm width and seven times the leg width of *O. simia* (also rendering diagnostic the two ratios, 'k' and 'l', that are based wholly on limb widths). In contrast, the two species resemble each other in dimensions of the remaining perianth segments, other than the slightly longer lateral petals of *O. purpurea*. With regard to other floral ratios, the labellum of *O. purpurea* is more equidimensional and has shorter legs relative to the lengths of the arms and torso, respectively. Labella of *O. purpurea* are more or less flat and held parallel to the stem, whereas those of *O. simia* are strongly concave and are inclined upward (i.e. subtend a substantial angle relative to the stem).

Orchis simia is more strongly pigmented than *O. purpurea*, both on the upper stems and flowers (Table 1). The anthocyanins in the limbs of both species are of a similar hue and chroma at Goring, but they average three times the density in *O. simia* relative to *O. purpurea* (mean reflectivity 17 vs. 55%). The colours of the abaxial surfaces of the sepals contrast even more strongly; they are far denser and red rather than purple in *O. purpurea*. The 'chest' of *O. purpurea* bears on average twice as many

Table 1. Comparison of values for characters and ratios of the suspected hybrids at Goring compared with co-existing populations of both putative parents (*Orchis purpurea* and *O. simia*), two nearby populations of the closely related species *O. militaris* from Marlow and Turville, and an archetypal population of *O. purpurea* from its UK heartland in East Kent. Characters are numbered as in Appendix 1. Boldface characters (and ratios) are statistically diagnostic of the two parents and italicized characters are taxonomically informative but less reliable, whereas the remaining characters are uninformative

				purpurea	hybrids			*simia*			*militaris* (Marlow)			*militaris* (Turville)			*purpurea* (Kent)		
Organ Category	Character	Units	Hybrid Category	x	x	σ	CV	x	σ	CV	x	σ	CV	x	σ	CV	x	σ	CV
A	**1**	mm	1	340	409	55	13	163	45	28	273	76	28	334	86	26	423	69	16
	2	mm	2	6.60	5.69	1.33	23	3.04	0.45	15	4.32	1.20	28	5.22	1.32	25	6.12	0.84	14
	3		4	0.5	1.4	0.5	36	1.4	0.8	60	0.9	0.6	67	1.0	0	0	0.5	0.7	142
	4	mm	2	72.5	73.6	25.0	34	25.7	4.9	19	65.0	20.1	31	91.0	46.8	51	71.7	22.7	32
	5		2	32.0	26.9	9.4	35	14.3	5.6	39	23.2	11.7	50	29.6	15.0	51	28.1	7.4	26
	6	mm	4	4.0	3.3	0.8	23	3.5	1.8	51	4.9	1.6	33	4.2	0.9	21	3.9	0.8	20
	7	mm	2	11.5	11.9	0.7	6	9.1	1.1	12	11.3	1.4	12	11.6	2.5	22	13.0	1.6	12
B	*8*		2	4.5	4.7	0.5	11	3.1	0.6	20	4.1	1.0	24	4.6	1.1	24	5.5	0.7	13
	9		2	2.0	1.9	0.7	37	1.0	0.3	30	0.9	0.3	33	0.6	0.5	83	0.8	0.4	53
	10		2	0.5	0.6	0.5	83	0.7	0.5	77	0.3	0.5	167	0.6	0.5	83	0.3	0.5	161
	11	mm	3	137	105	13	13	62	11	18	108	25	23	113	32	28	169	24	14
	12	mm	2	40	37	4	11	19	3	16	33	9	27	36	8	23	49	8	17
	13	mm	2	42	38	4	10	19	3	16	33	9	27	38	11	34	51	8	16
	14		1/5	1.5	1.7	0.5	29	1.5	0.5	35	1.8	0.4	22	2.0	0	0	1.9	0.3	17
C	**15**	mm	1	15.8	17.5	1.2	7	11.5	1.5	13	13.1	1.3	10	12.5	1.5	12	15.7	2.2	14
	16	mm	3	4.50	2.60	0.18	7	1.89	0.48	25	2.95	0.41	14	2.44	0.42	17	3.56	0.58	16
	17	mm	1	11.4	14.0	1.4	10	10.7	1.2	11	12.5	0.9	7	12.2	1.4	12	11.9	1.8	15
	18	mm	2	8.9	8.4	0.7	9	6.0	0.8	13	9.7	0.6	6	9.9	1.0	11	7.9	1.0	13
	19		NA	1.0	1.0	0	0	1.0	0	0	0.9	0.3	33	1.0	0	0	1.0	0	0
	20	mm	1	1.5	2.04	0.34	17	1.25	0.47	38	0.99	0.47	48	1.04	0.21	20	0.74	0.37	50
	21	mm	1	6.8	9.0	0.8	9	6.4	1.2	19	7.6	0.7	9	7.7	1.8	23	9.4	1.6	17
	22	mm	3	1.55	1.09	0.23	21	0.58	0.12	21	1.57	0.28	18	1.62	0.26	16	1.80	0.46	26
	23	mm	4	4.1	6.5	0.9	15	6.0	0.8	13	4.4	0.5	12	4.1	0.2	6	5.8	1.2	21
	24	mm	3	5.35	2.37	0.39	17	0.77	0.26	34	3.43	0.63	18	3.18	0.40	13	4.28	0.98	23
	25	CIE	1	308	274	1	< 1	314	5	2	273	5	2	269	3	1	312	4	1
	26	CIE	5	281	174	5	3	202	25	12	169	22	13	154	15	10	281	22	8
	27	%	5	55.0	11.9	2.3	19	17.0	6.3	37	10.6	6.1	58	8.4	0.5	6	59.7	14.5	24
	28		4	60	34	8	22	27	12	44	25	5	21	25	5	20	36	11	31
	29		2	3.0	3.0	0	0	2.5	0.6	25	2.0	0	0	2.0	0	0	2.8	0.4	15
	30		3	0.5	1.0	0.4	36	2.6	0.5	20	3.1	0.7	23	2.8	0.4	14	2.4	0.5	22
	31		4	1.5	3.7	0.5	14	3.6	0.5	14	3.3	0.5	15	3.0	0	0	2.2	0.4	19

