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Title:
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Date:
2020-02-01

Citation:
Yang, Q., Umina, P. A., Rašić, G., Bell, N., Fang, J., Lord, A. & Hoffmann, A. A. (2020). Origin of resistance to pyrethroids in the redlegged earth mite (Halotydeus destructor) in Australia: repeated local evolution and migration. Pest Management Science, 76 (2), pp.509-519. https://doi.org/10.1002/ps.5538.

Persistent Link:
https://hdl.handle.net/11343/286102

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Origin of resistance to pyrethroids in the redlegged earth mite (*Halotydeus destructor*) in Australia: repeated local evolution and migration

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This is the author manuscript accepted for publication and has undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process, which may lead to differences between this version and the Version of Record. Please cite this article as doi: 10.1002/ps.5538

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Abstract

BACKGROUND: *Halotydeus destructor* is a major pest of crops and pastures across southern parts of Australia. This invasive mite has been chemically controlled for over 50 years, but resistance to synthetic pyrethroids and organophosphates is developing. Understanding processes behind the emerging resistance is important for effective management efforts. We undertook a ddRAD pool-sequencing approach to analyse genome-wide SNP variation in *H. destructor* population samples at two scales: local resistance across a set of fields, and regional resistance across their Australian range, along with toxicology bioassays to screen for pyrethroid resistance.

RESULTS: Spatial patterns of genomic variation and resistance at a local scale indicated genetic similarity among samples were more closely correlated with distance along roads and fence-lines than with straight-line geographic distance. This pattern was particularly strong in resistant samples, which were also more related than the susceptible samples, suggesting local spread of resistance within an area after it emerged. In contrast, regional data suggests resistance has repeatedly emerged within parts of Australia. Our *de novo* annotation of the *H. destructor* draft genome sequence and Bayesian analysis identified several candidate loci strongly associated with population-level resistance to pyrethroids, located in genomic

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regions that code for transmembrane transport and signalling proteins which have been previously linked to insecticide resistance in other arthropods.

CONCLUSION: Our findings highlight multiple independent evolutionary events leading to resistance in *H. destructor*, and demonstrate the utility and cost-effectiveness of a cross-population, genome-wide association study to reveal processes underlying adaptive evolution in a non-model invasive species.

Key words: Resistance, *Halotydeus destructor*, evolution, population genetic structure, SNP

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Introduction

Pesticide resistance continues to increase across invertebrate agricultural pests and disease vectors as their control remains reliant on chemical pesticides [1]. Genetic research on resistance has tended to focus on the identification of dominance patterns and relatively simple genetic mechanisms that underlie resistance, which can assist in developing markers for rapid diagnostics [2-4]. Genetic information can also help in identifying population processes that are involved in the evolution of resistance. For instance, an important question is whether resistance develops repeatedly at a local scale or develops rarely and then spreads across regions. There is evidence for both these scenarios being important; long distance migration of genes has been implicated in the spread of resistance to pyrethroids in *Culex pipiens* [5], whereas local evolution of resistance has been critical in *Bemisia tabaci* resistance to organophosphates [6] and *Tetranychus urticae* resistance to various chemicals [7]. In other pests, such as *Spodoptera frugiperda,* an intermediate situation seems to occur where long distance migration plays a role only in some cases [8].

The relative importance of resistance evolving locally, rather than by migration, informs management programs designed to minimize the incidence and spread of resistance. If resistance readily evolves at a local scale, it is important to reduce selection pressures for resistance genes [9] through processes such as the rotation of pesticides with different modes of

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action. Such a strategy, for instance, underlies attempts to reduce pyrethroid resistance in *Plutella xylostella.* Conversely, if resistance persists despite periods when there is no local application of pesticides, immigration of resistant individuals may play an important role in the persistence of resistance [10] and the management of pesticide applications in regions away from target areas may need to be considered. In regions where resistance is absent, it becomes particularly important to reduce the risk of inadvertent importation of resistance genes, as in *Aedes aegypti* and *Aedes albopictus*[11].

Halotydeus destructor, is a phytophagous pest of field crops in Australia which likely invaded from South Africa, although the exact pathway of invasion remains unclear [12]. This sexually-reproducing species can occur at high densities and is particularly damaging to oilseed and clover crops. Pesticide application is the main tool for controlling this pest, particularly the use of pyrethroid and organophosphate chemicals, although the treatment of seed with neonicotinoids has become commonplace. There is increasing evidence for the emergence of resistance to the applied chemicals in *H. destructor*, with tolerance to organophosphates first noted in 1997 [13], resistance to pyrethroids identified in 2006 [14] and resistance to organophosphates in 2016 [15].

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Pyrethroid and organophosphate resistance were thought to be confined to growing regions in Western Australia, but have recently been detected several thousand kilometres away in South Australia [16]. In response to these developments, a resistance management program has been established [17] but needs to be supported by science aimed at understanding the patterns of spread as well as other factors like dominance and fitness costs associated with resistance. While active adult movement of this tiny arthropod (1 mm in length) is limited[18], *H. destructor* has potential for higher rates of passive dispersal of diapausing and non-diapausing eggs through agricultural machinery, livestock and fodder.

Genetic studies of *H. destructor* population structure around Australia using allozymes [19, 20] and DNA microsatellite markers[12] pointed to high rates of long-range movement and limited population structure. However, these markers can have low power to detect local genetic structure, whereas high density markers like SNPs can reveal population structure in cases where previously limited structure had been detected with microsatellites and other markers [21-23]. Furthermore, high density markers can also track patterns of gene flow across space to understand movement patterns at different scales [24-26].

Here we use genome-wide SNPs to investigate the genetic structure of resistant and susceptible *H. destructor* populations in different regions where resistance has recently

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evolved, and also in areas that have so far remained susceptible. We adopt a low-cost approach where pools of mites (rather than individuals) were sequenced to test the alternative hypotheses: independent emergence of resistance *versus* spread of resistance *via* migration across spatial scales. We initially undertook a detailed study of one site where pyrethroid resistance has been carefully documented, to contrast patterns of resistance development at a highly localized scale with patterns across a broad regional scale, focussing on multiple areas where resistance has now arisen. Using our genome annotation for *H. destructor*, we tested if putative pyrethroid resistance associated loci are shared among different regional populations. We were interested in elucidating if resistance tends to emerge in the same parts of the mite's genome, or if it evolves uniquely in different populations. We additionally utilised the rich genomic data generated as part of this study to explore putative genes involved in pyrethroid resistance in *H. destructor*.

Materials & Methods

Local sampling and bioassays for evaluating pyrethroid resistance

Halotydeus destructor samples from 16 locations within a single field located at Boyup Brook (BB) in Western Australia (WA) were collected in 2014 (Table 1, Fig. 2a). In this area, fields used for cropping and pasture were separated from each other by permanent contours and roads which could be used to move machinery and where weeds and other

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vegetation is permanently found. Fence-lines also followed contours and roads. Samples of mites (21-50 mites per sample) were collected *via* vacuum (Hoffmann et al. 1997) from a variety of host plants (including weeds) at locations where toxicology bioassays demonstrated the presence of resistant or susceptible individuals. These bioassays included a discriminating dose of the pyrethroid bifenthrin (Talstar 100EC®; bifenthrin 100 g/L; CropCare, Muarrie, Qld, Australia) and a control of water. The discriminating dose of bifenthrin was 0.01 g a.i. L^{-1} (approximately the LC_{99} value multiplied by 2), following Umina *et al.*[27].

Bioassays followed the method developed by Hoffmann et al. [13], which has become the standard approach for determining chemical sensitivities in *H. destructor*[14, 15]. In brief, 15-ml glass vials were coated with bifenthrin and left to dry overnight. Control vials were treated in the same way, with water used instead of pesticide solution. Once dry, a leaf of common vetch (*Vicia sativa*) was added to each vial to provide food and moisture. Individual mites were then placed into each vial, sealed with parafilm (Pechiney Plastic, Menasha, WI, USA) and placed in a controlled temperature cabinet at 18˚C. Mortality was assessed after 24 h.

