The colonisation of canola crops by the diamondback moth, *Plutella xylostella* L., in southern Australia

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For Kate,
April, Ethan and William

Declaration

I certify that this work contains no material which has been accepted for the award of any other degree or diploma in my name in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text. In addition, I certify that no part of this work will, in the future, be used in a submission in my name for any other degree or diploma in any university or other tertiary institution without the prior approval of the University of Adelaide and where applicable, any partner institution responsible for the joint award of this degree. The author acknowledges that copyright of published works contained within this thesis resides with the copyright holder(s) of those works. I give permission for the digital version of my thesis to be made available on the web, via the University's digital research repository, the Library Search and also through web search engines, unless permission has been granted by the University to restrict access for a period of time. I acknowledge the support I have received for my research through the provision of an Australian Government Research Training Program Scholarship.

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Preface

At submission this thesis contained, in addition to the Introduction and Synthesis chapters, four chapters comprising two published and two unpublished manuscripts. One examiner provided critical comments on elements of Chapter 3, a published manuscript. Following examination, an Addendum was added to Chapter 3 containing some explanatory commentary addressing these points.

Abstract

Background: Mobile insect pests cause substantial losses to the yields and economic value of annual crops throughout the world. A sound knowledge of the movement patterns and origins of seasonal pest populations is essential for tactical management, yet these are difficult to identify and often poorly characterised. Further complexities for management arise when pest species carry insecticide-resistant genotypes or when cryptic diversity creates uncertainty around species identity. The diamondback moth, *Plutella xylostella* L., the most destructive pest of *Brassica* crops worldwide, seasonally colonises winter canola crops in southern Australia leading to intermittent outbreaks. Little is known about regional movement and crop colonisation patterns of *P. xylostella* in the Australian canola agro-ecosystem, or the contribution of movement to seasonal outbreaks and the spread of insecticide resistance. The recent discovery of an endemic cryptic species, *Plutella australiana* Landry & Hebert, raised questions about the relative pest status of the two *Plutella* species in Australian *Brassica* crops. Sympatric populations of Australian *P. xylostella* and *P. australiana* were compared using multiple complementary approaches that included genomic datasets, *Wolbachia* infections, inter-species crosses and insecticide bioassays. The seasonal colonisation of Australian canola by *P. xylostella* was investigated using molecular markers and field studies over three years to infer the most likely source populations and the management implications.

Results: Although some laboratory hybridisation occurred, deep divergence between the genomes and biology of the two *Plutella* species was clear, implying contrasting colonisation histories and pest status. Population genetic structure in Australian *P. xylostella* was assessed using RAD-seq, first using nine field and laboratory populations to refine methods, then in 59 field populations collected throughout southern Australia in two separate years from wild and cultivated brassicas. A statistically powerful genome-wide SNP marker set revealed no spatial, temporal or host-related genetic structure among Australian *P. xylostella* populations despite a geographic sampling scale $>$ 3000 kilometres, reflecting a recent range expansion and/or high gene flow and confirming a previous microsatellite assessment. In a three year study, wild brassicaceous potential host plants were sampled for *P. xylostella* in autumn, then subsequent crop colonisation was measured across a regional network of sentinel canola crops and using a derived temperature-dependent development model to back-predict initial

oviposition in each crop. Each year, wild brassicas harboured *P. xylostella* in canola-growing areas prior to sowing, then most canola crops were colonised soon after germination. The autumn abundance of the insect and its hosts, and the subsequent colonisation timing in canola, varied widely between years driven by pre-season rainfall. A CLIMEX model explored spatio-temporal fluctuations in the potential distribution and abundance of *P. xylostella* based on climate suitability. CLIMEX predictions and light trapping at four locations confirmed that *P. xylostella* most likely persists in canola production areas throughout the year.

Conclusions: *Plutella xylostella* is the predominant pest in Australian *Brassica* crops while *P. australiana* is of minor importance. Low genetic diversity in Australian *P. xylostella* precludes the use of neutral markers to identify dispersal patterns. In South Australia, insecticide-resistant *P. xylostella* populations persisting in the local area, rather than arriving from distant source regions, appear to be the major source of seasonal colonisation of winter canola, suggesting local factors may determine outbreak risk.

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Perry KD, Baker GJ, Powis KJ, Kent JK, Ward CM, Baxter SW. Cryptic *Plutella* species show deep divergence despite the capacity to hybridize. BMC Evol Biol. 2018;18(1):77.

Perry KD, Pederson SM, Baxter SW. Genome-wide SNP discovery in field and laboratory strains of Australian *Plutella* species. In Srinivasan R, Zalucki MP, Shelton AM, Kumar ARV, Chandrashekara K, editors. The management of diamondback moth and other crucifer pests: Proceedings of the seventh international workshop; Mysore Journal of Agricultural Sciences, 2017; 51(A), p. 18–31. http://web.entomology.cornell.edu/shelton/diamondback-moth/

Chapter 1

Introduction and literature review

1.1 Aims and scope of the review

The seasonal invasion of annual crops by migratory herbivorous insects threatens food production and causes substantial economic losses throughout the world [163]. The diamondback moth, *Plutella xylostella* L., is a globally important insect pest that seasonally colonises canola crops in Australia. In a comprehensive review of the ecology of *P. xylostella* in the Australian canola agro-ecosystem, Furlong et al. [77] identified five research areas that must be addressed to improve the management of this insect in these crops. A key priority was the need to gain deeper knowledge of the regional movement patterns of *P. xylostella* in Australia and identify the sources of seasonal populations that colonise canola. This information is essential if the capacity to forecast the seasonal risk of *P. xylostella* outbreaks is to be developed, and to inform the scale and design of insecticide resistance management strategies for the Australian canola industry. In 2013, the reporting of a cryptic species of *Plutella* in Australia, *Plutella australiana* Landry & Hebert [131], raised uncertainty about the ecology and relative pest status of the two *Plutella* species in Australian *Brassica* crops, which required investigation. This review focuses on aspects of the biology and ecology of *P. xylostella* relevant to its dispersal and colonisation of canola crops in southern Australia as context for the thesis chapters.

1.2 Biology and pest status of the diamondback moth in Australian canola

1.2.1 The diamondback moth, *Plutella xylostella*, global pest

The family Brassicaceae contains about 338 genera and 3705 wild and cultivated species distributed worldwide [3]. *Brassica* vegetables are grown in temperate and tropical climates and form an important component of the human diet [228]. Over the past several decades, the intensity of *Brassica* vegetable production has increased dramatically in Asian countries through the wide uptake of continuous cropping rotations [78, 72]. In 2016, *Brassica* vegetables were grown over an area of approximately 3.82 million hectares worldwide, with Eastern and Southern Asia contributing nearly 70 % of total production and Europe, the Americas, Africa and Oceania (Australia and New Zealand) contributing 14.1 %, 4.7 %, 3.9 % and 0.3 %, respectively [72]. Canola, *Brassica napus* L., is grown in temperate climates around the world. Canola seed contains oil used in cooking and margarine while the crushed meal by-product is used in animal feed and hence seed quantity, oil content and protein content contribute to crop value. The global area of canola production has expanded steadily since the 1960s, reaching approximately 33.71 million hectares in 2016, with the majority of total production contributed by Europe (34 %), Eastern and Southern Asia (29.8 %), North America (27.7 %), and Australia (4.9 %) [72].

The diamondback moth, *Plutella xylostella* L., is a specialist of plants within the family Brassicaceae, including a wide range of cultivated crops and wild species [228, 173]. It has become the most destructive pest of brassicaceous crops worldwide, costing an estimated $US4-5 billion annually in damage and control costs [257] and $US0.77 billion in China alone [134, 171]. The larvae feed on leaves and fruiting material, reducing yield and marketability, and heavy infestations may result in complete crop destruction [228, 209]. *Plutella xylostella* is notorious for rapidly evolving resistance to insecticides, which have long been used for its control throughout horticultural production regions, and more recently to control periodic outbreaks in canola crops [55, 66]. This species was the first insect to evolve field resistance to DDT and *Bacillus thuringiensis* cry-toxins [227] and since then, resistance to all insecticide classes used for its control has been recorded in field populations [228, 78]. The development of integrated management strategies for *P. xylostella* has been a major focus of research over a number of decades, as evidenced by a series of seven international workshops since the 1980s [68, 210, 220] and a large global literature including several comprehensive reviews of its biology, ecology and management [228, 78, 173, 134]. In recent decades, the pest status of *P. xylostella* has continued to rise due to the increasing

availability of *Brassica* vegetable and canola host resources and a continuing reliance on insecticides for control [78]. The ongoing ecological and evolutionary success of *P. xylostella* is attributed to its ability to survive under a wide range of temperatures, migratory capacity, large population sizes, short generation times, and genotypic and phenotypic adaptibility [228, 137, 252].

Plutella xylostella may originate from Europe [228], southern Africa [124] or Asia [118], but has spread throughout temperate and tropical regions of the world to become one of the most widely distributed lepidopteran species [228]. *Plutella xylostella* does not enter diapause, and hence its seasonal distribution depends on the availability of suitable host plants, climatic conditions to enable population growth and its capacity to disperse and colonise new habitats during favourable seasons [254, 255]. In tropical countries where *Brassica* crops are continuously available, *P. xylostella* persists throughout the year and develops through more than 20 generations annually. In temperate regions at higher latitudes, environmental extremes prevent the establishment of permanent populations, but these areas are frequently recolonised following successful migration [243, 38, 55].

High dispersal ability enables *P. xylostella* to readily invade *Brassica* crops when they become available. In *Brassica* vegetable crops, where suitable hosts of a range of ages are available, flight is mainly trivial and most moths disperse less than several hundred metres from their natal hosts [152]. However, when host quality deteriorates [33], moths may utilize high-altitude air currents to migrate long distances, sometimes initiating outbreaks in *Brassica* crops [75, 38]. In Canada, where cold temperatures prevent local overwintering, *P. xylostella* outbreaks in canola crops are though to originate from populations migrating on northerly airstreams from Mexico during spring [55]. Similarly, insecticide-resistant *P. xylostella* that seasonally infest *Brassica* vegetable crops in northern China are migrants arriving from its overwintering range in southern China [243, 76, 250].

1.2.2 The Australian *Brassica* production system

The Australian continent is characterised by diverse climates, ranging from Mediterranean in southern regions to tropical in northern regions, and high climate variability from year to year. Canola is mainly grown in winter-dominant rainfall environments of southern and western Australia between 30°S and 38°S [43]. It has become an important part of rotations with wheat and barley crops due to its benefits as a disease break and in improving soil structure and yield in subsequent crops. Commercial canola production commenced in Australia in the late 1960s, but has rapidly expanded since the 1990s following improved crop varieties and favourable export prices [43]. Canola is now Australia's third largest winter crop. The

area under canola production reached 3.27 million hectares in 2013-14 before declining to around 2.32 million hectares in 2016-17 [1].

Winter canola crops are planted following season-opening rainfall in autumn (April-May) and harvested in late spring or early summer (October-November) following a 5-7 month growing period [43]. In higher rainfall areas of south-eastern South Australia and Victoria, small areas of canola are planted in spring and harvested in late summer, and some graziers plant *Brassica* forage crops in spring to provide summer feed [185]. Approximately 6000–13000 hectares of *Brassica* vegetable crops per annum, less than 1 % of the area devoted to canola production, are commercially grown in horticultural districts concentrated around the major urban centres in the mainland states and Tasmania [15].

1.2.3 Invertebrate pests of canola and their management

Australian canola crops are attacked by at least 30 invertebrate pest species that vary in importance with region and crop growth stage [89]. Earth mites, Lucerne flea, false wireworms, weevils and molluscs threaten canola establishment in southern regions. *Plutella xylostella* is among a transient pest complex including aphids, native budworm, *Helicoverpa puntigera*, and Rutherglen bug, *Nysius vinitor*, that attacks canola sporadically during spring, potentially causing flower abortion and reducing seed quantity and quality. Several aphid species damage canola indirectly by transmitting plant viruses [239]. A complex of natural enemies including hymenopteran parasitoids, generalist predators and entomopathogens, regulates pest populations in canola during spring. Insecticides are commonly applied to manage invertebrate pests in Australian canola crops. At establishment, insecticidal seed treatments in combination with bare earth or foliar applications of broad spectrum insecticides (synthetic pyrethroids and organophosphates) are applied prophylactically to control the redlegged earth mite, *Halotydeus destructor*, and other pests while in spring, foliar insecticides are sometimes applied in response to seasonal pressure from aphids, *H. punctigera* or *P. xylostella*. Reliance on broad-spectrum insecticides in Australian canola crops is considered unsustainable due to the destruction of natural enemies and selection for insecticide resistance in target and non-target pest species. Insecticide resistance is now widespread in several major pest species of Australian grain crops, including *H. destructor* [236], green peach aphid, *Myzus persicae* [237] and *P. xylostella* [66, 67, 17].

