Selbyana 26(1,2): 217–228. 2005.

MOLECULAR INVESTIGATION OF THE *DIURIS* "*PUNCTATA*" GROUP IN SOUTH-EASTERN AUSTRALIA

Z.F. SMITH*

University of Melbourne, Institute of Land and Food Resources, School of Resource Management, Burnley College, 500 Yarra Boulevard, Richmond, Victoria, 3121 Australia.
Email: z.smith@pgrad.unimelb.edu.au

D.J. MURPHY AND E.A. JAMES

Royal Botanic Gardens, Melbourne, Birdwood Avenue, South Yarra, Victoria, Australia.

C.B. MCLEAN

University of Melbourne, Institute of Land and Food Resources, School of Resource Management, Burnley College, 500 Yarra Boulevard, Richmond, Victoria, 3121 Australia.

ABSTRACT. *Diuris* species exhibit a large amount of morphological variation, resulting in a lack of unique characters and hence difficulties in assessing phylogenetic relationships. This study used direct sequencing of the nuclear ITS and plastid *trn*T-F regions for assessment of the phylogenetic relationships among some closely related *Diuris* species from the state of Victoria, Australia. Yellow- and purple-flowered *Diuris* species formed well-supported monophyletic sister clades. Relationships within the purple-flowered clade, including *Diuris fragrantissima,* were unresolved and purple-flowered species in the "*punctata*" group sampled for this study are genetically very closely related. This study will have implications for the conservation of *Diuris fragrantissima* and the location of a suitable mycorrhizal symbiont for reintroduction of this critically endangered species.

Key words: Orchidaceae, *Diurideae, Diuris,* ITS, *trn*T-*trn*F, conservation

INTRODUCTION

The sunshine diuris, *Diuris fragrantissima* D.L. Jones & M.A. Clem., is among Victoria's most endangered orchid species, suffering a severe population decline since the 1930s. Once abundant in numbers of tens of thousands on the native grassland plains west of Melbourne, by 1980 only one population of less than 100 individuals remained. In 2001, three plants existed at this site, but these plants have not recurred since 2002. The decline of plant numbers is largely the result of habitat destruction and degradation from agricultural, industrial, and urban development. Other factors are grazing, weed invasion, and illicit collection. At one stage, the last remaining site was guarded during daylight hours in the flowering season, because media publicity attracted thieves and vandals. In the late 1970s to early 1980s, plants were propagated symbiotically from seed, with some plants collected directly from the wild and placed in cultivation at the Royal Melbourne Zoo. Seed from these original plants has been germinated asymbiotically, resulting in a flourishing ex-situ collection of the plants. To date, however, attempts at (symbiotic) reintroductions have been unsuccessful.

The genus *Diuris* Smith was first placed in tribe Diurideae by Endlicher in 1842 (Kores et al. 2000, 2001). With the exception of a single species in East Timor, the genus is entirely restricted to Australia with more than 55 species distributed through every state except the Northern Territory (Bishop 1996, Jones 2000). Although the genus has been recognized consistently during 160 years of taxonomic treatments of the family Orchidaceae, the genus remained under revision in 2001 (Jones 2000). Hence the exact number of species within the genus was still unknown almost 160 years after its circumscription.

Although the genus as a whole is easily recognized by its attractive flowers (Backhouse & Jeanes 1995), many species and even plants growing in close proximity can exhibit an extraordinary range of floral forms. This variation causes great difficulties in defining taxa (Bishop 1996, Jones & Clements 2001, Kores et al. 2001). Adding to this are frequent hybridization (Bishop 2000, Jones & Clements 2001) and the ability of species to mimic other plants growing sympatricly that tricks pollinators into visiting the unrewarding flowers (Dafni & Bernhardt 1990, Bishop 1996).

* Corresponding author.

The limited number of unique characters and the great morphological diversity within and between species have made morphological studies difficult within the genus *Diuris.* Groups within the genus are being revised progressively, based on morphology (Jones 2000). The 55 species currently recognized have been placed into seven morphological groups, with *Diuris fragrantissima* placed in the *Diuris punctata* group along with other eastern Australian species *D. alba, D. arenaria, D. dendrobioides, D. oporina, D. parvipetala, D. punctata,* and *D. tricolor* (Jones 2000). Species within this group are characterized by forked to palmate tubers held vertically in the soil, white to purple flowers, and long lateral sepals (Jones 2000).

Once known as the white form of the purple diuris (*Diuris punctata* var. *albo-violacea* Rupp ex Dockr.), *Diuris fragrantissima,* the sunshine diuris, was first recognized as a separate species by Jones and Clements (Clements 1989, Murphy et al. 2002). The species is shorter in stature than other purple-flowered *Diuris* species and has generally pale-colored, fragrantly scented flowers (Backhouse & Jeanes 1995). The genetic distinction between *D. punctata* and *D. fragrantissima,* however, has not been investigated.

Substantial morphological variation occurs within populations of *Diuris punctata* across Victoria. Since 1989 *D. fragrantissima* has been recognized as a separate species based on morphological characters. The ex-situ collection of this species is also very morphologically variable. The relationships between *D. fragrantissima* and other species in the "*punctata*" group must be fully understood to achieve successful reintroductions and especially to locate suitable reintroduction sites. Fay and Krauss (2003) stated, "Until the relationships between species and delimitation of species in a group are fully understood, it can be difficult or inappropriate to formulate conservation strategies."

To date, molecular methods have not been employed to better understand relationships exclusively within the genus *Diuris.* An amplified fragment length polymorphism (AFLP) study of an ex-situ collection of *D. fragrantissima,* however, found genetic variation in this species, which has been in cultivation for 20 years (E. Pryde pers. comm.).

Most classifications of *Diuris* species, even at the tribal and sub-tribal levels, do not include more than one *Diuris* species (Cameron et al. 1999, Kores et al. 2000). In their molecular study of the tribe Diurideae, Kores et al. (2001) include only two species of *Diuris.* Clements et al. (2002) include four *Diuris* species in their phylogenetic analysis of tribe Diurideae, but none of these is placed within the same group based on morphology (Jones & Clements 2001). Species "complex" relationships within the genus *Diuris* (such as the *D. punctata* complex of large purple-flowered species) have not been resolved on the basis of molecular data. If the molecular relationship between species within this group is close, it may be possible to use fungi isolated from the more widespread species for use in the reintroduction of the rarer *D. fragrantissima.*