<u>*Regional sampling*</u>

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We obtained 28 *H. destructor* population samples from four regions across Australia (Fig. 1) between 2015-2017 for the analyses of genome-wide variation by ddRAD pool-sequencing. This included 14 samples from five distinct regions in WA, and 14 samples across South Australia (SA), Victoria (VIC) and New South Wales (NSW) (Table 2). Samples from the same region were collected in the same season. Mites from these locations were characterized for pyrethroid resistance status using toxicology bioassays described above and a discriminating dose of bifenthrin of 0.01 g a.i. L^{-1}. We only included populations that were clearly resistant or susceptible to bifenthrin [27].

ddRAD-seq library preparation

Because the available draft genome assembly for *H. destructor* was fragmented (36,016 contigs, provided by Tom Walsh, CSIRO), we used two approaches to determine optimal restriction enzyme combination that would maximize the number of RAD tags and the number of biological pools. First, we used *DDsilico* [22] to test different enzyme combinations *in silico* digestion of the *H. destructor* draft genome assembly. Second, we used BioAnalyzer traces of different single and double enzyme digestions to approximate the number of sequenceable fragments [28]. Both approaches indicated the *NlaIII* and *MluCI* enzyme combination to be optimal, estimated to produce 40,000 RAD tags within a fragment length

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range 235-385bp per genome. This setup allowed for more than 60 pools of 50 individuals each to be sequenced to the desired average read depth > 50 in just one HiSeq2500 lane.

We performed an initial digestion of 120 ng of pooled genomic DNA in a 40 μL reaction, using 10 units each of *Mlu*CI and *Nla*III restriction enzymes (New England Biolabs, Beverly MA, USA), NEB CutSmart buffer, and water. Digestions were run for 3 hours at 37˚C without a heat kill step, and the products were cleaned with 1.5× volume of Ampure XP paramagnetic beads (Beckman Coulter, Brea, CA). The digestion fragments were ligated to modified Illumina P1 and P2 adapters [22] overnight at 16˚C with 1000 units of T4 ligase (New England Biolabs, Beverly, MA, USA), followed by a 10-minute heat-deactivation step at 65˚C. Isolation of fragments between 235-385 bp in length was done using a Pippin-Prep 2% gel cassette (Sage Sciences, Beverly, MA). Final libraries were created by pooling eight 10 μL PCR reactions per library, each consisting of 1 μL size-selected DNA, 5 μL of Phusion High Fidelity 2× Master mix (New England Biolabs, Beverly MA, USA) and 2 μL of 10 μM standard Illumina P1 and P2 primers, run for 12 PCR cycles. These were cleaned and concentrated using an 0.8× concentration of Ampure XP paramagnetic beads (Beckman Coulter, Brea, CA) to make the final libraries. Pool-seq libraries were sequenced in three Illumina HiSeq2500 lanes using 100 bp paired-end chemistry.

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Data processing and genotyping

We processed raw fastq sequences within a customized pipeline [22], to retain reads with a minimum Phred score of 13 and trimmed them to equal length of 90 bp. High-quality reads were then passed to the program *Bowtie* [29] to be aligned against the draft *H. destructor* genome sequence. Uniquely aligned sequences in each pool were processed to achieve the same per-individual number of reads in all pools (normalization by random removal of reads). Normalized bowtie files were converted into the SAM format, and then to sorted BAM format and a pileup file using *SAMTools* [30]. We then used *Popoolation2* scripts (*mpileup2sync.pl*, *snp-frequency-diff.pl, fst-sliding.pl*) to create files with major and minor allele counts as well as F_{ST} for each SNP. Finally, this file was processed through our custom R script to produce different input files for downstream population genomic analyses.

Principal Component Analysis

We created data sets of minor allele frequency (MAF) for different *H. destructor* population scales by removing SNPs with MAF less than 0.05. To visualize the overall distribution of genetic diversity across sites, Principal Component Analyses (PCAs) were performed on informative SNPs using MAF and the R package '*adegenet*'. Sites that are genetically similar should appear close together based on their scores for Principal Components that account for most of the variance in MAF across all loci. PCAs were undertaken for 16 local locations

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within a single field in WA, for the entire collection of *H. destructor* samples (n=28) across Australia, for the WA samples only (n=8) where resistance first arose and where resistant samples were present from multiple sites, and for the samples collected from SA, VIC and NSW (n=14).

Phylogenetic analysis

Historical relationships among *H. destructor* populations at 4 scales were reconstructed in the program *PHYLIP* package v3.695 (http://evolution.genetics.washington.edu/phylip.html). The matrix of fixation indices (F_{ST}s) between population pairs was used to create a Neighbor-Joining tree with *PHYLIP*. Trees were produced by *FigTree* v.1.42 [31].

Isolation-by-distance

Isolation-by-distance (IBD) can be regarded as a null hypothesis when migration is spatially limited and there are no additional effects of landscape features. To test IBD, we generated six different spatial F_{ST} matrices to characterize the spatial relationship among 16 *H. destructor* samples within the field: geographic straight-line distance across all samples, geographic straight-line distance in susceptible and resistant samples treated separately (null expectations, IBD), distance along fences/contours/roads (f/c/r) among all samples, and f/c/r distance among resistant only and susceptible only samples.

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Genome annotation

We evaluated the assembled draft genome of *H. destructor* (49.67 Mb in size with 36,016 contigs and contig N50 of 1.91Kb, Table 3) using *CGEMA* (v2.5). Four software packages, *LTR-FINDER* (v1.0.5), *MITE-Hunter* (v1.0.0), *PILER* (v1.0), and *Repeat Scou*t (v1.0.5) were used to build a *de novo* repeat library from our assembly, using default settings. The predicted repeats were then classified using *PASTEClassifier* (v1.0) and merged with Repbase (v19.06). *RepeatMasker* (v4.0.5) was used to identify repetitive sequences in the *H. destructor* genome and create a database for the final repeat library.

To identify the protein-coding genes in the genome assembly, Augustus (v2.4), *GlimmerHMM* (v3.0.4), *GeneID* (v1.4) and *SNAP* (v2006-07-28) with the default parameters were used for the *ab initio*-based gene prediction. *GeMoMa* (v1.3.1) was applied in homolog-based gene annotation, with the protein databases of *Drosophila melanogaster* and *Tetranychus urticae* from GenBank provided references. The results from the three methods were integrated with *EVM* (v1.1.1). Genes were annotated by aligning to the non-redundant protein sequences, Nt (Nucleotide collection (NR, Nt)), eukaryotic orthologous groups of proteins (KOG), Kyoto Encyclopedia of Genes and Genomes (KEGG), Swissprot, and TrEMBL databases using the Basic Local Alignment Search Tool (BLAST) with cut-off set

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at $1E^{-5}$ and also aligned to the Pfam database with hmmer (V3.0). Gene Ontology (GO) terms were assigned to the genes under the *BLAST2GO* pipeline.

Outlier identification

Spatial heterogeneity of pesticide applications among fields would likely exert strong selection over small spatial scales. To test this hypothesis, we used a Bayesian approach developed by Gautier [32] implemented in the program *BayPass* which calculates an F_{ST} analog called *XtX* and can perform association tests between genomic outliers and population-specific covariables. The *BayPass* feature was particularly useful for analysing our pool-seq dataset because it considers read count information when estimating allele frequencies (pool-seq analysis mode) [32]. We used a subset of the data from the regional sampling, which consisted of five sets of 2 pool-seq population samples, each phenotyped for resistance and susceptibility to pyrethroids. As detailed in Table 3, the final data set consisted of allele counts of 130,736 - 249,758 SNPs. We employed the core model approach provided in *BayPass,* with a threshold of 97.5% and 95% respectively based on these filtered SNP loci for each set of pool-seq samples. Then we intersected the outliers from the five sets to identify candidate genes and assess if independent/repeated emergence of resistance could be driven by the changes is the same genomic regions.

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Results

Local sampling

Of the 16 samples collected from Boyup Brook, 22,688 SNPs (after filtering) were used to calculate minor allele frequencies and pairwise F_{ST} values. A PCA showed the genomic similarity increased when the samples were located nearby, particularly when they fell onto the same f/c/r line (Fig. 2). A plot of scores on PC1 (accounting for 27.09% of the variance) versus PC2 (19.28% of variance) showed that nearby susceptible samples of S-1, S-2 and S-3 formed one group, as did S-14, S-15 and S-16. Likewise, resistant samples R-5 and R-6 were closely connected, as were R-4 and R-8.