1.2.4 Cryptic *Plutella* species in Australia

Plutella xylostella has been a widespread pest of Australian *Brassica* vegetable crops since the late 1800s [65], and since the late 1990s has become a periodic but highly damaging pest

of Australian canola crops [77]. It was previously assumed that *P. xylostella* was the only representative of the genus *Plutella* in Australia, however, a cryptic mitochondrial lineage endemic to Australia was recently described as a new species, *Plutella australiana* Landry & Hebert [131]. The discovery of *P. australiana* has raised questions about the relative distribution and pest status of the two *Plutella* species in Australian *Brassica* crops. In the following sections, the present review assumes the species referred to in the literature is *P. xylostella*, recognizing that some Australian records may be in question [131].

1.2.5 Population ecology and outbreaks

Plutella xylostella has four larval instars. In *Brassica* vegetable crops, the neonate larvae are leaf miners while older instars mainly feed on leaf surfaces [228]. In canola crops, the 2^{nd} to 4^{th} larvae feed on the surfaces of leaves, causing "windowing" and small holes. They also feed on floral structures and graze stems and pods [89]. At low densities larval feeding does not reduce seed yield or quality, but at high densities can result in complete defoliation and removal of photosynthetic material from stems and pods, preventing grain development.

Since the 1990s, the rapid expansion in canola production has created vast new host resources for *P. xylostella*, leading to a rise in its pest status in both canola and *Brassica* vegetable crops [77]. Winter canola crops are colonised by *P. xylostella* each season, but the severity of infestation varies widely between regions and years. In some years, under favourable seasonal conditions, larval populations can rapidly increase to high densities during spring, causing the destruction of maturing plants and high yield losses. The first serious outbreaks in Australian canola were recorded in Western Australia between 1999-2002 and in New South Wales in 2002. Since then, outbreaks in canola have occurred periodically in Western Australia, South Australia, New South Wales and Victoria [77].

Outbreaks are thought to be driven by climate, but *P. xylostella* outbreaks recorded between 1999 and 2005 did not show a clear association with broad averages of climate variables [77]. While higher populations were associated with relatively warm and dry conditions during winter and spring, above-average pre-cropping season rainfall, which can proliferate the growth of brassicaceous weeds, preceded some outbreaks but not others [77]. The locations and timing of rainfall may be important in driving the seasonal abundance of brassicaceous hosts and *P. xylostella*. Furthermore, because *P. xylostella* is migratory, environmental factors driving emigration from source areas might be some distance away [31]. The relative contributions of local population growth versus immigration to spring densities of *P. xylostella* in Australia canola are uncertain, as datasets describing its population dynamics in these crops virtually do not exist [77].

The capacity to forecast the seasonal risk of *P. xylostella* outbreaks in *Brassica* crops could provide a foundation for the integrated management of this species [134, 78]. For mobile pests, predictive models require thorough knowledge of the timing, frequency and magnitude of immigration events, taking into account source areas [166]. In Australian canola crops, the regional movement patterns of *P. xylostella* and origins of source populations that seasonally colonise Australian canola crops are unknown [77]. Identifying these is essential for understanding the seasonal dynamics and likely resistance status of colonists.

1.2.6 *P. xylostella* management and insecticide resistance

The management of *P. xylostella* in Australian *Brassica* crops has long been reliant on insecticides. In *Brassica* vegetable crops, synthetic pyrethroids were widely used between the late 1970s to the late 1990s, and since the mid-1980s resistance to pyrethroid and organophosphate insecticides has been widespread in all vegetable production regions [18]. The introduction of several new insecticides in the late 1990s, and diamides in 2009-2010, enabled the deployment of rotation strategies to manage resistance [15]. However, these products continue to be intensively used, creating high selection pressure and leading to a continuing decline in the susceptibility of *P. xylostella* populations in *Brassica* vegetable regions [16].

In Australian canola crops, insecticide applications specifically targeting *P. xylostella* have been relatively infrequent [15]. However, the widespread use of synthetic insecticides to control other pests in canola has likely selected for resistance development in *P. xylostella* [77], and resistance to pyrethroid and organophosphate insecticides is now widespread throughout all Australian canola production regions [66, 17]. Two newer insecticides, emamectin benzoate and spinetoram, became available for control of *P. xylostella* in Australian canola in 2013-2014. Despite relatively infrequent use, *P. xylostella* populations collected from canola crops in all production regions have shown decreasing susceptibility to emamectin benzoate and diamides, even though diamides have never been used in canola [17]. Local insecticide use patterns appear to drive levels of pyrethroid resistance in Australian *P. xylostella* populations [66]. For newer chemistries in less frequent use, cross-resistance or gene flow from horticultural areas might contribute to the spread of insecticide resistance in canola-growing areas [17].

1.2.7 Dispersal and migration

In Australia, *P. xylostella* is thought to disperse frequently, but there is little knowledge of seasonal movement patterns. A study using six microsatellite markers found no genetic

differentiation among 17 Australian populations from different geographic locations and brassicaceous hosts, consistent with frequent intermixing or a recent population expansion [65, 202]. However, homogeneous allele frequencies precluded identification of spatial movement patterns or *Brassica* host sources. Indirect evidence for frequent dispersal includes collections of moths in light traps [131], pheromone trapping studies in vegetable crops [185], and seasonal colonisation of annual canola crops [77]. Mark-capture studies [152, 203] and localised insecticide resistance patterns [66] show that populations in *Brassica* vegetable crops, where hosts are continuously available, have limited propensity to emigrate. Moths move into *Brassica* vegetable regions seasonally in spring [95, 65, 185], coinciding with the senescence of canola crops and weeds. Insecticide resistance profiles are relatively homogeneous in populations across Australian canola-growing regions and between crops and weeds, reflecting regular intermixing [17], and evidenced by the seasonal colonisation of canola over vast areas on an annual basis.

1.2.8 Summer hosts and local persistence

When canola is harvested, *P. xylostella* must disperse and exploit alternative brassicaceous host plants in the landscape, which may either be nearby or long distances away and colonised through migration. These hosts provide a refuge for *P. xylostella* populations that subsequently colonise winter canola crops. Summer-active brassicas include geographically restricted areas of *Brassica* vegetable and forage crops, and a large diversity of native and introduced wild brassicas distributed throughout Australia. In Australia, *P. xylostella* has been recorded on a range of introduced brassicaceous weeds, including turnip weed, *Rapistrum rugosum* L., wild radish, *Raphanus raphanistrum* L., Ward's weed, *Carrichtera annua* L., wild turnip, *Brassica tournefourtii* L., weedy canola, *Brassica napus* L. and turnip, *Brassica rapa* spp., Indian hedge mustard, *Sisymbrium orientale* L., hedge mustard, *Sisymbrium officinale* L. [77, 88, 95], and sea rocket, *Cakile maritima* (Michael Keller, Pers. Comm.). Because the host range of the cryptic *P. australiana* is unknown, some Australian host records for *P. xylostella* may be in question [131].

The geographic distribution of *P. xylostella* changes seasonally in response to changing host resource availability and climate suitability. *Plutella xylostella* can develop and survive over a wide range of temperatures [137], but climatic conditions over vast areas of Australia are marginal for its growth and survival [254, 255]. In canola-growing regions of southern Australia, *P. xylostella* survives winter [88] but summer populations may be limited by heat extremes and a lack of host plants [77]. The extent to which *P. xylostella* can over-summer locally in canola-growing regions has not been assessed. Local survival of resistant genotypes

could maintain insecticide resistance alleles in an area [228], and provide source populations that may subsequently colonise canola crops when they become available [77].

1.3 Measuring movement and colonisation

1.3.1 Observational methods for tracking insect movement

Measuring dispersal is a highly challenging field of ecology [157]. For insects, techniques such as mark-release-recapture [98] or mark-capture using fluorescent compounds [203], rare elements [141] or protein-based immunomarkers [91, 199] can a provide direct measure of dispersal of marked individuals at relative small scales up to several kilometres. At larger scales, intrinsic isotopic markers are an emerging tool for inferring the natal origins of migrating insects [104, 103]. Tracking the aerial movements of insects directly has numerous practical difficulties due to their small body size, short life span and aerial dilution effects [140]. For larger-bodied taxa, use of harmonic radars, radio telemetry [129, 248], vertical-beam entomological radars [39] and weather radars [208] can reveal aerial movements at landscape, regional and continental scales. Trapping studies employing sex pheromone traps, light traps or suction traps can measure the temporal dynamics of flight activity or population size at a given location, while trapping networks enable inferences about spatial dynamics [12] and population re-distributions across a region [206]. In general, observational methods are limited in the spatio-temporal scale at which data can be practically collected and interpreted, and are problematic for estimating the frequency of rare long distance dispersal events [159]. A variety of genetic tools exists for quantifying large-scale insect dispersal both directly and indirectly [30].

Mark-recapture studies have investigated local dispersal of *P. xylostella* among *Brassica* vegetable crops and surrounding fields [152, 203]. Around the world, most evidence for larger-scale *P. xylostella* movement is indirect and comes from unexpectedly high trap catches [185], sudden population increases [75], seasonal poleward range expansions [78], colonisation of remote islands [44], and genetic homogeneity among populations across neutral marker loci [65, 243]. Wind trajectory modelling analysis has also been used to infer the origins of suspected migrations of *P. xylostella* in Canada and the UK [55, 75].

1.3.2 Species distribution modelling

Species distribution models can aid in generating hypotheses about dispersal and assist with interpreting observational data. Bioclimatic models, such as CLIMEX [226], can be used to predict temporal changes in the geographic distribution and abundance of a species based on

its known responses to climate variables. This approach could be used in conjunction with local climate data to identify possible source and sink areas and determine when certain areas may become suitable for colonisation [164, 253, 78]. A CLIMEX model for *P. xylostella* [254, 255, 135] reasonably describes its worldwide distribution and seasonal abundance in parts of the UK and northern Asia. The model predicts that most of the Australian continent is climatically unsuitable for year-round survival of *P. xylostella* [254, 255]. Temporal analysis of locations suitable for *P. xylostella* across its Australian range might be used to identify when and where population re-distribution becomes likely.

1.3.3 Molecular markers for population genetic studies

Molecular markers are a useful tool for estimating the levels of gene flow and population structure among geographically distinct insect populations, which can provide insights into likely dispersal patterns at spatio-temporal scales that are impractical to measure using direct methods [190]. Gene flow occurs through "effective" dispersal resulting in successful reproduction in a new location. It influences the spatial distribution of alleles and hence local adaptation, including the evolution of insecticide resistance [30, 66].

Around the world, population structure in *P. xylostella* has been investigated using various genetic markers including allozymes, ISSRs, microsatellites, and mitochondrial genes. Early studies reported limited sequence variation among populations within regions of Asia [133, 128, 125] and the USA [37, 34, 125], consistent with high levels of gene flow. Evidence of genetic isolation has been reported among populations from different continents [174, 196, 65]. Earlier studies were largely inconclusive due to limitations in field sampling sizes and scale, use of laboratory-reared populations or inconsistent findings [reviewed in 64]. More recent studies using representative field sampling and independent mtDNA and nuclear markers have provided new insights into seasonal movement patterns of *P. xylostella* in China [243, 250]. In a study of 27 geographically distinct populations across China, Wei et al. [243] found little genetic differentiation across eight microsatellite loci but geographic differences in mtDNA haplotype frequencies revealed evidence for northward migrations and occasional reverse migrations, which is supported by light trapping [76].

In Australia, Endersby et al. [65] found a lack of genetic differentiation across six microsatellite loci among 17 *P. xylostella* populations from Australia and one from New Zealand, and concluded that *P. xylostella* forms a single panmictic population across an Australian range spanning several thousand kilometres. However, low genetic diversity provided evidence that Australian and New Zealand *P. xylostella* have undergone a recent bottleneck [202, 52] and therefore nuclear homogeneity may reflect recent common ancestry [77]. Whether the six microsatellite markers had adequate statistical power to detect weak

population structure was uncertain. The levels of contemporary gene flow among Australia *P. xylostella* remain to be fully resolved.