Table 1. *Continued*

Organ Category	Character	Units	Hybrid Category	*purpurea* x	hybrids x	hybrids σ	hybrids CV	*simia* x	*simia* σ	*simia* CV	*militaris* (Marlow) x	*militaris* (Marlow) σ	*militaris* (Marlow) CV	*militaris* (Turville) x	*militaris* (Turville) σ	*militaris* (Turville) CV	*purpurea* (Kent) x	*purpurea* (Kent) σ	*purpurea* (Kent) CV
D	*32*	mm	2	6.25	6.07	0.82	14	4.5	0.9	20	5.4	0.6	10	5.7	0.7	13	4.6	0.4	9
	33	mm	3	2.00	1.79	0.29	16	1.50	0.25	17	2.42	0.27	11	2.46	0.09	4	2.51	0.14	6
	34		1/5	4.0	5.0	0	0	4.0	0	0	4.2	0.4	10	4.2	0.4	10	5.0	0	0
E	*35*	mm	2	9.0	8.6	1.3	15	7.2	0.8	11	10.9	0.8	8	11.1	0.9	8	6.5	0.5	8
	36	mm	1/5	1.30	1.40	0.19	14	1.31	0.34	26	1.72	0.29	17	1.60	0.12	8	1.01	0.14	14
F	37		2	11.2	10.8	1.5	14	9.9	1.6	16	12.2	1.2	9	12.2	1.2	10	8.5	0.7	8
	38		3	4.4	4.2	0.2	6	4.1	0.5	12	4.9	0.4	9	4.9	0.3	7	4.1	0.2	5
	39	CIE	4	455	313	12	4	312	0	0	303	0	0	303	0	0	415	26	6
	40	CIE	1	282	237	41	17	292	0	0	270	0	0	270	0	0	257	23	9
	41	%	3	6	32	20	62	62	0	0	48	0	0	48	0	0	7	3	44
	42		NA	1.0	1.0	0	0	1.0	0	0	0.4	0.5	125	0.4	0.5	125	0.9	0.3	35
	43		NA	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Ratios	a		5	0.019	0.014	0.03	19	0.018	0.003	17	0.016	0.003	21	0.016	0.002	12	0.013	0.002	15
	b	%	3	21.3	17.7	4.7	27	16.0	3.9	24	23.9	3.5	15	26.3	7.6	29	16.7	4.2	25
	c	fls/cm	1	4.4	3.6	0.4	11	5.5	1.8	33	3.5	1.0	28	3.3	0.3	9	4.1	0.8	21
	d		1	0.26	0.22	0.05	21	0.28	0.12	43	0.30	0.05	18	0.27	0.07	26	0.23	0.04	16
	e		NA	0.35	0.34	0.04	10	0.33	0.05	15	0.32	0.02	7	0.33	0.03	8	0.27	0.03	10
	f		2	0.23	0.26	0.02	7	0.22	0.03	14	0.23	0.04	18	0.25	0.03	11	0.23	0.03	15
	g		2	0.58	0.56	0.03	6	0.48	0.03	6	0.51	0.02	3	0.50	0.01	2	0.43	0.04	9
	h		4	0.43	0.52	0.03	5	0.51	0.05	10	0.44	0.03	6	0.44	0.04	8	0.54	0.04	7
	i		3	0.32	0.43	0.03	7	0.50	0.05	10	0.31	0.03	8	0.29	0.01	5	0.42	0.05	12
	j		3	0.63	0.59	0.02	4	0.51	0.05	10	0.63	0.03	4	0.65	0.04	6	0.62	0.03	4
	k		3	0.19	0.11	0.01	12	0.08	0.01	16	0.17	0.03	15	0.18	0.02	12	0.16	0.04	24
	l		3	0.56	0.27	0.03	10	0.11	0.04	35	0.44	0.06	13	0.44	0.03	6	0.43	0.09	21

CIE, Commission Internationale de l'Eclairage; NA, not applicable.

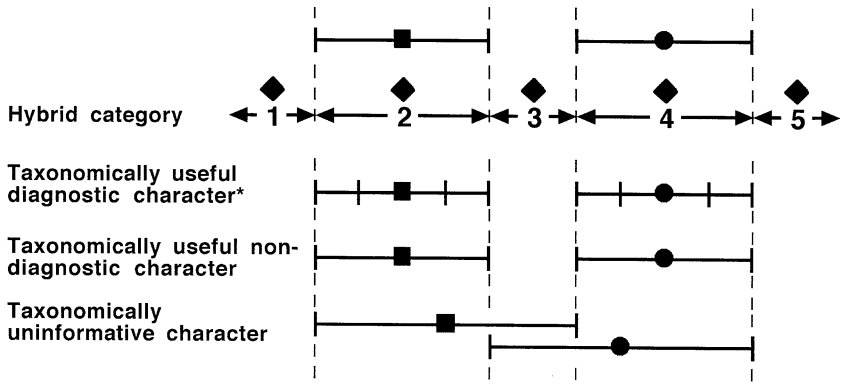

Figure 5. Explanation of the five hybrid categories used in Table 1 and of classes of diagnostic characters (modified after Bateman & Farrington, 1987, fig. 2).

purple-stained clusters of papillae (mean 60 vs. 27), although, given that *O. purpurea* has a more extensive chest than *O. simia*, the density of papillae appears broadly similar in the two species.

Two additional characters of relevance subsequently described by Raper (2006–2008) were not formally scored during the present analysis. Firstly, the leaves of *O. purpurea* are bright green, whereas those of *O. simia* are grey–green, apparently a consequence of their less reflective cuticle. Secondly, the inflorescences of most populations of *O. simia*, including that at Goring, open from the top downward, whereas *O. purpurea* reliably exhibits a more conventional bottom-up phenology (Summerhayes, 1968; Bateman & Farrington, 1989; Bateman & Rudall, 2006).

Turning briefly to morphological variation within the anthropomorphic *Orchis* species, several significant differences between the *O. simia* populations of Oxfordshire and Kent were discussed in detail by Bateman & Farrington (1989), who identified six taxonomically informative (although no diagnostic) characters. In contrast, the present study revealed strong morphological similarity between the populations of *O. militaris* in the Chilterns at Turville and Marlow; this will be discussed in greater detail elsewhere (R. Bateman & P. Rudall, unpublished). Perhaps the most informative comparison is that between the two plants of *O. purpurea* from Goring and the large population at Covert Wood, East Kent. The modest vegetative differences between the two populations evident in Table 1 could be attributed to the shadier habitat of the Kentish plants, but this is unlikely to explain the floral differences observed. Specifically, the Goring plants have rounder labella and contrasting proportions of appendages (Figs 1D, 2): they have wider torsos richer in purple-coloured papillae, shorter arms, shorter but wider legs and a longer tail; also, the entire labellum is presented perpendicular to the stem. The spur is longer but narrower, and all five of the perianth segments that form the hood are longer.

Comparison of the hybrids with their parents

In order to explore inheritance patterns in the single Kentish plant of *O. simia* × *anthropophora*, Bateman & Farrington (1987 *et seq.*) defined five categories of character state found in the hybrid plant according to its relative similarity to the two parental populations (Fig. 5). This approach is slightly more problematic to apply to the present data, as we are analysing seven hybrid plants rather just one, so that we are attempting to categorize an (admittedly limited) range of variation rather than an invariant value for each character. Nonetheless, by focusing on mean values for hybrids and parents, the established principles remain applicable.

In this case, the hybrids are morphologically more extreme than either parent (categories 1 and 5) in four taxonomically informative characters and eight taxonomically uninformative characters. They have slightly taller stems and wider labella than *O. purpurea* and are slightly darker flowered than *O. simia*. They are intermediate to the parents (category 3) in seven informative characters and one uninformative character; these include the widths of the spur, torso, arms and legs, together with leaf length and density of pigment on the reverse of the sepals. The hybrids resemble *O. purpurea* (category 2) in eleven informative and three uninformative characters. These are mostly vegetative: stem diameter, inflorescence length, number of flowers, ovary length, leaf width and numbers of basal leaves and sheathing leaves. Floral characters include the lengths of the torso, spur and lateral petals. In contrast, the hybrids resemble *O. simia* (category 4) in a rather heterogeneous suite of only five characters: vegetative anthocyanins, leg length, number of papillae on the chest, outward curvature of the limbs and redness of

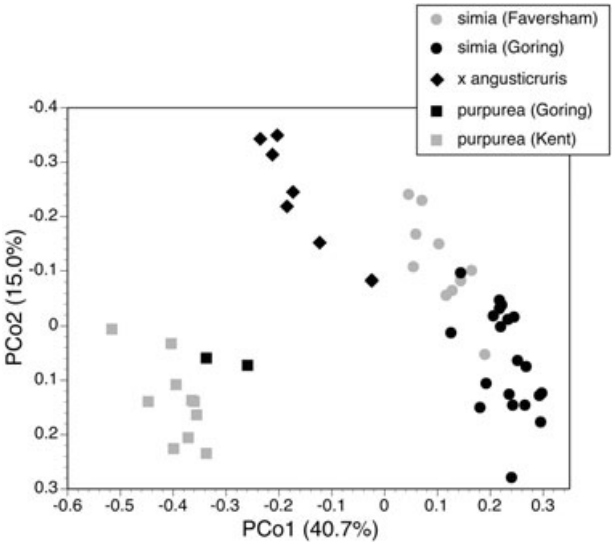

Figure 6. Principal coordinates plot of the first two axes for morphometric data from the putative hybrid and both parents (*O. purpurea* and *O. simia*) at Goring, together with ten additional plants of *O. purpurea* from East Kent.

the reverse of the sepals. The three remaining characters show insufficient variation to be meaningfully categorized.

