Uncovering the sexual tricks of orchid flowers

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Abstract

Rather than the conventional offer of a food reward such as nectar or pollen, many orchid species from the Australian *Chiloglottis* genera use sexual deception to attract male pollinators. These orchids emit an odorous pheromone very similar to the sexual pheromone produced by females of the pollinator species, thereby luring the male to the flower with the false offer of sex. The high degree of specificity between sexually deceptive orchids and their pollinators indicates that there must be subtle but important differences in the pheromones produced among even closely related orchids. This paper investigates whether the *PACPT* (Palmitoyl-Acyl Carrier Protein Thioesterase) gene, found to be highly expressed in the pheromone-producing part of *Chiloglottis* orchids, may be involved in the production of sexual mimicry pheromones. An analysis of the distribution of genetic variation in the gene sequence, as well as its possible effects on the amino acid it encodes was conducted across five species within the *Chiloglottis* genera. The results are not only highly encouraging for future research into the role of this gene in pheromone production, but may also provide helpful diagnostic tools for cryptic species that pervade the sexually-deceptive orchids.

Figure 1. *Chiloglottis valida* with sexually attracted pollinators.

Credit: Rod Peakall.

Sexual deception of pollinators is surprisingly common and diverse in terrestrial orchids, being the method of pollination in nine of thirty-nine Australian orchid genera within the Diuridea tribe (Dafni & Bernhardt 1990; Mant *et al.*, 2002; Schiestl & Peakall 2005). By mimicking the morphology and sex pheromones of female pollinators, these sexually deceptive orchids are able to lure male pollinators with the false offer of sex and accomplish cross-pollination without the cost of a food reward (Schiestl *et al.*, 2003; Bower & Brown 2009). This type of sexually deceptive pollination has been well studied in many Orchidaceae families (Dafni & Bernhardt 1990; Soliva & Widmer 2003; Vela *et al.*, 2007; Mant, Peakall & Schiestl 2005), with extreme specificity between orchid and pollinator species the norm. Recent investigations into the sexual deception of *Neozeleboria* (Tiphiiidae) thynnine wasp by orchids in the Australian *Chiloglottis* genera have also revealed very species-specific pollination patterns, such that

closely related *Chiloglottis* species are usually pollinated by closely related thynnine wasp species (figure 1) (Mant *et al.*, 2002; Schiestl & Peakall 2005; Bower & Brown 2009).

Attraction of specific pollinator species maintains the species borders of sexually deceptive orchids and is accomplished through variation in pheromone traits between species (Mant, Peakall & Schiestl 2005). The array of odour compounds involved in the mimicry of thynnine wasp sex pheromones have only recently begun to be discovered within the *Chiloglottis* genera (Schiestl & Peakall 2005). For instance, a single chemical compound termed ‘Chiloglottone’ has recently been found to be the sole sex pheromone responsible for the attraction of male pollinators in two sister thynnine wasp species (Schiestl *et al.*, 2003). Interestingly, this pheromone is produced by two distantly related orchid species; *Chiloglottis trapeziformis* and *Chiloglottis valida* (Schiestl *et al.*, 2003). The joint production and attraction to the Chiloglottone pheromone in the wasps can easily be explained by a shared ancestry, however, the mimicry in distantly related orchids has raised interestingly evolutionary questions regarding the extent to which compounds involved in pheromone production vary among *Chiloglottis* orchid species (Mant, Peakall & Schiestl 2005). This variation and flexibility in pollinator pheromone mimicry may explain the lack of genetic differentiation among *Chiloglottis* species within subgeneric clades (compared to among clades) i.e. the joint attraction of the same thynnine wasp pollinator by *C. trapeziformis* and *C. valida* has led to gene flow and low genetic variation between the orchid species (Mant, Peakall & Weston, 2005).

To fully understand the evolutionary processes involved in the mimicry of pollinator sex pheromones by *Chiloglottis* orchids and the subsequent effects on gene flow and pollinator specificity, the active compounds involved in pheromone production across species and populations must be identified (Mant, Peakall & Schiestl, 2005). A cDNA library has been constructed containing nucleotide gene sequences which are highly expressed in the pheromone-producing labellum of *Chiloglottis* orchids (Schiestl & Peakall, 2005). Analysis of the taxonomic variation in these gene sequences will assist in identifying the multiple genes involved in pheromone production and the subsequent variation that results in subtle but important differences in the pheromones produced by different *Chiloglottis* orchids. This paper aims to measure the genetic variation in the exon and intron nucleotide sequences of the highly expressed Palmitoyl-Acyl Carrier Protein Thioesterase (*PACPT*) gene across five distantly related *Chiloglottis* species. The protein encoded in this gene is known to play a crucial role in the biosynthesis of plant fatty acids (Jones *et al.*, 1995), hence any genetic variation within the expressed exon sequence that results in nonsynonymous changes between species in the encoded amino acid sequence may alter the biosynthesis pathway of the sex pheromones.

