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A chromosome-scale genome assembly of the pollen beetle, *Brassicogethes aeneus*, provides insight into cytochrome P450-mediated pyrethroid resistance

Rob King[1,], Debora Boaventura[2,**], Benjamin J. Hunt[3,**], Angela Hayward[3,**], Kumar Saurabh Singh[3], Oliver Gutbrod[2], Christoph T. Zimmer[3,4], Martin S. Williamson[1], Linda M. Field[1], Chris Bass[3,*], and Ralf Nauen[2,*]**

[1] Department of Biointeractions and Crop Protection, Rothamsted Research, Harpenden, UK
[2] Bayer AG, Crop Science Division, Alfred Nobel-Strasse 50, 40789 Monheim, Germany
[3] College of Life and Environmental Sciences, Biosciences, University of Exeter, Penryn Campus, Penryn, Cornwall, UK
[4] Current address: Syngenta Crop Protection, Werk Stein, Schaffhauserstrasse, Stein CH4332, Switzerland
[*] Corresponding authors: ralf.nauen@bayer.com; c.bass@exeter.ac.uk
[**] These authors contribute equally to this work

With 4 figures

Abstract: The pollen beetle, *Brassicogethes aeneus*, is an economically important pest of oilseed rape (*Brassica napus*) throughout Europe. The control of *B. aeneus* has relied heavily on the use of chemical insecticides leading to the evolution of resistance. However, investigation of the molecular basis of resistance has been hampered by an absence of genomic resources for this species, including the lack of a reference genome assembly. Here we address this need by generating a chromosome-scale genome assembly for *B. aeneus*. A combination of long-read single-molecule sequencing and *in vivo* chromatin conformation capture (Hi-C) sequencing was used to generate an assembly of 585 Mb, comprising 11 chromosome sized scaffolds (scaffold N50 of 61.6 Mb) and containing 13,381 protein-coding genes. We leveraged the new assembly, in combination with post-genomic functional approaches to investigate the molecular basis of metabolic resistance to pyrethroid insecticides in *B. aeneus*. Our data confirmed that two P450s, CYP6BQ23 and CYP6BQ25, have the capacity to metabolise the pyrethroid deltamethrin in *B. aeneus* and thus have the potential to confer resistance. However, the relative expression of these P450s in pyrethroid susceptible and resistant strains suggests that CYP6BQ23 plays a much more significant role in resistance than CYP6BQ25. In summary, the high-quality genome assembly for *B. aeneus* reported here provides a valuable resource for future research on this species. Our findings on P450-mediated resistance to insecticides are of applied relevance for the development of strategies for the sustainable control of this important pest.

Keywords: *Brassicogethes aeneus*, genome, P450, resistance, pyrethroid

1 Introduction

Oilseed rape (*Brassica napus*) is a globally important crop grown for the production of animal feed, vegetable oil and more recently for alternative usages such as liquid biofuel. The pollen beetle, *Brassicogethes aeneus* (formerly known as *Meligethes aeneus*) (Coleoptera: Nitidulidae), is the most economically important arthropod pest of oilseed rape throughout Europe (Williams 2010). Adult beetles feed almost exclusively on pollen and cause most damage during the early (green to yellow) stages of bud formation when, in attempts to access this food source, they chew through flowering structures resulting in damage to sepals, petals and ovaries (Seimandi-Corda et al. 2021). Feeding may completely destroy smaller buds while destruction of embryos in larger buds leads to abortion and blind (podless) stalks. As a result, pollen beetle infestations can cause significant reduction in yields, as high as 80% in spring rape (Hansen 2004).

The main method of protection of oilseed rape against pollen beetle is the application of chemical insecticides, however, their intensive use has led to the development of resistance. The most high-profile example of this is the development of resistance to pyrethroids, first described in 1999 in Northeastern France (Thieme et al. 2010). Since then, resistance has been reported in numerous European countries and has had serious economic consequences, as demonstrated in Germany in 2006 when resistant pollen beetles could no longer be controlled by pyrethroids, leading to

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E. Schweizerbart'sche Verlagsbuchhandlung, 70176 Stuttgart, Germany, www.schweizerbart.de

the destruction of 30,000 ha and serious damage to 200,000 ha of winter oilseed rape (Nauen 2007).

Insecticide resistance has been shown to most frequently arise via two main mechanisms: 1) structural changes (mutations) in the insecticide target protein that make it less sensitive to the toxic effects of the insecticide, and 2) enhanced production or activity of enzymes (such as esterases, glutathione-S transferases and cytochrome P450s) which break down, or bind to (sequester), the insecticide. For pollen beetle both mechanisms have been described in pyrethroid resistant populations (Nauen et al. 2012; Zimmer et al. 2014a; Zimmer & Nauen 2011). In the case of target-site resistance, the cloning and sequencing of a section of the pyrethroid target, the voltage-gated sodium channel, identified a single amino acid change (L1014F, known as knockdown resistance, or kdr) in domain IIS6 of the channel in resistant pollen beetles (Nauen et al. 2012). In the case of metabolic resistance, the best documented evidence implicates enhanced oxidative detoxification by cytochrome P450 monooxygenases (P450s) (Philippou et al. 2011; Zimmer et al. 2014a, b; Zimmer & Nauen 2011). Firstly, the efficacy of pyrethroids was shown to be synergised by the P450 inhibitor piperonyl butoxide (PBO) in resistant strains of pollen beetle (Philippou et al. 2011; Zimmer & Nauen 2011). Secondly, microsomal preparations from resistant pollen beetle strains showed a significantly increased rate of deltamethrin metabolism in the presence of NADPH, which was inhibited by PBO, and two other P450-inhibitors, tebuconazole and 1-aminobenzotriazole (Zimmer & Nauen 2011). Thirdly, the level of 4-hydroxy deltamethrin formation in microsomal preparations of several pollen beetle strains as assessed by LC-MS/MS analyses was found to be significantly correlated with the level of pyrethroid resistance (Zimmer & Nauen 2011).