Multivariate analyses of morphometric data

Not surprisingly, the principal coordinates analysis confined to *O. simia*, *O. purpurea* and their putative hybrids (Fig. 6) encapsulated an unusually high degree of the total variance (over 40%) in the first coordinate. Many characters contribute to the axis, reflecting the more vigorous plants, broader labellar limbs and dark reddish-brown hoods of *O. purpurea* (left) relative to *O. simia* (right). These characters also permit partial separation of the Kentish from the Oxfordshire populations of both parents, which are in both species more robust in Kent. The substantial discontinuity separating the two parents is largely bridged by the seven putative F1 hybrids; of these, all but the smallest plant clearly show greater similarity to *O. purpurea* than to *O. simia*. The second coordinate, which is substantially weaker, partially separates the hybrids from both parents and, to a lesser extent, the Kentish from the Oxfordshire populations of *O. simia*, on the basis of their relatively tall stems and the long, dark red limbs of their labella. The third axis revealed no biologically interesting trends.

As anticipated, adding *O. militaris* to the previous analysis (Fig. 7) yielded a more multidimensional result and so slightly weakened the first axis. The first two coordinates (Fig. 7A) gave very similar relative placements of *O. purpurea*, *O. simia* and their

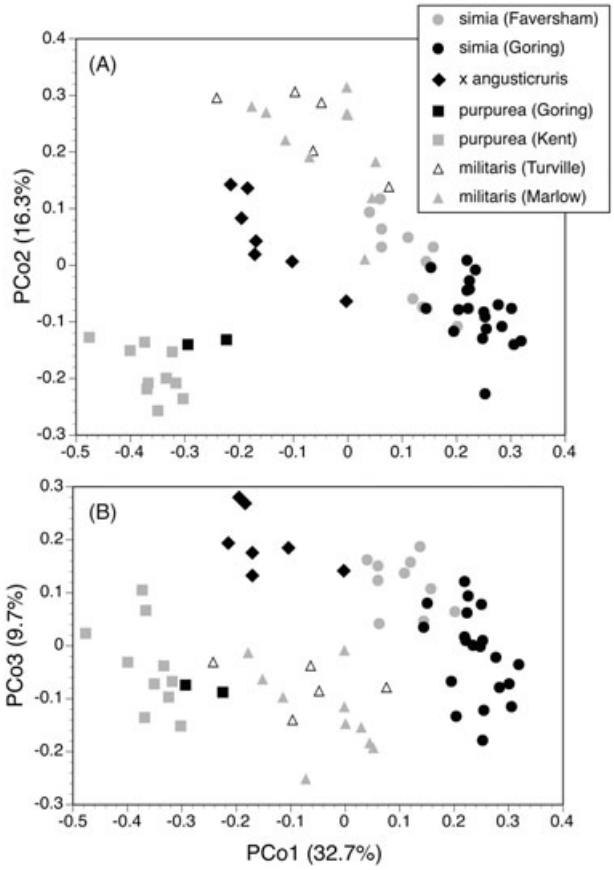

Figure 7. Principal coordinates plot of (A) the first and second axes and (B) the first and third axes for morphometric data from the putative hybrid and both parents (*O. purpurea* and *O. simia*) at Goring, plus samples of *O. purpurea* from East Kent and of *O. militaris* from Marlow and Turville (cf. Fig. 6).

hybrids, with *O. militaris* almost precisely coinciding with the hybrids. However, the somewhat strengthened second axis successfully separates *O. militaris* from the hybrids, although slight morphological overlap is evident between the smallest individuals of *O. militaris* from the Chilterns and largest individuals of *O. simia* from Kent. The weaker third coordinate (10%) serves primarily to separate the hybrids from *O. militaris*, the two parental species yielding intermediate scores (Fig. 7B). Thus, in overall morphology, the hybrids more closely resemble *O. militaris* than either parent, but nonetheless they can readily be distinguished from *O. militaris* using characters contributing to the second axis.

Genetics

Amplified fragment length polymorphism

Presence/absence data for genomic fragments generated during AFLP analysis were subjected to princi-

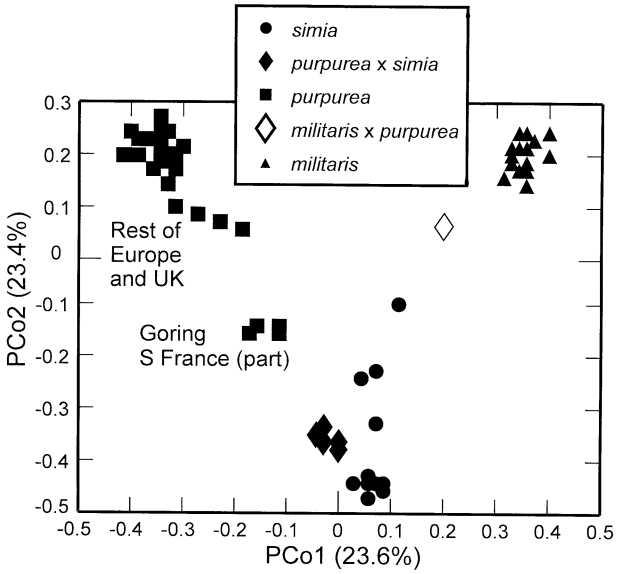

Figure 8. Principal coordinates plot of the first two axes for amplified fragment length polymorphism (AFLP) data from the putative hybrid and both parents (*O. purpurea* and *O. simia*) at Goring, plus representative samples from several other locations in Western Europe.

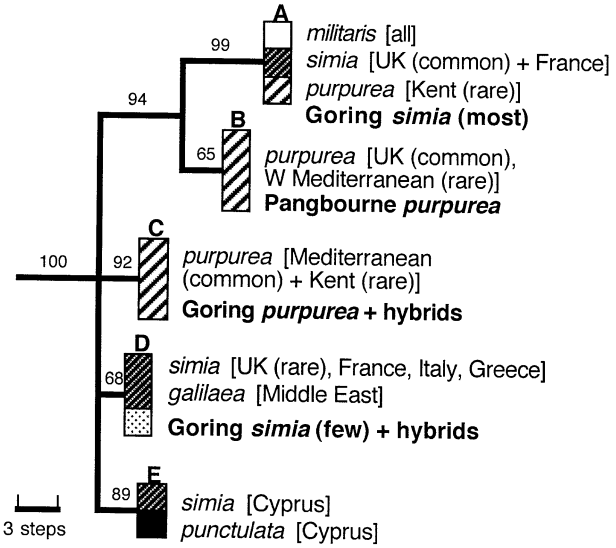

Figure 9. Summary of most-parsimonious trees for internal transcribed spacer (ITS) alleles from 69 accessions of *Orchis* s.s. sampled across Europe and Asia Minor. Figures show percentage bootstrap support.

pal coordinates analysis (Fig. 8). Individual plants of the three species group strongly, although plants of *O. purpurea* form two clusters; the smaller cluster, which consists of both plants from Goring plus two from southern France, is intermediate between the main cluster of *O. purpurea* (which contains all other English plants) and that of *O. simia*. The first coordinate (29%) separates *O. purpurea* (especially the larger cluster) from *O. militaris*. *Orchis simia* occupies an intermediate position on the first coordinate but it is readily separated from the other two species on the second coordinate, which accounts for 23% of the total variance – almost as much as the first axis. The broad discontinuities separating the three species offer excellent prospects of identifying any F1 hybrids. A specimen of *O. militaris* × *purpurea* from France is significantly closer to *O. militaris* (most likely its ovule-parent) than to either cluster of *O. purpurea*. In contrast, the seven plants of *O. purpurea* × *simia* from Goring are placed remarkably close to *O. simia*, even allowing for the presence of the presumed *O. purpurea* parents in the more proximal cluster.