A secondary aim of this investigation into genetic variation is the development of primers for highly variable intron sequences within the *Chiloglottis* genera. Species-specific variation is expected to occur most highly within the intron sequences of the *PACPT* gene as these are relatively isolated from selection. As a result, introns accumulate single nucleotide polymorphisms (SNPs) and nucleotide insertions and deletions (indels) more frequently than exons (Campbell *et al.*, 2006). Therefore, identification of species-specific variation in intron sequences may not only complement existing markers for phylogenetic analysis of *Chiloglottis* orchids, but also aid in the identification of morphologically similar or cryptic orchid species (such as *Chiloglottis valida* subsp.) that have so far confounded and limited many studies on sexually deceptive orchid-pollinator interactions (Mant, Peakall, & Schiestl, 2005; Mant, Peakall, & Weston, 2005; Bower & Brown 2009).

Methods

Primer Design

Primers were designed using web-based PRIMER 3 software (Rozen & Skaletsky 2000) to amplify the gene sequence encoding for the production of palmitoyl-acyl carrier protein thioesterase (*PACPT*) in five *Chiloglottis* species based on a consensus cDNA sequence of *PACPT* from *C. trapeziformis* (Purdue Genomics Facility, 2010). I predicted the location of four introns in the cDNA sequence (exons only) by comparison with the complete genomic sequence of *PACPT* in the genomic model plant *Arabidopsis* (GenBank). The four sets of primers were designed to amplify and sequence four overlapping sections of the *PACPT* gene sequence, avoiding intron/exon junctions. The total exon length was estimated to be around 1362 nucleotide bases with four introns estimated to be between 30 and 150 bases in length each.

Orchid DNA sequencing

Each of the five *Chiloglottis* species included in this study, has a distinct pollinator that is distinguishable by morphological characteristics (Mant *et al.*, 2002). A simplified phylogenetic tree of the *Chiloglottis* genera (Mant, Peakall, & Weston, 2005), illustrating the placement of the five sampled *Chiloglottis* species across the three main clades is presented in figure 2. Multiple species samples were included to allow comparison of genetic variation among and within species and clades (table 1).

Orchid DNA was extracted using DNeasy Plant Mini Kit (Qiagen, Melbourne, Australia), with confirmation of DNA extract obtained by gel electrophoresis (1% agarose, 40 min at 110 volts). Polymerase chain reactions (PCRs) (40µl) contained: 24.9 µl milliQ H₂O; 4.0 µl 10X PCR buffer (Qiagen, containing 15 mM MgCl₂); 2.5 mM dNTPs, 2.0 µl; 25 mM MgCl₂, 2.4 µl (Qiagen); 1% (w/v) BSA(Biolabs), 0.5 µl 2mM of each primer in a pair, 2.0 µl; 5 units/µl *Taq* polymerase, 0.2 µl; and 100 nm/µl of template DNA; 2.0 µl.

Taxon	Location
C. diphylla	NSW: Buladelah fire trail
C. formicifera 1	NSW: Nattai National Park
C. trapeziformis 1	ACT: Black Mountain
C. trapeziformis 2	ACT: Black Mountain
C. valida 1	ACT: Lowden Road
C. valida 2	ACT: Black Mountain
C. aff. plaricallate	NSW: Kanangra-Boyd National park

Table 1. Taxon and location of the *Chiloglottis* samples

Figure 2. Simplified *Chiloglottis* phylogeny of the five sample species.

Source: Mant, Peakall & Weston, 2005

The PCR amplification was performed in CP-002 Palm Cycler (Corbett Research) with a touch-down thermal cycling program: 30 sec denaturation at 94°C followed by 40 secs at 66°C for primer annealing and then 90 secs at 72°C for extension. The annealing temperature decreased by 4 °C at every cycle until it reached 54°C, where it then reduced to 49°C for the last 10 cycles. Successful amplification of primer products in each sample was confirmed with gel electrophoresis (2%, 20min 130 volts then 10 min at 145 volts).