Following these studies, the P450 gene, *CYP6BQ23*, was shown to be highly overexpressed (up to ~900-fold) in adults and larvae of pyrethroid resistant strains compared to susceptible strains, with expression levels correlating with both the level of resistance and with the rate of deltamethrin metabolism in microsomal preparations of these populations (Zimmer et al. 2014a). Furthermore, functional expression of CYP6BQ23 *in vitro* demonstrated its capacity to efficiently metabolise deltamethrin to 4-hydroxy deltamethrin (Zimmer et al. 2014a). The prominent role of CYP6BQ23 in pyrethroid resistance was further confirmed by a proteomic study comparing dead and alive pollen beetle after deltamethrin treatment (Kocourek et al. 2021).

A study of European pollen beetle populations employing transcriptomic approaches, confirmed the overexpression of *CYP6BQ23* but also discovered a second P450 gene, *CYP6BQ25*, with a high similarity to *CYP6BQ23*, that was also found to be overexpressed in pyrethroid resistant strains (Zimmer et al. 2014b). However, to date the functional role of CYP6BQ25 in detoxifying pyrethroids has not been demonstrated and thus its role in resistance remains unclear.

Recently the interaction between target-site resistance (kdr, L1014F) and P450-mediated resistance (CYP6BQ23 overexpression) has been investigated (Samantsidis et al. 2020). Elegant work combining genetic transformation and CRISPR/Cas9 genome modification of *D. melanogaster* provided strong evidence that the metabolic and target-site resistance mechanisms identified in *B. aeneus* can act synergistically to confer strong resistance.

To date, characterisation of the molecular bases of insecticide resistance in *B. aeneus* has been hampered by the paucity of genomic resources for this species, most notably the lack of a reference genome assembly. Here we addressed this need by sequencing, assembling, and annotating a high-quality chromosome-scale assembly of *B. aeneus*. We then leveraged this resource, in combination with post-genomic functional approaches, to investigate the role of the CYP6BQ subfamily of P450s in resistance to pyrethroids.

2 Materials and methods

Details on chemicals and insect collection, DNA extraction and genome sequencing, *de novo* genome assembly and annotation, ortholog analysis, species level phylogeny and divergence time estimation, curation, phylogenetic and syntenic analyses of genes involved in detoxification of xenobiotics, molecular and functional characterisation of *CYP6BQ23* and *CYP6BQ25*, computational modeling and deltamethrin docking, and insecticide bioassays is provided in supplementary methods.

2.1 Data availability

The genome assembly and raw sequencing data generated in this study has been deposited at NCBI under accession PRJEB48953.

3 Results

3.1 A chromosome scale genome assembly of *B. aeneus*

Sequencing of *B. aeneus* DNA generated 1,046,296 PacBio HiFi reads with a read length N50 of 16,775 bp, and 709,616,444 150 bp paired-end Illumina Hi-C reads representing 182× coverage. This data was assembled into 11 chromosome-scale scaffolds (Fig. S1) resulting in a final assembly of 585 Mb, with a scaffold N50 of 61.6 Mb (Table S1). The completeness of the gene space in the assembled genome was assessed using the Benchmarking Universal Single-Copy Orthologues (BUSCO) pipeline, with 98.6% of the Insecta reference gene set found to be present as complete copies (Table S1). Structural genome annotation using a workflow incorporating RNAseq data predicted a total of 13,381 protein-coding genes in the assembly. Of these, 13,290 were successfully assigned functional annotations

based on BLAST searches against the non-redundant protein database of NCBI and the InterPro database.

Ortholog analysis generated 16,094 gene clusters (Fig. 1), of which 4,632 were found in all species, of which 1,156 consisted of single-copy genes. A total of 138 genes were specific to *B. aeneus*, and 8,465, 8,547, 7,837, 7,362, 7,777, 7,073, 7,979, 8,301 and 8,488 genes were shared with *A. tumida, A. planipennis, A. glabripennis, L. decemlineata, N. vespilloides, O. taurus, P. pyralis and T. castaneum* respectively. Modelling of global gene gain and loss revealed a gene turnover rate of 0.0015 gains and losses per gene per million years in *B. aeneus*, slower than that reported for *D. melanogaster* (0.0023 duplications/gene/million years) (Lynch & Conery 2000). Estimation of gene gain and loss in gene families across the 10 arthropod species revealed a negative average gene family expansion (–0.226) in *B. aeneus*, with a greater number of gene families contracted (3,566) and genes lost (4,091) than expanded (660) and gained (1,159) (Table S2). Gene ontology (GO) enrichment analysis of orthogroups specific to *B. aeneus* identified GO:0005549, odorant binding, (Table S3) while the rapidly evolving and expanding gene families were significantly enriched for xenobiotic transport functions and defence response to fungi (Table S4, S5).

3.2 Curation and phylogeny of P450 genes

We leveraged the new genome assembly to identify and curate genes encoding P450s as these have been frequently implicated in the detoxification of natural and synthetic insecticides in a range of insect species including *B. aeneus* (Nauen et al. 2022; Zimmer et al. 2014a). A total of 103 P450s, were identified in the *B. aeneus* assembly, a gene count consistent with that of other beetle species (Table S6). The curated P450 gene set of *B. aeneus* was compared to the CYPomes of *T. castaneum, D. melanogaster, A. gambiae* and *A. mellifera.* Phylogenetic analyses revealed that, as for other insect species, *B. aeneus* P450 genes group into four main clades (CYP2, CYP3, CYP4 and mitochondrial clans) (Fig. S2A, B, C, D), which could be further sub-divided into 25 families and 57 subfamilies (Table S7).

P450s from the CYP2 and mitochondrial clans show a high degree of 1:1 orthology across the five species, which may indicate possible functional conservation of these P450s (Zhu et al. 2013). *B. aeneus* has large and diverse CYP3 (23 subfamilies, 50 genes) and CYP4 (20 subfamilies, 38 genes) P450 clans. With the exception of the *CYP4AA* and *CYP4AG* subfamilies, there appears to be a lack of 1:1 orthology across the five species in these two clans, with most families and subfamilies exhibiting marked species-specific expansions (Fig. S2C, D). This is particularly apparent in the CYP3 clan, with marked species-specific radiations obvious even within orders. For example, *T. castaneum* has only one main family of CYP6 sequences (*CYP6B*), whereas *B. aeneus* has three (*CYP6B, CYP6G* and *CYP6V*) (Fig. S2C).