DNA sequence data are now used widely to investigate phylogenetic relationships of orchid taxa at varying taxonomic levels and in the delimitation of orchid species (Fay & Krauss 2003). For reconstructing phylogenetic relationships at various taxonomic levels within Orchidaceae, the following DNA regions have been used successfully: the internal transcribed spacer (ITS) region of nuclear ribosomal DNA (nrDNA), *trn*T-*trn*F (including the *trn*T-*trn*L intergenic spacer, *trn*L intron, and region between *trn*L and *trn*F) and *mat*K regions of the chloroplast DNA (cpDNA). Analyses of plastid and nuclear DNA sequences, as well as combined analyses of these sequences, have been used in many molecular systematic studies to date: Douzery et al. (1999), Sierra et al. (2000), Whitten et al. (2000a, 2000b), Gravendeel et al. (2001), Kores et al. (2001), Pridgeon and Chase (2001), Pridgeon et al. (2001), Soliva et al. (2001), Gravendeel and Vogel (2002), Carlsward et al. (2003). The use of combined data sets using regions with different levels of variation increases phylogenetic resolution, hence providing insight into evolutionary changes within taxa rather than genes (Whitten et al. 2000b, Soliva et al. 2001). Therefore the aims of this study were to identify phylogenetic relationships within the purple-flowered "*D. punctata*" species complex and to determine their relationship to some yellow-flowered *Diuris* species within Victoria.

Materials and Methods

Species sampled and collection sites are listed in Table 1. *Diuris fragrantissima, D. dendrobioides,* and *D. punctata* form the "*punctata*" group. The remaining species were selected for the analyses, because they are all placed in different morphological groups (Jones 2000) and hence provide a good comparison for closeness of relationships between species in the "*D. punctata*" group. They also were selected, because they are found in a similar geographical range within Victoria. Plant material did not need to be collected for all *D. fragrantissima,* two *D. sulphurea,* and two *D. punctata,* as their stored DNA samples were provided by Liz

TABLE 1. Sample sites, species sampled, and accession numbers.

Sample site	Species sampled	Genbank accession number	
		*trn*T-F	ITS
Melbourne Zoo	*Diuris fragrantissima**	AY851051	AY851068
Munro	*D. punctata*	AY851059	AY851076
Rockbank	*D. chryseopsis*	AY851046	AY851063
Parwan	*D. chryseopsis*	AY851045	AY851062
Wooragee	*D. chryseopsis*	AY851053	AY851070
	D. punctata	AY851060	AY851077
Bonegilla	*D. dendrobioides*	AY851047	AY851064
Boorhamen	*D. punctata*	AY851056	AY851073
	D. dendrobioides	AY851048	AY851065
Grampians	*D. punctata*	AY851057	AY851074
Lake Fyans	*D. punctata*	AY851058	AY851075
	D. orientis	AY851054	AY851071
	D. pardina	AY851055	AY851072
Research	*D. sulphurea*	AY851061	AY851078
Genbank	*Orthoceras strictum*	AJ409433	AF348048

* Plant material collected and DNA extracted by Liz Pryde, RMIT University, Melbourne, Victoria, Australia, 2002.

Pryde, RMIT University. These samples had been extracted from plants held in ex-situ collections at Melbourne Zoo and the Royal Botanic Gardens Melbourne and private collections of Dick Thomson and Helen Richards. For DNA extraction, ca. 5 cm of one young leaf was collected from each plant, placed on moist paper toweling, and transported to the laboratory on ice.

DNA Extraction, Amplification, and Sequencing

DNA was extracted from fresh or silica-gel-dried leaf tissue, using Dneasy extraction kits (Qiagen), following the manufacturer's instructions. Amplification of all DNA regions was carried out in 40 ul polymerase chain reactions (PCR), including 20 ul HotStarTaq® MasterMix (Qiagen), 0.8 ul each primer, 1.6ul template DNA, and 16.8 ul MilliQ H_2O. Three regions between the *trn*T and *trn*F genes (including the *trn*T-L and *trn*L-F intergenic spacers and *trn*L intron) were amplified in separate reactions, using the forward primers **a** (CATTACAAATGC GATGCTCT), **c** (CGAAATCGGTAGACGCT ACG), and **e** (GGTTCAAGTCCCTCTATCCC), along with their reverse primers, **b** (TCTACC GATTTCGCCATATC), **d** (GGGGATAGAGG GACTTGAAC), and **f** (ATTTGAACTGGTGA CACGAG) (Taberlet et al. 1991). Nuclear ribosomal ITS1and ITS2 spacers along with the 5.8S gene were amplified with the primers 17SE (A CGAATTCATGGTCCGGTGAAGTGTTCG) and 26SE (TAGAATTCCCCGGTTCGCTCGC CGTTAC) (Sun et al. 1994). The thermal cycling protocol, conducted using a Biometra™ thermocycler was as follows: for ITS, 94°C 2 min., (94°C 1 min., 52°C 1 min., 72°C 2 min) 30 cycles, 72°C 7 min. extension; for *trn*T-F, 95°C 15 min., (94°C 30 sec., 57°C 30 sec., 71°C 1 min.) 30 cycles, 72°C 5 min. extension. PCR products were purified using the Qiaquick PCR Purification Kit (Qiagen) following the manufacturers protocol. Cycle sequencing was carried out directly on the purified PCR product, using the ABI Prism Big Dye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Foster City, California, USA) with 3 µl PCR product, 4 µl Big Dye Terminator mix, 4 µl of sequence dilution buffer, 1.0 µl primer, and 8 µl MilliQ H_2O. Cycle sequencing conditions for both nrDNA ITS and plastid *trn*T-F regions was comprised as follows: 96°C 30 sec., 50°C 15 sec., 60°C 4 min. (24 cycles) 4°C 30 min., using a Biometra™ thermal cycler. Sequencing reactions were purified using ethanol precipitation, and both forward and reverse sequences were analyzed on an ABI 377 automated sequencer, using standard dye-terminator chemistry (conducted by the Microbiology Department, Monash University, Clayton, Victoria).

Phylogenetic Analyses

Programs made available by the Australian National Genomic Information Service, AGIC 1996–1998) (ANGIS) were used to edit and align sequences (ClustalX). All sequences were submitted to Genbank, and accession numbers are provided in TABLE 1. Individual gaps in the sequence data were coded as missing values; insertions/deletions were coded as present/absent characters; and individual base positions were

TABLE 2. Values and statistics from parsimony analyses of plastid and nuclear sequences and combined data.