The regression between genetic differentiation and geographic distance of the 16 samples indicated evidence for IBD overall (Fig. 3a), but the relationship was weak and the association between these variables was only marginally significant by a Mantel test (P = 0.049). A stronger association was obtained with f/c/r, with an increase in R^2 (Fig. 3b) and a highly significant Mantel test (P = 0.009). When the resistant and susceptible samples were considered separately, the regression for IBD showed a weaker relationship for the susceptible samples compared with the resistant samples (Fig. 3c), with a Mantel test indicating borderline significant associations (P = 0.05) in both cases. However, the relationship between genetic distance and f/c/r distance was stronger (Fig. 3d), particularly in

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the case of the resistant samples ($P = 0.003$) and to a lesser extent in the susceptible samples ($P = 0.026$). These patterns point to a stronger IBE when the connecting environment is considered to run along the fence, contour and road lines, particularly for resistant mite samples. This was further confirmed by a multiple regression analysis that involved fitting geographic distance and f/c/r distance to genetic distance, which showed that once f/c/r was included in the analysis, there was no additional signature of geographical distance ($P = 0.049$).

Regional samples

When considering the 28 *H. destructor* samples collected across Australia, a PCA and phylogenetic tree analysis indicated independent emergence and local spread of pyrethroid resistance. When all samples are considered in a PCA (Fig. 4), there is clear evidence for susceptible and resistant samples co-clustering for the SA locations of Tintinara and Myponga, and also for the Boyup Brook susceptible and resistant samples. The susceptible samples from VIC and NSW cluster separately, while some SA samples cluster close to WA. While the Boyup Brook susceptible and resistant samples are clustered together, they are separate from other samples from WA, including Dandaragan, East Capel, West Williams and NE-Williams. Clustering of resistant and susceptible samples from these sites is less

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clear-cut. A separate PCA with only 8 samples from these 4 sites from WA (Fig. 5) shows the susceptible and resistant East Capel samples locate close to each other. However, the Dandaragan susceptible and resistant are more distant from each other than expected given that they are geographically located well away from the NE Williams and West Williams samples (Fig. 5). For the South Australian samples, the resistant and susceptible populations from two locations are closely associated, reflecting local emergence of resistance (Fig. 6). The susceptible SA populations are distinguishable genetically from other susceptible populations in Victoria and NSW.

Highly related and spatially close resistant and susceptible mites from several locations in WA and SA (Figs. 5, 6) suggest resistance has emerged locally (independently), but movement of resistant individuals may have contributed to some regional patterns within southern WA. These patterns of relationships between samples are further supported by the phylogenetic trees (Fig. 7). These show the clear separation of the Tintinara and Myponga samples from others in SA and local relatedness between the susceptible and resistant samples at each of the SA locations (Figs. 7a & 7d). They also emphasize the strong patterns of relatedness between susceptible and resistant samples from Boyup Brook and East Capel and the confusing picture around the Dandaragan samples in WA (Figs. 7b & 7c). The

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phylogenetic analysis also confirms the diverged pattern of samples from SA, with samples west of Tintinara (with the exception of Myponga) falling with the WA samples, and the rest being relatively isolated or falling with the other east coast samples, in agreement with the earlier PCAs (Figs. 4 & 6).

Assembly genome annotation

Genome annotation revealed that 74.08% of the genome is composed of repetitive elements and 53.44% is composed of long terminal repeat elements. We predicted 37,188 protein-coding genes, of which 96.53% were functionally annotated. Using *CGEMA*, 403 of the 458 core eukaryotic genes (CEGs) (87.99%) and 193 of the 248 highly conserved CEGs (77.82%) could be found in the *H. destructor* genome assembly (Table S1). Higher weights were assigned to the GeMoMa-predicted homologous transcripts than to the *ab initio*-predicted transcripts when conducting the EVM integration. In total, a gene set with 11,280 protein-coding genes was predicted from the *H. destructor* genome assembly (Table S2). 74% of these predicted genes could be classified into families according to their putative functions (Table S3).

Candidate genes

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We used an outlier identification approach, *BayPass*, to infer positive selection for resistance to synthetic pyrethroids. In total, we identified 11 candidate genes as contributing to the genomic basis of pyrethroid adaptation from the intersection of five comparisons, using a threshold of 97.5%. Enriched functions among these candidate genes involved the cytochrome P450 and G protein-coupled receptor family, as well as the sodium channel gene previously related to pyrethroid resistance (Table S4). We also used a threshold of 95% as a lower boundary to identify more informative markers. This resulted in 126 candidate genes being identified and enriched for GO function (Fig. S1). Cell, organelle, and cell part were significant enrichment terms in the cellular component. Catalytic activity, binding and cellular process were the main functional terms in molecular function.

Discussion

Pyrethroid resistance in *H. destructor* first arose in WA[30] and is thought to be mainly associated with mutations in the sodium channel gene, although pyrethroid resistance in other mites can include other mechanisms as well[33]. The documented occurrence of resistance locally across fields and more widely across regions of WA and other areas, provides an opportunity to explore the role of local evolution versus gene flow in the origin of resistance, with implications for resistance management in *H. destructor*.

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Local patterns

We explored the local origin of pyrethroid resistance at Boyup Brook, where two sodium channel mutations associated with resistance in WA occurred in 2013: a common TTC mutation and a rare TTT mutation[30]. We did not distinguish these mutations because we sequenced pooled samples of mites that individually yielded little DNA. Nevertheless, with one mutation vastly predominating, the level of genomic divergence between samples from Boyup Brook should reflect two processes: a common origin of mites associated with the evolution of resistance, and ongoing gene exchange between mites from adjacent locations. If resistance arises locally at one location, a high level of genomic similarity between the resistant sample from this location and adjacent susceptible samples is expected as a result of ongoing gene flow and a common origin of the mites. If resistant mites move to other areas and are favoured by selection due to pesticide applications, higher genomic differentiation between the resistant sample and nearby susceptible mites is expected to occur until gene flow eventually breaks it down at a rate dependent on mite movement and levels of recombination/independent assortment.

The strong IBE patterns compared to IBD point to contours, roads and fence-lines being a more important predictor of *H. destructor* movement than geographic distance alone. Such a

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pattern would not be expected if resistance repeatedly and independently evolved in each location. The fields bordering these barriers are treated with pesticides to reduce mite (and other arthropod pest) populations, with applications of organophosphates rather than pyrethroids being increasingly applied since the development of resistance (P. Mangano, pers. comm.). These pesticide applications and other practices should diminish mite populations in fields, and it is therefore not surprising that the contours provide a better measure of genetic isolation given that weeds and other vegetation maintained around fence-lines are a source of *H. destructor* [34].

The findings at this fine spatial scale emphasize the power of a SNP-based approach in making inferences about population processes, consistent with previous research on other invertebrate pests and disease vectors. For instance, in *A. aegypti* mosquitoes, SNP markers were able to detect even small barrier effects of major roads on the local movement of mosquitoes within part of a local area [26]. In that study, sequencing was carried out on individuals rather than population samples, but the *H. destructor* data suggests that local patterns can be discerned from sequencing pooled samples when resources are limiting.

Regional patterns

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Comparisons point to regionally-specific resistance rather than movement of resistance genes across large distances. In several cases, there is close similarity genomically between susceptible and resistant mites from the same region. Intensive monitoring of pyrethroid resistance, stemming from industry concerns [16, 27], has meant that new cases of resistance have been rapidly detected, such as in SA in 2016 [35]. Appearance of resistance in SA soon after it was originally detected in WA raised concerns that there is an extensive movement of resistance mites across regions. However, our genomic analyses found no indication that the resistant mites in SA are highly related to those from WA (i.e. that they have a recent common origin). We did, however, find an indication of some movement of resistance genes within WA, given the high similarity between the Dandaragan resistant sample and mites collected from Williams (approximately 400 km apart). This gene flow is likely driven by the passive movement of diapause eggs blown by winds, or through human assisted movement (e.g. contaminated produce, livestock, farm machinery) [36, 37].

The genomic data also indicate a more complex pattern of genetic relationships across Australian *H. destructor* populations than previously inferred from microsatellites and allozyme data. Namely, allozyme research suggested high rates of gene flow in *H. destructor* without evidence of regional structuring [19, 20]. Subsequent microsatellite research [12] also supported inference of limited structure across the entire invasive Australian range. Our work

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supports the notion that eastern Australia populations of *H. destructor* are similar to each other and likely have a recent common origin distinguishable from the western populations. But we also detected a complex pattern of relatedness in SA: some SA populations are more genomically similar to WA populations, suggesting a degree of gene flow between west and east, while other SA populations are more distinct and may have a different origin.