Phylogenetic analyses of the CYPomes of *B. aeneus* (superfamily: Cucujoidea) and *T. castaneum* (superfamily: Tenebrionoidea) affords the opportunity to compare two Coleopteran species that shared a last common ancestor approximately 200 million years ago (Zhang et al. 2018) (Fig. 2A). As with the broader phylogeny across five species, there is a high degree of 1:1 orthology in the CYP2 and mitochondrial clans and almost none within the other two P450 clans. Indeed, the CYP3 and CYP4 clan sequences of the two beetle species appear as sister clades rather than groups of orthologous genes. Sequences from the CYP4 family account for half of the *B. aeneus* CYP4 clan (9 subfamilies, 19 genes) and the species-specific *CYP3016* family is the second largest gene group (4 subfamilies, 10 genes) (Fig. 2A).

In the CYP3 clan the CYP6 family accounts for over half of the complement of sequences (12 subfamilies, 26 genes), with the CYP9 family the second largest (5 subfamilies, 13 genes) (Fig. 2A). Most of the *B. aeneus CYP9* genes are from the *CYP9BZ* subfamily (7 genes), and these form a species-specific clade with the *CYP9BK* sequences (3 genes). Of the three remaining *B. aeneus* CYP9 genes two are possible 1:1 orthologs of *T. castaneum* sequences (*B.a_CYP9BN1*: *T.c_CYP9Y1* and *B.a_CYP9BM2*: *T.c_CYP9AD1*) (Fig. 2A).

The largest species-specific subfamily expansions occur in the CYP6 family and there are several large sister groups of genes. For example, *B. aeneus CYP6GN* and *CYP6GM* form a sister group with *T. castaneum CYP6BK/L/M/N/P* genes (Fig. 2A). The *CYP6BQ* subfamily contains *T.c_CYP6BQ9* and *B.a_CYP6BQ23* both of which have been linked to pyrethroid resistance (Zhu et al. 2010; Zimmer et al. 2014b). However, there is a large discrepancy in the number of *CYP6BQ* genes between the two Coleopteran species. *T. castaneum* has 12 functional *CYP6BQ* genes and one pseudogene (*CYP6BQ3P*), whereas *B. aeneus* only has two: *CYP6BQ23* and *CYP6BQ25*. In *T. castaneum*, with the exception of *CYP6BQ13*, the *CYP6BQ* genes all occur in a large cluster on chromosome LG4, made up of three clans: I (*CYP6BQ2, CYP6BQ4*), II (*CYP6BQ6, CYP6BQ7, CYP6BQ12*) and III (*CYP6BQ1, CYP6BQ5, CYP6BQ8, CYP6BQ9, CYP6BQ10, CYP6BQ11*). The Bayesian phylogeny places the two *CYP6BQ* exemplars from *B. aeneus* as a sister group to the *T. castaneum* clan I sequences, *CYP6BQ2* and *CYP6BQ4* (Fig. 2B). However, *B. aeneus CYP6GJ/GS/VE* genes form a second sister clade to the *T. castaneum CYP6BQ* sequences and *CYP6GL1* and *CYP6VD1* appear more closely related to *T. castaneum CYP6BQ13,* which is located as a solitary P450 gene on chromosome LG2.

CYP6BQ23 is located on chromosome 11 of the *B. aeneus* genome, and this chromosome is also the putative site of *CYP6BQ25* (see below). Analyses of locally colinear blocks (LCBs) show that there is poor macro-synteny between chromosome LG4 of *T. castaneum* (which contains the *CYP6BQ* cluster) and chromosome 11 of *B. aeneus* (17 LCBs, LCB weight 879 bp) (Fig. S3). However, there is a high degree of macro-synteny between chromosome LG4 of *T. castaneum* and chromosome 7 of *B. aeneus* which contains a cluster of *CYP6GJ* genes (216 LCBs, LCB weight 895 bp) (Fig. S3).