Values and statistics	*trn*T-F	ITS	Combined matrix
Taxa (No.)	8	8	8
Characters (No.)	1745	836	2581
Variable characters (No.)	141	137	278
Phylogenetically informative characters (No.)	44	60	104
MPTs (No.)	6	1	4
Tree length (steps)	79	81	164
CI	0.6552	0.8148	0.7134
RI	0.8272	0.9453	0.8922
Clades in bootstrap consensus with >85% support (No.)	4	4	6

Note: MPTs = maximum parsimony trees. CI = consistency index. RI = retention index.

coded as unordered multistate characters. Eight indel characters were coded for the *trn*T-F and two for the ITS region. All characters were treated as independent, unordered, and equally weighted. Maximum parsimony (MP) analyses were conducted using PAUP* version 4.0b10 (Swofford 1998) for separate and combined *trn*T-F and ITS datasets, using heuristic searches with 100 random addition sequence replicates, tree bisection-reconnection (TBR) branch swapping, and MULPARS on. *Orthoceras strictum* was used as outgroup in all analyses based on previous molecular studies by Kores et al. (2001) and Clements et al. (2002). The consistency indices (CI) and ensemble retention indices (RI) (Kitching 1998) were calculated. Internal support values of clades in separate and combined analyses were calculated using bootstrap analyses (Felsenstein 1985), with 1000 full heuristic bootstrap replicates, TBR branch-swapping, 10 random addition sequence replicates, and MULPARS on. Bootstrap values were considered as strong (85–100%), moderate (75–84%), and weak (50–74%) following Pridgeon et al. (2001).

RESULTS

Results of this study are presented under the following headings: the *trn*T-*trn*F alignment, nuclear ribosomal ITS1–5.8S–ITS2, and combined nuclear and plastid sequences.

*trn*T-*trn*F

The *trn*T-*trn*F alignment had a total of 1745 sites, of which 141 were variable and 44 were phylogenetically informative (TABLE 2). Eight indels were scored in this region. Greater variation was observed in the non-coding *trn*T-*trn*L intergenic spacer than in the combined sequence data of the *trn*L intron and *trn*L-F intergenic spacer, which was unexpected. FIGURE 1 shows the strict consensus of the six most parsimonious trees (MPTs) obtained (length = 79, CI = 0.66, RI = 0.83) and the bootstrap percentages of supported clades. Although few groups were resolved, the majority of clades were supported by high bootstrap percentages (BP > 85%). Four out of six clades received strong support: Clade C, *Diuris chryseopsis—D. pardina* (BP = 99); Clade E, *D. dendrobioides* (BP = 84); Clade D, *D. chryseopsis* (BP = 96), and Clade F, *D. punctata* from The Grampians and Lake Fyans (BP = 91). The entire ingroup, with the exception of *D. sulphurea,* was weakly supported as monophyletic (Clade A, BP = 57). Within this clade, yellow-flowered species (*D. chryseopsis, D. pardina,* and *D. orientis*) formed a separate clade (B), and purple-flowered species (*D. punctata, D.* sp. aff. *punctata,* and *D. fragrantissima*) were unresolved. *Diuris pardina* formed the sister taxon to *D. chryseopsis,* and these two species formed a sister clade to *D. orientis. Diuris dendrobioides* was moderate–strongly resolved (BP = 84) and was the sister group to *D. punctata* from Boorhamen.

The relationships of *Diuris fragrantissima* were not resolved within the purple-flowered species. *Diuris sulphurea* was unresolved at the basal node of the cladogram without bootstrap support, hence the relationship of this species or whether it is a sister group to the remaining taxa was unclear.

Nuclear Ribosomal ITS1–5.8S–ITS2

The alignment had a total of 836 sites, of which 137 were variable and 60 were phylogenetically informative (TABLE 2). Two indels were scored in this region. The MP analyses yielded 1 MPT (length = 81, CI = 0.81, RI = 0.95), and resolution of this single MPT (FIGURE 2) was moderate, again with four out of six

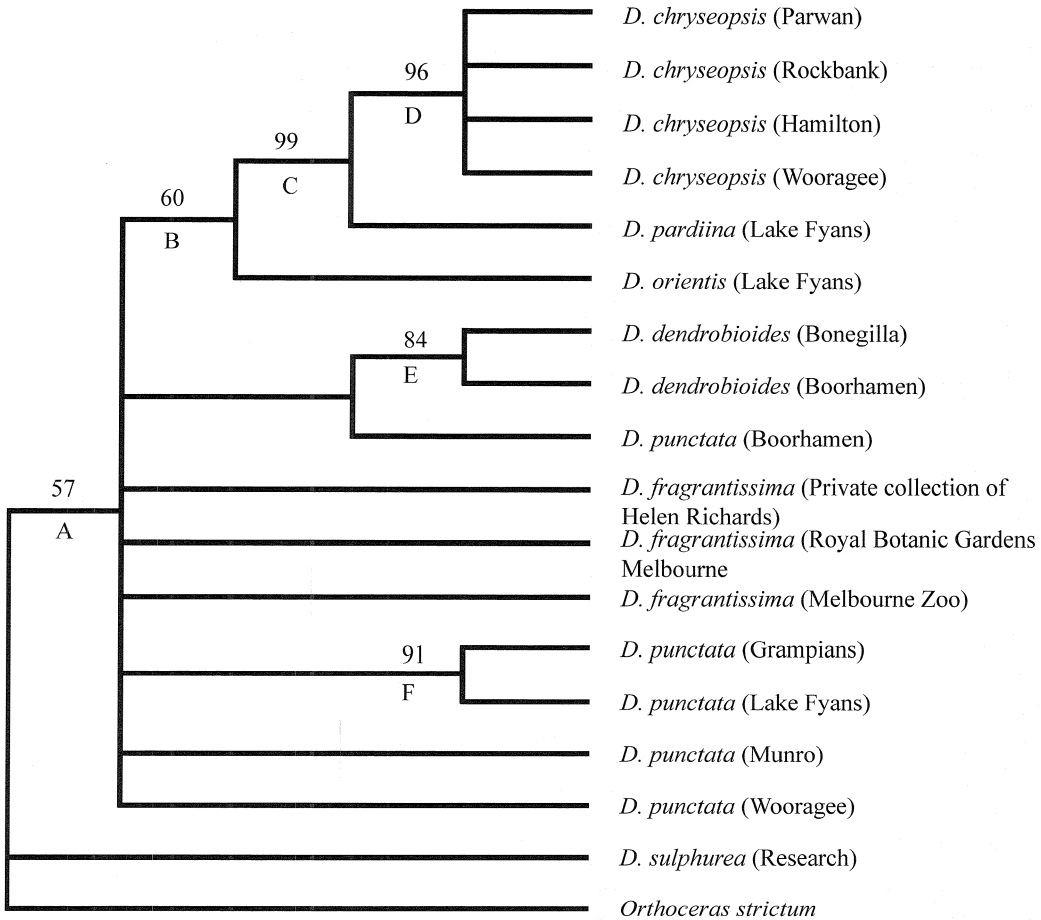

FIGURE 1. Strict consensus of six trees (length = 79, consistency index = 0.66, retention index = 0.83) derived from a parsimony analysis of the *trn*T-F sequence data. Bootstrap percentages are indicated above branches. Letters indicate major clades.