<u>*Candidate genes*</u>

Previous research involving shallow sequencing of the *H. destructor* genome from resistant and susceptible mites identified mutations in the sodium channel gene associated with pyrethroid resistance [30]. Our work, based on outlier detection across multiple samples and regions, has added a list of candidate genes that may also be involved in the evolution of resistance in this pest. Alleles in the sodium channel gene undoubtedly account for a substantial component of resistance [30] as is the case in other mites [33], but other mechanisms have been implicated [33] and our work provides pointers to these possible mechanisms. The P450 genes and G-protein coupled receptor kinases are particularly strong candidates that are worth exploring further given their previous association with resistance in other taxa (Table S4). Several SNPs identified in our study provide useful markers that can be further tested through targeted sequencing of the surrounding genomic regions and through high-throughput genetic screening.

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Conclusions

These findings have implications for the management of pyrethroid resistance in *H. destructor*. The genetic analysis at Boyup Brook suggests mites tend to move along fence-lines and contours, therefore reservoirs of susceptible mites could be maintained in the fence-lines rather than being removed through pesticide applications to ensure that resistance does not easily spread within and among farms *via* active movement of mites. Conversely, if resistance has only recently been detected on a farm along a fence-line, it might be contained by applying chemicals other than pyrethroids.

At the regional level, the repeated evolution of resistance in new areas highlights the importance of reducing local selection pressures to prevent resistance evolution. Recent surveys of pyrethroid resistance alleles through high throughput molecular approaches suggests an extremely low frequency of resistance could exist in *H. destructor* populations (X. Cheng, unpublished data), which may reflect some fitness costs associated with resistance. Rotations of pesticides as well as non-chemical control strategies that reduce selection pressures will therefore be important in decreasing the risk of further local resistance evolution, as advocated in the current National Resistance Management Strategy for this pest [17].

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Acknowledgements

This research was supported by the Grains Research and Development Corporation project UM00057. We thank Owain Edwards and Xuan Cheng for technical assistance, Tom Walsh for providing the draft genome, and Jessica Chung, Shujun Wei, Lijun Cao and Igor Filipovic for bioinformatics assistance.

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Tables

Table 1. Sample information for local resistance monitoring study at Boyup Brook, Western Australia. All samples were collected in 2014.

Sample	Pool size	Pyrethroid resistance status†
1	50	S
2	50	S
3	50	S
4	50	R
5	50	R
6	50	R
7	50	R
8	50	R
9	50	S
10	50	S
11	50	R
12	41	R
13	21	R
14	20	S
15	19	S
16	50	S

†R = resistant, S = susceptible.

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Table 2. Sample information for broader regional surveys of *H. destructor*. Codes indicate resistance status and Australian State – see Fig. 1. Samples were collected between 2014 - 2017. Pool size represents the number of mites combined in a sample.

Sample code	Sampling location †	Pool size	Pyrethroid resistance status ‡
R-WW	West Williams, WA	50	R
S-WW	West Williams, WA	50	S
R-NEW	North-East Williams, WA	50	R
S-NEW	North-East Williams, WA	50	S
S-BB1	Boyup Brook, WA	50	S
S-BB2	Boyup Brook, WA	50	S
S-BB3	Boyup Brook, WA	50	S
R-BB4	Boyup Brook, WA	50	R
R-BB5	Boyup Brook, WA	50	R
R-BB6	Boyup Brook, WA	50	R
S-GOO1	Goorambat, VIC	50	S
S-GOO2	Goorambat, VIC	30	S
S-MOU	Moutajup, VIC	50	S
S-NAR	Naracoorte, SA	50	S
R-MYP	Myponga, SA	20	R
S-MYP	Myponga, SA	20	S
R-TIN	Tintinara, SA	20	R
S-TIN	Tintinara, SA	20	S
S-JAM	Jamestown, SA	50	S
S-HAL	Hallett, SA	30	S
S-MC	Mount Compass, SA	50	S
S-IV	Inman Valley, SA	50	S
S-COO	Cookardinia, NSW	50	S
S-MOR	Morven, NSW	30	S
R-EC	East Capel, WA	50	R
S-EC	East Capel, WA	50	S

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R-DAN	Dandaragan, WA	50	R
S-DAN	Dandaragan, WA	50	S

† Australian State codes: WA = Western Australia, VIC = Victoria, SA = South Australia, NSW = New South Wales.

‡ R = resistant, S = susceptible.

Table 3. Sample information for *BayPass* analysis. Resistant and Susceptible groups were compared from 5 locations. Different numbers of SNPs were available for each comparison. Further information on the samples is provided in Table 2.

Samples in *BayPass* run †	Sample code	Pyrethroid resistance status ‡	SNPs
BB1 (WA)	R-BB5 S-BB12	R S	130,736
BB2 (WA)	R-BB6 S-BB11	R S	154,791
EC (WA)	R-EC S-EC	R S	147,404
MYP (SA)	R-MYP-SA S-MYP-SA	R S	244,216
TIN (SA)	R-TIN-SA S-TIN-SA	R S	249,758

† Australian State codes: WA = Western Australia, SA = South Australia.
‡ R = resistant, S = susceptible.

Table S1. Statistics for the genome assembly of RLEM and its evaluation

	Variable	**Number**
Statistics	Contig number	36,016
	Contig length (bp)	49,562,335
	Contig N50 (bp)	1,970
	Contig N90 (bp)	647

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	Contig max (bp)	55,349
	GC content	45.76%
Evaluation	Number of 458 CEGs present in assembly	403
	% of 458 CEGs present in assembly	87.99%
	Number of 248 highly conserved CEGs present	193
	% of 248 highly conserved CEGs present	77.82%

Contig N50: length-weighted median, at least 50% of the nucleotides in the assembly belongs to contigs with the N50 length or longer. Contig N90: at least 90% of the nucleotides in the assembly belongs to contigs with the N90 length or longer. Contig max: maximum contig length. Number of 458 CEGs present in assembly: 403 genes in assembly were found within the set of 458 core eukaryotic genes (CEGs) defined by CEGMA v2.5, % of 458 CEGs present in assembly: proportion of the 403 genes in the set of 458 CEGs, Number of 248 highly conserved CEGs present: 193 genes in assembly were found 248 highly conserved CEGs defined by CEGMA v2.5, % of 248 highly conserved CEGs present: proportion of the 193 genes in the set of 248 highly conserved CEGs.

Table S2. Summary of genome annotation from different databases.

Method	Software and gene set	Species	Gene number
Ab initio	Augustus	-	14,613
	GlimmerHMM	-	14,253
	GeneID	-	18,010
	SNAP	-	15,152
Homology-based	GeMoMa	*Drosophila melanogaster*	9,467
		Tetranychus urticae	8,834
Integration	EVM		11,280

Table S3. Summary of functional annotation for the predicted genes.

Annotation database	Annotated gene number	Percentage (%)
GG	3,415	30.27
KEGG	4,116	36.49
KOG	5,982	53.03
TrEMBL	7,928	70.28
NR	7,767	68.86

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All	8,347	74.00

Table S4. Details of relevant functions that were significantly enriched among candidate genes (97.5% threshold) of pyrethroids resistant samples. Literature is given if the same or closely related function was previously identified in insects.