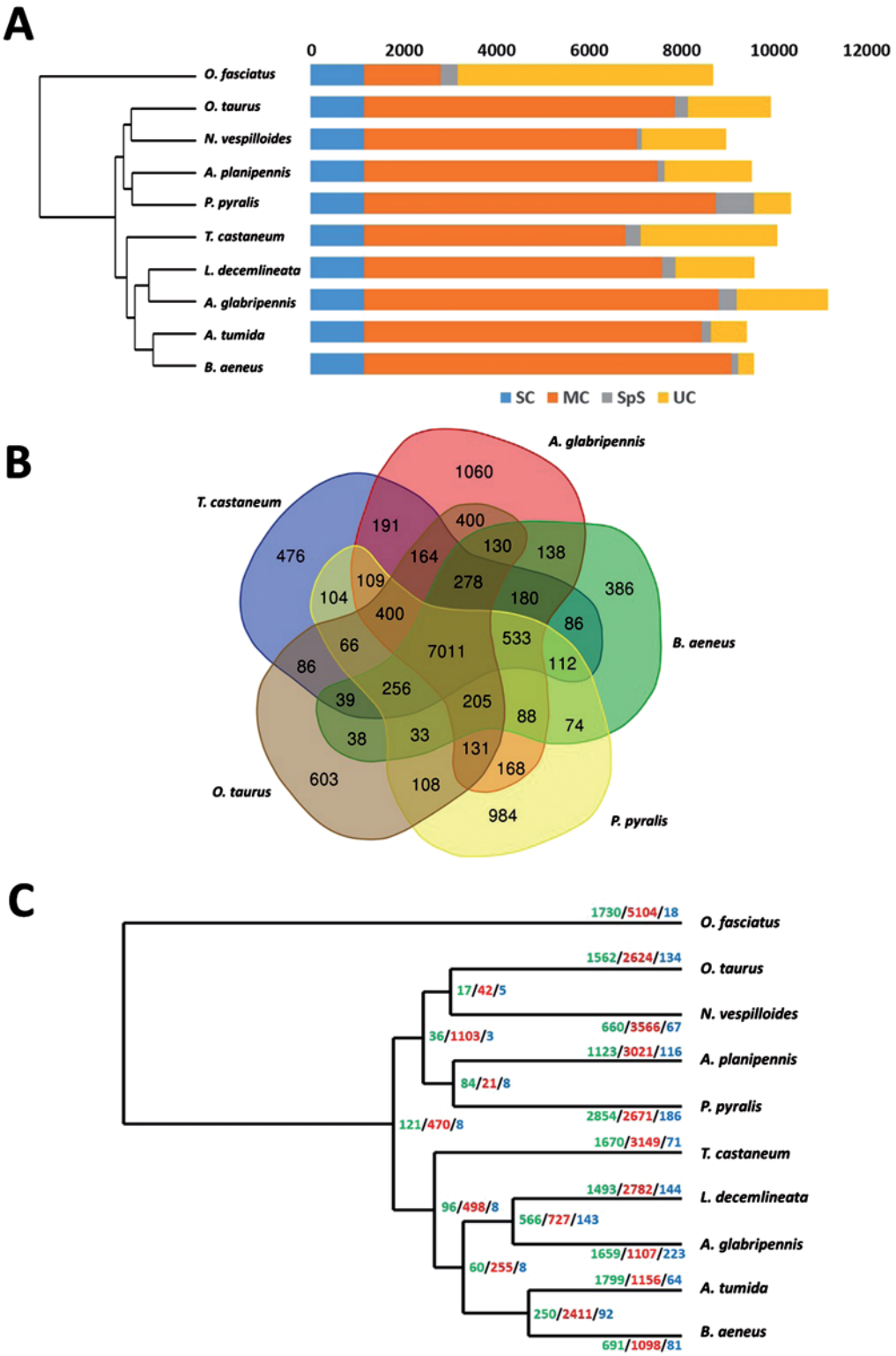

Fig. 1. Phylogenomic analysis of the genome of *B. aeneus* and 9 other arthropod species. **(A)** Phylogenetic relationship and gene orthology of *B. aeneus* and other arthropods. SC indicates common orthologs with the same number of copies in different species, MC indicates common orthologs with different copy numbers in different species. SpS indicates species-specific paralogs, UC indicates all genes which were not assigned to a gene family. **(B)** Gene families shared by selected species. **(C)** Species dated phylogenetic tree and gene family evolution. Numbers on the branch indicate counts of gene families that are expanding (green), contracting (red) and rapidly evolving (blue).