clades receiving strong support. The bootstrap consensus tree was mostly congruent with the *trn*L-F data set. Clade A, including all ingroup taxa except *D. sulphurea* (as for *trn*T-F) was strongly supported as monophyletic (BP = 91) and contained two well-supported monophyletic clades (B, BP = 100, C, BP = 87) that divide yellow- and purple-flowered species, with the exception of *D. orientis*. *Diuris pardina* formed a sister group to *D. chryseopsis*. The topology of the strict consensus obtained was very similar to *trn*T-F, but the BPs of some clades were slightly higher, with stronger resolution of ingroup clades obtained. The only incongruence between the ITS and *trn*T-F tree was that *D. orientis* was resolved as sister group to the purple-flowered species (BP = 87) rather than to the yellow-flowered species, for which it received lower support (BP = 60). The ITS region produced considerably more resolution and more well supported groups (BP > 85) than *trn*T-F, however less resolution was obtained between the purple-flowered species (within clade E). Almost no variation was observed between purple-flowered species in this region. *Diuris sulphurea* was resolved outside remaining clades, hence its relationship to remaining taxa remained unclear.

Combined Nuclear and Plastid Sequences

Comparative observations of the strict consensus trees and bootstrap percentage (BP) of the clades recovered in the separate nuclear and chloroplast analyses identified no conflicting well-supported clades (see FIGURES 1, 2). The total aligned length of the combined ITS and *trn*T-F sequences was 2581 bp, which contained 278 (10.8%) variable and 104 (4.0%) phyloge-

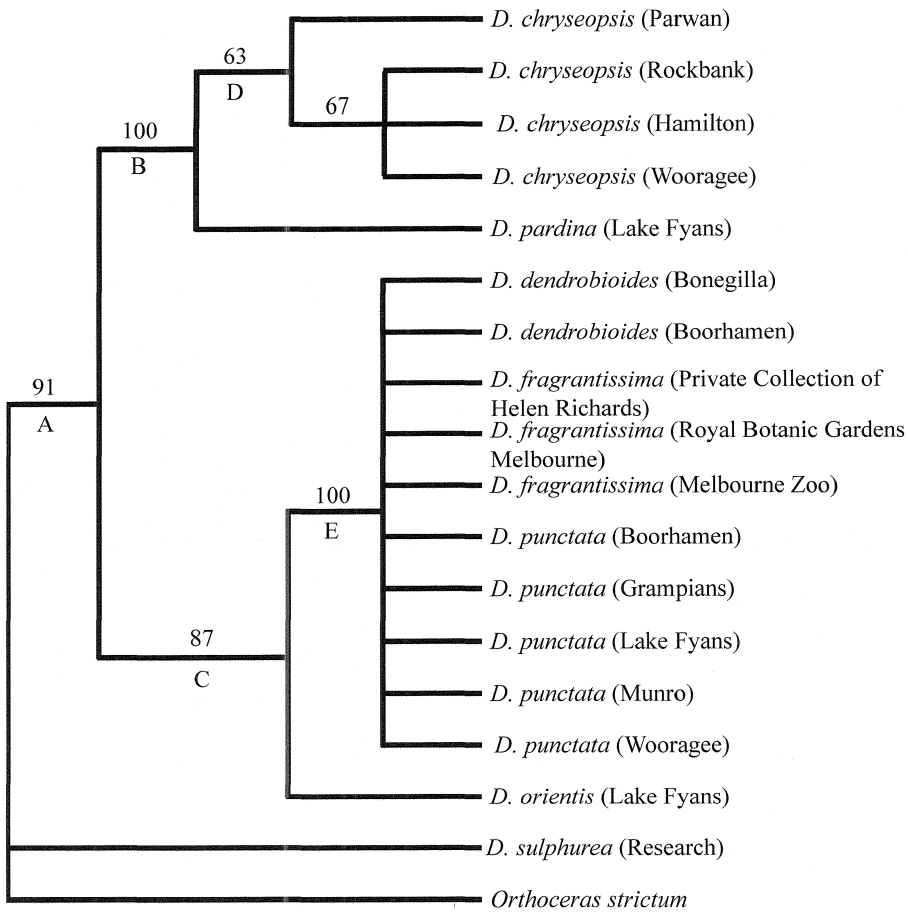

FIGURE 2. Single most parsimonious tree (length = 81, consistency index = 0.81, retention index = 0.95) resolved from analysis of the ITS sequence data. Bootstrap percentages are indicated above branches. Letters indicate major clades.

netically informative characters. Parsimony analysis of combined DNA sequences produced four MPTs with a length of 164 steps, CI of 0.71 and RI of 0.89. The strict consensus of the four trees, including the bootstrap percentages (BP) for each clade, is depicted in FIGURE 3. Resolution in the consensus tree was higher than for the individual *trn*T-F and ITS trees; however, *Diuris fragrantissima* remained unresolved. Six out of ten clades received strong support (BP > 85). Both purple- and yellow-flowered species formed well-supported monophyletic sister clades in this analysis (BP = 100). *Diuris orientis* was weakly supported as sister to the yellow-flowered species (clade A), as found in the analysis of the *trn*T-F region (FIGURE 1). *Diuris dendrobioides* formed a close relationship (BP = 86) with *D. punctata* weakly supported as sister taxon (BP = 57). *Diuris pardina* was a well-supported sister taxon to *D. chryseopsis* (clade A, BP = 100). *Diuris sulphurea* was placed at the basal node of the cladogram, outside the remaining ingroup taxa. Remaining ingroup taxa formed a well-supported monophyletic clade (BP = 94). Branch lengths on a randomly chosen MPT are depicted in FIGURE 4. More variation was observed within the yellow-flowered species clade than within purple-flowered species (clade B), as shown by the short branch lengths in the purple-flowered clade. Very little variation was observed between the purple-flowered *Diuris* species sampled in this study.

DISCUSSION

Separate and combined analyses showed that yellow- and purple-flowered *Diuris* species sampled in this study formed two monophyletic