Over the past decade, rapid advances in next generation sequencing (NGS) technology have led to a revolution in the field of molecular ecology. The development of new reduced-representation sequencing methods that combine the power of high-throughput NGS with targeted sequencing of short genomic regions has made it possible to generate vast quantities of genomic data at high depth of coverage across sequenced loci [156, 84]. One reduced representation genotyping method, restriction-site-associated DNA sequencing (RAD-seq) [13], has been widely adopted for ecological and evolutionary genetics [4]. RAD-seq facilitates low-cost discovery and genotyping of genome-wide markers in a wide range of organisms and is able to generate high densities of single nucleotide polymorphism (SNP) markers across multiple individuals for population genetic studies [49]. Despite some limitations, microsatellites remain popular for population genetic studies because they are highly polymorphic [177]. While SNPs are individually less informative, large panels of SNPs can potentially outperform microsatellites in resolving population structure [90, 181, 240]. Genome-wide markers derived from NGS may provide novel insights into the population structure of *P. xylostella*.

1.4 Research aims

This thesis addressed the question "What are the source areas of *P. xylostella* populations that seasonally colonise winter canola crops in southern Australia, and what are the implications for management?" The related research questions were:

1. Does population genetic structure exist among Australian *P. xylostella* from different geographic locations, host plants and seasons?

2. Can *P. xylostella* populations persist locally in canola-growing areas of southern Australia during summer and autumn when canola crops are unavailable?

3. When is winter canola colonised by *P. xylostella* in southern Australia, and what are the likely source areas?

Here, a combined approach was used to investigate movement from multiple angles, including molecular markers, a bioclimatic model and field observation. The thesis contains six chapters: *Chapter 1* comprises the present review. *Chapter 2* describes a study investigating the utility of the restriction-site-associated DNA sequencing (RAD-seq) method for SNP marker discovery and genotyping in field and laboratory strains of Australian *Plutella*

species, and its potential to generate new insights into the genetic structure among field populations of Australian *P. xylostella*. This study was also used to refine laboratory methods and bioinformatics analysis pipelines for the genetic studies described in subsequent chapters. *Chapter 3* describes a study of aspects of the biology of the cryptic species, *P. australiana*, the distribution and relative abundance of *P. australiana* and *P. xylostella* on brassicaceous crops and weeds throughout southern Australia, and their pest status in Australian *Brassica* vegetable and canola crops. Although this work was not central to the thesis, molecular species identification of *Plutella* samples was required prior conducting genetic studies on *P. xylostella*. Extensive field sampling and genotyping provided an opportunity to generate novel information on the two cryptic species that has consequences for their management. *Chapter 4* describes a study of genetic structure among 59 populations of *P. xylostella* collected from locations throughout southern Australia and from the four *Brassica* host types, canola crops, *Brassica* vegetable crops, *Brassica* forage crops and wild brassicaceous species, and genotyped using RAD-seq. *Chapter 5* describes a study combining field observation, phenological modelling and CLIMEX modelling to investigate the timing and likely origins of canola colonisation at a regional scale in South Australia across three consecutive seasons from 2014–2016. *Chapter 6* offers a general synthesis and discussion of the research findings.

Statement of Authorship - Chapter 2

Principal Author

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Overall percentage (%):	80 %
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i the candidate's stated contribution to the publication is accurate (as detailed above);
ii permission is granted for the candidate to include the publication in the thesis; and
iii the sum of all co-author contributions is equal to 100 % less the candidate's stated contribution.

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Chapter 2

Genome-wide SNP discovery in field and laboratory populations of Australian *Plutella* species

Abstract

Understanding dispersal and gene flow is an important focus of evolutionary biology, conservation biology and pest management. The diamondback moth, *Plutella xylostella*, is a worldwide pest of *Brassica* vegetable and oilseed cropping systems. This insect has high dispersal ability, which has important consequences for population dynamics and the potential spread of insecticide resistance genes. Population genetic studies of the diamondback moth have found little evidence of population structure, suggesting that frequent intermixing occurs within regions, however the patterns of local and regional dispersal remain to be identified. For this and many other pest species, understanding dispersal is crucial for developing integrated management tactics such as forecasting systems and insecticide resistance management plans. In recent years, next generation sequencing (NGS) methods have provided previously unparalleled resolution for population genetic studies in a wide range of species. Here, we assessed the potential of NGS-derived molecular markers to provide new insights about population structure in the diamondback moth. We use restriction-site-associated DNA sequencing (RAD-seq) to discover hundreds to thousands of single nucleotide polymorphism (SNP) markers in nine field and laboratory-reared populations collected from Australia. Genotypic data from RAD-seq markers identified a cryptic species, *Plutella australiana*, among individuals collected from a wild host, *Diplotaxis* sp., indicating strong divergence in the nuclear genomes of two Australian *Plutella* lineages. Significant genetic differentiation

was detected among populations of *P. xylostella* used in our study, however this could be explained by reduced heterozogosity and genetic drift in laboratory-reared populations founded by relatively few individuals. This study demonstrates that RAD-seq is a powerful method for generating SNP markers for population genetic studies in this species.

Keywords

Gene flow, population structure, RAD-seq, *Plutella australiana*, insecticide resistance

2.1 Introduction

Dispersal is a fundamental life history trait with important consequences for the spatial and temporal dynamics of populations. Effective dispersal (resulting in reproduction) also affects allele frequencies and the genetic structure of populations, and consequently, evolutionary processes [30]. For example, adaptation and speciation depend on a balance between selection and gene flow [233, 66]. For pest species, quantifying dispersal and its effects on genetic structure is crucial to developing integrated management tactics, such as forecasting systems [253, 140] and insecticide resistance management strategies. Molecular markers are a powerful tool for assessing the geographic structure of populations and inferring patterns of gene flow at large scales [190]. Population genetic approaches have been successfully employed to infer patterns of long distance dispersal in a range of insect pests [127, 222].

The diamondback moth, *Plutella xylostella* L., is a worldwide pest of *Brassica* vegetable and oilseed crops [77, 257, 78]. The success of this insect is due in part to its remarkable genetic plasticity [100], large effective population sizes and high genetic diversity [252] that enable it to rapidly adapt to local environments and evolve insecticide resistance [78]. Furthermore, the diamondback moth is highly mobile and well-adapted to exploit its short-lived brassicaceous hosts. It displays predominantly short-range dispersal within high quality host patches [152] but when habitat quality deteriorates, may utilize high altitude air currents to migrate long distances and colonise new habitats [40, 38, 132, 76]. In some years, large scale migration events instigate damaging outbreaks in *Brassica* crops [55, 243]. In most regions, the dispersal ecology of diamondback moth is poorly understood, yet this knowledge is critical for the development of forecasting systems and insecticide resistance management plans [77]. One reason for this is the challenging nature of studying long distance dispersal in small insects [140].

Previous population genetic studies in diamondback moth have employed a range of molecular markers, including allozymes [34, 162, 128, 174], ISSRs [196], microsatellites

[65] and mitochondrial genes [37, 125, 133, 202, 161]. Several authors have reported population differentiation at inter-continental scales [65, 174, 196]. However, most studies from around the world have found little evidence of population structure within regions, including China [128, 133], Korea [128, 126, 125, 133], the USA [34, 37] and Australia [65, 202]. These findings suggests that frequent intermixing occurs within regions, however the local and regional patterns of dispersal remain to be identified. More recently, studies using mitochondrial markers, or complementing these with microsatellite [243] or ISSR [250] nuclear markers, have provided new insights into seasonal migration routes from southern to northern regions of China, and identified potential geographic barriers to gene flow [161]. Mitochondrial markers have also recently identified a novel *Plutella* lineage in Australia [131].

In recent years, next generation sequencing (NGS) has revolutionized the fields of molecular ecology and population genetics. Reduced representation sequencing methods [156] combined with the power of high-throughput NGS platforms [81] facilitate rapid and cost-effective marker discovery and genotyping in a wide range of organisms [50]. Restriction-site-associated DNA sequencing (RAD-seq) [13] is one of several reduced-representation methods for sequencing targeted regions across the genome at high sequencing depth, providing numerous advantages over traditional markers for population genetic studies [49]. RAD-seq studies have provided new insights into previously undetected population genetic structure in a wide range of contexts [156, 183] including terrestrial invertebrates [155, 138].

Here, we assess the potential of NGS methods to provide new insights about population structure in the diamondback moth. We use RAD-seq to discover single nucleotide polymorphism (SNP) markers in nine field and laboratory-reared populations collected from Australia. The RAD-seq markers facilitate an initial assessment of genetic diversity within and among these populations.

2.2 Materials and methods

Sample collection

Samples of diamondback moth were collected from *Brassica* vegetables, canola or wild *Brassica* hosts from nine locations in Australia between September 2012 and April 2014 (Table 2.1, Figure 2.1b). At each location, individuals were collected using a sweep net or by direct sampling. Seven populations were reared in laboratory cages on cultivated cabbage and 10 % honey solution for between one and six generations. Individuals from

two field populations and six of the laboratory-reared populations were preserved in 20 % DMSO, 0.25 M EDTA salt saturated solution [251] and stored at −80 °C. Individuals from the Nundroo population were stored in USP Grade propylene glycol and stored at −20 °C.

RAD library preparation and sequencing

Libraries for RAD sequencing were prepared following a protocol modified from Baird et al. [13]. Genomic DNA was extracted from individual larvae or pupae by homogenizing tissue in DNA isolation buffer [261] followed by two phenol and one chloroform extractions. DNA was treated with RNase A then precipitated and re-suspended in TE buffer. Genomic DNA was quantified using a Qubit 2.0 fluorometer (Invitrogen) and 200 ng digested with 10 units of *Sbf1* in Cutsmart Buffer (NEB) for 1 hour at 37 °C then heat inactivated at 80 °C for 20 minutes.

P1 adapters with one of six molecular identifiers (MIDs) (AATTT, AGCTA, CCGGT, GGAAG, GTCAA or TTCCG) were annealed then ligated to digested DNA (top strand 5′-GTTCAGAGTTCTACAGTCCGACGATCxxxxxTGCA-3′, bottom strand 5′-Phos-xxxxxGATCGTCGGACTGTAGAAC-3′, x represents sites for MIDs) using 1 µL T4 DNA ligase (Promega), 1 mM ATP, Cutsmart Buffer. Six individuals from different populations were pooled to form 12 library groups, each containing different P1 adapters to facilitate sample multiplexing. Library pools were then sheared using a Bioruptor sonicator (diagenode), ends blunted (NEB), adenine overhangs added then P2 adapters ligated (top strand 5′-Phos-TGGAATTCTCGGGTGCCAA-3′, bottom strand 5′-CCTTGGCACCCGAGAATTCCAT-3′). DNA purification between each step was performed with magnetic beads (AMPure). PCR library amplification conditions were 16 cycles of 98 °C for 10 seconds, 65 °C for 30 seconds and 72 °C for 30 seconds using RP1 (forward) 5′-AATGATACGGCGACCACCGAGATCTACACGTTCAGAGTTCTACAGTCCGA-3′ and 12 unique RPI-indexed (reverse) 5′-CAAGCAGAAGACGGCATACGAGATxxxxxxGTGACTGGAGTTCCTTGGCACCCGAGAATTCCA-3′ primers. Libraries were run on agarose gel to size select DNA fragments 300-700 base pairs in length. Paired end sequencing using 100 bp reads was performed over two lanes of Illumina HiSeq2500 at the Australian Cancer Research Foundation (ACRF) Cancer Genomics Facility.

Read filtering and variant calling

A total of 131.6 million raw sequence reads were de-multiplexed using RADtools v1.2.4 [21] then 50.3 million PCR duplicates were removed using the clone_filter tool in STACKS v1.19 [35]. Read trimming, adapter removal and quality filtering were performed in TRIM-

MOMATIC v0.32 [26]. First, a thymine base overhang added during P2 adapter ligation was trimmed from reverse reads, then paired end trimming was performed using the ILLUMINACLIP tool to remove adapter, trailing low quality bases (quality score < 3), bases within a 4-base sliding window with average quality below 15 and trimmed reads shorter than 40 bp. Paired reads were aligned to the *Plutella xylostella* reference genome (version 1.1, modified to include the mitochondrial genome, accession number: JF911819) using STAMPY v1.0.21 [139] with –baq and –gatkcigarworkaround options and expected substitution rate set to 0.005. Genotypes were called using the Genome Analysis Toolkit (GATK) v3.3-0 [149, 53] HaplotypeCaller tool following the GATK Best Practices Workflow for GVCF-based Cohort Analysis. Sites with a genotype quality (GQ) > 30 were retained. Filtering was performed using VCFtools v0.1.12a [48] to identify a set of variant sites for population genetic analysis. We removed indels and retained bi-allelic SNPs that passed the following quality filters: genotyped in at least 60 of 72 individuals, QUAL > 400, average read depth between 20 and 100 across individuals, minor allele frequency > 0.2 and in Hardy-Weinberg equilibrium with p-value set to 0.05. To avoid closely linked markers, variants were separated by a minimum distance of 2 kb using the VCFtools –thin function. A final set of 1285 SNP variants were retained after filtering. In addition, from the GATK HaplotypeCaller output, we generated a set of all confidently called variant and invariant sites (GQ > 30). Filtering was performed using VCFtools v0.1.12a [48] to remove indels and sites located within transposons, and retain sites genotyped in 60 of 72 individuals with mean depth between 20 and 100 across individuals. After filtering, we retained 491 831 confidently called variant and reference sites, including 623 sites from the mitochondrial genome.