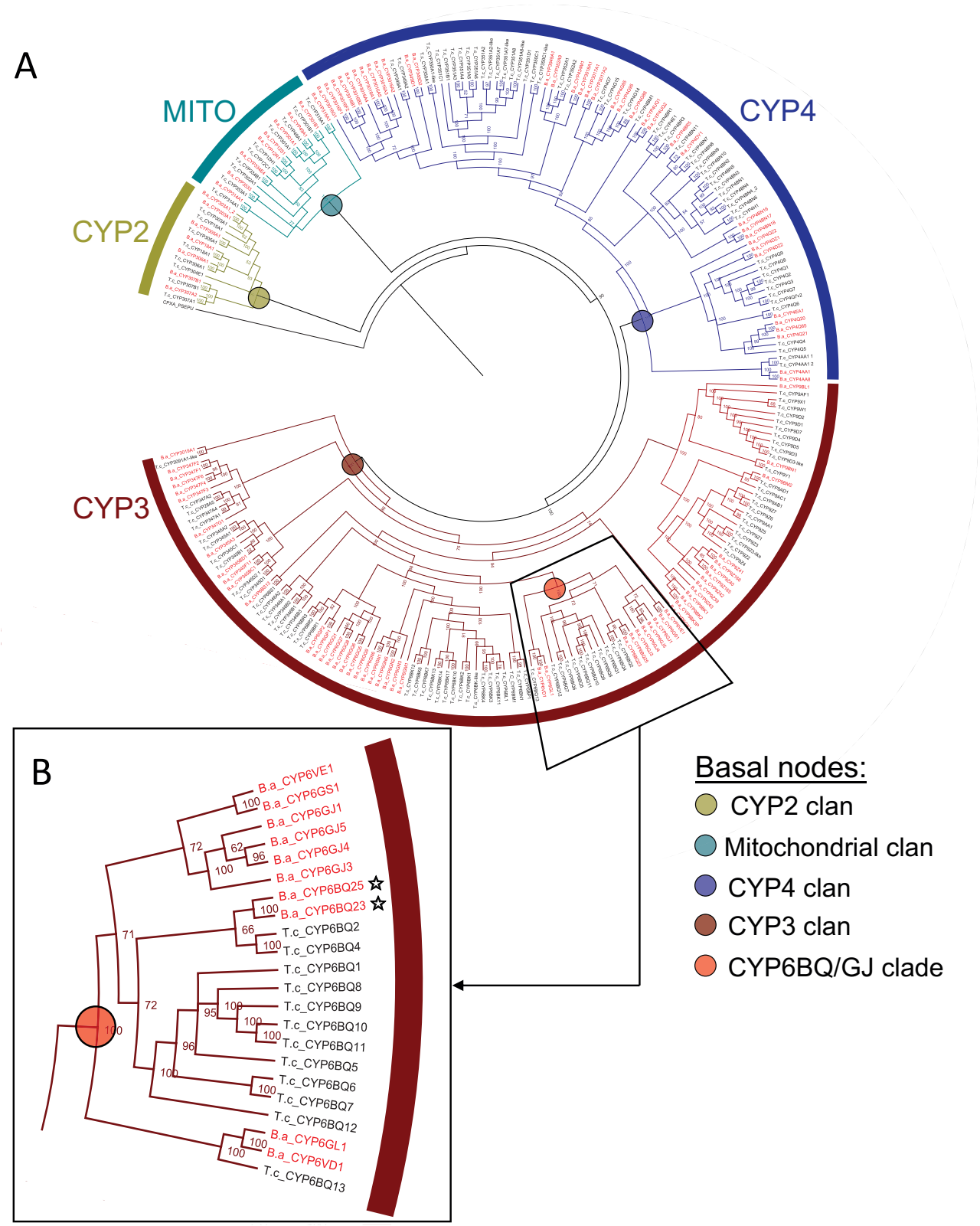

Fig. 2. Phylogenetic relationship of P450s of *Brassicogethes aeneus* and *Tribolium castaneum*. **(A)** Phylogenetic relationship of the four P450 clans of the two species; **(B)** Focus on the clade containing the *T. castaneum* CYP6BQ cluster. P450s were aligned with the outgroup P450cam, the camphor hydroxylase from *P. putida* and phylogeny estimated using Bayesian inference. Branches are coloured by P450 clan and leaves by species (red for *B. aeneus* and black for *T. castaneum*). *B. aeneus* CYP6BQ sequences marked with a star in figure (B).

B. aeneus chromosome 11 shows good macro-synteny to *T. castaneum* chromosome LG6 (117 LCBs, LCB weight 845 bp) (Fig. S4).

There are 38 annotated named genes in the region ~250Kbp upstream and downstream of the *CYP6BQ* cluster on chromosome LG4 of *T. castaneum.* When these genes were used as query sequences in a discontiguous BLAST search of the *B. aeneus* genome 30 (~84%) returned a top hit of chromosome 7 (Table S8). It appears that while there is conservation of global chromosomal gene content (inter-chromosomal synteny or macro-synteny) between *T. castaneum* chromosome LG4 and *B. aeneus* chromosome 7, the order of genes has not been conserved (Fig. S3, Table S8). Although there is evidence of some micro-synteny, overall, it appears that gene shuffling has occurred and intra-chromosomal synteny is present at a low level. This is in keeping with other research into syntenic patterns in Coleoptera which revealed that there is a high level of macro-synteny, a low level of translocation events and modest levels of micro-synteny across the order (Van Dam et al. 2021). The apparent translocation of *B. aeneus CYP6BQ* genes to a new genomic position therefore is potentially an unusual evolutionary event.

As detailed in the introduction, two P450s have been identified as overexpressed in pyrethroid resistant pollen beetle populations (Zimmer et al. 2014a, b). The first, CYP6BQ23, was shown to hydroxylate the alcohol moiety of pyrethroids to a less toxic hydroxy-metabolite *in vitro* (Zimmer et al. 2014a). The second, CYP6BQ25, is closely related to CYP6BQ23 (86.1% amino acid identity), however, to date, its causal role in pyrethroid detoxification has not been demonstrated (Zimmer et al. 2014b). In the present study, *CYP6BQ23* was found to occur on chromosome 11 of the *B. aeneus* genome assembly and was not found in a gene cluster with other P450s (Fig. S3). Surprisingly, however, BLAST searches of the previously described *CYP6BQ25* sequence failed to identify this gene in the genome assembly. Similarly, BLAST searches of the raw PacBio reads did not return hits that corresponded to this P450. In contrast, mapping HiC Illumina reads to the open reading frames of the two P450s identified reads that corresponded to *CYP6BQ25* (Fig. S5). The finding that *CYP6BQ25* was not present in our *de novo* genome assembly may result from technical reasons, such as reduced coverage of PacBio reads at the *CYP6BQ25* loci, or the fact that the loci occurs in a repetitive, or difficult to sequence or assemble, region of the genome. In support of this, we exploited HiC Illumina reads to extend beyond the 5′ end of the transcriptome coding sequence, with an initial consensus subsequently degenerating into unique sequences for each read (Fig. S5). Of these, the most extensive sequence that could also be found in the genome was from a read which aligned to position 2,476,049–2,476,193 on chromosome 11, diverging from the genome prior to the start of the *CYP6BQ25* coding sequence (Fig. S5B). Moreover, at the 3′ end, iterative extensions using the Hi-C reads ended with a repeat sequence with high coverage (Fig. S5C). This repeat is found 17 times across the genome, including at position chr11:2,585,957–2,586,047, in reverse complement form. Other variations of this repeat taken from the Hi-C alignments can be found nearby. Given this closeness to the putative 5′ location detailed above, we conclude this region is the likeliest candidate for where *CYP6BQ25* is located. Thus, the association of *CYP6BQ25* with highly repetitive sequences, which can be difficult to sequence and assemble, may explain its absence in the final assembly.

An alternative explanation is that the *CYP6BQ25* gene copy may segregate in populations of *B. aeneus*. Further experimental work is required to investigate this possibility, however, *CYP6BQ25,* was found to be expressed in samples from Ukraine, Germany, Poland and Sweden in a previous study (Zimmer et al. 2014b), and was found to be expressed in 22 pollen beetle samples from Germany in this study (see below and Table S9). This suggests that *CYP6BQ25* is present at high frequency in European populations of *B. aeneus*.

3.3 The role of CYP6BQ P450s in pyrethroid metabolism and resistance

The high level of amino acid sequence similarity of CYP6BQ25 with CYP6BQ23, a known pyrethroid metaboliser, suggests that it may also be capable of metabolising pyrethroid insecticides or their primary metabolites. To investigate this, and gain insight into the relative importance of these two P450s in resistance, we first deduced the full-length coding sequence of *CYP6BQ25* by 3′ RACE to obtain the complete coding sequence of 1,575 bp encoding a predicted protein of 525 amino acids (Fig. S6). Both CYP6BQ25 and CYP6BQ23 were then heterologously expressed with *D. melanogaster* CPR in an insect cell line. Both recombinant P450s were catalytically active against BOMFC, confirming the functional expression in both cases, although the specific activity of CYP6BQ23 for this coumarin probe was significantly ($p < 0.05$) greater than that of CYP6BQ25 (Fig. 3A). Incubation of microsomal preparations containing each P450 and CPR with the pyrethroid deltamethrin and subsequent LC-MS/MS analyses revealed that both CYP6BQ25 and CYP6BQ23 can hydroxylate deltamethrin resulting in 4-hydroxy deltamethrin (Fig. 3B). The rate of deltamethrin hydroxylation by both recombinantly expressed P450s was found to be time-dependent and followed Michaelis–Menten kinetics in response to deltamethrin concentration (Fig. 3B) resulting in a K_m value of 8.19 ± 1.48 and 6.59 ± 1.48 μM and a V_{max} value of 2.77 ± 0.21 and 2.11 ± 0.18 pmol/min/mg protein for CYP6BQ23 and CYP6BQ25 respectively. The co-incubation of CYP6BQ23 and CYP6BQ25 revealed no enhancement of deltamethrin metabolism (V_{max} = 2.52 ± 0.31 pmol/min/mg protein). In addition, we incubated recombinantly expressed CYP6BQ25 with 4-hydroxy deltamethrin but failed to detect any depletion of this metabolite (data not shown).