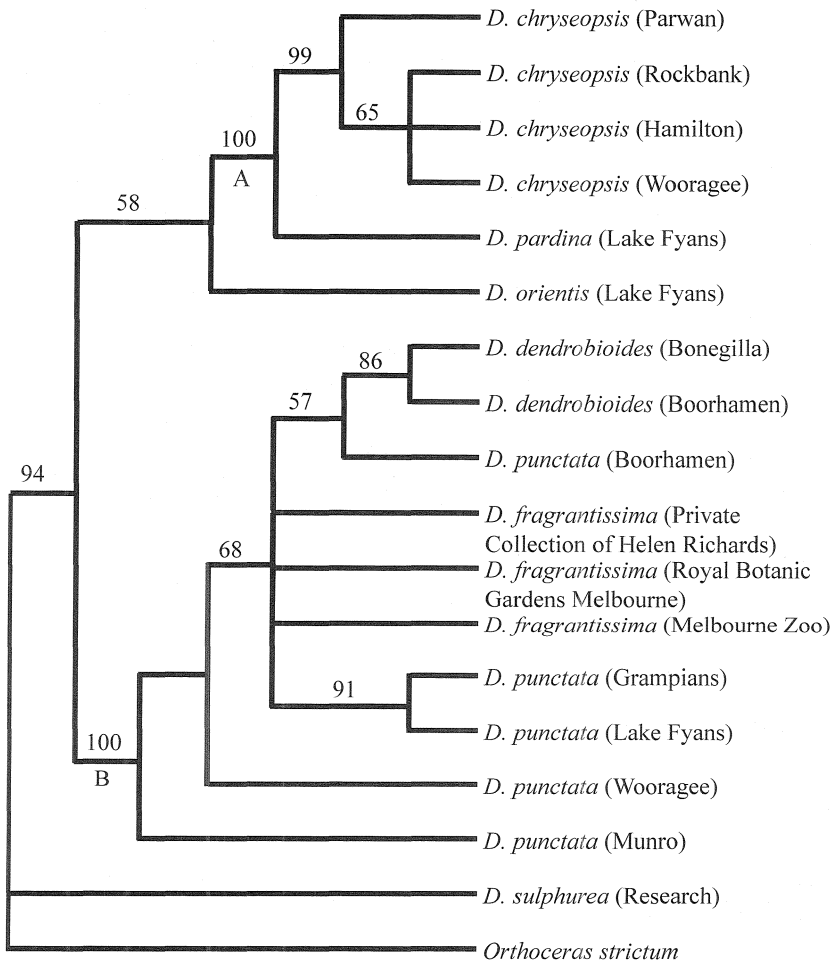

FIGURE 3. Strict consensus of four trees (length = 164, consistency index = 0.71, retention index = 0.89) derived from a parsimony analysis of the combined *trn*T-F and ITS sequence data. Bootstrap percentages are indicated above branches. Letters indicate major clades.

clades. The placement of *D. sulphurea,* unresolved at the basal node in all analyses, was placed outside the two major clades of purple- and yellow-flowered species (FIGURE 3). *Diuris orientis* formed a weakly supported sister group to clade A (*D. chryseopsis* and *D. pardina*) in the *trn*T-F and combined analysis. This species, however, was sister to the "*punctata*" group based on ITS characters. The different gene trees may be indicative of potential hybridogenic origins of *D. orientis,* with the cpDNA *trn*T-F tracking the maternal line and the nuclear ITS sequence tracking the paternal line.

All populations of *Diuris chryseopsis* sampled during this study showed a large amount of morphological variation, particularly in plant height and floral structure, color and fragrance. This variation also was observed in all populations of *D. punctata,* and *D. dendrobioides* sampled during this study. Because of its late flowering time and small population numbers, however, *D. dendrobioides* was rarely seen in flower. *Diuris punctata* generally exhibited large variation in plant stature and floral color, ranging from white to mottled purple to profuse dark purple. Flowers were variably fragranced, which may reflect the flowering stage rather than a useful taxonomic character. Hence fragrance is not a defining character for *Diuris fragrantissima,* as noted in Backhouse and Jeanes (1995) and Jones and Jones (2000). Little variation (evolutionary change) was observed within the purple-flowered "*punctata*" group, and the clade (B) has very short branch lengths compared to the yellow-flowered species used in this study (FIGURE 3). These taxa were unresolved in the ITS anal-

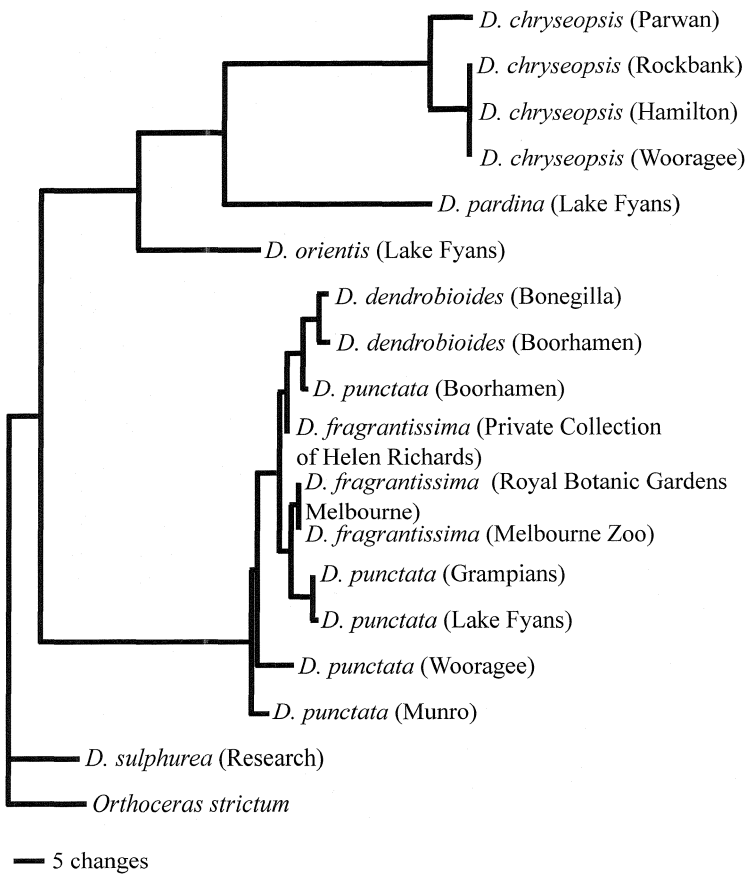

FIGURE 4. Phylogram derived from a parsimony analysis of the combined *trn*T-F and ITS sequence data.

ysis, with almost no variation being detected. The *trn*T-F and combined analyses revealed a small amount of variation, but this was still low. The variation in the purple-flowered clade was lower than that between individuals of *D. chryseopsis* (FIGURE 3). This indicates the very close relationships between species in the '*punctata*' group defined by Jones (2000). Within clade B, *D. dendrobioides* is well supported as being closely related (BP = 86), and one sample of *D. punctata* forms a sister taxon to this species. *Diuris punctata* is paraphyletic within clade B, with two *D. punctata* individuals from the Wooragee and Munro sites, forming the two basal nodes of the purple-flowered clade. *Diuris fragrantissima,* also paraphyletic, is resolved as sister to the two *D. punctata* species from The Grampians and Lake Fyans, and also as the basal taxon of a clade containing a monophyletic *D. dendrobioides* and *D. punctata* from the Boorhamen site.