Purification of PCR products for sequencing followed ExoSAP-IT protocol (USB Corporation). The primers used for amplification were also used for sequencing, and all DNA fragments were sequenced in both directions. Sequencing reactions (20µl) contained 12.93 µl milliQ H₂O, 3.0 µl 5X PCR buffer (Qiagen), 2 mM of one primer, 0.32 µl; 0.75 µl of ABI Big Dye (Perkin Elmer Boston, MA); and 50 ng/µl of PCR Product, 3 µl. PCR sequence products were precipitated and purified with sodium acetate: ethanol followed by centrifuging and a 70% ethanol wash (x3).

Sequence Analysis

Alignment and editing of sequences was done in Geneious basic 4.6.4. PCR products for each sample were aligned to form a continuous sequence, except in *C. formicifera* which was constructed with only the reverse primer sequence for the second DNA segment as the forward primer sequence was corrupted. The *PACPT* sequences for each sample were aligned with the cDNA sequence from *C. trapeziformis* to identify intron location, size and polymorphisms. Ambiguities in the identity of nucleotide bases were resolved with visual analysis of sequence chromatographs. All sequences were trimmed to 1645 base in length, commencing and ending at the same exon sequence. SNPs and insertions/deletions (indels) within the exons and introns of each sample were identified as species or clade specific. Extra nucleotide bases were classified as an insertion if present only in that species or clade. Likewise, if more than one clade shared the extra nucleotide bases, the species or clade without the extra nucleotide bases was classified as having a deletion. Phylogenetic trees were constructed using complete, trimmed sequences, as well as exon and intron only sequences (bootstrapping was not conducted). Finally, exon-only sequences of each sample were translated into amino acid sequences to identify the SNPs that may produce non-synonymous changes in the encoded amino acid sequence.

Results

Not only did the four primer pairs successfully amplify their intended products, producing a continuous *PACPT* gene sequence length (ranging from 1150 to

1623 base pairs in length between species) in all of the *Chiloglottis* samples, but there was substantial interclade and interspecific genetic variation within the exon and intron sequence: rejecting the null hypothesis of no variation. Surprisingly, comparison of the *C. trapeziformis* cDNA sequence with nuclear sequence from the seven *Chiloglottis* samples (including 2 *C. trapeziformis* samples) revealed the location of 6 introns, 2 more than predicted (figure 3).

Figure 3. A) Four predicted introns in the PACPT sequence when compared to the complete *Arabidopsis* sequence. B) Actual position of 6 introns identified in the *Chiloglottis* consensus sequence.

The intron and exon-only phylogenetic trees showed identical genetic relatedness among samples, as did the phylogenetic tree constructed from the entire sequence (figure 4). The location and relatedness of species samples within the *PACPT* phylogenetic tree almost mimics that of Mant, Peakall, & Weston's (2005) phylogeny constructed from comprehensive DNA fingerprinting methods (figure 2), except for *C. formicera*'s position within the *C. trapeziformis* clade. This is due to the extremely high similarity (99.8%) in the *PACPT* sequence between the two species. Such low genetic variation was not observed even between the two *C. valida* samples, who shared just 98.4% of their nucleotide bases. Similarity between the *C. valida* sampled from Black Mountain and *C. aff. plaricallata* was also very high, sharing 97.9% of their nucleotide bases. Overall, the *PACPT* gene sequence was fairly conserved between clades, with 91.3% of the sequenced nucleotide bases being shared amongst all seven *Chiloglottis* samples.

Figure 4. Phylogenetic tree constructed from ACPT gene sequences from seven *Chiloglottis* samples (cDNA is exon-only sequence from a *C. trapeziformis*)

Analysis of intron-only sequences revealed taxonomic differences in the distribution of variation, with 64% of variation accounting for differences between clades. Overall, 86% of nucleotide bases were shared across all seven samples with 49 SNPs and 5 indels distinguishing *Chiloglottis* species among the three clades (table 3 & figure 5). There was no variation in the intron sequence between *C. formicifera* and *C. trapeziformis* samples (as previously mentioned), but there was surprising intra-species variation within the *C. valida* clade. The *C. valida* sampled from Black Mountain shared 7 SNPs and a 7bp deletion with the *C. aff. plaricallata* sample, which were not present in the *C. valida* sampled from Lowden Road.

Figure 5. Intron-only phylogenetic tree illustrating the distribution of genetic variation within the intron sequences of the PACPT gene in seven *Chiloglottis* samples.