To explore the potential impact of amino acid differences between CYP6BQ25 and CYP6BQ23 on pyrethroid metabolism, homology models were generated for both P450s

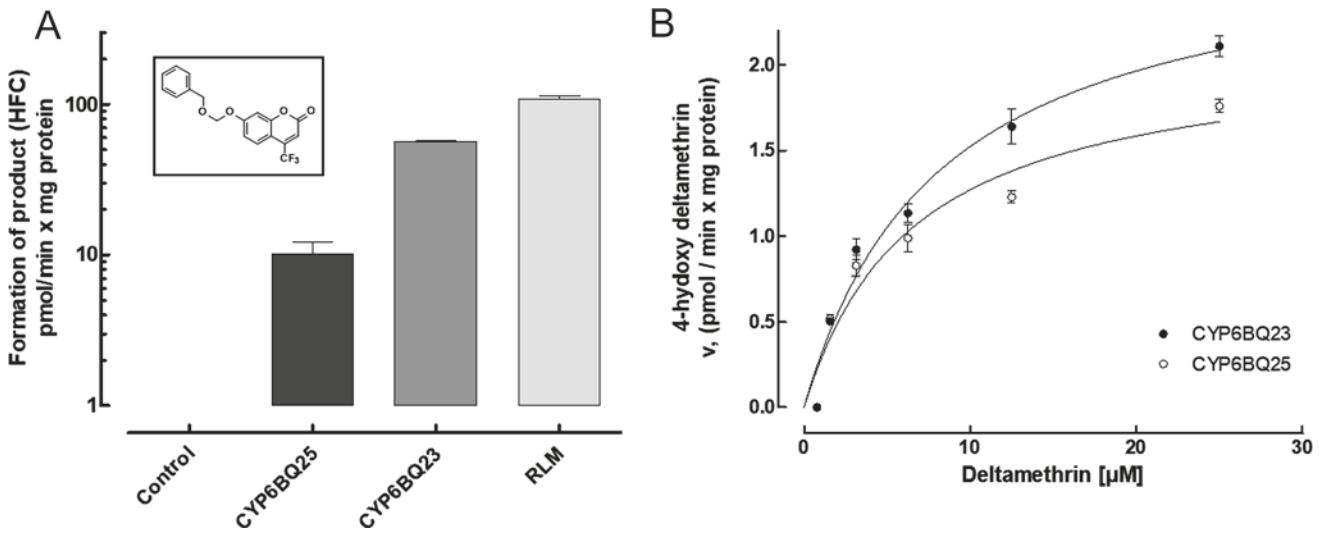

Fig. 3. Catalytic activity of CYP6BQ23 and CYP6BQ25 of *Brassicogethes aeneus* against a model fluorescent substrate and the insecticide deltamethrin. **(A)** O-dearylation of the artificial model substrate BOMFC (see inset for chemical structure) by recombinant CYP6BQ23 and CYP6BQ25 in High Five™ cells as compared to rat liver microsomes (RLM). High Five cells with empty baculovirus (control) and reactions without NADPH were used as negative controls. Data shown are mean values ± SD (n = 9). **(B)** Michaelis-Menten kinetics of the formation of 4-hydroxy deltamethrin by recombinantly expressed CYP6BQ23 and CYP6BQ25 of *B. aeneus*. Data points are mean values ± SEM (n = 3).

(Fig. 4). Investigation of the putative substrate binding site identified just two amino acid residues (S222L and V323I) that vary between CYP6BQ25 and CYP6BQ23 (Fig. 4A–C). However, both of these residues are not predicted to be readily accessible to substrates in the active site and thus are unlikely to play a major role in ligand docking. Computational docking of deltamethrin in the catalytic site of the two P450 models identified preferred docking poses of this insecticide and provided additional evidence that the S222L and V323I substitutions have minimal impact on deltamethrin position and orientation in the active site (Fig. 4D, E). These analyses also rationalised the activity and metabolite formation observed for CYP6BQ25 against deltamethrin in vitro by illustrating a putative mode of oxidative attack by the heme iron-oxygen centre of this P450 that would result in the hydroxylation of the phenoxybenzyl para position of deltamethrin leading to the observed 4-hydroxy metabolite (Fig. 4F).

To further explore the relative importance of *CYP6BQ25* and *CYP6BQ23* in the resistance of field populations of *B. aeneus* to pyrethroids we examined the level of expression of these P450s in 22 pollen beetle populations collected in Germany falling into two different pyrethroid resistance classes as defined by IRAC (Slater et al. 2011). Specifically, qPCR was used to compare the expression of 7 samples categorised as 'moderately resistant' (IRAC code 3) and 15 samples categorized as 'resistant' (IRAC code 4) relative to a sample from Ukraine categorized as 'highly susceptible' (IRAC code 1). These susceptibility categories have been validated alongside pyrethroid efficacy field trials to demonstrate that reduced efficacy in insecticide bioassays correlates with equivalent reduced performance in the field (Brandes et al. 2014). *CYP6BQ23* was found to be highly overexpressed in both the moderately resistant (47-fold to 368-fold) and resistant (180-fold to 1191-fold) samples compared to the pyrethroid susceptible sample (Fig. S7A). Furthermore, the overall level of expression between the two categories of resistant samples was significantly (p = 0.0083) greater in the resistant samples than the moderately resistant samples (Fig. S7A). Thus, the pattern of expression of *CYP6BQ23* in the samples tested is consistent with an important role in pyrethroid resistance. In contrast, *CYP6BQ25* was not found to be consistently overexpressed in pyrethroid resistant *B. aeneus* compared to the susceptible reference, with expression in the moderately resistant and resistant samples ranging from 0.2-fold to 3.1-fold and 0.2-fold to 6.0-fold respectively relative to the pyrethroid susceptible sample. Furthermore, no significant difference in expression levels (p >0.05) was observed between the moderately resistant and resistant samples (Fig. S7B). Thus, these data suggest that *CYP6BQ25* plays a much less significant role in pyrethroid resistance in *B. aeneus* than does *CYP6BQ23*.