Population genetic analysis

We used the 491 832 sites to generate a phylogeny for 72 individuals using a neighbor-joining clustering method implemented in the program GENEIOUS v7.1.9 [122]. An individual representing a cryptic species, *P. australiana*, Calca-6, was used as the out-group. We assumed the Tamura and Nei [229] model and resampled with 1000 bootstraps to generate a consensus tree displaying nodes with at least 50 % consensus support and visualized the resulting tree in the program FigTree v1.4.2 (http://tree.bio.ed.ac.uk/software/figtree/). Summary population statistics were calculated for variant SNPs ($n = 1285$) and separately for all confident variant and invariant sites ($n = 491\,832$). The VCFtools [48] –depth function was used to calculate average site depth, and vcf-stats used to calculate the average number of genotyped sites and private alleles. Expected and observed heterozygosity and the inbreeding coefficient, F_{IS}, were calculated and significance determined by bootstraps (1000 bootstraps for the SNP variants, and 100 bootstraps for the 491 832 sites due to computational limits) using modified

functions in the R package diveRsity [123]. To examine the effects of laboratory-rearing on genetic diversity, we plotted the distributions of average heterozygosity in R [179]. To investigate population differentiation, a global estimate of Weir and Cockerham's (1984) F_{ST} with 99 % bootstrap confidence intervals (10 000 bootstraps) was calculated in the R package diveRsity [123]. Pairwise F_{ST} values [245] were calculated and significance determined using exact *G* tests in GENEPOP v4.3 [195] after Bonferroni correction for multiple comparisons [60]. To test for genetic isolation by distance [249], we performed a Mantel test [144] using 10 000 permutations on the regression of Slatkin's 1995 linearized F_{ST} transformation ($F_{ST}/(1-F_{ST})$) onto the natural log of geographic distance [194] using the R package ade4 [58]. Geographic distances were calculated using the Geographic Distance Matrix Generator [70].

We used the 1285 SNP variants to analyse population structure using a Bayesian clustering method in the program STRUCTURE v2.3.4 [176]. Variant data were converted from Vcf to Structure file format using PGDSpider v2.0.8.2 [136]. STRUCTURE analysis was used to infer the number of genotypic clusters and assign individuals to clusters. Analyses were performed for all individuals ($n = 72$) and separately for *P. xylostella* individuals only ($n = 69$). For each analysis, we assumed the *admixture* model with correlated allele frequencies and the *locprior* model, specifying nine geographic populations. For each analysis, we performed ten independent STRUCTURE runs for each value of $K = 1-10$, where K is the number of genotypic clusters. For all runs, we used 500 000 burn-ins and 500 000 MCMC replicates. The optimal K was determined using the delta K method of Evanno et al. [71] visualized in the program STRUCTURE HARVESTER [62]. Individual and population Q-matrices (containing posterior probability of assignment to genotypic clusters) across replicate structure runs were aligned in the program CLUMPP v1.1.2 [110] and visualized in DISTRUCT v1.1 [192].

PCR genotyping assays

To examine the frequency of mutations associated with pyrethroid resistance, we performed PCR-based genotyping assays for three point-mutations in the voltage gated sodium channel, T929I (*skdrl*), L1014F (*kdr*) and F1020S (*cdr*) according to Endersby et al. [67]. MyTaq polymerase (Bioline) was used for amplification in a Verity thermocycler (ABI). To distinguish between *P. xylostella* and *P. australiana* lineages, we developed a PCR-RFLP genotyping assay using COI sequence published by Landry and Hebert [131]. Genomic DNA was amplified using a modified LCO1492_Px primer (5′-TCAACAAATCATAAAGATATTGG-3′) and HCO2198 (5′-TAAACTTCAGGGTGACCAAAAAATCA-3′) [73]. Ten microliter reactions were run with 2 µL of MyTaq 10x buffer, 0.4 µL of each primer (10 mM stocks),

1 µL of DNA (approx. 5 ng) and 0.05 µL of MyTaq polymerase (Bioline). Samples were amplified at 95 °C for 2 minutes, then 35 cycles at 95 °C for 10 seconds, 52 °C for 20 seconds, 72 °C for 30 seconds) followed by a 5-minute final extension at 72 °C. PCR products were then digested at 37 °C for 1 hour with *AccI* restriction enzyme with 2 µL Cutsmart Buffer and 1 unit of *AccI* (NEB) to a final volume of 20 µL. Following digestion, products were separated using agarose gel electrophoresis (1.5 %). *Plutella xylostella* products are approximately 517 bp and 191 bp and *P. australiana* products are 348 bp and 360 bp (Figure 2.1c).

2.3 Results

Population genetic analysis

Using RAD sequencing, we identified a set of 491 832 variant and invariant sites, representing 0.146 % of the *P. xylostella* reference genome, and a subset of 1285 SNP variants, for population genetic analysis. Individuals from nine field and laboratory-reared populations from Australia were genotyped at an average read depth of 45 (Table 2.2).

The 491 832 confident sites were used to generate a neighbour-joining phylogeny for all 72 individuals (Figure 2.1a). Seven individuals collected from South Australian locations, three from Calca, three from Nundroo and one from Picnic Beach, were confidently resolved with 100 % consensus support. Three individuals, Calca-4, -6 and -7, showed particularly long branch lengths, although the branch length estimate for Calca-4 is inflated due to missing data (48 % sites genotyped). We performed PCR mitochondrial genotyping assays to assess whether the dataset contained multiple *Plutella* lineages. The assays identified the three divergent individuals, Calca-4, -6 and -7, as a cryptic species, *P. australiana*, and confirmed that all other individuals ($n = 69$) were *P. xylostella* (Figure 2.1c). These results based on RAD-seq markers indicate strong divergence in the nuclear genome between the two Australian *Plutella* lineages, consistent with high mitochondrial sequence divergence already reported [131]. Four basal *P. xylostella* individuals, Picnic Beach-5, -6, -9 and Nundroo-8, were also confidently resolved (100 % consensus support), and relative branch length estimates suggest strong divergence from other *P. xylostella*. All remaining *P. xylostella* individuals could not be confidently resolved (<50 % consensus support), despite some evidence of individuals grouping according to geographic location.

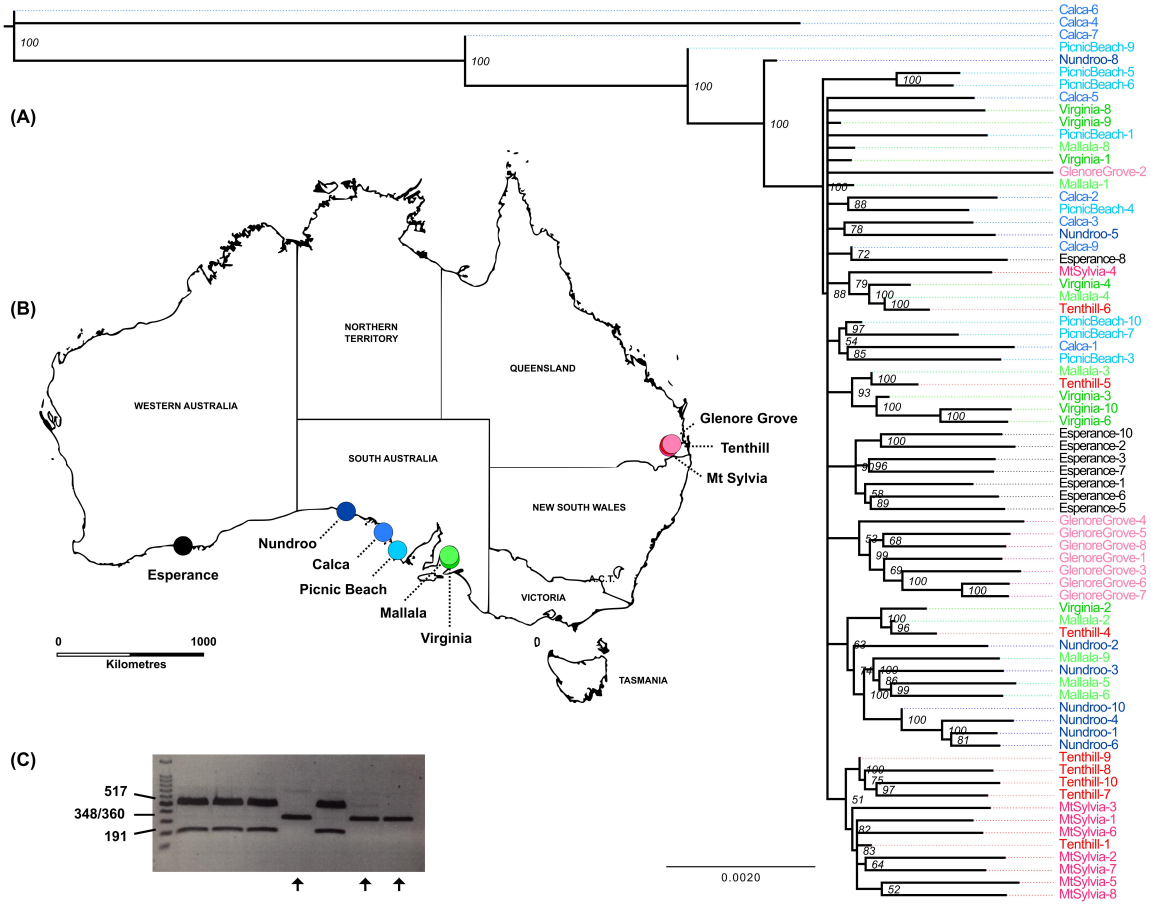

Fig. 2.1 (A) Neighbour-joining consensus tree generated from 491 832 variant and invariant sites for 72 *Plutella* individuals displaying nodes with at least 50 % consensus support. Node labels are the percentage consensus support for 1000 bootstrap replicates. (B) Map of collection sites for nine *Plutella* populations from Western Australia ($n = 1$), South Australia ($n = 5$) and Queensland ($n = 3$). (C) Gel electrophoresis image showing the results of a mitochondrial CO1 gene assay to distinguish the two *Plutella* lineages. *Plutella australiana* is genotyped as a single band, shown by black arrows, and *P. xylostella* as two bands.

Table 2.1 Collection details of *Plutella* populations from Australia genotyped using RAD-seq.