The distribution of genetic variation in the exon sequences was similar to that within introns, with 78% of the variation accounting for differences between clades, and each clade is being distinguishable by unique SNP combinations (although there were no indels within the exon regions) (figure 6). Again there was no species-defining genetic variation within the *C. trapeziformis* clade in the exon sequence, making it impossible to uniquely identify these two species based on these *PACPT* gene sequences alone. As expected, the exon sequences were less variable than the intron sequences, with 93.5% of nucleotides shared by all samples (this is due to the absence of indels within exon regions). Approximately 50% of all the SNPS within exons translate into nonsynonymous changes in the encoded amino acid (AA) sequence across the seven *Chiloglottis* samples, with each sample from the *C. diphylla* and *C. valida* clades encoding for at least 1 unique AA change (figure 6).

Figure 6. Exon-only phylogenetic tree illustrating the distribution genetic variation within the exon sequences of the PACPT gene in seven *Chiloglottis* samples.

Discussion

An analysis was conducted of the genetic variation in the exon and intron nucleotide sequences within the *PACPT* gene, which is found to be highly expressed in the pheromone-producing labellum of *Chiloglottis* orchids (Schiestl & Peakall 2005). Genetic variation that codes for changes to the *PACPT* amino acid sequence were identified both among and within clades (figure 6). In addition, species-specific genetic variation within the (unexpressed) intron segments were mapped, thereby providing an additional genetic marker to complement existing phylogenetic markers and possibly aid in the identification of cryptic *Chiloglottis* taxa and other Orchideae (figure 5).

The high expression of the *PACPT* gene within the pheromone-producing labellum has lead researches to suspect that it may be involved in the production of pheromone compounds. As a metabolically essential protein that determines the length of plant fatty acids (Jones *et al.*, 1995), it is certainly possible that *PACPT* plays a role in the production of the active compounds used to mimic pollinator sex pheromones across the *Chiloglottis* genera. As such, the high

percentage (50%) of SNPs identified within the expressed exon sequences that result in nonsynonymous changes in the encoded amino acid sequence of *PACPT* (at least 5 per species excluding *C. formicifera*) may create differences in the form and function of the protein between species and certainly between genetically divergent clades (figure 6). Unfortunately, the poorly understood effects of post-transcription and post-translation regulatory mechanisms (Campbell *et al.*, 2006) on *PACPT* function prevent any conclusions as to how, and what degree, these identified SNPs might change the role of *PACPT* in the plants. However, given the variety of SNPs, and subsequent variation in the amino acid sequence within species (see *C. valida* figure 6), between species and particularly between clades, there is evidence to suggest that *PACPT*'s metabolic function may vary between *Chiloglottis* clades even if the protein is not involved in pheromone production.

Although exon variation was identified (figure 6), the level of conservatism within the coding regions of *PACPT* is high enough to allow four pairs of primers designed from *C. trapeziformis* cDNA sequence to successfully amplify the intended gene sequence (1,645 nucleotide bases) across the five *Chiloglottis* species. The conservation of the *PACPT* gene sequence is also illustrated in the way that its phylogeny mirrors the *Chiloglottis* phylogeny constructed from a more comprehensive AFLP fingerprinting method (Mant, Peakall & Weston's (2005)) (figure 2, 4 & 5). Congruency between phylogeny and variation in sex pheromone compounds is reported in Lepidopteran moths (Lofsdedt & Kozlov, 1996 as cited by Mant *et al.*, 2002) and within some species of thynnine wasps (Mant *et al.*, 2002). However there was one exception to this pattern; the sequence of *PACPT* in *C. formicifera* is identical to that of *C. trapeziformis* even though they are relatively distantly related species. While there is less certainty in the sequence of *C. formicifera* due to one forward primer segment being discarded, it is unlikely that the reverse sequence was misinterpreted. In addition, there was no cross-species contamination evident in other PCR products or the negative control. Lastly, increased species sampling could only reposition *C. formicifera* into its taxonomically correct *C. valida* clade if there is extremely high intra-species variation within the *PACPT* gene, which has not been observed within this study. Instead, it is likely that the *PACPT* gene is highly conserved between this two distantly related species, either due to pollinator-driven selection (Mant, Peakall, & Schiestl, 2005) or other selection pressures on its metabolic functions (Jones *et al.*, 1995).