4 Discussion

Here we present the first genome sequence assembly for *B. aeneus*. Measures of assembly contiguity (N50, L50 etc.) and gene representation (BUSCO and OrthoDB assessment) are indicative of a highly contiguous chromosome scale assembly with a high degree of gene content completeness. Thus, the new genome assembly of *B. aeneus* represents a powerful resource for further research on this important

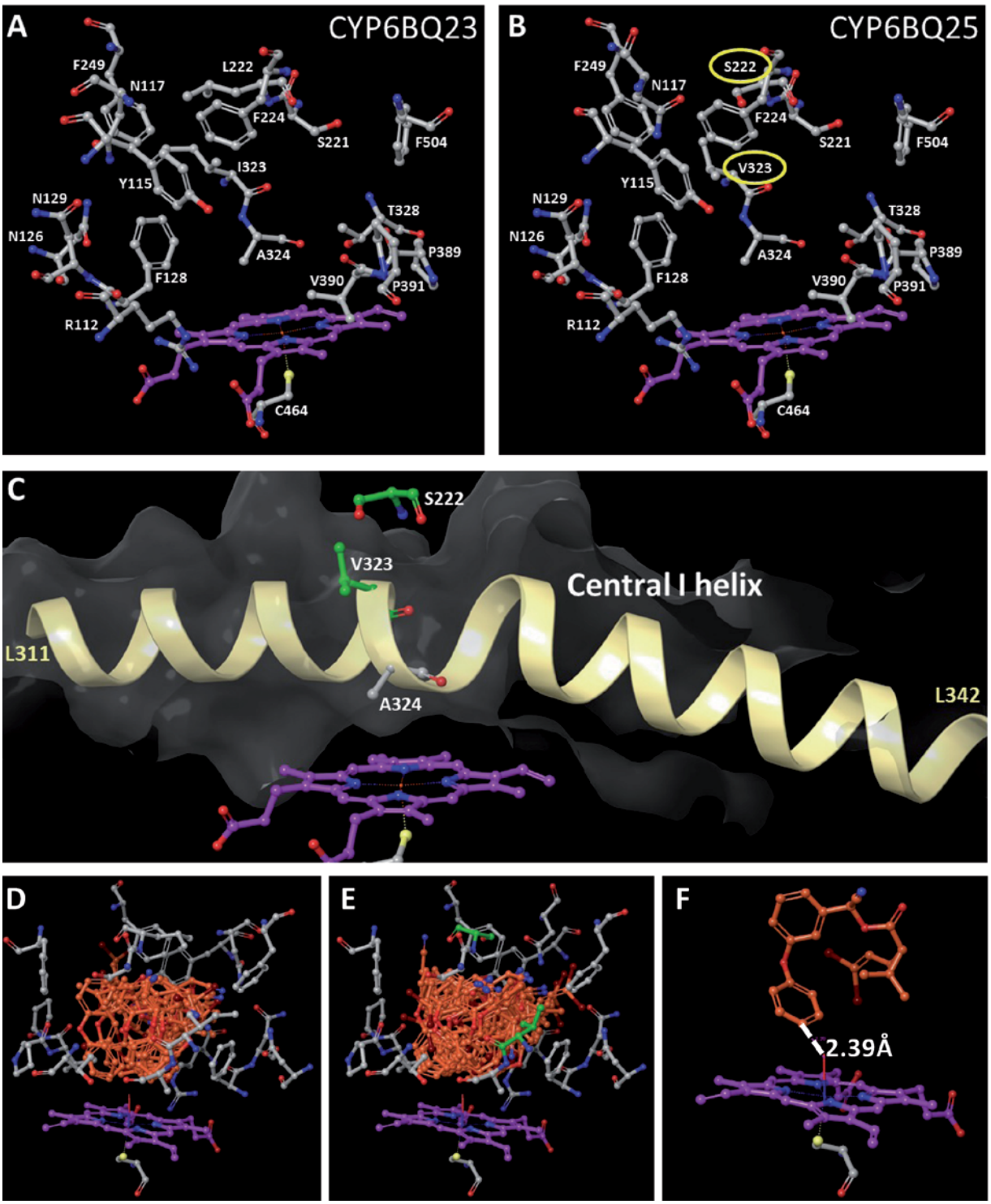

Fig. 4. Binding site homology modeling based on the crystal structure of human CYP3A4 (PDB-ID: 4D6Z co-crystallized with a ritonavir-derived inhibitor and covalently bound to imidazole) of *Brassicogethes aeneus* CYP6BQ23 **(A)** and CYP6BQ25 **(B)**. Amino acid residues shown constitute the putative substrate binding site defined by a distance of 4Å from any atom of the co-crystallized inhibitor from reference cited above. Amino acids different between CYP6BQ23 (A) and CYP6BQ25 (B) are encircled. For orientation the conserved I helix residue A324 is shown along with the two amino acid residues (S222 and V323) that differ in CYP6BQ25 compared to CYP6BQ23 **(C). (D)** Catalytic site model of CYP6BQ23 with 11 identified deltamethrin docking poses derived from a hierarchical conformational clustering; additionally displayed are all amino acid residues in a 3.5 Å radius around the heme iron-oxygen. **(E)** Catalytic site model of CYP6BQ25 with 17 deltamethrin docking poses as described above; differences in the catalytic sites when compared to CYP6BQ23 are indicated by green-coloured amino acid residues. **(F)** Representative deltamethrin docking pose inside the CYP6BQ25 catalytic site, illustrating a plausible mode of oxidative attack by the heme iron-oxygen center resulting in 4-hydroxy deltamethrin. Carbons are generally colour-coded in orange.

pest species. In the current study, we leveraged this genomic resource to investigate the molecular basis of P450-mediated resistance to pyrethroids.