Location	Latitude	Longitude	Host	Collection date	Lifestage[a] collected	F_1 individuals	Generation sequenced
Esperance, Western Australia	−33.8588	121.8931	Canola	26/09/2012	L, P	59	F6
Nundroo, South Australia	−31.7516	132.0565	Canola	19/08/2013	L	86	F2
Calca, South Australia	−33.0492	134.3729	Wild *Brassica*[c]	08/04/2014	L	–	Field
Picnic Beach, South Australia	−34.1696	135.2744	Wild *Brassica*[d]	07/04/2014	L	40	F1
Mallala, South Australia	−34.4383	138.5099	Canola	11/09/2013	L	173	F5
Virginia, South Australia	−34.7150	138.5570	Cauliflower	07/03/2013	L, P	–	Field
Glenore Grove, Queensland	−27.5280	152.4070	Cabbage	11/10/2012	L, P	25	F5
Mt Sylvia, Queensland	−27.7170	152.2200	Cabbage	03/10/2012	L, P	59	F5
Tenthill, Queensland	−27.5660	152.2350	Red cabbage	11/10/2012	L, P	40	F5

[a] Larvae (L), pupae (P)
[b] Number of F_1 individuals used to establish laboratory populations
[c] *Diplotaxis* sp.
[d] Sea rocket, *Cakile maritima*

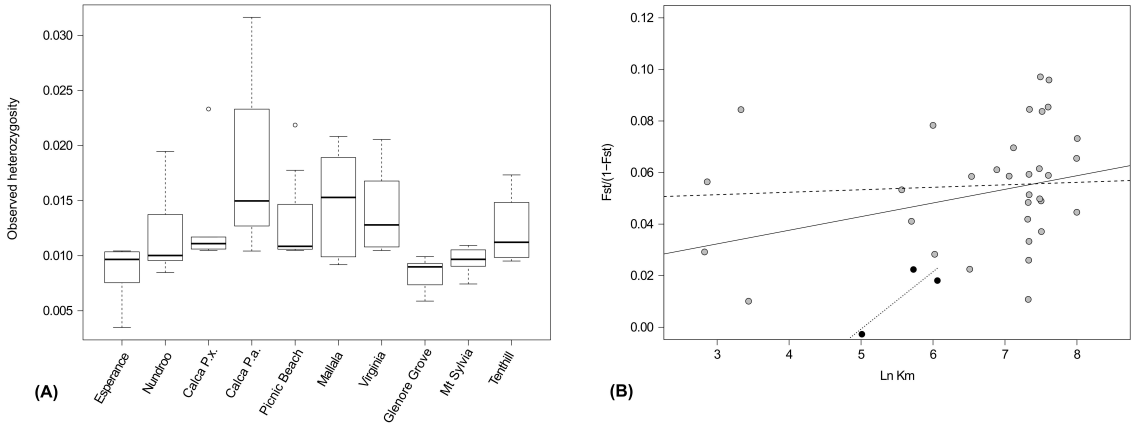

Fig. 2.2 (A) Distributions of observed heterozygosity for *Plutella* populations. Each population contains eight individuals. Calca is split into two lineages, Calca *P.x.* ($n = 5$) and Calca *P.a.* ($n = 3$). (B) Regressions of Slatkin's linearized genetic distance ($F_{ST}/(1-F_{ST})$) against the natural log of geographic distance (*Ln* km) for all pairwise population comparisons of *P. xylostella* ($n = 69$ individuals). Lines represent the fitted linear regression model for pairwise comparisons for "all populations" (grey and black circles, solid line, $y = 0.00529x + 0.01646$, Mantel's $r = 0.3048$, $p = 0.0215$), "cage" populations (grey circles, dashed line, $y = 0.00096x + 0.04854$, Mantel's $r = 0.07388$, $p = 0.36226$) and "field" populations (black circles, dotted line, $y = 0.02224x - 0.11197$, Mantel's $r = 0.88862$, $p = 0.33307$).

Table 2.2 Population statistics calculated for the SNP variant sites (top) and for all confidently called (GQ$\geqslant$ 30) variant and invariant sites (bottom) for populations of *Plutella* species collected from Australia. Populations each contain eight sequenced individuals with Calca split into *P. xylostella* ($n = 5$) and *P. australiana* ($n = 3$) individuals. Statistics include the population means for the number of individuals genotyped per locus (N), number of sites genotyped, site depth, number of sites unique to each population (private alleles), the proportion of observed (H_O) and expected (H_E) heterozygosity, and Wright's inbreeding coefficient (F_{IS}).

Population	N	Sites genotyped	Site depth	Private alleles	H_O	H_E	F_{IS}
SNP variants ($n = 1285$)							
Esperance	6.7	1073	32	0	0.3485	0.3650	0.0451
Nundroo	7.6	1215	54	0	0.4008	0.3763	−0.0653
Calca_Px	4.8	1246	65	0	0.4327	0.3844	−0.1256
Calca_Pa	2.0	855	34	0	0.5249	0.3233	−0.6173
Picnic Beach	7.7	1238	73	0	0.3977	0.3821	−0.0408
Mallala	7.7	1229	42	0	0.5619	0.4263	−0.3181
Virginia	7.4	1194	30	0	0.5115	0.4032	−0.2685
Glenore Grove	7.2	1158	34	0	0.3052	0.3469	0.1202
Mt Sylvia	7.3	1169	33	0	0.3490	0.3667	0.0482
Tenthill	7.3	1175	32	0	0.4827	0.4026	−0.1991
All variant and invariant sites ($n = 491\,832$)							
Esperance	7.5	462 076	33	41	0.0090	0.0108	**0.1715***
Nundroo	7.8	479 764	54	32	0.0120	0.0120	0.0041
Calca_Px	4.9	482 269	65	91	0.0135	0.0129	−0.0492
Calca_Pa	2.4	398 538	39	1476	0.0220	0.0185	−0.1923
Picnic Beach	7.9	482 882	72	77	0.0132	0.0133	0.0113
Mallala	7.9	484 254	43	20	0.0150	0.0129	−0.1651
Virginia	7.8	480 662	31	53	0.0142	0.0129	−0.1017
Glenore Grove	7.6	464 262	35	28	0.0085	0.0103	**0.1800***
Mt Sylvia	7.7	473 837	34	30	0.0098	0.0112	**0.1313***
Tenthill	7.8	479 215	33	20	0.0126	0.0118	−0.0658

* F_{IS} values in bold are significantly different from zero according to bootstrapped 95 % CL.

Table 2.3 Pairwise comparisons of genetic distance measured by Weir and Cockerham's (1984) F_{ST} (lower diagonal) and geographic distance in km (upper diagonal) for all *P. xylostella* population pairs.

	Esp (F_6)	Nun (F_2)	Cal_Px (F_0)	Pic B. (F_1)	Mal (F_4)	Vir (F_0)	Gle G. (F_5)	Mt. Syl (F_5)	Ten (F_5)
Esperance, WA	–	979	1162	1234	1530	1534	2993	2968	2975
Nundroo, SA	**0.0576*****	–	261	403	672	689	2021	1997	2004
Calca_Px, SA	0.0554	0.0506	–	150	413	429	1836	1811	1819
Picnic Beach, SA	**0.0651*****	**0.0726*****	−0.0027	–	299	307	1793	1767	1776
Mallala, SA	0.0322	0.022	0.0275	0.0395	–	31	1531	1504	1515
Virginia, SA	0.0489	0.0553	0.0178	0.0219	0.01	–	1541	1514	1525
Glenore Grove, Qld	**0.0682*****	**0.0875*****	**0.0772*****	**0.0885*****	**0.0560****	**0.0779*****	–	28	18
Mt Sylvia, Qld	**0.0615*****	**0.0787*****	0.0467	**0.0579*****	0.0402	0.0462	**0.0778*****	–	17
Tenthill, Qld	0.0427	0.0556	0.0358	0.0474	0.0108	0.0253	**0.0534****	0.0284	–

Significance of exact *G* tests after Bonferonni correction: $p < 0.05^{*}$, $p < 0.001^{**}$, $p < 0.0001^{***}$

The levels of heterozygosity were used to assess genetic diversity within and among populations. For all genotyped sites, the average observed heterozygosity ranged from 0.0085 to 0.0150 for *P. xylostella* populations but was notably higher at 0.0220 for the three *P. australiana* individuals (Table 2.2). The average number of private alleles among *P. xylostella* populations ranged from 20–91, while three *P. australiana* individuals had a much higher average of 1476 private alleles (range 606–2063), consistent with strong divergence. For the variant SNP dataset, the average observed heterozygosity ranged from 0.3052 to 0.5115 for *P. xylostella* populations, and again was higher at 0.5619 for the three *P. australiana* individuals (Table 2.2). Private alleles were filtered out of the SNP variant dataset. Average levels of heterozygosity were variable within and among populations (Figure 2.2). Laboratory-reared populations, Esperance, Glenore Grove and Mt Sylvia, show the lowest levels of genetic diversity (Figure 2), and were founded with relatively low numbers of individuals, ranging from 25-59 (Table 2.1). The values for the inbreeding coefficient, F_{IS}, were significantly different from zero for these three populations (Table 2.2). The global estimate of F_{ST} across all *P. xylostella* populations and loci was significantly different from zero ($F_{ST} = 0.0487$, 99 % CL 0.0187–0.0905), indicating significant genetic differentiation among the nine populations. The pairwise F_{ST} values indicate that the differentiation is associated with populations from the three regions, Western Australia, South Australia and Queensland (Table 2.3). However, patterns of differentiation did not clearly relate to geographic proximity and may reflect inbreeding in laboratory populations. Glenore Grove, for example, was highly differentiated from all other populations including those from Tenthill and Mt Sylvia in close proximity (18-28 km). Lower F_{ST} values among some South Australian populations suggests high levels of gene flow within this region, however these populations were also not differentiated from Tenthill in Queensland despite large geographic separation (1515–1819 km). The Mantel test for all pairwise population comparisons indicated a weak but significant effect of isolation by distance ($n = 69$ individuals, $r = 0.3048$, $p = 0.0215$) (Figure 2.2b). When analysed separately however, there was no relationship between genetic and geographic distance for pairwise comparisons of either "cage" populations ($n = 33$, $F_2 - F_6$; Mantel's $r = 0.07388$, $p = 0.36226$) or "field" populations ($n = 3$, $F_0 - F_1$; Mantel's $r = 0.88862$, $p = 0.33307$).

We performed an analysis of population structure using a Bayesian clustering approach in the program STRUCTURE. For the analysis of all population samples ($n = 72$), the data most likely formed three genotypic clusters ($K = 3$) (Figure 2.3a). Inspection of STRUCTURE bar plots shows that individuals from Calca and Picnic Beach populations were assigned to similar genotypes clusters, however STRUCTURE did not identify the three *P. australiana* individuals from Calca at that value of K. Although statistically less likely, K values of 2 and 4 are also presented. At $K = 4$, the *P. australiana* individuals are more clearly resolved, as

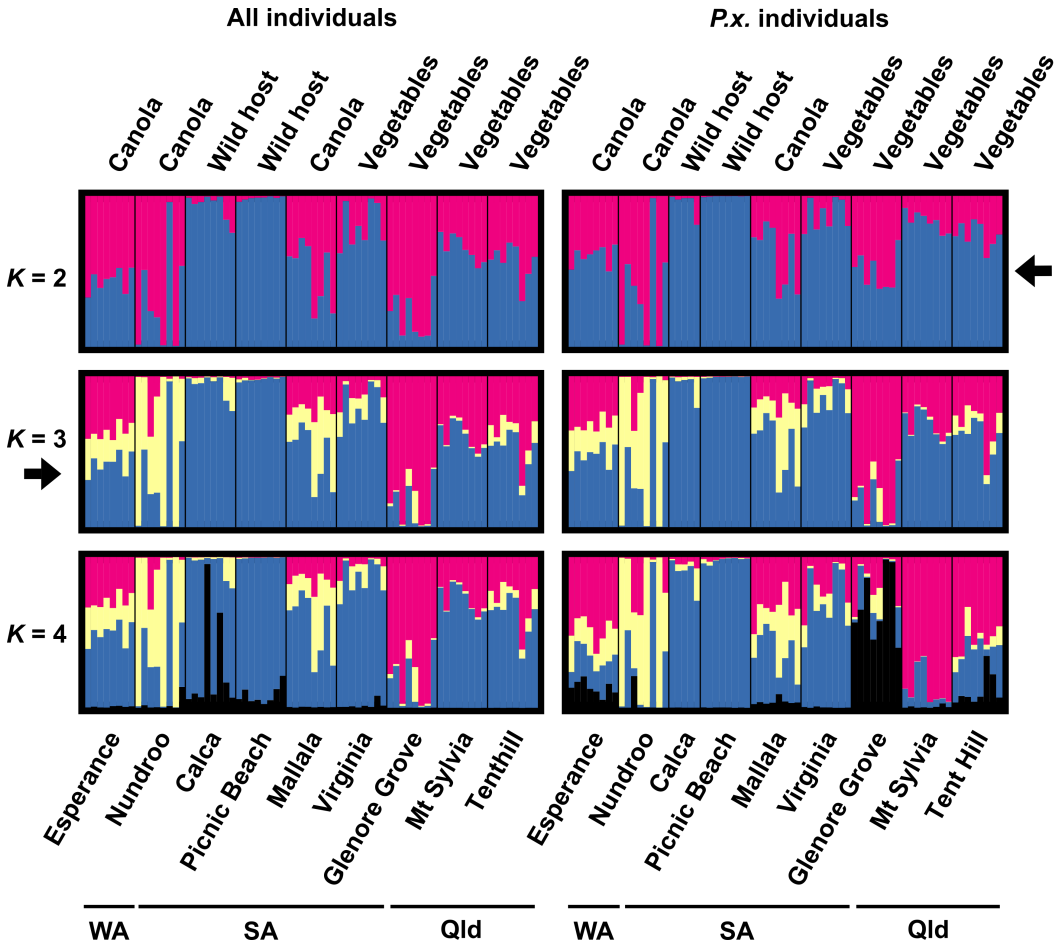

Fig. 2.3 Posterior probability of assignment to inferred genotypic clusters, K, generated in the program STRUCTURE for all individuals ($n = 72$), and separately for *P. xylostella* individuals ($n = 69$) where three *P. australiana* individuals from the Calca population were excluded. The most likely K for "all individuals" and "*P.x.* only" is indicated by black arrows. Each vertical bar represents a single individual, populations are separated by black vertical lines, and different genotypic clusters for $K = 2 - 4$ are represented by different colours.

seen by tall black bars for individuals Calca-4 and -6 (Figure 2.3). Moderate sharing of the black-coloured genotype cluster occurs across all individuals from Calca and Picnic Beach, and rarely elsewhere. For the analysis excluding the three *P. australiana* individuals, the most likely number of clusters was reduced to two ($K = 2$). At this value of K, a high degree of admixture is evident among most populations, as seen by sharing of pink and blue-coloured genotypic clusters. The two populations collected from wild hosts in a similar region, Calca and Picnic Beach, share similar genotypic clusters. At $K = 3$, the assignment of individuals to genotypic clusters is consistent with patterns of population differentiation inferred from pairwise F_{ST} values (Table 2.3).