Nuclear ITS and Plastid Sequence Data

The ITS region had 60 (7.2%) phylogenetically informative characters, compared to 44 (2.5%) for *trn*T-F in this study. The *trn*T-F region is more than twice the length of ITS, but ITS had almost three times the amount of informative variation than did *trn*T-F, on a percentage basis. Other authors who have used combined ITS and *trn*L-F data (e.g., Whitten et al. 2000b, Williams et al. 2001a, Salazar et al. 2003) have found similar results. The CI and RI values for the ITS region were considerably higher than for *trn*T-F (CI = 0.81 and 0.66 respectively; RI = 0.95 and 0.83 respectively). Overall tree length (*trn*T-F = 79 steps, ITS = 81 steps) and topology, however, were similar in the two analyses.

The high level of congruence between the nuclear and plastid data sets (which also included coding and non-coding regions), and high bootstrap values for clades in all analyses support the resulting consensus tree of the combined data as

a good hypothesis of phylogenetic relationships among the taxa sampled.

Within the *trn*T-F region, the *trn*T-L intergenic spacer was found to be more variable than the *trn*L intron and *trn*L-F spacer collectively. Most authors have used the *trn*L intron and *trn*L-F spacer only, in phylogenetic analyses at varying taxonomic levels of Orchidaceae (Whitten et al. 2000a, 2000b, Kores et al. 2001, Pridgeon & Chase 2001, Soliva et al. 2001, Williams et al. 2001a, 2001b, Carlsward et al. 2003, Salazar et al. 2003). In their analysis of phylogenetic relationships of section *Moniliformes* within the genus *Coelogyne,* Gravendeel and Vogel (2002) include the trnT-trnL intergenic spacer, and discovered 43 phylogenetically informative characters from 1643 sites (2.62%). In comparison, Soliva et al. (2001), in their phylogenetic study of the genus *Ophrys* obtained only 11 parsimony informative characters from 766 sites (1.44%) in *trn*L-*trn*F, concluding that ITS and *trn*L-F sequence data provided a limited insight into the interspecific relationships and that more variable DNA regions are required for better resolution of phylogenetic relationships. The inclusion of the *trn*T-*trn*L intergenic spacer may aid future analyses.

Diuris fragrantissima in the '*punctata*' group

Diuris fragrantissima was considered a variant of *D. punctata* until 1989, when less than 100 individuals remained at a single site. It is not known whether the distributions of these two species have ever overlapped. This study did not attempt to resolve phylogenetic relationships within the whole genus *Diuris,* but rather aimed to provide an insight into the relationships of *Diuris fragrantissima* within the "*punctata*" group and of the "*punctata*" group within the genus. The results of all analyses support placement of *D. fragrantissima* within the "*punctata*" group of purple-flowered *Diuris* species, but the clade is unresolved in terms of species relationships. Yellow-flowered species are clearly distinct from purple-flowered species, with high bootstrap percentage values, apart from *D. orientis,* which has yellow flowers suffused with reddish brown. Although major clades are well supported, very little variation was detected within the purple-flowered clade. This low level of variation compares with that shown between *D. chryseopsis* individuals. Yet *Diuris fragrantissima* is recognized as a distinct species to *D. punctata.*

Diuris pardina and *D. orientis,* which are placed in "*maculata*" and "*longifolia*" groups respectively (Jones 2000), show up to six times the number of character changes between their closest relative than do any of the purple-flowered taxa exhibit. (This could be the result, however, of gaps in sampling.) The low levels of variation between all purple-flowered species in this study suggest that morphological variation may be a gradational change across the geographic range of these species and may be overlapping and not taxonomically informative. Although closely related to its purple-flowered counterparts, *D. fragrantissima* was unresolved in all analyses. To confirm the taxonomic status of the species and further investigate gradational morphological change, additional sequence data of the *mat*K region will be included with the analyses in this study. The goal will be to investigate whether further phylogenetically informative variation is discovered and to further resolve the closely related taxa in the "*punctata*" complex.

Van den Berg et al. (2002) found in their molecular study of the tropical orchid genus *Cymbidium* that *mat*K had a much higher percentage of phylogenetically informative characters than did ITS, hence *mat*K might be informative in resolving the close relationships between purple-flowered species in the "*punctata*" complex. Clements et al. (2002) resolved four species of *Diuris* (*D. punctata, D. aurea, D. drummondii,* and *D. magnifica*), using only the ITS sequence region, with high bootstrap support (BP = 98–100). The very low resolution of purple-flowered *Diuris* species in our study, which also incorporates the plastid *trn*T-F region, is another indication of the close relationships between the purple-flowered species sampled. A method for investigating levels of genetic variation with potentially high levels of variation is required to examine the "*D. punctata*" group, for example, AFLP or inter-simple sequence repeat (ISSR) methods. These methods will resolve levels of variation at the intra-specific level and within and between populations and may also be more effective for determining geographical gradational morphological variation.

Whitten et al. (2000b) effectively used the criteria of Backlund and Bremer (1998) to recognize subtribes within Maxillarieae from their resultant cladograms: (1) subtribes and clades must be monophyletic and highly supported, (2) nodes defining subtribes preferably should include morphological synapomorphies that permit recognition of members, (3) the classification should be as consistent as possible with previous systems. Based on these criteria, *Diuris chryseopsis, D. pardina,* and *D. orientis* are recognized as separate, distinct species. These three species also fall into separate morphological groups as defined by Jones (2000). *Diuris chryseopsis* has bright lemon-yellow flowers with a

few brown streaks and markings. In contrast, flowers of *D. pardina* are yellow with heavy dark red-brown blotching, and *D. orientis* flowers are yellow with reddish-brown suffusions. *Diuris dendrobioides* is a strongly supported monophyletic clade that probably warrants recognition at the species level; however further analysis of genetic relationships between closely related taxa is required for formal taxonomic assignment.

The resultant cladograms in these analyses supported all previous studies including *Diuris* species and appear to support the Jones (2000) morphological grouping of *Diuris* species. All purple-flowered species group closely together, while species that belong to separate morphological groups (the yellow-flowered species) group more distantly. No formal morphological study, however, has been conducted for these species to determine unique morphological synapomorphies for each taxon. Morphological characters that were thought to distinguish species (such as paler and fragrant flowers and short stature in *D. fragrantissima*) were discovered during this study to be questionable (e.g., fragrance was noticed variably in all populations of *D. punctata*). Until a thorough morphological investigation is conducted and the taxonomy of the "*punctata*" group is resolved, including genetic relationships, formulating taxonomies from the results of this study is inappropriate.

Sequence Variation and Geographical Distance

This study also investigated whether the nuclear ITS and plastid *trn*T-F regions would detect intraspecific geographical variation. Such variation observed between taxa sampled during this study did not correlate strongly with geographical distance. Within clade B in the combined analysis (FIGURE 3), *Diuris punctata* from Boorhamen was more closely related to *D. dendrobioides* than to other *D. punctata* individuals. Two *D. chryseopsis* individuals were sampled from populations in NE Victoria (Hamilton and Wooragee sites) that were separated by less than 20 km. No variation was observed between these two individuals; however, these individuals showed no sequence variation to *D. chryseopsis* sampled from Rockbank, near Melbourne in southern Victoria.