Internal transcribed spacers

ITS alleles yielded broadly similar results to those obtained from AFLP but show weaker clustering of accessions according to taxonomy (Fig. 9), suggesting that there has been significant and relatively recent gene flow among all three species of interest. The results show that the typological phylogeny generated by Bateman *et al*. (2003) is highly simplistic, obscuring a much more interesting pattern (cf. Figs 3, 9).

Much the largest, most divergent and best-supported ITS clade (A) includes all of the British and Continental accessions of *O. militaris*, together with most of the British and some Continental accessions of *O. simia*, plus several cloned alleles from two accessions of *O. purpurea*: one from Kent and the other from France. The sister group (B) contains only *O. purpurea* accessions, including the great majority of the accessions analysed from England and a few French samples. Three further groups (C–E) are less divergent and form a polytomy; in total they encompasses four widely recognized species. The first of these groups (C) contains a mixture of English and Continental *O. purpurea*. The two remaining groups require more intensive sampling but currently suggest strong geographic constraints. Group E contains the only available accessions of both *O. simia* and *O. punctulata* from Cyprus. Lastly, Group D (poorly supported) contains accessions of *O. simia* from France, Italy and Greece, plus the closely related *O. galilaea* from the eastern Mediterranean. Interestingly, Group D alleles also occur in a minority of individuals of *O. simia* from Goring; of eight individuals analysed in the present study, five were uniformly Group A, one was uniformly Group D and two were polymorphic for Groups A and D.

Thus, *O. purpurea* is represented by several accessions in each of the three main allelic groups (A–C);

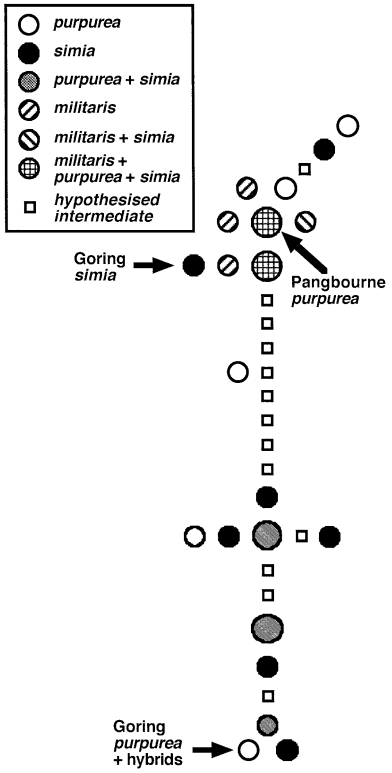

Figure 10. Simplified plastid haplotype network for *c*. 200 accessions of *Orchis* s.s. sampled across Europe, showing the relative positions of Goring accessions of *O. simia*, *O. purpurea* and their hybrids, and of the nearby populations of *O. purpurea* from Pangbourne and Bix. The larger circles indicate the more common haplotypes.

moreover, just one East Kent population (Stockbury Hill Wood) is represented by alleles occurring in all three clusters. The Goring *O. simia* contained mainly Group A alleles and the nearby *O. militaris* yielded only Group A alleles. The Goring *O. purpurea* yielded only Group C alleles, whereas the *O. purpurea* nearby at Pangbourne and Bix produced only Group B alleles. Predictably, the two cloned *O. purpurea* × *simia* hybrids from Goring contained ITS alleles from Group C, presumably derived from co-occurring *O. purpurea*. However, less predictably, the hybrids also inherited (presumably from co-occurring *O. simia*) Group D alleles, which were eventually shown to occur in *O. simia* at Goring but are far less common there than Group A alleles.

Plastid microsatellites

Data for plastid microsatellites showed the weakest correlation with taxonomic assignment. Four variable regions yielded a total of 19 haplotypes (Fig. 10), although these formed two main clusters. The first cluster, epitomized by haplotype 15, is dominated by *O. simia* and lacks *O. militaris*. The second cluster, epitomized by haplotype 6, is dominated by *O. militaris* but contains a few individuals assigned to *O. simia*. Surprisingly, individuals of *O. purpurea* were distributed approximately equally between the two main clusters. Continental populations showed a wider range of haplotypes from both the *miltaris* and *simia* clusters, whereas most English populations were dominated by haplotype 6 (*militaris* cluster).

Interestingly, the *O. purpurea* plants from Goring were the one exception; they possessed haplotype 4, a slight modification of haplotype 6 that was otherwise found only at low frequencies at Stockbury Hill Wood in East Kent and more often, apparently at higher frequencies, in several populations from southern France. This haplotype was also inherited from these *O. purpurea* plants by the *O. purpurea* × *simia* hybrids at Goring, where the *O. simia* plants reliably yielded haplotype 8 – an unusual haplotype similar to the more widespread haplotype 7 (Fig. 10).

DISCUSSION

Predisposition of anthropomorphic *Orchis* to hybridization

Like almost all orchids, anthropomorphic *Orchis* lack strong intrinsic sterility barriers (e.g. Scopece *et al*., 2007). In addition, the karyotypes (Moore, 1982) and DNA (Bateman *et al*., 2003; Fay *et al*., 2007) of species in this group are sufficiently similar to allow them to cross with relative ease, a fact that has been demonstrated repeatedly in cultivation. In the wild, the species in this group appear to rely for pollination on a range of bees, bumble bees and, occasionally, flies (Kretzschmar *et al*., 2006), most of which are seeking nectar. The insects are engaged in a fruitless search, as all anthropomorphic *Orchis* species lack nectar despite the presence of substantial labellar spurs in all species but *O. anthropophora* (reviewed by Box *et al.*, 2008); they deceive pollinators into transferring pollinia between flowers (e.g. Cozzolino & Widmer, 2005).

Certainly, hybrids among anthropomorphic *Orchis* are encountered throughout the ranges of the parents (Wollin, 1972; Bournérias & Prat, 2005; Delforge, 2006; Kretzschmar *et al*., 2006) and involve any pair of species – indeed, three-way combinations of genomes have occasionally been inferred in single plants (e.g. Wollin, 1972), although with inadequate supporting evidence. In some cases, large populations of the parents apparently yield extensive hybrid swarms, but, even when numbers of flowering plants of both parents are small, hybrids can result. For example, at Faversham in Kent, typically a dozen flowering plants of *O. simia* grew alongside an even

smaller number of *O. anthropophora*, yet an F1 plant was generated (Bateman & Farrington, 1987) (Fig. 1G, H). Our results for AFLP analysis, and especially ITS alleles and plastid haplotypes, all suggest that hybridization progressed beyond the F1 generation and that the anthropomorphic *Orchis* species thereby remain evolutionarily interconnected by significant gene flow.

Phylogenetic context of the parents

The essentially typological nuclear ribosomal ITS phylogeny of Bateman *et al*. (2003, fig. 2) placed *O. purpurea* as sister to *O. militaris*, differing by eight steps and separated from *O. simia* by a similar phylogenetic distance, but substantially more phylogenetically removed from *O. italica* and *O. anthropophora* (Fig. 3), which are united morphologically by possession of acute labellar lobes. Similar relationships and relative degrees of molecular disparity were inferred using an unrooted AFLP tree by Qamaruz-Zaman *et al*. (1998, fig. 3), except that *O. purpurea* was placed closer to *O. simia* than to *O. militaris* (cf. Fig. 8). In addition, the 24 plants of *O. simia* sampled from Goring by Qamaruz-Zaman *et al.* were separated from, and significantly less molecularly divergent than, five individuals sampled from various localities in Italy and Greece.