Candidate loci	Candidate gene nr_annotation	GO term	Biological plausibility	Reference
contig_1352.1	LDL receptor-related protein		The Vitellogenin (Vg) and Vitellogenin receptors (VgRs) belong to the low-density lipoprotein receptor (LDLR) super family with no clear relationship to pesticide resistance.	Yao et al., 2018
contig_1352.2	conserved oligomeric Golgi complex subunit 1-like		Golgi vesicles play roles in cellular detoxification processes.	Rebecca et al., 2003; Cheng et al., 2012
contig_152.4	cytochrome P450 3A29-like	binding, oxidoreductase activity	Cytochrome P450 monooxygenases (P450s) have been identified as resistance mechanisms linked to detoxification.	Li et al., 2007
contig_152.3	cytochrome P450 354A12			

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contig_152.6	G protein-coupled receptor kinase 2-like	G-protein coupled receptor kinase activity, ATP binding, protein phosphorylation, termination of G-protein coupled receptor signaling pathway	GPCRs are involved in the regulation of resistance P450 gene expression.	Liu et al., 2015
contig_867.1	sodium channel	voltage-gated ion channel activity, sodium channel activity, sodium ion transport, transmembrane transport, integral component of membrane	The voltage-sensitive sodium channel is the primary target of pyrethroid insecticides.	Devonshire et al., 1996
contig_152.2	Sodium-dependent glucose transporter 1			
contig_3088.1	Amiloride-sensitive sodium channel			
contig_6312.1	F-actin-capping protein subunit beta	actin binding, F-actin capping protein complex, WASH complex, actin cytoskeleton organization	F-actin-capping/severing proteins Cofilin, CapZ, and Gelsolin are associated with mechanical stress but not chemical stress.	Thompson, et al., 2016
contig_2838.1	collybistin		Collybistin is a guanine nucleotide exchange factor with unknown links to resistance in invertebrates.	Brose et al., 2015; Papadopoulos et al., 2013

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contig_3088.2	stearoyl-CoA desaturase		Stearoyl-CoA desaturases are key enzymes in the synthesis of monounsaturated fatty acids with unknown links to pesticide resistance.	Bellosta et al., 2017; Wang et al., 2013

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Figure Legends

Figure 1. Sampling locations across Australia

Figure 2. (a) Location of local samples at Boyup Brook East and (b) Results of Principal Components Analysis assessing overall genomic similarity among the samples. “R” are resistant samples, “S” are susceptible samples based on bioassays from mites from the same locations.

Figure 3. Regression analysis of IBD (isolation by distance) and IBE (isolation by environment as measured through fence/contours/roads) at the local level when all the samples are considered and when split into resistant and susceptible samples. In comparisons, “R” are resistant samples, “S” are susceptible samples, and lines indicate linear regressions. (a) all samples considered by geographic distance to test for IBD. (b) All samples considered by fence/contour/road distance to test for IBE. (c) IBD tests where susceptible and resistant

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samples are considered separately. (d) IBE tests where susceptible and resistant samples are considered separately.

Figure 4. Principal components analysis assessing genomic similarity of 28 samples of *H. destructor* from across Australia. Note separation between resistant and susceptible samples as described in Table 2. WA = Western Australia, SA = South Australia, NSW = New South Wales, VIC = Victoria.

Figure 5. (a) Principal components analysis assessing genomic similarity of 8 sites in Western Australia with codes in Table 2 and the position of samples also shown. (b) Sample location map

Figure 6. (a) Principal components analysis assessing genomic similarity of 14 sites across the south-eastern grain belt with codes following Table 2 and the position of samples also shown. (b) Sample location map

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Figure 7. Phylogenetic trees of samples with codes given in Table 2: (a) all regional samples, (b) Western Australia samples apart from Boyup Brook, (c) North eastern Boyup Brook samples, (d) south-eastern Australian samples.

Figure S1. Functionally enriched GO terms of 126 candidate genes (95% threshold from *BayPass*). These analyses show a preponderance of candidates in catalytic activity and binding belonging to molecular function, including candidate genes of cytochrome P450, and sodium channel processes.

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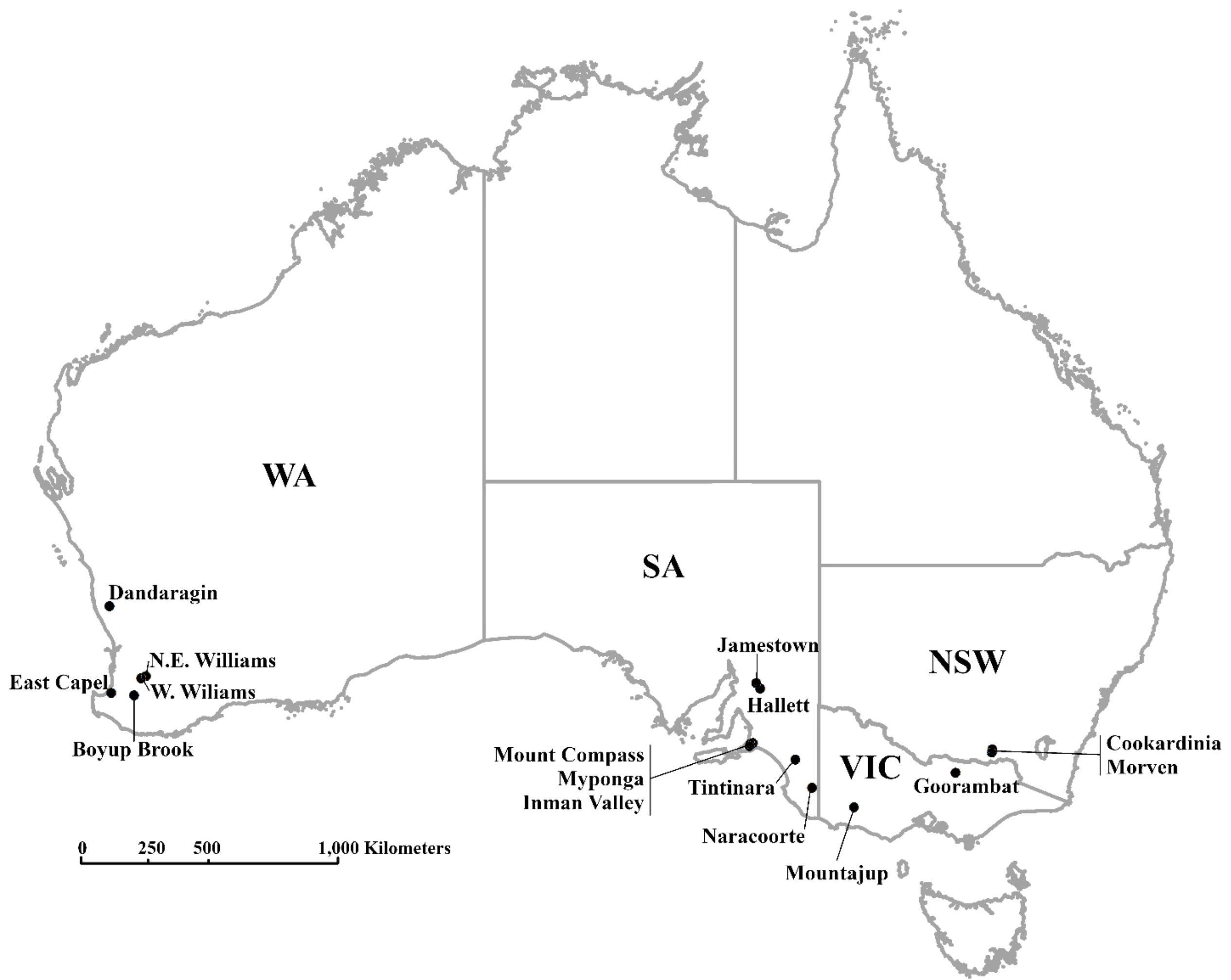