Variation was observed between individuals of *Diuris punctata* from different sites but this variation did not reflect geographic distance between populations sampled. *Diuris punctata* individuals sampled from the relatively close Grampians and Lake Fyans populations were monophyletic in the combined consensus (FIGURE 3), but *D. punctata* individuals from Wooragee and Boorhamen populations, which are separated by a similar distance, were resolved as paraphyletic. *Diuris dendrobioides* is resolved as monophyletic, but with some variation. The two individuals of this species included in this study were sampled from populations within 20 km distance.

Conclusions and Implications for Conservation

The extremely close molecular relationships of the purple-flowered species sampled for this study indicate that a shared mycorrhizal symbiont might exist. Warcup (1971, 1973, 1975, 1981) concluded that genus-level specificity might exist between Australian terrestrial orchids and their fungal isolates. In addition, he discovered in 1981 that seed of six *Diuris* species germinated with *Tulasnella* (perfect stage *Rhizoctonia*) spp. isolates but did not germinate with *Sebacina,* a species commonly associated with *Caladenia* species. Hence closely related *Diuris* species, potentially all *Diuris* species, may germinate in the presence of similar fungi.

Many authors have used molecular methods to successfully reconstruct phylogenies within tribe Diurideae (Kores et al. 2000, 2001; Clements et al. 2002), but this study is the first to use molecular methods to assess "complex" relationships (defined by Jones 2000) within the genus *Diuris.* This is surprising, considering the high level of morphological variation and few unique characters observed in the majority of *Diuris* populations during this study. Many other authors have used sequence information for delimitation of species within subtribes and genera (Cox et al. 1997; Ryan et al. 2000; Pridgeon et al. 2001; Pridgeon & Chase 2001; Soliva et al. 2001; Williams et al. 2001a, 2001b; Gravendeel & Vogel 2002; van den Berg et al. 2002; Carlsward et al. 2003; Makarevitch et al. 2003). Also the resolution of species in this study, such as *D. orientis, D. pardina,* and *D. chryseopsis* from *D. punctata* reveals that the small amount of variation between the purple-flowered species sampled may well reflect their close genetic relationships. Further analyses of population-level variation, however, are required to assess genetic relationships and taxonomy. In addition, a morphological study with high sample numbers within each species would aid in the classification and taxonomic status of the morphologically variable species. Such studies are complicated by the threatened status of many of the species. Plants sampled for this study cannot be removed from the wild, requiring in-situ measurements. Morphological diversity within the whole tribe

Diurideae, however, is known to be high (Kores et al. 2001), resulting in a lack of easily identifiable morphological synapomorphies, which has made taxonomy and classification within *Diuris* difficult. The phylogenetic trees generated in this study show high levels of bootstrap support and demonstrate the potential use of ITS and *trn*T-F sequence data for the resolution of taxonomic groups within *Diuris.*

Acknowledgments

This study was funded in part by the Australian Orchid Foundation. Travel to the Second International Orchid Conservation Congress was partly funded by a Melbourne Abroad Traveling Scholarship. The authors wish to thank Colin Knight, Roger Cousens, Glen Johnson, Karen Lester, Russell Mawson, Dick Thomson, Helen Richards, and Andrew Pritchard for their assistance with the collection of plant material and Liz Pryde for DNA samples of *Diuris fragrantissima.*

Literature Cited

Backhouse, G. and J. Jeanes. 1995. The Orchids of Victoria. The Miegunyah Press, Carlton, Victoria.

Backlund, A. and K. Bremer. 1998. To be or not to be: principles of classification and monotypic plant families. Taxon 47: 391–400.

Bishop, T. 1996. Field Guide to the Orchids of New South Wales and Victoria. University of New South Wales Press, Sydney, NSW.

———. 2000. A Field Guide to the Orchids of New South Wales and Victoria. University of New South Wales Press Ltd., Sydney, NSW.

Cameron, K.M., M.W. Chase, W.M. Whitten, P.J. Kores, D.C. Jarrell, V.A. Albert, T. Yukawa, H.G. Hills, and D.H. Goldman. 1999. A phylogenetic analysis of the Orchidaceae: evidence from *rbc*L nucleotide sequences. Amer. J. Bot. 86: 208–224.

Carlsward, B.S., W.M. Whitten, and N.H. Williams. 2003. Molecular phylogenetics of neotropical leafless Angraecinae (Orchidaceae): reevaluation of generic concepts. Inter. J. Pl. Sci. 164: 43–51.

Clements, M.A. 1989. Catalogue of Australian Orchidaceae. Austral. Orchid Res. 1: 1–160.

Clements, M.A., D.L. Jones, I.K. Sharma, M.E. Nightingale, M.J. Garratt, K.J. Fitzgerald, A.M. Mackenzie, and B.P.J. Molloy. 2002. Phylogenetics of Diurideae (Orchidaceae) based on the internal transcribed spacer (ITS) regions of nuclear ribosomal DNA. Lindleyana 17: 135–171.

Cox, A.V., A.M. Pridgeon, V.A. Albert, and M.W. Chase. 1997. Phylogenetics of the slipper orchids (Cypripedioideae, Orchidaceae): nuclear rDNA ITS sequences. Pl. Syst. Evol. 208: 197–223.

Dafni, A. and P. Bernhardt. 1990. Pollination of terrestrial orchids of southern Australia and the Mediterranean region. Systematic, ecological and evolutionary implications. Evol. Biol. 24: 193–253.

Douzery, E.J.P., A.M. Pridgeon, P. Kores, H.P. Linder, H. Kurtzweil, and M.W. Chase. 1999. Molecular phylogenetics of Diseae (Orchidaceae): a contribution from nuclear ribosomal ITS sequences. Amer. J. Bot. 86: 887–899.

Fay, M.F. and S.L. Krauss. 2003. Orchid conservation genetics in the molecular age. Pp. 91–112 *in* K.W. Dixon, S.P. Kell, R.L. Barrett and P.J. Cribb, eds. Orchid Conservation. Natural History Publications, Kota Kinabalu, Sabah.

Felsenstein, J. 1985. Confidence limits on phylogenies: an approach using the bootstrap. Evolution 39: 783–791.

Gravendeel, B. and E.F. de Vogel. 2002. Revision of *Coelogyne* section Moniliformes (Orchidaceae) based on morphology, plastid, and nrDNA ITS sequences. Blumea 47: 409–462.