A far more complex pattern has emerged from the present study (Figs 8, 9). AFLP data show greater dissimilarity between *O. militaris* and *O. purpurea* than either shows from *O. simia*, but reveal two clusters within *O. purpurea*. ITS alleles suggest that *O. simia* may share alleles with *O. punctulata* in the eastern Mediterranean and it often shares alleles with *O. militaris* in the west (presumably because of past introgression); moreover, *O. purpurea* plants are admixed with both of the other species. Plastid haplotypes show a similar pattern: distinct clusters nucleate on *O. simia* and *O. militaris*, but with *O. simia* infiltrating *O. militaris* and *O. purpurea* haplotypes overlapping with those of both of the other species (Fig. 10).

Overall, the impression gained is that *O. simia* is the most distinct species, but in western Europe it acts as the ovule parent in apparently frequent hybridization with *O. militaris*. The most difficult species to characterize is *O. purpurea*, which is comparatively genetically heterogeneous; possessing two distinct ITS types but lacking distinctive plastid haplotypes, it is the subject of ongoing research. For the present, the anthropomorphic *Orchis* species pose serious challenges to both molecular phylogenetics and molecular identification through DNA barcoding (e.g. Savolainen *et al.*, 2005; Cowan *et al.*, 2006).

The mother was *Orchis purpurea* and the father was *Orchis simia*

The morphometric analyses (Figs 6, 7), AFLP study (Fig. 8), ITS sequences (Fig. 9) and microsatellite data (Fig. 10, Table 2) all show that the study plants from Goring were correctly identified in the field as hybrids between *O. purpurea* and *O. simia*; this is the first time that this hybrid combination has been formally recorded in Britain (cf. Stace, 2009).

The stronger phenotypic similarity of the hybrids to *O. purpurea* initially suggested that this species was the mother (Bateman, 2006b). Subsequent univariate analyses demonstrate that, vegetatively, the hybrids are near-identical to *O. purpurea* and most floral characters are either intermediate between the parents or closer to *O. purpurea*; they more closely resemble *O. simia* only in the relatively long legs, inrolled (welcoming) arms, fewer, larger clusters of papillae on the chest, dark-coloured limbs and pale purple (rather than dark red) abaxial surfaces to the sepals (cf. Fig. 1B–E; Table 1). Similarly, the first axis of the multivariate ordination of hybrids plus parents places the hybrid cluster significantly closer to *O. purpurea* than to *O. simia*, although the weaker second axis has the converse effect (Fig. 6).

Some of the molecular data supported the morphological inference of maternity. Plastids are typically, and perhaps universally, maternally inherited in orchids (Corriveau & Coleman, 1988; Cafasso, Widmer

Table 2. Comparison of plastid haplotypes found in *Orchis simia*, *O. purpurea* and their hybrids from Goring, demonstrating that *O. purpurea* was the ovule-parent of the hybrids

		Plastid microsatellite				
Taxon	No. analysed	Om16	Orch1	OsppA	Arpl16	Brpl16
simia	6	217	101	175	94	185
Hybrid	7	217	100	175	93	183
purpurea	2	217	100	175	93	183

& Cozzolino, 2005). Those of the hybrids yielded haplotypes that matched those of the two co-occurring plants of *O. purpurea*, which share a haplotype that is rare in populations of this species in England (Fig. 10). Predictably, the hybrids contains ITS alleles characteristic of both parental species at the Hartslock site, although the copy paternally inherited from *O. simia* is the less common of the two alleles found in the population (Table 2). Admittedly, the AFLP profiles of the hybrids show greater similarity to *O. simia* than to *O. purpurea*; nonetheless, the weight of evidence strongly favours *O. purpurea* as the ovule parent.

This maternity could perhaps be predicted from the relative phenologies and population sizes of the anthropomorphic *Orchis* at Goring. In recent years, the *O. simia* population has typically yielded hundreds of flowering plants, whereas *O. purpurea* has not exceeded two inflorescences, thus offering pollinators far fewer pollinia for collection (but many targets for deposition, in the form of *O. simia* inflorescences). In addition, at Goring the peak flowering of *O. purpurea* precedes that of *O. simia* by 10–15 days (Raper, 2006–2008), whereas the peak flowering of the hybrids is intermediate between those of the parents. Other studies suggest that the lower flowers in an orchid inflorescence are much more likely to be the pollen recipients, whereas the upper flowers are more commonly the pollen donors, presumably because pollinators typically work upwards along the inflorescence (e.g. Tremblay, 2006; see also Bateman & Rudall, 2006) – in this case making *O. purpurea* the more probable ovule parent. The two-week phenological separation of the parents is sufficiently narrow to allow successful cross-pollination. For example, the much-discussed early and late-flowering populations of *Neotinea* (formerly *Orchis*) *ustulata* differ in phenology by an average of 5–6 weeks and are reliably separated spatially by kilometres, yet they show evidence of continued gene flow between early and late-flowering populations across their European range (Tali, Fay & Bateman, 2006).

In addition, the F1 hybrid plants are sufficiently similar in appearance to each other (Figs 1, 2) to suggest that they resulted from a single pollination event between the two mis-matched parents, most likely representing a lapse of concentration on the part of a passing bee engaged in a (fruitless) search for nectar. Genetic similarity among the hybrids is further suggested by tight clustering of their AFLP profiles (Fig. 8) and the uniformity of their ITS and plastid genotypes (Figs 9, 10). Cross-fertilization most likely occurred within four years of *O. purpurea* first flowering at Goring in 1999, given that the closely related *O. militaris* can reach flowering size from seed in two years under *in vitro* cultivation (R. Manuel, pers. comm., 2007).

Asymmetrical inheritance of phenotype favouring the mother is a widespread phenomenon among European orchids

We now have available for consideration three recent reports of rare hybrids between anthropomorphic *Orchis* species in Britain, two of them subject to detailed case studies (Bateman & Farrington, 1987; present study). These studies are supported by less detailed investigations of natural hybrids on the Continent (Peitz, 1970) and the generation of artificial hybrids of known parentage by orchid enthusiasts (e.g. Malmgren, 2004). Together, they should allow some generalizations to be made regarding the relative heritability of particular characters within the group. For example, each of the English examples of such hybrids has exhibited denser, darker floral pigments than either parent, suggesting reinforcement of the relevant biosynthetic pathway. Also, discrete markings on various floral organs are reliably inherited, so that in the case of the Kentish *O.* × *bergonii*, the sepals of the hybrid combined the coloured margin that characterizes *O. anthropophora* with the more central spots that typify *O. simia*. However, even these 'rules' of inheritance appear less reliable when sufficient examples of a particular hybrid combination are available (cf. Bournérias & Prat, 2005: 67).

In fact, the most striking, and certainly to one of us (RMB) the most interesting, generalization evident from these case studies is that in each case the hybrid showed strong asymmetry in inheritance of traits from its two parents. In the case of the Kentish *O.* × *bergonii*, of 28 taxonomically useful, non-overlapping characters that could be categorized, the hybrid was intermediate in 13, resembled *O. simia* in 10, but resembled *O. anthropophora* in only five. And in the present example of *O.* × *angusticruris*, of 26 taxonomically useful characters that could be categorized, the hybrid was intermediate in seven characters, resembled *O. purpurea* in 12, but *O. simia* in only seven. Thus, in both cases, the hybrids resemble one parent twice as strongly as the other.