Figure 1. Sampling locations across Australia

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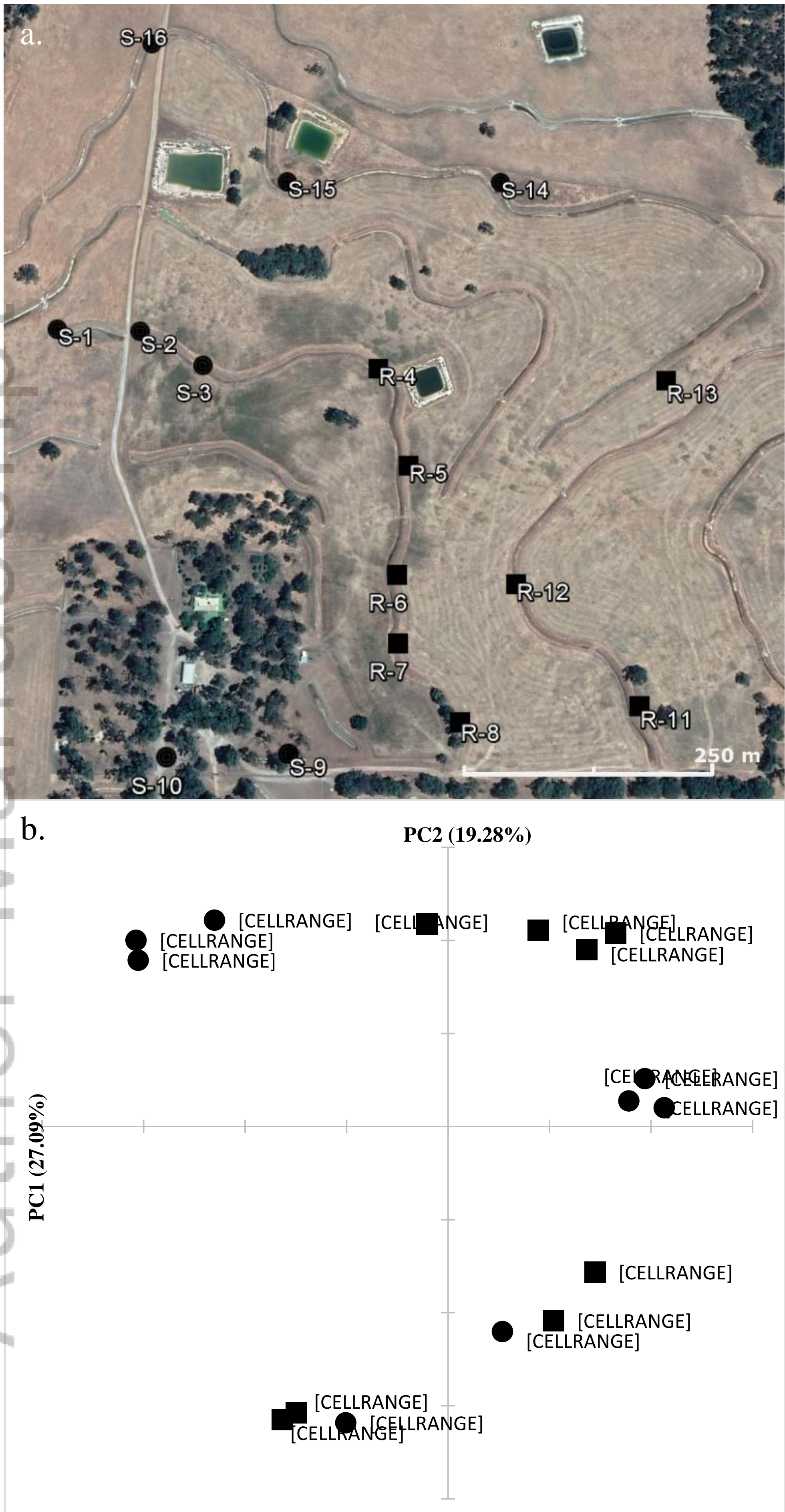

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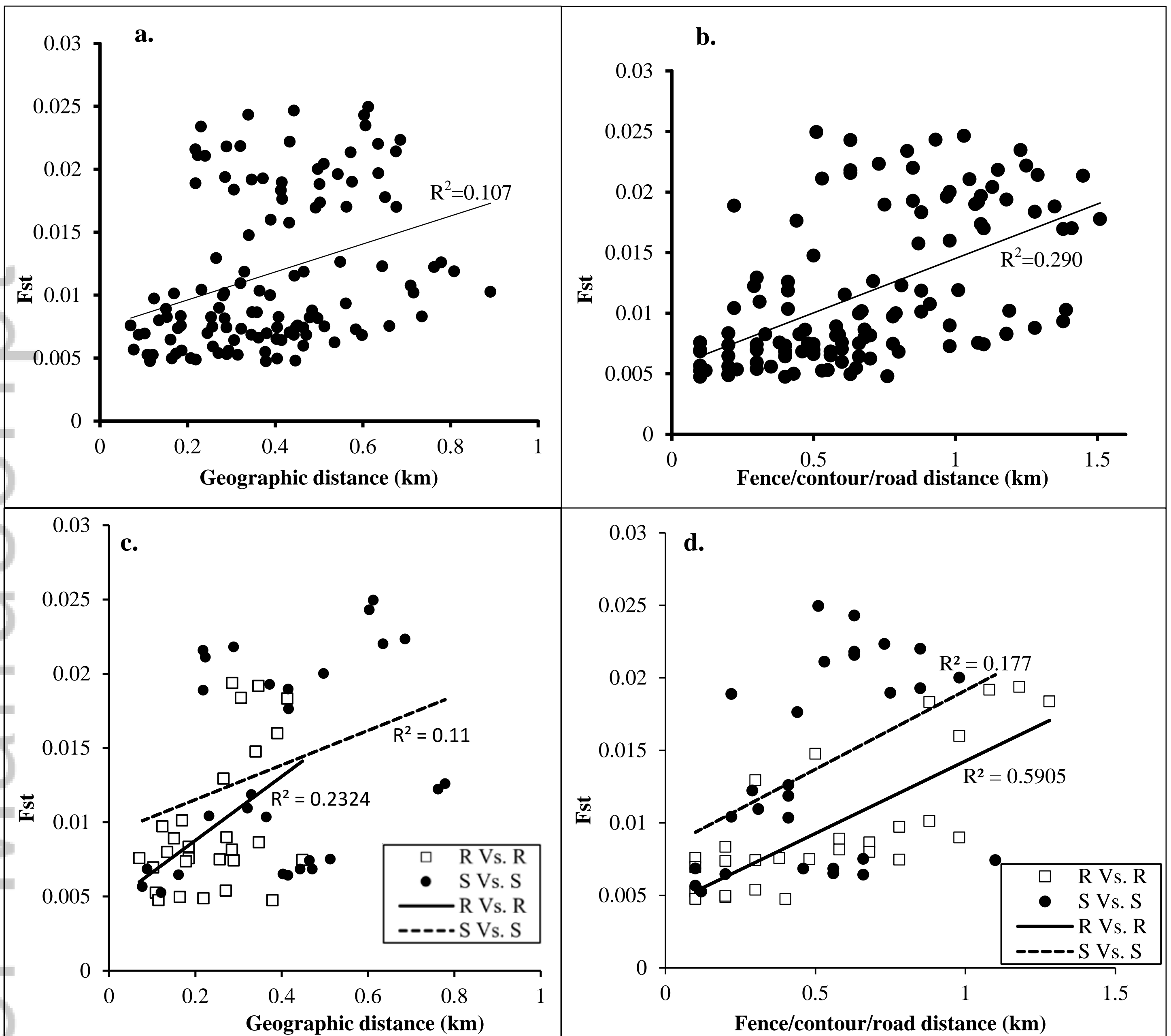

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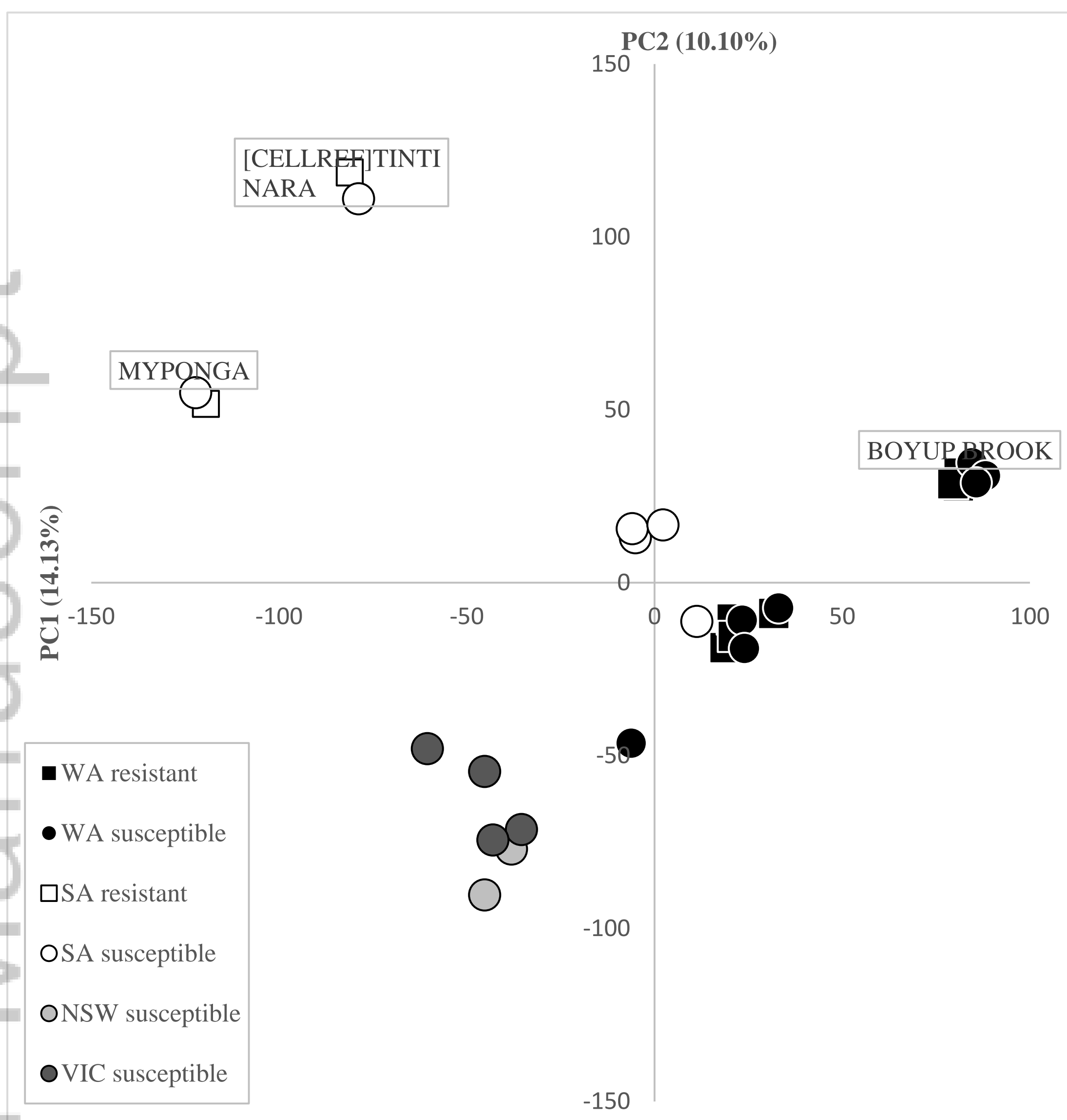