Gravendeel, B., M.W. Chase, E.F. de Vogel, M.C. Roos, T.H.M. Mes, and K. Bachmann. 2001. Molecular phylogeny of *Coelogyne* (Epidendroideae; Orchidaceae) based on plastid RFLPs, *mat*K, and nuclear ribosomal ITS sequences: evidence for polyphyly. Amer. J. Bot. 88: 1915–1927.

Jones, D.L. 1993. Native Orchids of Australia. William Heinemann, Australia.

———. 2000. Natural groups in *Diuris* Sm. Pp. 83–88 *in* Proceedings of the Fourth Australian Orchid Conference and Show, Ringwood, Melbourne, Oct. 5–8.

Jones, D.L. and B. Jones. 2000. A Field Guide to the Native Orchids of Southern Australia. Bloomings Books, Hawthorn, Victoria.

Jones, D.L. and M.A. Clements. 2001. Tribe Diurideae. *In* A.M. Pridgeon, P.J. Cribb, M.W. Chase and F.N. Rasmussen, eds. Genera Orchidacearum. Oxford University Press, Oxford, UK.

Kitching, I.J. 1998. Cladistics: The Theory and Practice of Parsimony Analysis. Oxford University Press, Oxford, UK.

Kores, P.J., P.H. Weston, M. Molvray, and M.W. Chase. 2000. Phylogenetic relationships within the Diurideae (Orchidaceae): inferences from plastid *mat*K DNA sequences. Pp. 449–456 *in* K.L. Wilson and D.A. Morrison, eds. Monocots: Systematics and Evolution. CSIRO, Melbourne.

Kores, P.J., M. Molvray, P.H. Weston, S.D. Hopper, A.P. Brown, K.M. Cameron, and M.W. Chase. 2001. A phylogenetic analysis of Diurideae (Orchidaceae) based on plastid DNA sequence data. Amer. J. Bot. 88: 1903–1914.

Makarevitch, I., K. Golovnina, S. Scherbik, and A. Blinov. 2003. Phylogenetic relationships of the Siberian Iris species inferred from noncoding chloroplast DNA sequences. (Systematics and Evolution). Inter. J. Pl. Sci. 164: 229–237.

Murphy, A.H., C. Knight, G. Backhouse, and A. Webster. 2002. Recovery Plan for the Sunshine Diuris, *Diuris fragrantissima* (Orchidaceae: Diuridinae) 2002–2007, Department of Sustainability and Environment, Melbourne, Victoria.

Pridgeon, A.M. and M.W. Chase. 2001. A phylogenetic reclassification of Pleurothallidinae (Orchidaceae). Lindleyana 16: 235–271.

Pridgeon, A.M., R. Solano, and M.W. Chase. 2001. Phylogenetic relationships in Pleurothallidinae

(Orchidaceae): combined evidence from nuclear and plastid DNA sequences. Amer. J. Bot. 88: 2286–2308.

Ryan, A., W.M. Whitten, M.A.T. Johnson, and M.W. Chase. 2000. A phylogenetic assessment of *Lycaste* and *Anguloa* (Orchidaceae: Maxillarieae). Lindleyana 15: 33–45.

Salazar, G.A., M.W. Chase, M.A. Soto Arenas, and M. Ingrouille. 2003. Phylogenetics of Cranichideae with emphasis on Spiranthinae (Orchidaceae, Orchidoideae): evidence from plastid and nuclear DNA sequences. Amer. J. Bot. 90: 777–795.

Sierra, S.E.C., B. Gravendeel, and E.F. de Vogel. 2000. Revision of *Coelogyne* section Verrucosae (Orchidaceae): a new sectional delimitation based on morphological and molecular evidence. Blumea 45: 275–318.

Soliva, M. and A. Widmer. 2003. Gene flow across species boundaries in sympatric, sexually deceptive *Ophrys* (Orchidaceae) species. Evolution 57: 2252–2261.

Soliva, M., A. Kocyan, and A. Widmer. 2001. Molecular phylogenetics of the sexually deceptive orchid genus *Ophrys* (Orchidaceae) based on nuclear and chloroplast DNA sequences. Molec. Phylogen. & Evol. 20: 78–88.

Sun, Y., D.Z. Skinner, G.H. Liang, and S.H. Hulbert. 1994. Phylogenetic analysis of *Sorghum* and related taxa using internal transcribed spacers of nuclear ribosomal DNA. Theoret. Appl. Genet. 89: 26–32.

Swofford, D.L. 1998. PAUP* 4.0b: Phylogenetic Analyses Using Parsimony (and Other Methods). Sinauer, Sunderland, Massachusetts.

Taberlet, P., L. Gielly, G. Pautou, and J. Bouvet. 1991. Universal primers for amplification of three noncoding regions of chloroplast DNA. Pl. Molec. Biol. 17: 1105–1109.

van den Berg, C., A. Ryan, P.J. Cribb, and M.W. Chase. 2002. Molecular phylogenetics of *Cymbidium* (Orchidaceae: Maxillarieae): sequence data from internal transcribed spacers (ITS) of nuclear ribosomal DNA and plastid *mat*K. Lindleyana 17: 102–111.

Warcup, J.H. 1971. Specificity of mycorrhizal association in some Australian terrestrial orchids. New Phytol. 70: 41–46.

———. 1973. Symbiotic germination of some Australian orchids. New Phytol. 72: 387–392.

———. 1975. Factors affecting symbiotic germination of orchid seed. Pp. 87–104 *in* F.E. Sanders, B. Mosse and P.B. Tinker, eds. Endomycorrhizae. Academic Press, London.

———. 1981. The mycorrhizal relationships of Australian orchids. New Phytol. 87: 371–381.

Whitten, W.M., M.A.T. Johnson, and M.W. Chase. 2000a. A phylogenetic assessment of *Lycaste* and *Anguloa* (Orchidaceae: Maxillarieae). Lindleyana 15: 33–45.

Whitten, W.M., N.H. Williams, and M.W. Chase. 2000b. Subtribal and generic relationships of Maxillarieae (Orchidaceae) with emphasis on Stanhopeinae: combined molecular evidence. Amer. J. Bot. 87: 1842–1856.

Williams, N.H., M.W. Chase, and W.M. Whitten. 2001a. Phylogenetic positions of *Miltoniopsis, Caucaea,* a new genus, *Cyrtochiloides,* and *Oncidium phymatochilum* (Orchidaceae: Oncidiinae) based on nuclear and plastid DNA sequence data. Lindleyana 16: 272–285.

Williams, N.H., M.W. Chase, T. Fulcher, and W.M. Whitten. 2001b. Molecular systematics of the Oncidiinae based on evidence from four DNA sequence regions: expanded circumscriptions of *Cyrtochilum, Erycina, Otoglossum,* and *Trichocentrum* and a new genus (Orchidaceae). Lindleyana 16: 113–139.