Nor is this phenomenon confined, within subtribe Orchidinae, to the re-circumscribed genus *Orchis*. For example, Bateman & Hollingsworth (2004) showed that *Anacamptis* × *albuferensis* was intermediate between its parents in the majority of morphometric characters, but resembled *A. fragrans* in 14 characters and *A. robusta* in eight, again approximating a 2 : 1 ratio. Using RFLP and sequence data for biparentally inherited nuclear ITS and sequencing of the maternally inherited plastid region *trnL*, they were able to demonstrate that the 'mother' (ovule parent) contributed substantially more morphological traits to her offspring than did the 'father' (pollen parent). This has

also proven to be the case in the present study, and we would hazard a guess that *O. simia* was the mother of the hybrid with *O. anthropophora* found in Kent in the 1980s. In addition, one of us (RMB) has frequently been presented with artificially generated hybrids of parentage unknown to him but known to the breeder and, in most cases, he has experienced little difficulty in identifying the stronger phenotypic influence of the maternal parent.

We cannot currently explain this consistent result of a substantially stronger contribution to phenotype from the mother relative to the father. It is tempting to attribute this phenomenon to greater contributions from the maternally inherited organelles (i.e. plastids and/or mitochondria), but neither is known to exert significant influence over morphology. We are more likely witnessing either a strong influence of the cytoplasm on nuclear gene expression or possibly an unusually strong form of cytoplasmic inheritance.

Given that AFLP data (Vos *et al*., 1995) are supposedly a random selection of genic regions, potentially sampling all three genomes within each plant (although predominantly nuclear), one might expect the hybrid cluster to be placed midway between the two parental clusters in the AFLP ordination (Fig. 8). However, as with morphology, the hybrids occur substantially closer to one parent than the other. Moreover, in this case, the hybrids more closely resemble the paternal parent, *O. simia*, than the maternal parent, *O. purpurea*. Similar behaviour has been recorded in other AFLP studies (for discussion of similar situations in *Schoenoplectus*, *Sorbus* and the temperate orchid *Dactylorhiza*, see Fay *et al.* (2003, 2007), where it has variously been attributed to (1) larger numbers of fragments generated from the larger genome, in cases where the two differ substantially in size, or (2) biased back-crossing toward one of the two parents. However, in the present case, the two parents are known to have identical chromosome numbers ($2n = 42$: Moore, 1982) and are likely to have similar genome sizes; in addition, weight of evidence gives us confidence that the seven flowering plants of the hybrid are F1 individuals that most likely were derived from a single mis-matched cross-pollination event. This leaves as the most likely explanation for the AFLP asymmetry transmission ratio distortion – the over-representation of alleles from one parent in the offspring of intraspecific or interspecific crosses. This phenomenon has been widely reported in the plant kingdom (Fishman *et al*., 2001; Myburg *et al*., 2004; Hall & Willis, 2005). A form of reproductive isolation, it can be attributed to interactions between the heterospecific genomes as a result of substantial genetic divergence between the parental genomes (e.g. Fishman *et al*., 2001).

The questionable origins of *Orchis purpurea* and *Orchis* × *angusticruris* at Goring

We commenced this study entertaining six competing hypotheses regarding the origin of the population of *O. purpurea* at Goring, the first four involving natural agencies and the last two mankind's deliberate intervention:

1. Seed and/or tubers lay undetected at Goring for perhaps a century since the apparent disappearance of previously extensive native populations of *O. purpurea* from the area.

The remaining five theories specify recent arrival:

2. Seed arrived in air currents from the small nearby population of *O. purpurea* at Pangbourne.
3. Seed arrived in air currents from more extensive but more distant English populations of *O. purpurea* in Kent.
4. Seed arrived in air currents from Continental populations of *O. purpurea*.
5. Seed was deliberately or accidentally spread at the site.
6. Tubers were deliberately planted at the site.

We begin by rejecting Hypothesis 1. First principles suggest that seed and/or tubers could not have lain undetected at Goring for perhaps a century since the apparent disappearance of *O. purpurea* from the area. Firstly, such long-term viability is highly improbable; embryos in the seeds are too small and too fragile to survive for a century underground (Rasmussen, 1995) and most demographic studies of terrestrial orchids suggest that tubers very rarely survive 'blind' for more than two successive years (e.g. Wells, 1981; Hutchings, 1987; Hutchings, Mendoza & Havers, 1998). Secondly, for much of the 20th century, Goring was believed to be the only population of *O. simia* to have persisted in Britain (Paul, 1965; Harrap & Harrap, 2005). Given its rarity and consequent high conservation status, the population has been monitored increasingly carefully since its demographic nadir in 1950. However, the first plant of *O. purpurea* known to flower at the site did so only in 1999.

Turning to Hypothesis 2, one possible source population of *O. purpurea* still exists near Hartslock. In 1961, a single flowering plant of *O. purpurea* was found just 4 km from the Goring site, near Pangbourne (Paul, 1965; Kemp, 1987); this eventually bulked up to a maximum of 29 flowering plants in 1997 (Harrap & Harrap, 2005). Another plant was found 12 km east of Goring near Bix, flowering for the first time in 2006. Single leaf tips from each of these localities were included in the present molecular analysis. Plants from both sites proved identical and had the commonest ITS and plastid genotypes found

in the species in England, but neither population showed a close genetic similarity to the two plants of *O. purpurea* sampled from Goring (Figs 8–10). Thus, we reject Hypothesis 2.

Our data also challenge Hypothesis 3, as the genotype of the Pangborne *O. purpurea* is also that most commonly found in its UK heartland in Kent (described in detail by Rose, 1949). Although the plastid haplotype found in the Goring *O. purpurea* was also found (albeit at a low frequency) in one Kentish population, the unusual ITS genotype of the Goring plants has only otherwise been found thus far in Continental (specifically, French) plants, and the AFLP data also tightly grouped the Goring *O. purpurea* with two French plants. Admittedly, the single Kentish sample of *O. purpurea* that contained the typically French ITS allele was not included in the AFLP analysis. Nonetheless, we are confident that the weight of evidence shows that the Goring *O. purpurea* plants are highly unlikely to have originated from pre-existing British populations; we therefore reject Hypotheses 1–3.

Thus, we conclude that the plants originated from Continental stock, arriving either via long-distance wind transport or by the agency of man. *Orchis purpurea* is widespread across much of Europe and it is substantially more common than in Britain over much of its range (e.g. Bournérias & Prat, 2005). Also, one of us (RMB) has long speculated on the potential for dispersal of orchid dust seed (perhaps deep frozen) in high-level air currents (e.g. Bateman & Hollingsworth, 2005), making Hypothesis 4 a viable proposition. Given that the anthropomorphic *Orchis* species most likely share the same range of mycorrhizal fungi to aid germination and provide nutrition, there is a reasonable probability that any viable seed arriving at the Goring site would successfully establish itself. This statement would apply equally to dust-like seeds deliberately spread at the site by man and those arriving by their own devices, most likely carried by the wind.

Realistically, deliberate introduction by man seems the most likely explanation for the presence of *O. purpurea* at Hartslock. We would like to believe that even the most enthusiastic aficionado would quail at deliberately planting foreign tubers at such a conservationally sensitive site (Hypothesis 6). However, some orchid enthusiasts have certainly been known to deliberately spread seed of rare native orchids in conducive habitats (Hypothesis 5: e.g. Lousley, 1976: 359–360). Although this practice brings a degree of 'sink-or-swim' fatalism to the introduction that mimics the vicissitudes of natural migration, it should nonetheless be discouraged, as it assists neither science nor conservation. In this particular case, we appear to have expended considerable time and effort examining a classic red herring, although fortunately the 'side benefits' of this study have proven considerable.

One notable example was our increasing sense of obligation to explore in greater detail genetic variation within *O. simia* at Goring. Previous analysis of two individuals (Hooper, 2004) suggested that the population contained only Group A ITS alleles, which also characterize the Kentish locality. Thus, the paternal inheritance from *O. simia* of the apparently wholly Continental Group D alleles by *O.* × *angusticruris* strongly suggested that the hybrids had, like their mother, most likely been deliberately introduced into the Goring site. However, our subsequent analysis of eight individuals of *O. simia* from Goring during the present study revealed five individuals with only Group A alleles, one with only Group D alleles and two apparently polymorphic for both alleles. Only the rarest of these three genotypes – uniform Group D – is likely to have fathered the *O.* × *angusticruris* at Goring, suggesting that hypotheses considered improbable should nonetheless be taken seriously and appropriately tested. However, given that Group D alleles are common in *O. simia* in Continental Europe, we can no longer use the ITS sequences to confidently reject the possibility that the hybrids were bred from parents that were both of Continental origin and were deliberately introduced to Goring alongside their mother, the Continental *O. purpurea*.