Frequency of insecticide resistance alleles

We examined the frequency of mutations associated with pyrethroid resistance among nine populations collected from canola crops, *Brassica* vegetable crops and wild hosts. The average frequencies for *skdrl*, *kdr* and *cdr* were 0.29, 0.51 and 0.27 respectively (Table A.1). The frequencies were comparable among populations for *skdrl* (range = 0.2–0.38) and *kdr* (range = 0.25–0.83), but more variable for *cdr* (range = 0.05–0.6). Interestingly, the populations collected from wild hosts, Calca and Picnic Beach, had a moderately high frequency of the *cdr* mutation relative to most other populations. In contrast, the three *P. australiana* samples were all found to be susceptible for *skdrl* and *cdr* however the *kdr* assay failed, possibly due to variation in primer binding sites.

2.4 Discussion

RAD sequencing was used to identify thousands of SNP markers and hundreds of thousands of invariant loci from across the genome of two *Plutella* species. These markers facilitated an initial assessment of genetic diversity within and among nine *Plutella* populations collected from different locations and host plants across Australia.

Analysis of RAD-seq markers and mitochondrial genotyping identified three individuals of a cryptic *Plutella* lineage, *P. australiana*, among our 72 *Plutella* individuals. The relative branch length estimates in the neighbouring-joining tree, differences in heterozygosity and high numbers of private alleles within the *P. australiana* populations (albeit only three individuals) provide the first evidence that the *P. australiana* and *P. xylostella* lineages are strongly divergent in their nuclear genomes. Among the three *P. australiana* individuals, it was interesting to note that individual Calca-7 had the fewest number of private alleles (606, compared to 1760 and 2062) despite having the highest heterozygosity (3.2 %, compared

to 1.5 % and 1.04 %). The phylogeny also shows this individual most closely related to *P. xylostella* individuals Picnic Beach-9 and Nundroo-8, which grouped separately from all other *P. xylostella* individuals. As yet, the potential for hybridization between these lineages remains to be tested.

The original discovery of *P. australiana* in Australia was made through sequencing the mitochondrial COI gene from moths collected in light traps, rather than from known host plants [131]. Hence, the fundamental biology of this species and its potential pest status remain to be understood. In our study, individuals of *P. australiana* and *P. xylostella* were collected from a wild brassicaceous host, *Diplotaxis* sp., at the same location and date, indicating that these lineages can co-exist in similar environments and exploit at least one common host. Considering that there have been several previous genetic studies of *P. xylostella* in Australia [65, 174, 196, 66], including mitochondrial markers [202], the discovery of this novel lineage only recently is intriguing. It is possible that differences in sampling strategies (direct sampling from plants vs trapping), times or locations between studies, or differences in the biology of *Plutella* lineages (e.g. host range), meant that *P. australiana* was not collected in previous studies. Alternatively, some molecular markers designed for *P. xylostella* may not amplify efficiently in *P. australiana*. These questions require further investigation.

We examined genetic diversity in field and laboratory-reared populations of *P. xylostella*. Significantly reduced heterozygosity was observed in the laboratory populations, Glenore Grove, Mt Sylvia and Esperance, as measured by the inbreeding co-efficient, F_{IS} (Table 2.2). These populations were founded by 25–59 individuals at the F_1 generation and then reared for five to six generations. The population from Mallala was also reared for five generations but established from a higher number of individuals ($n = 173$), and maintained higher levels of heterozygosity in culture, comparable with the field (F_0) populations.

We assessed genetic structure among our population samples using a range of approaches. The global estimate of F_{ST} (0.0487, 99 % CL 0.0187–0.0905) indicated significant genetic differentiation among our populations. Pairwise F_{ST} comparisons showed that most differentiation was associated with the three most inbred laboratory populations, Glenore Grove, Mt Sylvia, Esperance, but also Nundroo (F_2). There was no evidence for isolation by distance in pairwise population comparisons among laboratory populations only. Hence, the estimates of genetic isolation are inflated by inbreeding in population cages. The STRUCTURE analysis for all population samples ($n = 72$) inferred that individuals most likely form three genotypic clusters, however failed to resolve the two lineages at that value of K. This result could reflect that alleles unique to *P. australiana* individuals were filtered out of the variant SNP dataset used for this analysis. Removing *P. australiana* reduced the optimal value of K to two.

At this K value, a large degree of admixture was observed among populations, supporting the neighbor-joining phylogeny which failed to clearly resolve clusters for the majority of *P. xylostella* individuals (<50 % consensus support). Overall, these findings are consistent with high levels of gene flow previously reported for Australian populations of diamondback moth [65].

The frequency of pyrethroid resistance alleles has previously been documented for Australian *P. xylostella* populations collected from 2003–2005 [67]. Endersby et al. [67] reported average resistance allele frequencies for *skdrl* (0.139), *kdr* (0.609) and *cdr* (0.305), however considerable spatial variation was observed. To assess the potential change in frequencies over time, we re-examined these frequencies from populations collected between 2012 and 2014 (Table A.1). The average frequency for *skdrl*, *kdr* and *cdr* were 0.29, 0.51 and 0.27 respectively, which is comparable between studies for *kdr* and *cdr*, however somewhat higher for *skdrl*. The stability of these frequencies may reflect that synthetic pyrethroid insecticides continue to be widely used to control a range of invertebrate pests in Australian crops.

Conclusion

RAD-seq is a powerful method for generating SNP markers for population genetic studies in the diamondback moth. To aid biological inference, we recommend that future studies focus on field sampling design and wherever possible strive to use field-collected (F_0) populations to adequately represent genetic diversity.

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Name of Principle Author (Candidate):	Kym D Perry
Contribution to the Paper:	Organised collection of *Plutella* samples across Australia, performed RAD-seq, COI genotyping and data analysis with the exception of *Wolbachia*, wrote the manuscript, acted as corresponding author.
Overall percentage (%):	75 %

Signature: _____ Date: _____

Co-Author Contributions are listed on the next page.

Statement of Authorship - Chapter 3 ... continued

Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

i the candidate's stated contribution to the publication is accurate (as detailed above);
ii permission is granted for the candidate to include the publication in the thesis; and
iii the sum of all co-author contributions is equal to 100 % less the candidate's stated contribution.

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Signature:	Date:

Chapter 3

Cryptic *Plutella* species show deep divergence despite the capacity to hybridize

Abstract

Background: Understanding genomic and phenotypic diversity among cryptic pest taxa has important implications for the management of pests and diseases. The diamondback moth, *Plutella xylostella* L., has been intensively studied due to its ability to evolve insecticide resistance and status as the world's most destructive pest of brassicaceous crops. The surprise discovery of a cryptic species endemic to Australia, *Plutella australiana* Landry & Hebert, raised questions regarding the distribution, ecological traits and pest status of the two species, the capacity for gene flow and whether specific management was required. Here, we collected *Plutella* from wild and cultivated brassicaceous plants from 75 locations throughout Australia and screened 1447 individuals to identify mtDNA lineages and *Wolbachia* infections. We genotyped genome-wide SNP markers using RADseq in coexisting populations of each species. In addition, we assessed reproductive compatibility in crossing experiments and insecticide susceptibility phenotypes using bioassays.

Results: The two *Plutella* species coexisted on wild brassicas and canola crops, but only 10 % of *Plutella* individuals were *P. australiana*. This species was not found on commercial *Brassica* vegetable crops, which are routinely sprayed with insecticides. Bioassays found that *P. australiana* was 19-306 fold more susceptible to four commonly-used insecticides than *P. xylostella*. Laboratory crosses revealed that reproductive isolation was incomplete but directionally asymmetric between the species. However, genome-wide nuclear SNPs

revealed striking differences in genetic diversity and strong population structure between coexisting wild populations of each species. Nuclear diversity was 1.5-fold higher in *P. australiana*, yet both species showed limited variation in mtDNA. Infection with a single *Wolbachia* subgroup B strain was fixed in *P. australiana*, suggesting that a selective sweep contributed to low mtDNA diversity, while a subgroup A strain infected just 1.5 % of *P. xylostella*.

Conclusions: Despite sympatric distributions and the capacity to hybridize, strong genomic and phenotypic divergence exists between these *Plutella* species that is consistent with contrasting colonization histories and reproductive isolation after secondary contact. Although *P. australiana* is a potential pest of brassicaceous crops, it is of secondary importance to *P. xylostella*.

Keywords

Plutella australiana, *Plutella xylostella*, Lepidoptera, hybridization, sympatric, insecticide resistance, *Wolbachia*

3.1 Background

Cryptic species can show remarkable diversity in aspects of their ecology, behaviour, and at the level of the genome. They exist across metazoan taxa [172], including globally important arthropod pest taxa, such as whiteflies [51], disease-vectoring mosquitoes [42], fruit flies [99], thrips [197, 108] and mites [216, 150], some of which are characterised by cryptic species complexes. Discovering cryptic diversity has important consequences for estimates of global biodiversity, conservation planning, and the management of pests and diseases. Morphologically similar species can vary in pest status due to differences in genotypic and/or phenotypic traits that influence their host range and specificity, geographic distribution, the ability to vector diseases, or insecticide resistance [150, 238, 8]. Therefore, recognising cryptic species and the differences in their biology and ecology are essential for effective management, with important implications for public health, agriculture and trade.

The diamondback moth, *Plutella xylostella*, is the major pest of brassicaceous crops worldwide, costing an estimated US$4 to US$5 billion annually in direct losses and management costs [78, 257]. Insecticide resistance is widespread in *P. xylostella* populations around the world, fuelling wide-ranging research to develop alternative management tactics [78, 134]. *Plutella xylostella* was initially recorded in Australia in the late 1800s and rapidly became a widespread pest of *Brassica* vegetables, and then canola following its expanded

production from the 1990s [65, 77]. Recently, Landry and Hebert [131], through mtDNA barcoding, identified a cryptic lineage of *Plutella* in Australia not detected in previous molecular studies of *P. xylostella* [65, 202, 52, 196, 174, 67]. Although external morphology was indistinguishable from *P. xylostella*, deep mtDNA divergence (8.6 %), differences in genital morphology and endemism in Australia led them to describe a new species, *Plutella australiana* Landry & Hebert. *Plutella australiana* was originally collected together with *P. xylostella* in light trap samples in eastern Australia, suggesting at least some ecological overlap [131], but its biology, ecology and pest status were unknown.