Curation of the CYPome of *B. aeneus* revealed a contracted complement of P450s belonging to the CYP6BQ subfamily compared to other beetle species, with just two members in *B. aeneus* compared to 12 members in *T. castaneum*. This is significant as there is growing evidence that P450s belonging to this subfamily may be important metabolisers of xenobiotics. Specifically, previous work has shown that *CYP6BQ9* is 200-fold upregulated in QTC279, a deltamethrin-resistant *T. castaneum* strain, compared to a lab susceptible strain, with functional analyses demonstrating the causal role of this P450 gene in resistance (Zhu et al. 2010). Furthermore, subsequent work on the *CYP6BQ* gene cluster in *T. castaneum* has provided evidence that the majority of P450 genes in this subfamily may contribute to pyrethroid resistance. Specifically, 10 out of 12 of these genes were found to be significantly upregulated in QTC279 compared to a susceptible strain, and the expression of 6 of these was induced by exposure to deltamethrin (Zhu et al. 2013). The reasons for the marked difference in *CYP6BQ* gene content between *B. aeneus* and *T. castaneum* is unclear but may reflect differences in the life histories of the two species and in their pattern of exposure to xenobiotics. Intriguingly, in *T. castaneum* the majority (~67%) of P450 genes in the CYPome are found in gene clusters with the largest of these containing 12 CYP6BQ genes with the same orientation located within a 30 kb region on the LG4 chromosome (Zhu et al., 2013). In contrast *CYP6BQ23* was identified in our assembly as a singleton on chromosome 11, while *CYP6BQ25* was not incorporated in our assembly but inferred, using short sequence read information, to reside at a distant locus on the same chromosome in association with repetitive sequences. Thus, the distribution of members of this P450 gene family in *T. castaneum* is consistent with expansion by tandem gene duplication (Zhu et al. 2013), while a similar mechanism is not supported for *B. aeneus*.

Our functional analyses revealed that both of the CYP6BQ subfamily members in *B. aeneus* have the capacity to detoxify deltamethrin, by hydroxylating this compound to its 4-hydroxy metabolite. These results confirm the findings of previous work on CYP6BQ23 (Zimmer et al. 2014a) while also demonstrating the pyrethroid metabolising capacity of CYP6BQ25 for the first time. Homology modelling of the two P450s supported these findings suggesting the key amino acids that make up the substrate binding site are largely conserved between CYP6BQ23 and CYP6BQ25 resulting in very similar positioning of deltamethrin in the active site of the two enzymes.

The finding that both CYP6BQ23 and CYP6BQ25 metabolise deltamethrin with similar efficiency raises a question on their relative contribution to pyrethroid resistant phenotypes as defined earlier (Slater et al. 2011). Our investigation of the expression of the two P450s in highly susceptible, moderately resistant, and resistant pollen beetle samples provides insight into this question by revealing clear differences in the expression profile of the two P450s. The finding that *CYP6BQ23* is highly overexpressed in moderately resistant pollen beetles compared to a susceptible sample, and overexpressed to an even greater extent in the resistant category of pollen beetles, strongly supports a major role of this P450 in resistance in the field. Our findings are also consistent with previous studies which found this P450 to be from ~130- to >1800-fold overexpressed in resistant strains (Zimmer et al. 2014a, b). In contrast, our finding that *CYP6BQ25* was not consistently overexpressed in the resistant pollen beetle samples tested and exhibited no significant difference in overall expression between moderately resistant and resistant pollen beetle samples, suggests it plays, at best, a more minor role in resistance. A previous transcriptomics study also found *CYP6BQ25* to be overexpressed to a much lower extent than *CYP6BQ23* (Zimmer et al. 2014b), although the levels of overexpression of *CYP6BQ25* reported previously (>15-fold in samples from Sweden, Denmark and Poland compared to a susceptible sample from Ukraine) are higher than those observed in our study, which were all from Germany. Thus, there may be a degree of variation in the importance of CYP6BQ25 in pyrethroid resistance between geographically separated pollen beetle populations.

In conclusion, while uncovering the pyrethroid metabolising potential of CYP6BQ25 our data also provide further evidence of the important role of CYP6BQ23 in pyrethroid resistance in *B. aeneus*. Together with the results of previous studies (Zimmer et al. 2014a, b), these findings illustrate the potential for the overexpression of this P450 to serve as a molecular marker of resistance. Current estimates of *CYP6BQ23* overexpression require the extraction of RNA for use in qPCR, precluding the testing of samples stored in ethanol. Thus, the future identification of mutations linked to resistance (such as those in the promoter regions of *CYP6BQ23* that lead to its overexpression) would allow the development of DNA-based assays, enabling the testing of stored samples and increased throughput. In this regard, the genome assembly generated in our study provides a reference assembly for the future mapping of DNAseq data from resistant and susceptible pollen beetle samples at the *CYP6BQ23* locus, facilitating the identification of DNA markers linked to resistance. Diagnostics targeting these markers could then be used, in combination with diagnostic assays developed for the pyrethroid target-site mutation L1014F (Nauen et al. 2012), to determine the frequency and distribution of these key resistance mechanisms and inform resistance management and rational control strategies. Unfortunately, resistance to pyrethroids is now widespread in pollen beetle populations throughout much of Europe and only a few alternative insecticides with a different mode of action have been introduced for control. The new genome assembly will facilitate the rapid identification of the molecular basis of resistance against these compounds should it arise, while also aiding in the development and study of novel insecticidal and non-insecticidal control measures.

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