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MOLECULAR GENETIC DIAGNOSIS OF THE 'TAXONOMICALLY DIFFICULT' AUSTRALIAN ENDANGERED ORCHID, *MICROTIS ANGUSII*: AN EVALUATION OF THE UTILITY OF DNA BARCODING.

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As species are the common currency for conservation efforts, their accurate description is essential for efficient preservation of biological diversity. The use of DNA Barcodes, short DNA sequences that evolve fast enough to differentiate species, has been proposed both for the discovery of new species and the identification of previously described species. The first objective remains controversial, with a strong argument that species discovery be based on 'Integrated Barcodes', including multiple sources of data (see Rubinoff 2006a). In contrast, once a species has been described, including for molecular sequence data at barcoding loci, the use of a DNA barcode may facilitate the identification of the species, particularly in cases where recognition based on easily-visualised characters is problematic.

Nonetheless, the use of the DNA Barcoding approach for species diagnosis pre-supposes a comprehensive understanding of the circumscription of the species under study. In reality this is very rarely true, as it requires the combination of multiple lines of investigation, which, in the case of orchids include pollinator observations and manual cross pollination, in addition to morphological and molecular character analysis (Peakall 2007). Thus, while ideally the sole use of DNA Barcoding should be limited to species diagnosis, in practice it may often yield further data to be set against the working hypothesis of species status, thus contributing to species delineation and discovery.

We present a study of the application of barcoding to the endangered Australian orchid, *Microtis angusii* (Flanagan *et al.* 2006). This species was recently described from a single location in New South Wales, Australia, consisting of approximately 100 plants (Jones 1996). *Microtis* species commonly exhibit clonal growth (Peakall & Beattie 1989, 1991), and it was highly likely that the plants present at the type location represented a small number of clones. Additionally, the site had been subject to various threatening processes such as road improvements and encroachment by invasive plants. *Microtis angusii* was listed as a nationally endangered species on Schedule 1 of the Australian Commonwealth *Endangered Species Protection Act 1992* in 1997.

The genus *Microtis* has been relatively neglected taxonomically, possibly because of their inconspicuous small, green, often ant-pollinated flowers. *Microtis angusii* is morphologically very similar to more common, widespread relatives, and easily confused, even by experienced field biologists. In accordance with the New South Wales Threatened Species Conservation Act, a recovery plan for the species was prepared in order to ensure self-sustaining populations in the wild. For this, the identification of further populations of *M. angusii* was highly desirable, but hindered by difficulties in species recognition.

Conservation practitioners identified six potential populations of *M. angusii*, and requested a genetic study to provide confirmation of their con-specific sta-

tus for the recovery plan. We investigated patterns of molecular genetic variation in both Amplified Fragment Length Polymorphisms (AFLPs) and DNA sequence (rDNA ITS) loci, compared to that of the type population and known examples of potentially confounding, congeneric species. The type population was invariable across 122 AFLP markers. Of the six potential populations only two were identified unambiguously as *M. angusii*, having identical ITS sequences and highly similar AFLP profiles. Three populations collected showed a high genetic affinity to the related species *M. parviflora*, including identical ITS sequences, while a fourth population was diagnosed, on the basis of the molecular data, to be *M. rara*.

A subset of samples from one of the populations was most similar to, but not identical to *M. angusii* across the genetic loci. This genetically-distinct clade may represent an additional previously-unknown species. Alternatively, given the clonal nature of the *Microtis* genus, this geographically distant population may represent a highly differentiated conspecific population. Clonality, in combination with high selfing rates due to restricted ant pollination (Peakall & Beattie 1989, 1991) are traits that will act to reduce effective population size, thereby enhancing the effects of genetic drift and so promoting higher levels of genetic differentiation between isolated populations than expected in a predominantly outbreeding species.

Whilst barcoding based on complete DNA sequence data is preferred in order to identify rare, differentiated haplotypes, extensive sequencing projects are expensive and beyond the financial capacity of many conservation programmes. In order to provide an economical alternative to full sequence characterization, we designed a rapid, PCR-based assay for the effective identification of *M. angusii* from single nucleotide polymorphism (SNP) differences seen in the study of sequence variation at the ITS locus (Flanagan *et al.* 2007). The assay was designed to be easily visualized on a standard agarose gel, avoiding the use of expensive restriction enzymes and DNA sequencing reagents and equipment.

An important aspect of this assay was its validation through the application of a 'blind trial'. Here the assay was applied to samples of disguised identity, including all ITS haplotypes identified in the original genetic survey, and samples from a previously uncharacterized population. *Microtis angusii* samples were successfully discriminated from amongst the several congeners, and the further, previously unknown, population was diagnosed as *M. angusii*. Sequencing of the ITS locus for these individuals confirmed this PCR diagnosis.

While these studies demonstrate the application of DNA Barcoding for species diagnosis of the endangered *M. angusii*, it must be emphasized that further morphological and ecological studies of the genus *Microtis* are sorely needed in order to unambiguously define the species boundaries in the genus. As has been recognized by Rubinoff (2006b), amongst other authors, sole reliance on DNA sequence data at one, or a few loci, may mislead conservation efforts, either by making decisions on species status based on characters that are not species-specific, or by diverting resources from broader studies that are ultimately more capable of providing robust species circumscriptions. It is imperative that, in the reality of limited funds for conservation research, priority must be given to studies that will have direct practical outcome in conservation management. A recent review suggested that genetic studies of clonal plants, plants with uncertain taxonomic status, and plants targeted for translocation were most likely to result in practical outcomes (Hogbin *et al.* 2000). Nonetheless, as the case of *Microtis angusii* shows, even in these scenarios a genetic study is not necessarily sufficient by itself.

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Nicola Flanagan has a broad experience of evolutionary studies in the Orchidaceae, including species boundaries in the sexually-deceptive *Chiloglottis* orchids, orchid mycorrhizal specificity in the tropical *Ionopsis utricullariodes*, and patterns of genetic variation in *Vanilla* species.

Rod Peakall has interests spanning the fields of plant reproductive biology, population genetics, evolutionary biology and conservation biology and has worked on a range of plant and animals species. His current research is focused on the evolution of sexually deceptive orchids.

Mark Clements has extensively studied the taxonomy and evolutionary relationships of Australian native orchids.

Tupac Otero has interests in orchid biology and interactions, including reproductive biology, mycorrhizal interactions, evolutionary biology and conservation biology.