The management of *P. xylostella* in Australian *Brassica* crops has been a significant challenge for decades [77, 15], but the discovery of *P. australiana* has made the relative abundance and pest status of both species in these crops uncertain. With rare exception, *P. xylostella* and allied species feed on plants in the order Brassicales, mainly within the family Brassicaceae [131, 200, 189], implying that the host range of *P. australiana* may include cultivated brassicas. Widespread resistance to pyrethroid and organophosphate insecticides has been attributed to Australian populations of *P. xylostella* from all vegetable and canola production regions, which has led to ineffective control during outbreaks [15, 66]. *Plutella xylostella* is well known as a migratory insect with a high capacity for gene flow [78, 134], facilitating the rapid spread of resistance alleles. Australian *P. xylostella* are thought to disperse frequently, based on indirect evidence from ecological and genetic studies [65, 77, 185]. Most studies have found a lack of genetic differentiation at microsatellite loci and low sequence variation in mitochondrial DNA markers among Australian and New Zealand populations of *P. xylostella*, consistent with high gene flow and/or recent ancestry [65, 77, 202, 52]. While species identification was not in question in these studies, somewhat inconsistent findings in two studies from eastern Australia using allozymes or SSR markers [174, 196] might reflect the confounding presence of *P. australiana* samples [131]. Given these considerations, future management of *Plutella* in Australian crops will require thorough understanding of the ecological requirements, genetic traits and pest status of the two *Plutella* species. In addition, reproductive isolation between these two species is unknown but has implications for evolutionary inference and the potential for gene flow. The capacity for hybridization and introgression could lead to the exchange of insecticide resistance or other adaptive alleles [41, 97].

Although mtDNA markers are widely used in studies of species identity and population structure [96, 7, 219], mitochondrial variation within or between species can be influenced by direct and/or indirect selection, or introgressive hybridization [247, 61]. One factor that can confound mtDNA-based inference is interaction with inherited bacterial symbionts [106, 186]. *Wolbachia* is a widespread endosymbiont thought to infect at least half of arthropod [244]

and 80 % of lepidopteran [2] species. It is mainly transmitted vertically from infected females to their offspring through the egg cytoplasm, and inheritance is therefore linked with mtDNA. To facilitate its spread, *Wolbachia* manipulates host reproductive biology to favour the fitness of infected females by inducing host phenotypes that distort sex ratios (male-feminization, male-killing or induction of parthenogenesis) or cause sperm-egg cytoplasmic incompatibility (CI) [246, 69]. In the simple case involving a single CI-inducing strain, crosses with infected females are fertile but crosses between uninfected females and infected males fail to produce offspring. If maternal transmission is efficient and infected females have a reproductive advantage, *Wolbachia* infection can spread rapidly through an insect population [114], driving a selective sweep of a single haplotype and reducing mtDNA diversity [211]. Limited surveys to date have identified *Wolbachia* strains infecting *P. xylostella* at low frequency in populations from North America, Africa, Asia and Europe [52, 20, 112]. Because symbionts can contribute to reproductive isolation and influence mtDNA diversity [106, 232], assessing their role can provide important insights into host evolution and population structure [186, 59, 154, 224].

Here we investigated the biology, ecology and population genetic structure of two cryptic *Plutella* species by collecting *Plutella* from brassicaceous plants throughout Australia and screening individuals to identify mtDNA lineages and *Wolbachia* infections. For a subset of populations, we examined genetic diversity using thousands of nuclear SNPs from across the genome. In addition, we assessed reproductive compatibility in laboratory crosses and determined the susceptibility of each species to commercial insecticides.

3.2 Methods

Sample collection

Plutella larvae (rarely, eggs or pupae) were collected from canola crops, *Brassica* vegetable crops, forage brassicas and wild brassicas throughout Australia between March 2014 and December 2015 (Table 3.1). The wild species included wild radish, *Raphanus raphanistrum*, turnip weed, *Rapistrum rugosum*, sea rocket, *Cakile maritima*, Ward's weed, *Carrichtera annua*, African mustard, *Brassica tournefortii*, and mixed stands of sand rocket, *Diplotaxis tenuifolia*, and wall rocket, *D. muralis*. At each location, at least 25 individuals were collected from randomly selected plants to achieve a representative sample. Insect samples were collected from *Brassica* vegetables by hand, from sea rocket by beating plants over a collection tray and from other hosts using a sweep net. Each population sample was separately reared in ventilated plastic containers on leaves of the original host material for

1-2 days and thereafter on cabbage leaves. Non-parasitised pupae or late-instar larvae were fresh frozen at −80 °C.

DNA isolation and COI genotyping

For each population sample, we aimed to genotype a minimum of 16 individuals where possible after removing parasitized individuals. Individual pupae (but not larvae) were sexed under a dissecting microscope, then genomic DNA was isolated by homogenising whole individuals followed by two phenol and one chloroform extractions according to Zraket et al. [261]. DNA was treated with RNase A, then precipitated and re-suspended in TE buffer. *Plutella* lineages were distinguished using a PCR-RFLP assay [171]. A 707 bp COI region was amplified using a combination of two primer pairs: (i) PxCOIF (5′-TCAACAAATCA TAAAGATATTGG-3′) and PxCOIR (5′-TAAACTTCAGGGTGACCAAAAAATCA-3′), and (ii) PaCOIF (5′-TCAACAAATCATAAGGATATTGG-3′) and PaCOIR (5′-TAAACCT CTGGATGGCCAAAAAATCA-3′). Ten microliter reactions were run with 2 µL of MyTaq 5x buffer, 0.2 µL of each primer (10 mM stocks), 1 µL of DNA (approx. 5 ng) and 0.05 µL of MyTaq polymerase (Bioline). Samples were amplified at 95 °C for 2 min, then 35 cycles at 95 °C for 10 s, 52 °C for 20 s, 72 °C for 30 s followed by a 5 min final extension at 72 °C. PCR products were digested at 37 °C for 1 h with 1 unit of *AccI* (NEB) restriction enzyme with 2 µL Cutsmart Buffer in a 20 µL reaction. Following digestion, products were separated using agarose gel electrophoresis (1.5 %). *Plutella xylostella* products are approximately 516 bp and 191 bp and *P. australiana* products are 348 bp and 359 bp [171]. To examine mtDNA haplotypes, sequencing of the 707 bp COI amplicon was performed for 44 *P. xylostella* and 37 *P. australiana* individuals at the Australian Genome Research Facility (AGRF). In addition, we downloaded sequence trace files from Landry and Hebert [131] (dx.doi.org/10.5883/DS-PLUT1) and re-analysed, aligned and trimmed all sequences in GENEIOUS version 10.0.6 [122]. Haplotype networks were constructed using R package pegas version 0.9 [165].

Wolbachia screening and phylogenetics

Wolbachia infection was detected using two separate PCR assays of the 16S rRNA gene (16S-2 and 16S-6) according to Simoes et al. [214]. To identify *Wolbachia* strains, the *Wolbachia surface protein* (*wsp*) gene was sequenced in a subset of individuals. Amplification was performed using wsp81F and wsp691R sequence primers [259]. Amplicons were sequenced using the reverse primer and aligned in GENEIOUS version 10.0.6 [122]. We used a 493 bp alignment to construct a maximum likelihood phylogeny in RAxML version 8.2.4 [221] using a general time reversal substitution model [230] with 1000 bootstrap replicates.

RADseq library preparation and sequencing

Libraries were prepared for restriction-site-associated DNA sequencing (RADseq) according to a protocol modified from Baird et al. [13]. Genomic DNA was quantified using a Qubit 2.0 fluorometer (Invitrogen) and 200 ng digested with 10 units of high fidelity *SbfI* in Cutsmart Buffer (NEB) for 1 h at 37 °C, then heat inactivated at 80 °C for 20 min. One microlitre of P1 adapter (100 nM) with a 6-base molecular identifier (MID) (top strand 5′-TCGTCGGCAGC GTCAGATGTGTATAAGAGACAGxxxxxxtgca-3′, bottom strand 5′-[P]xxxxxxCTGTCTC TTATACACATCTGACGCTGCCGACGA-3′, x represents sites for MIDs) were then added using 0.5 µL T4 DNA ligase (Promega), 1 nM ATP and Cutsmart buffer. Library pools were sheared using a Bioruptor sonicator (Diagenode), then DNA fragments end-repaired using a Quick Blunting Kit (NEB), adenine overhangs added then P2 adapters (top strand 5′-[P]CTG TCTCTTATACACATCTCCAGAATAG-3′, bottom strand 5′-GTCTCGTGGGCTCGGAG ATGTGTATAAGAGACAGT-3′) ligated. DNA purification between steps was performed using a MinElute PCR purification kit (Qiagen). Libraries were amplified using KAPA HiFi Hotstart Readymix (Kapa Biosystems) and Nextera i7 and i5 indexed primers with PCR conditions: 95 °C for 3 min, two cycles of 98 °C for 20 s, 54 °C for 15 s, 72 °C for 1 min, then 15 cycles of 98 °C for 20 s, 65 °C for 15 s, 72 °C for 1 m followed by a final extension of 72 °C for 5 min. Libraries were size-selected (300-700 bp) on 1-1.5 % agarose gel and purified using a minElute Gel Extraction Kit (Qiagen), then Illumina paired-end sequencing was performed using HiSeq2500 (100 bp) or NextSeq500 (75 bp) at the AGRF.

Read filtering and variant calling

Sequence reads were demultiplexed using RADtools version 1.2.4 [21] allowing one base MID mismatch, then TRIMMOMATIC version 0.32 [26] was used to remove restriction sites, adapter sequences and thymine base from reverse reads introduced by the P2 adapter, and quality filter using the ILLUMINACLIP tool with parameters: TRAILING:10 SLIDINGWINDOW: 4:15 MINLEN:40. Paired reads were aligned to the *P. xylostella* reference genome (accession number: GCF_000330985.1) using STAMPY version 1.0.21 [139] with –baq and –gatkcigarworkaround options and expected substitution rate set to 0.03 for *P. xylostella* and 0.05 for *P. australiana* to reflect expected levels of sequence divergence relative to the *P. xylostella* reference genome. Duplicate reads were removed using PICARD version 1.71 [29]. Genotypes were called using the Genome Analysis Toolkit (GATK) version 3.3-0 [149, 53] HaplotypeCaller tool. We determined that base quality score recalibration using bootstrapped SNP databases was inappropriate for this dataset as it globally reduced quality scores. For downstream comparisons between species, we joint-genotyped *P. australiana* and

P. xylostella individuals using the GATK GenotypeGVCFs workflow. To examine finer scale population structure, we also joint-genotyped the *P. australiana* individuals alone. All variant callsets were hard-filtered with identical parameters using VCFtools version 0.1.12a [48]: We removed indels and retained confidently-called biallelic SNPs (GQ $\geqslant$ 30) genotyped in at least 70 % of individuals with a minimum genotype depth of 5, minQ $\geqslant$ 400, average site depth of 12–100, minimum minor allele frequency of 0.05, in Hardy-Weinberg equilibrium at an alpha level of 0.05. To avoid linked sites, we used the VCFtools –thin function to retain only SNPs separated by a minimum of 2000 bp. To estimate genetic diversity, we generated a set of all confidently-called variant and invariant sites (GQ $\geqslant$ 30), and hard filtered to remove sites within repetitive regions and retain sites genotyped in at least 70 % of individuals with an average site depth of 12–100. Sites from the mitochondrial genome were excluded from all datasets.

Genetic diversity and population structure

Genetic diversity was calculated for *Plutella* populations of both species from five locations. The R package hierfstat [85] was used to calculate observed heterozygosity, gene diversity and the inbreeding coefficient, F_{IS}, according to Nei [160]. Population means for site depth and number of SNPs, indels and private sites were calculated using the –depth function and vcfstats module in VCFtools version 0.1.12a [48]. The number of heterozygous sites within individuals was determined from all confidently called sites excluding indels using a custom python script parseVCF.py [147] and visualised using R [180]. To examine population structure in *P. australiana*, a global estimate of F_{ST} [245] with bootstrapped 99 % confidence intervals (10^4 bootstrap replicates) was calculated in R package diveRsity [123]. Pairwise F_{ST} values for all population pairs were calculated and significance determined using exact G tests (10^4 mc burnins, 10^3 batches, and 10^4 iterations per batch) in GENEPOP version 4.6 [195] after Bonferroni correction for multiple comparisons. Separate analysis of population structure was performed using the Bayesian clustering program STRUCTURE version 2.3.4 [176], first for all individuals of co-occurring *Plutella* species, and second for *P. australiana* alone. For all runs, we used a burnin length of 5×10^5 followed by a run length of 10^6 MCMC iterations and performed ten independent runs for each K value from 1 to 10, where K is the number of genotypic clusters, using a different random seed for each run, assuming the *locprior* model with correlated allele frequencies and λ set to 1. The optimal value of K was determined using the delta K method [71] implemented in STRUCTURE HARVESTER [62] and inspection of the likelihood distribution for each model. Q-matrices were aligned across runs using CLUMPP version 1.1.2 [110] and visualised using DISTRUCT version 1.1 [192].

Table 3.1 Collection details showing the frequency (*f*) of *Plutella* species and *Wolbachia* infections among *Plutella* populations from Australia.