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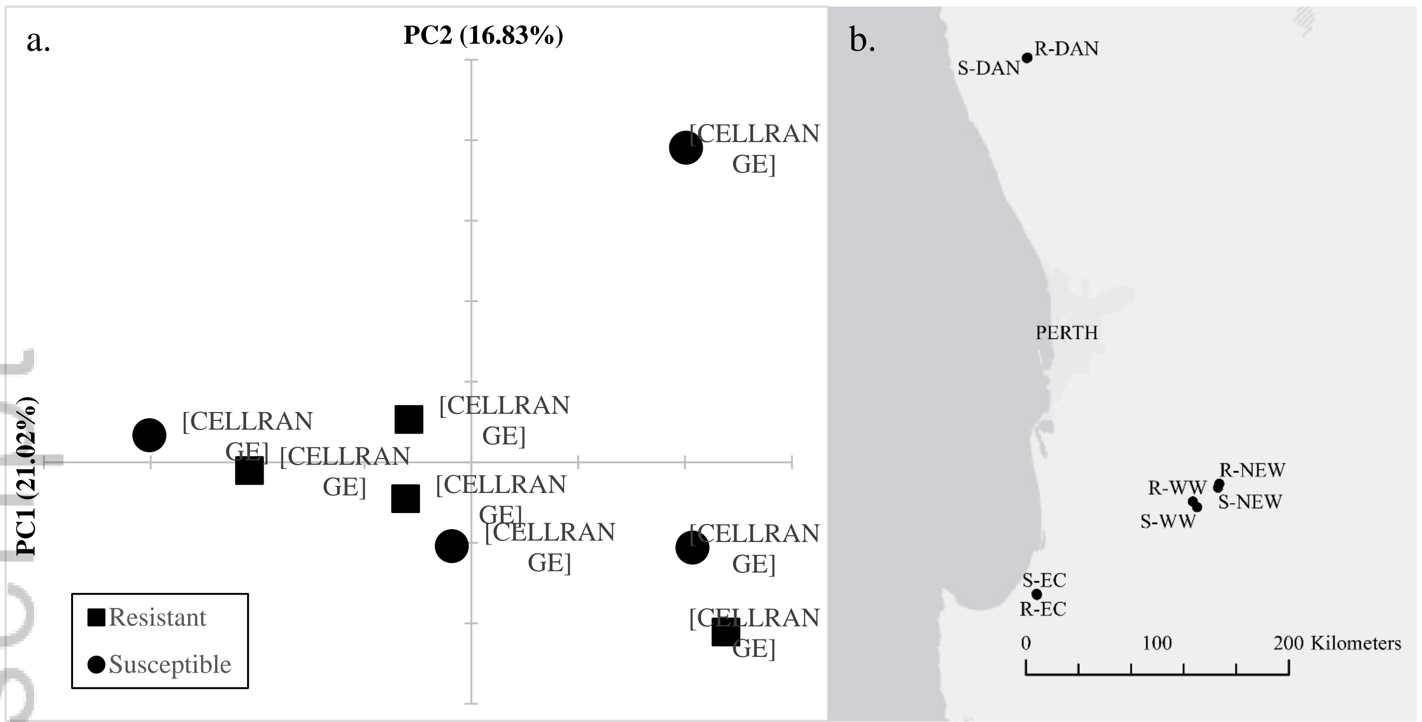

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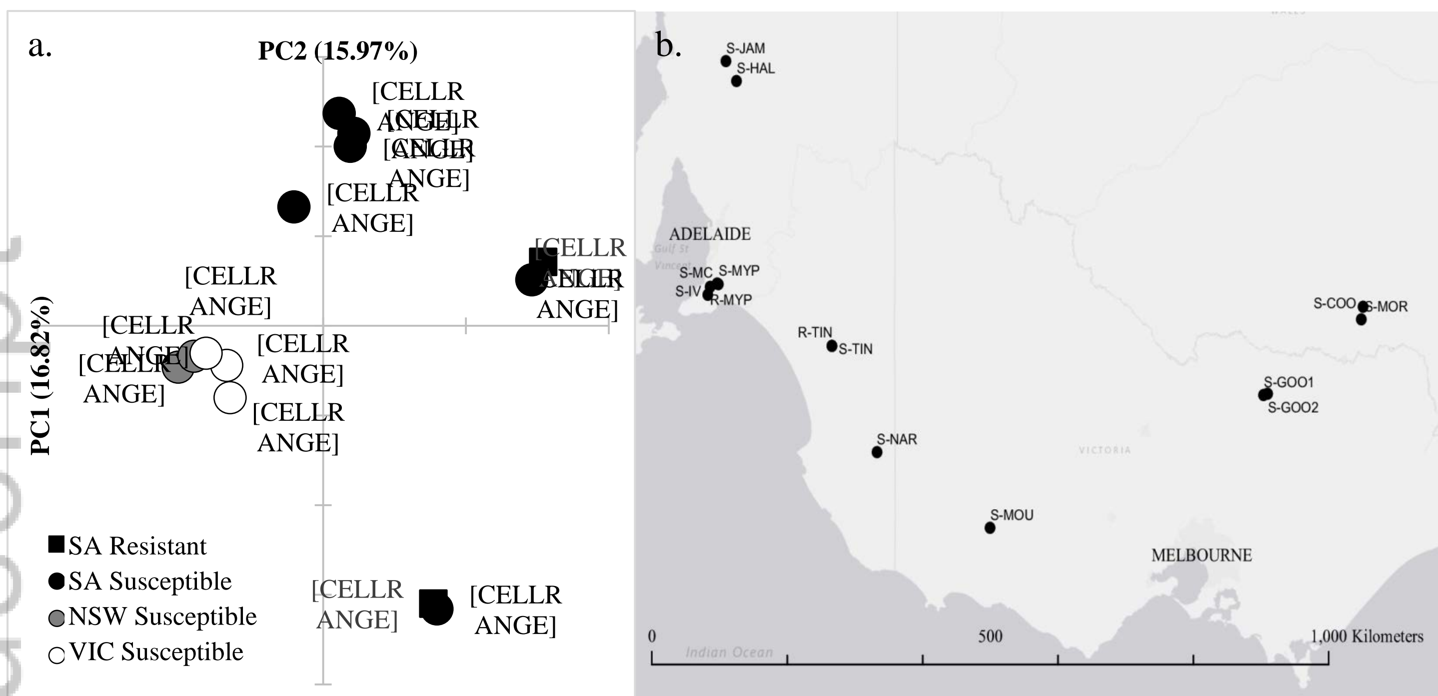