A final twist to this tale was provided by the recent suggestion that Continental pollinia of *O. simia* may have been deliberately introduced into the Hartslock population during the 1980s or 90s (cf. pers. comm. from R. Manuel, C. Raper and N. Phillips, 2008). This could potentially explain the presence of the Group D alleles at low frequencies.

Could hybridization between *Orchis purpurea* and *Orchis simia* have given rise to *Orchis militaris*?

While admitting that there was clearly considerable confusion during the 18th and 19th centuries, our current knowledge of the appearance of the anthropomorphic species means that few experienced field botanists would confuse *O. simia* with *O. purpurea* (cf. Fig. 1D, E). Rather, it is the partial morphological intermediacy of *O. militaris* (Fig. 1F) relative to the other two species that continues to cause identification problems (note that Linnaeus, and many later botanists, combined the three species under a more broadly circumscribed *O. militaris* L.). Indeed, this morphological intermediacy causes greater confusion in parts of mainland Europe, notably France and the Low Countries. In such regions, two or more of these anthropomorphic species commonly co-occur, sometimes in impressive numbers and often hybridizing

with apparent enthusiasm (Peitz, 1970; Wollin, 1972; Bournérias & Prat, 2005; Delforge, 2006; Kretzschmar *et al*., 2006).

Reconsidering the tendency of some initial observers of the Goring hybrids to view them as *O. militaris*, if one compares the two taxa in sufficient detail, several significant differences emerge (cf. Fig. 1B, C vs. 1F; Table 1). The limbs of the hybrids are more incurved, longer and narrower than those of *O. militaris* (rendering indices 'k' and 'l' especially diagnostic), although the torso is somewhat shorter; also, the tails of the hybrids are on average twice as long. The non-labellar perianth segments are shorter and slightly narrower, and the spurs are narrower and more downcurved. The hybrid plants show on average slightly greater vegetative vigour, as well as typically possessing two bracteose cauline leaves rather than one. The labellar margins of the hybrids are darker in colour and the pigmented papillae are on average more numerous and more widely distributed. Also, all of the hybrids bear dots and/or dashes on the reverse of the sepals, whereas less than half of the individuals of *O. militaris* bear any such markings.

Overall phenotypic similarity, as summarized in the principal coordinates analysis (Fig. 7), placed both *O. militaris* and the hybrids as intermediate between *O. purpurea* and *O. simia* on the strong first coordinate. The second axis served primarily to separate *O. militaris* from the remaining groups, although it shows some morphological overlap with both the hybrids and *O. simia*. The weaker third axis completely separates *O. militaris* from the hybrids. Thus, morphology does indeed indicate similarity between *O. militaris* and the hybrids of *O. purpurea* with *O. simia*. However, it also shows that the hybrids differ from *O. militaris* by approximately 10% of the total variance and so can be reliably distinguished, provided that the most effective diagnostic characters are known. Taken together, these phenotypic differences are sufficient to suggest, at least superficially, that an origin for *O. militaris* by hybridization between *O. purpurea* and *O. simia* is unlikely, although it would be interesting to determine whether hybrid phenotypes closer to that of *O. militaris* originate when *O. simia* is the mother and *O. purpurea* the father.

The hypothesis of a hybrid origin for *O. militaris* is further undermined by the genetic data, which show that *O. militaris* is the most genetically cohesive and least variable of the three species of anthropomorphic *Orchis* under scrutiny. It has the least variable plastid haplotypes, just one ITS allele, and forms the tightest cluster on the AFLP analysis, where the greatest genetic distance separates *O. militaris* from the more extreme of the two clusters of *O. purpurea* (Fig. 7). It would, in fact, be easier to make a genetic case for a hybrid origin for *O. purpurea*. The overall impression gained is that gene flow among the anthropomorphic *Orchis* species is asymmetric, *O. militaris* typically acting as donor rather than recipient.

Table 3. Chronology of numbers of flowering plants in, and key demographic events that affected the population of, *Orchis simia* at Goring (largely derived from Paul, 1965; Harrap & Harrap, 2005)

Date(s)	Number of flowering plants (or event)
1800s (early)	Many thousands
c. 1840	(Extensive ploughing)
Late 1800s	Few thousand
Late 1800s–early 1900s	(Extensive botanical collecting)
1920s–1930s	100–200
1949–1950	(Most of site ploughed)
1951	0
1952–1953	1
1959	9
1960–1965	1–5
1968–1977	*c.* 8
1977	(Began hand pollination)
1980s	*c.* 20
1995	72
1999	100
2000>	*c.* 150

Taken together, the various lines of evidence appear to refute the prior hypothesis of a hybridogenic origin for *O. militaris*, although further study is desirable.

The parents (and hybrids) were once more frequent in the Chilterns

Much can be learned about the traumatic history of anthropomorphic *Orchis* species in Britain by studying vast numbers of (mostly 19th century) pressed specimens held in British herbaria. Many herbaria are rich in specimens of *O. simia* from the Thames Valley (a significant proportion labelled 'Goring') and many of those specimens are far larger and more impressive than the plants that flower there today (Fig. 11A). Aided by conservation measures, the Goring population is large and expanding at present. However, it recovered from near-extirpation during the period 1950–1970 (Paul, 1965; Harrap & Harrap, 2005), which undoubtedly constituted a classic genetic bottleneck (Bateman & Farrington, 1989; Qamaruz-Zaman *et al*., 1998) (Table 3). This observation has led to credible suggestions that the intensive botanical collecting by Victorians favoured the more impressive of the available specimens and so constituted a form of reverse (or negative) directional selection – the fittest plants were eliminated and only the

Figure 11. A, *Orchis simia* collected from several Thames-side localities in Oxfordshire and Berkshire, subsequently variously assigned to *O. militaris*, *O.* '*macra*' and *O.* '*tephrosanthes*', remounted in 1867 (**K**). B, Individuals of *O. simia* (left), *O. militaris* (centre) and *O. militaris* × *simia* (right), all collected by L. Darwall as *O.* '*tephrosanthes*', from (inset) 'Hartslock Wood, Goring' on May 23rd 1831 (**K**). Horizontal dimension of each image is *c.* 25 cm.

weaker plants were left to engender subsequent generations. If so, today's meagre survivors could lack the diversity and/or quality of genes needed to recover the innate vigour of their more illustrious ancestors (Raper, 2006–2008). Certainly, the lack of vigour in the Goring plants relative to those from Kent is readily observed in Table 1 and Figures 6 and 7.

Moreover, even brief scrutiny of herbarium labels demonstrates that *O. militaris*, now confined in the Chiltern Hills to two sites east of Goring, once occurred far more frequently along the Thames Valley, extending from Pangbourne in the west (Fig. 11B) to the border of Hertfordshire and Middlesex in the east. *Orchis simia* similarly extended further east, these wider distributions thereby offering much greater potential for hybridization between the two species. Sheets of *O. simia* and *O. purpurea* held in the herbaria at **BM** and **K** consistently appear correctly identified, whereas a significant proportion of those filed with (misplaced) confidence under *O. militaris* raise serious questions of legitimacy of birth. Specimens of possible hybrids between *O. simia* and *O. militaris* occur from Goring through Pangbourne and Reading, stretching as far east as Beaconsfield. It would not, therefore, be surprising if Britain's few residual, supposedly 'pure' populations of *O. simia* and *O. militaris* proved on DNA evidence to support a residuum of genes from past hybridization with each other (as is suggested by the ITS and plastid data) and/or with other previously co-existing species of anthropomorphic *Orchis*, *O. purpurea* and *O. anthropophora* (M. F. Fay, unpublished).