The distribution of genetic variation was taxonomically unbalanced, with a high proportion of variation due to the differences between clades in both exons (78%) and introns (64%). Mant, Peakall & Weston (2005) found similarly large genetic differences between the three *Chiloglottis* clades (73% of the total variation) relative to species within the clades. The lack of genetic differentiation

between species is suggested to be the result of consistent (or episodic) gene flow among closely related orchids (Mant, Peakall & Weston, Schiestl 2005; Mant, Peakall & Weston, 2005). Schiestl and Peakall (2005) demonstrated that different species of *Chiloglottis* (*C. trapeziformis* and *C. valida*) can indeed attract each others pollinators through joint production of the sex pheromone Chilogottone, thereby facilitating hybridization and gene flow between species. The joint attraction of even distantly related pollinators and subsequent gene flow between sympatric orchid species has also been supported in microsatellite studies in *Ophrys* orchids by Soliva & Widmer (2003). Whether gene flow between species can account for the high genetic similarity in the *PACPT* gene among the sampled *Chiloglottis* species cannot be inferred from the present results. However, the genetic similarity of species within the spring and summer-flowering *C. trapeziformis* and *C. valida* clades, and their relatively higher genetic differentiation from the autumn-flowering *C. diphylla* clade, does match the predicted relatedness of cross-pollinating plants in accordance with their ecological circumstances (Mant, Peakall & Weston, 2005)

Comparing variation in exons versus introns in the *PACPT* sequence at all taxonomic levels supports my prediction of higher genetic variation in intron segments; the *Chiloglottis* species samples shared less nucleotide bases and had five indels in their intron sequences compared to the exon segments (which had no indels difference between species) (figure 5 & 6). However, further sampling and statistical tests would need to be done to confirm whether introns do have significantly higher variation than exons. Variation in the number of introns within the *PACPT* gene between *Chiloglottis* and *Arabidopsis* (figure 2) species is an important result, but not unusual as the two plants are not closely related (Campbell *et al.*, 2006). Nevertheless, it does provide an additional measure of the genetic variation that exists within the *PACPT* gene in plants and may be useful for phylogenetic studies.

The identification of cryptic *C. valida* and other *Chiloglottis* subspecies to date has been based on the responses of pollinators to their unique chemical signatures (Bower & Brown 2009), as have cryptic *Ophrys* species (Vela *et al.*, 2007). However, the use of short intron sequences for identification of cryptic species would be valuable in many instances, particularly sympatric species that may attract multiple pollinators (Schiestl & Peakall 2005). Aside from *C. formicifera*, I hypothesise that the four sampled species could be assigned to their correct taxonomic clade solely on species-specific variation within the intron sequence (varying from 2 to 21 SNPs and accompanying indels), based on multiple samples within the *C. formicifera* and *C. valida* clades and the overall genetic divergence of the *C. diphylla* clade (figure 5). Although clade-specific variation was observed in exon regions, the shorter length and higher proportion of variation within introns suggests that intron-based diagnostics

would be ideal for identifying morphologically similar or cryptic *Chiloglottis* species (such as *C. valida* subsp., Bower & Brown 2009). Future research would benefit from designing primers that target the diagnostic intron sequences in the sampled species followed by wide-spread testing in the *Chiloglottis* genera.

Conclusion

This study provides the first measure of the distribution of genetic variation in the *PACPT* gene sequence across the three main *Chiloglottis* clades. The high number of nonsynonymous changes to the encoded amino acid sequence of *PACPT* identified in four of the five *Chiloglottis* species sampled indicates that *PACPT*'s metabolic function may indeed vary within and between *Chiloglottis* clades. This genetic differentiation among species provides evidence in support of the hypothesis that *PACPT* is involved in the production of species-specific pheromone production. Future research will benefit by increasing the orchid sample size (including more taxa), as well conducting multiple extractions from each plant (to add consistency and statistical strength to the data). Continued differential analyses of *PACPT* expression between the labellum and other parts of the plant will also indicate whether *PACPT* is indeed expressed significantly higher in the pheromone-producing labellum than anywhere else in the plant; providing further evidence for the protein's potential role in the evolution and variation of pollinator sex pheromones in *Chiloglottis* orchids. An important secondary outcome from the design of novel primers for the partial sequencing of the *PACP* gene is the identification of species and clade-specific genetic variation. These genetic markers could be used for the identification of morphologically similar or cryptic species that so often characterise sexually deceptive genera (Mant, Peakall & Weston, 2005; Bower & Brown 2009). Aside from diagnostic purposes, the variability in these primer segments may complement existing markers in phylogenetic investigations in taxonomic groups with little genetic differentiation (Mant *et al.*, 2002).

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