Figure 6. (a) Principal components analysis of 14 sites across the south-eastern grain belt with codes following Table 2 and the position of samples also shown. (b) Sample location map

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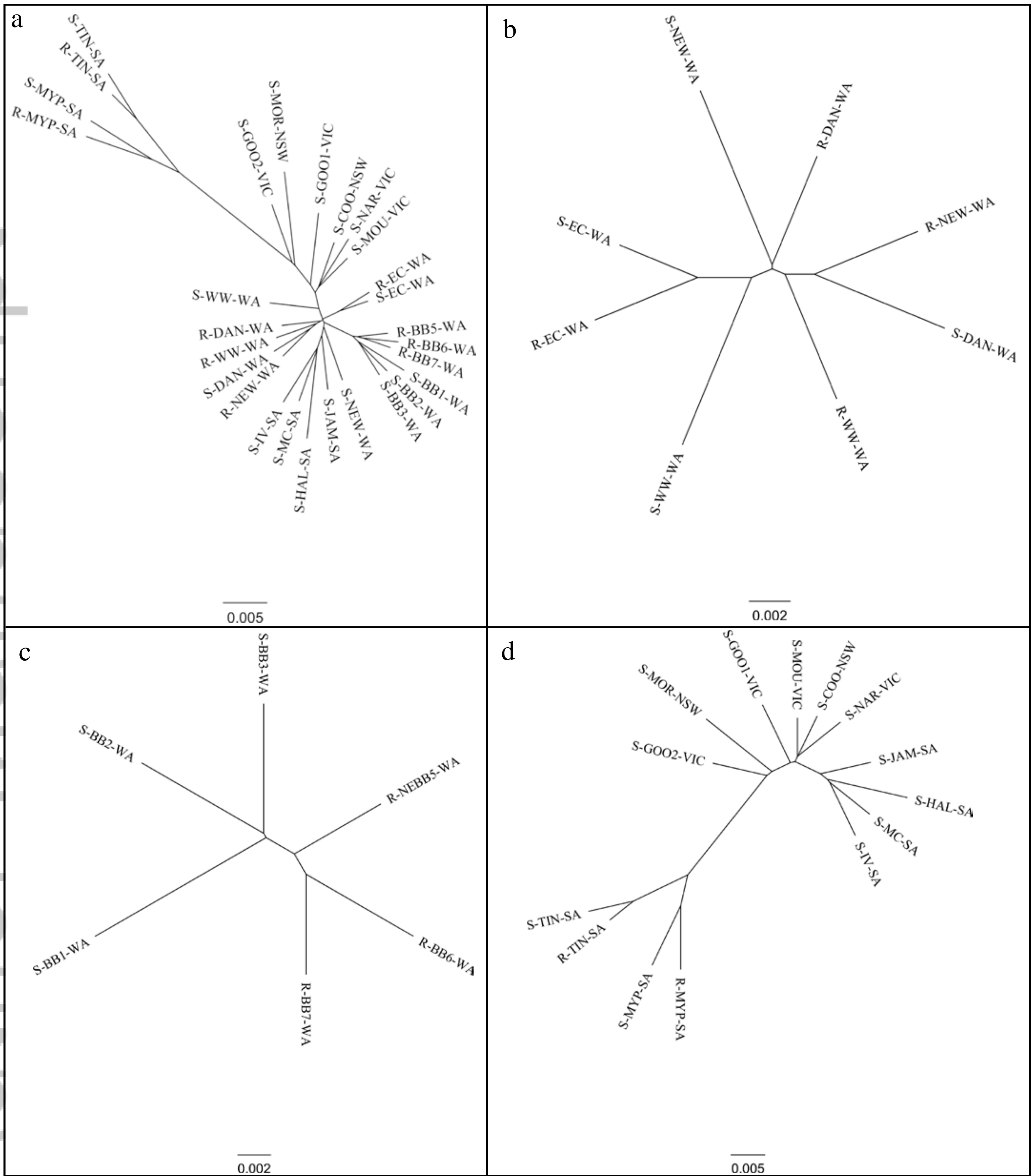

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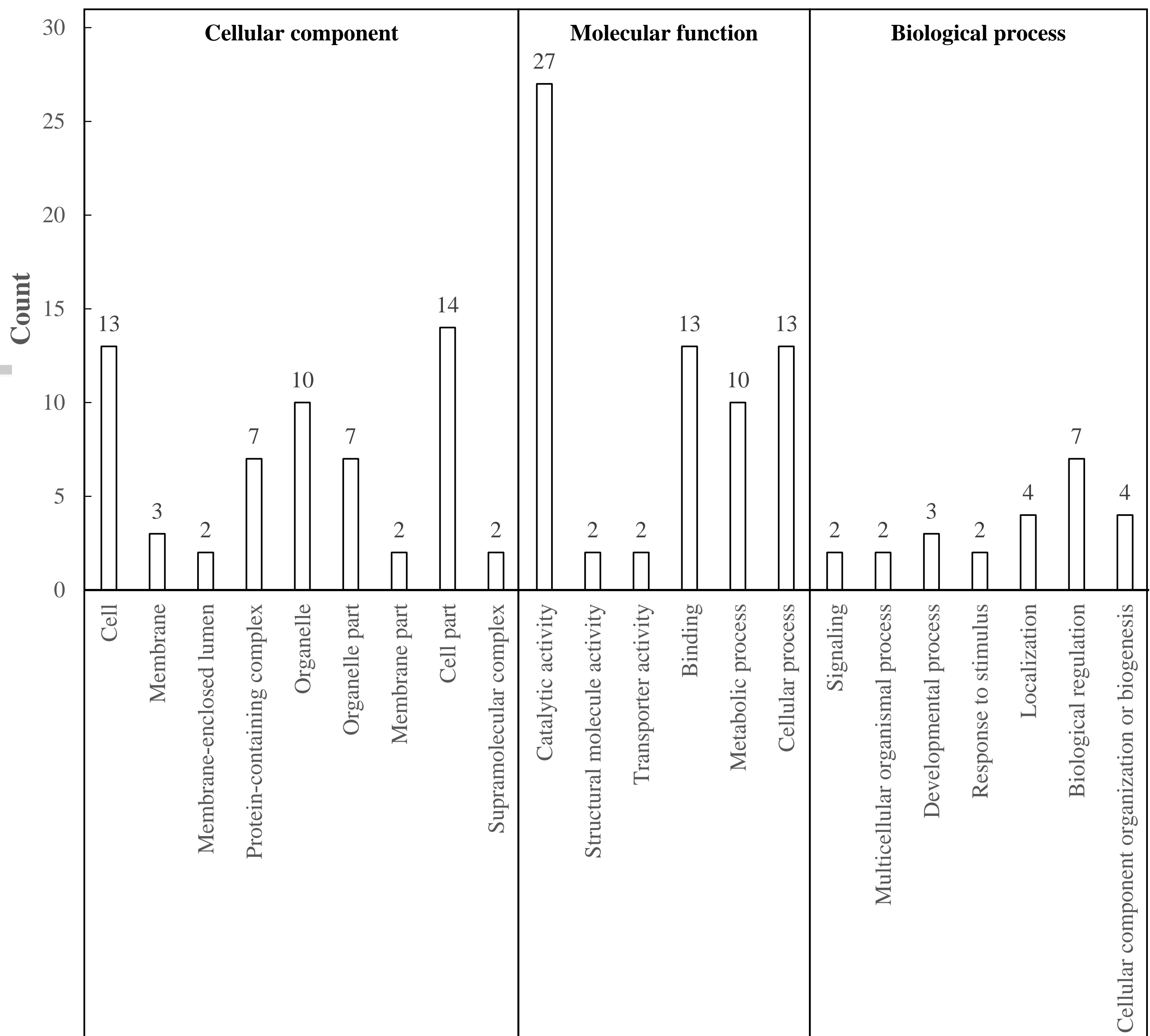

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Origin of resistance to pyrethroids in the redlegged earth mite (*Halotydeus destructor*) in Australia: repeated local evolution and migration

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Pyrethroid resistance in redlegged earth mite presents a substantial challenge to agriculture across southern Australia. Understanding the evolutionary processes driving the emergence of resistance is critical for effective management strategies.

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