Of particular interest is a herbarium sheet at **K** that was collected from 'Hartslock Wood, Goring' in

May 1831. It bears single specimens of *O. simia*, *O. militaris* and a reasonably convincing hybrid between them (Fig. 11B), suggesting that introgression between anthropomorphic orchids at Hartslock is by no means a recent phenomenon, and perhaps helping to explain the complexity of the genetic profiles shown in Britain by both *O. simia* and *O. purpurea*. One possible interpretation of the ITS data (Fig. 9) is that most of the remaining British, and many of the French, plants attributed morphologically to *O. simia* possess only an ITS copy previously captured from *O. militaris* – a genetic signature also evident in some British and French populations of *O. purpurea* (Fay *et al*., 2007).

Do the hybrids represent a serious conservation threat to their parents?

Our investigations suggest that the Goring hybrids generate at least partially fertile pollen and seed. This observation raises the spectre of possible introgression between one or both parents and the newly generated hybrids, which are increasing in numbers (12 such plants attempted to flower in 2008, compared with 11 in 2007 and seven in 2006: Raper, 2006–2008). Such gene flow could in theory, if sufficiently frequent and persistent, blur the genotypic and phenotypic discontinuities that currently distinguish these rare and conservationally sensitive species at Goring. A traditional view of conservation would likely prescribe eradication of the hybrids in order to prevent this perceived eugenic catastrophe, particularly given our conclusion that the arrival of *O. purpurea* at Goring most likely reflected deliberate introduction of Continental seed by person or persons unknown.

We, however, are inclined to share the more optimistic views of Chris Raper, currently warden at Goring: 'It is my theory that in the past the three species grew in colonies scattered all along the south Chilterns . . . They probably hybridized much more frequently and the resulting plants were [consequently] harder to split into three distinct species. Far from being a problem, [the new] hybrids might actually be returning the population to a more natural state where occasional mixing of genes between the species was normal' (Raper, 2006–2008). There is indeed ample evidence of such extensive introgression. In addition, we recognize the considerable degree of interference already exerted on the Hartslock site by mankind. This certainly applies to selective collecting of specimens for herbaria, the 1950 population crash caused by ploughing of most of the site, and the subsequent period of artificial pollination designed to bulk up the population.

An optimist might argue that a fresh, yet limited, injection of genes from demonstrably successful, expansive plants of a closely related species (albeit not representing British stock) could help to return the Goring population of *O. simia* to its former levels of collective diversity and individual vigour. A realist would argue that the continued presence at Goring of *O. purpurea* and *O.* × *angusticruris* will certainly constitute an interesting ongoing natural experiment in the phenotypic and genotypic effects of introgression – one benefiting greatly from the fact that, unlike previous cases of introgression among anthropomorphic *Orchis* species, it will have been monitored since very soon after its inception.

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APPENDIX 1

MORPHOMETRIC CHARACTERS MEASURED AND RATIOS CALCULATED FOR ANTHROPOMORPHIC *ORCHIS*

Asterisked figures were measured in the field, the remainder in the laboratory.

A. Stem and inflorescence (7 characters).

1.* Stem height, above ground level (including inflorescence).
2.* Stem diameter, above uppermost sheathing leaf.
3.* Anthocyanin pigmentation immediately below inflorescence, on a scale 0–2 (0 = none, 1 = diffuse, 2 = dense).
4.* Inflorescence length.
5.* Number of flowers/buds.
6.* Bract length.
7. Ovary length.

B. Leaves (7 characters).

The leaves of anthropomorphic *Orchis* are difficult to categorize. Basal leaves form a spreading rosette immediately above ground level. Sheathing leaves arise from the rosette but surround the stem (occasional leaves intermediate between these categories were arbitrarily classed as basal leaves). Cauline leaves arise from the stem above its base and are usually much smaller than the lower leaves; unlike many other genera in subtribe Orchidinae, individuals rarely possess more than one.

8.* Number of basal leaves.
9.* Number of sheathing leaves.
10.* Number of cauline leaves.
11.* Length of longest leaf.
12.* Width of longest leaf (value often = C13).
13.* Width of widest leaf.
14.* Shape of longest leaf, as determined by the position of maximum width relative to length, on a scale 1–2 (1 = 26–50%; 2 = 51–75%).

C. Labellum (17 characters).

Patches of pigmented epidermal outgrowths characteristic of the 'torsos' of most anthropomorphic *Orchis* are termed papillae (they are absent from *O. anthropophora*).

15. Maximum width.
16. Width of 'torso'.
17. Maximum length.
18. Length of 'torso'.
19. Presence (1) or absence (0) of 'tail'.
20. Length of 'tail' (if present).
21. Length of 'arm'.
22. Width of 'arm', measured halfway along length.
23. Length of 'leg'.
24. Width of 'leg', measured halfway along length.
25. Colour of 'limbs', x (arbitrary values potentially ranging from 100–600).
26. Colour of 'limbs', y (arbitrary values potentially ranging from 100–600).
27. Colour of 'limbs', percentage reflectivity (Y).
28. Number of papillae on 'torso'.
29. Distribution of papillae on 'torso' (if present), on a scale 0–3 (0 = absent, 1 = concentrated immediately below spur entrance, through to 3 = distributed over most of 'torso').
30.* Attitude of 'torso' relative to stem, on a scale 0–5 (0 = slightly recurved, 1 = parallel, through to 5 = perpendicular).
31. Attitude of 'limbs' relative to 'torso', on a scale 1–4 (1 = shallowly convex; 2 = planar; 3 = shallowly concave; 4 = deeply concave).

D. Spur (3 characters).

Note that the concavity that represents a vestigial spur in *O. anthropophora* is too shallow to be measured satisfactorily.

32. Length, from entrance to apex.
33. Diameter, halfway along length when viewed laterally.
34. Curvature, on a scale 1–5 (1 = strongly recurved, through to 5 = strongly decurved).

E. Lateral petals (2 characters).

35. Length.
36. Maximum width.

F. Lateral sepals (7 characters).

37. Length.
38. Maximum width.
39. Colour of reverse surface, x (arbitrary values potentially ranging from 100–600).

40. Colour of reverse surface, y (arbitrary values potentially ranging from 100–600).
41. Colour of reverse surface, percentage reflectivity (Y).
42. Presence (1) or absence (0) of dispersed dots and/or dashes on reverse surface.
43. Presence (1) or absence (0) of peripheral and median linear markings on reverse surface (this character is restricted to *O. anthropophora* and its hybrids).

Selected characters were used to calculate the following 12 ratios, which summarize the shapes of certain structures. The characters are numbered according to the above list and preceded by the letter 'C':

a. Robustness of stem. C2/(C1 + C2).
b. Percentage of stem bearing flowers. (100 × C4)/C1.
c. Density of inflorescence (fls/cm). C5/C4.
d. Length of bract relative to length of ovary. C6/(C6 + C7).
e. Length of spur relative to length of ovary. C32/(C32 + C7).
f. Shape of longest leaf. C12/(C11 + C12).
g. Roundness of labellum. C17/(C15 + C17).
h. Length of 'arms' relative to length of 'torso'. C21/(C18 + C21).
i. Length of 'legs' relative to length of 'torso'. C23/(C18 + C23).
j. Length of 'arms' relative to length of 'legs'. C21/(C21 + C23).
k. Width of 'arms' relative to length of 'arms'. C22/(C21 + C22).
l. Width of 'legs' relative to length of 'legs'. C24/(C23 + C24).