						P. australiana[2]	*P. xylostella*	
Location[1]	Collection date	Latitude	Longitude	Host	No. genotyped	No. (*f*)	No. (*f*)	No. (*f*) *wol*-infected
Boomi NSW	Sep-2014	−28.76°	149.81°	Canola	25	15 (0.60)	10 (0.40)	0 (0.00)
Gilgandra NSW	Sep-2014	−31.67°	148.72°	Wild turnip	23	21 (0.91)	2 (0.09)	0 (0.00)
Ginninderra NSW	Sep-2014	−35.19°	149.05°	Canola	15	2 (0.13)	13 (0.87)	0 (0.00)
Ginninderra NSW	Oct-2015	−35.19°	149.05°	Canola	34	27 (0.79)	7 (0.21)	0 (0.00)
Goulburn NSW	Nov-2015	−34.84°	149.67°	Canola	32	25 (0.78)	7 (0.22)	0 (0.00)
Henty NSW	Oct-2014	−35.60°	146.95°	Canola	18	1 (0.06)	17 (0.94)	0 (0.00)
Narromine NSW	Sep-2014	−32.22°	148.03°	Canola	26	0 (0.00)	26 (1.00)	1 (0.04)
Richmond NSW	Oct-2015	−33.60°	150.71°	Cabbage	21	0 (0.00)	21 (1.00)	0 (0.00)
Wagga Wagga NSW	Sep-2014	−35.04°	147.33°	Canola	21	5 (0.24)	16 (0.76)	0 (0.00)
Werombi NSW	Nov-2014	−33.99°	150.64°	Vegetables	16	0 (0.00)	16 (1.00)	0 (0.00)
Werombi NSW	Oct-2015	−34.00°	150.56°	Kale	13	4 (0.31)	9 (0.69)	0 (0.00)
Bundaberg QLD	Oct-2014	−24.80°	152.26°	Canola	14	1 (0.07)	13 (0.93)	0 (0.00)
Bundaberg QLD	Sep-2015	−24.80°	152.26°	Canola	30	0 (0.00)	30 (1.00)	0 (0.00)
Cunnamulla QLD	Sep-2015	−28.07°	145.68°	African mustard	17	17 (1.00)	0 (0.00)	0 –
Dalby QLD	Sep-2014	−27.28°	151.13°	Canola	30	0 (0.00)	30 (1.00)	0 (0.00)
Gatton QLD	Oct-2014	−27.54°	152.33°	Broccoli	16	0 (0.00)	16 (1.00)	0 (0.00)
Gatton QLD	Nov-2015	−27.54°	152.33°	Broccoli	15	0 (0.00)	15 (1.00)	0 (0.00)
Warwick QLD	Oct-2015	−28.21°	152.11°	Canola	16	0 (0.00)	16 (1.00)	0 (0.00)
Calca SA	Apr-2014	−33.02°	134.28°	Sand rocket, Wall rocket	13	8 (0.62)	5 (0.38)	0 (0.00)
Cocata SA	Sep-2014	−33.20°	135.13°	Canola	18	0 (0.00)	18 (1.00)	0 (0.00)
Colebatch SA	Feb-2015	−35.97°	139.66°	Forage brassica	18	0 (0.00)	18 (1.00)	0 (0.00)
Coonalpyn SA	Oct-2015	−35.62°	139.91°	Wild radish	11	0 (0.00)	11 (1.00)	0 (0.00)
Cowell SA	Sep-2014	−33.66°	137.16°	Canola	16	0 (0.00)	16 (1.00)	0 (0.00)
Keith SA	Oct-2014	−36.09°	140.29°	Canola	32	0 (0.00)	32 (1.00)	6 (0.19)
Lameroo SA	Sep-2014	−35.32°	140.51°	Canola	16	0 (0.00)	16 (1.00)	0 (0.00)
Lameroo SA	Oct-2015	−35.17°	140.48°	Canola	14	0 (0.00)	14 (1.00)	0 (0.00)
Littlehampton SA	Oct-2014	−35.06°	138.90°	Cabbage	34	0 (0.00)	34 (1.00)	6 (0.18)
Littlehampton SA	Sep-2015	−35.06°	138.90°	Brussels sprouts	8	0 (0.00)	8 (1.00)	0 (0.00)
Loxton SA	Sep-2014	−34.37°	140.72°	Canola	31	0 (0.00)	31 (1.00)	0 (0.00)
Loxton SA	Oct-2015	−34.50°	140.80°	Canola	14	1 (0.07)	13 (0.93)	0 (0.00)
Mallala SA	Sep-2015	−34.38°	138.50°	Canola	26	0 (0.00)	26 (1.00)	0 (0.00)
Meribah SA	Sep-2014	−34.74°	140.82°	Canola	16	0 (0.00)	16 (1.00)	0 (0.00)
Millicent SA	Apr-2015	−37.61°	140.34°	Canola	9	0 (0.00)	9 (1.00)	2 (0.22)
Minnipa SA	Oct-2015	−32.81°	135.16°	Canola	22	1 (0.05)	21 (0.95)	0 (0.00)
Moonaree SA	Aug-2014	−31.99°	135.87°	Ward's weed	16	0 (0.00)	16 (1.00)	0 (0.00)
Mt Hope SA	Sep-2014	−34.14°	135.33°	Canola	29	0 (0.00)	29 (1.00)	0 (0.00)
Mt Hope SA	Sep-2015	−34.20°	135.34°	Canola	16	0 (0.00)	16 (1.00)	0 (0.00)
Padthaway SA	Oct-2015	−36.56°	140.43°	Canola	18	2 (0.11)	16 (0.89)	0 (0.00)
Picnic Beach SA	Apr-2014	−34.17°	135.27°	Sea rocket	2	0 (0.00)	2 (1.00)	0 (0.00)
Picnic Beach SA	Sep-2014	−34.17°	135.27°	Sea rocket	16	0 (0.00)	16 (1.00)	0 (0.00)
Redbanks SA	Oct-2014	−34.49°	138.59°	Canola	38	0 (0.00)	38 (1.00)	1 (0.03)
Sherwood SA	Oct-2014	−36.05°	140.64°	Wild radish	8	0 (0.00)	8 (1.00)	0 (0.00)
Southend SA	Apr-2015	−37.57°	140.12°	Sea rocket	18	0 (0.00)	18 (1.00)	0 (0.00)
Tintinara SA	Oct-2015	−35.97°	139.66°	Forage brassica	17	0 (0.00)	17 (1.00)	0 (0.00)
Ucontichie SA	Sep-2014	−33.22°	135.31°	Canola	3	0 (0.00)	3 (1.00)	0 (0.00)
Virginia SA	Oct-2014	−34.64°	138.54°	Broccoli	18	0 (0.00)	18 (1.00)	1 (0.06)
Virginia SA	Sep-2015	−34.64°	138.54°	Cabbage	23	0 (0.00)	23 (1.00)	0 (0.00)
Walkers Beach SA	Sep-2014	−33.55°	134.86°	Sea rocket	16	0 (0.00)	16 (1.00)	0 (0.00)
Walkers Beach SA	Mar-2015	−33.55°	134.86°	Sea rocket	16	0 (0.00)	16 (1.00)	0 (0.00)
Walkers Beach SA	Sep-2015	−33.55°	134.86°	Sea rocket	19	0 (0.00)	19 (1.00)	0 (0.00)
Wirrabara SA	Oct-2014	−32.99°	138.31°	Canola	28	2 (0.07)	26 (0.93)	0 (0.00)
Wokurna SA	Sep-2015	−33.67°	137.96°	Wild radish	24	1 (0.04)	23 (0.96)	0 (0.00)
Wurramunda SA	Apr-2014	−34.30°	135.56°	Wild canola	16	0 (0.00)	16 (1.00)	0 (0.00)
Deddington TAS	Nov-2014	−41.59°	147.44°	Kale	16	0 (0.00)	16 (1.00)	0 (0.00)
Launceston TAS	Nov-2014	−41.47°	147.14°	Wild mustard	16	0 (0.00)	16 (1.00)	0 (0.00)
Newstead TAS	Nov-2015	−41.59°	147.44°	Cauliflower	22	0 (0.00)	22 (1.00)	0 (0.00)
Cowangie VIC	Oct-2015	−35.10°	141.33°	Canola	19	0 (0.00)	19 (1.00)	0 (0.00)
Ouyen VIC	Sep-2014	−35.00°	142.31°	Canola	28	1 (0.04)	27 (0.96)	0 (0.00)
Robinvale VIC	Sep-2014	−34.81°	142.94°	Canola	16	0 (0.00)	16 (1.00)	0 (0.00)
Werribee VIC	Oct-2014	−37.94°	144.73°	Cauliflower	16	0 (0.00)	16 (1.00)	0 (0.00)
Werribee VIC	Nov-2015	−37.94°	144.73°	Cauliflower	16	0 (0.00)	16 (1.00)	0 (0.00)
Yanac VIC	Sep-2014	−36.06°	141.25°	Canola	17	0 (0.00)	17 (1.00)	0 (0.00)
Boyup Brook WA	Sep-2014	−33.64°	116.40°	Canola	26	2 (0.08)	24 (0.92)	0 (0.00)
Dalwallinu WA	Sep-2015	−30.28°	116.66°	Canola	20	0 (0.00)	20 (1.00)	0 (0.00)
Dalyup WA	Oct-2015	−33.72°	121.64°	Wild radish	22	3 (0.14)	19 (0.86)	0 (0.00)
Esperance WA	Sep-2014	−33.29°	121.76°	Canola	23	8 (0.35)	15 (0.65)	1 (0.07)
Esperance WA	Oct-2015	−33.79°	122.13°	Canola	16	0 (0.00)	16 (1.00)	0 (0.00)
Gingin WA	Dec-2014	−31.28°	115.65°	Red cabbage	23	0 (0.00)	23 (1.00)	1 (0.04)
Kalannie WA	Sep-2015	−30.00°	117.25°	Canola	18	0 (0.00)	18 (1.00)	0 (0.00)
Manjimup WA	Dec-2014	−34.18°	116.23°	Chinese cabbage	17	0 (0.00)	17 (1.00)	0 (0.00)
Manjimup WA	Nov-2015	−34.18°	116.23°	Vegetables	13	0 (0.00)	13 (1.00)	0 (0.00)
Narrogin WA	Oct-2015	−32.95°	117.32°	Wild radish, Wild canola	15	0 (0.00)	15 (1.00)	0 (0.00)
Narrogin WA	Oct-2015	−32.96°	117.33°	Canola	32	0 (0.00)	32 (1.00)	0 (0.00)
Walkaway WA	Sep-2014	−28.94°	114.83°	Canola	19	0 (0.00)	19 (1.00)	0 (0.00)
Walkaway WA	Sep-2014	−28.16°	114.63°	Canola	16	0 (0.00)	16 (1.00)	0 (0.00)
Total					1447	147 (0.10)	1300 (0.90)	19 (0.01)

[1] Australian states: NSW = New South Wales, QLD = Queensland, SA = South Australia, TAS = Tasmania, VIC = Victoria, WA = Western Australia.
[2] All *P. australiana* individuals were infected with *Wolbachia*.

Laboratory cultures of *Plutella* species

Laboratory cultures of *P. australiana* and *P. xylostella* were established from field populations and used for crossing experiments and insecticide bioassays. *Plutella* adults were collected at light traps at Angle Vale and Urrbrae, South Australia, in October–November 2015. Females were isolated and allowed to lay eggs, then identified using PCR-RFLP and progeny pooled to produce separate cultures of each species. A laboratory culture of the Waite Susceptible *P. xylostella* strain (S) has been maintained on cabbage without insecticide exposure for approximately 24 years ($\approx$ 310 generations) and was used as a bioassay reference strain. All cultures were maintained in laboratory cages at $26 \pm 2.0\,^{\circ}\mathrm{C}$ and a 14:10 (L:D) hour photoperiod at the South Australian Research and Development Institute, Waite Campus, Adelaide, South Australia. The *P. australiana* culture was maintained on sand rocket, *Diplotaxis tenuifolia*, and the *P. xylostella* culture was maintained on cabbage, *Brassica oleracea* var. *capitata*. The purity of cultures was assessed regularly using PCR-RFLP.

Crossing experiments

Plutella australiana and *P. xylostella* pupae were sexed under a stereo microscope, then placed into individual 5 mL clear polystyrene tubes with fine mesh lids and gender visually confirmed after eclosion. Enclosures used for crossing experiments were 850 mL polypropylene pots (Bonson Pty Ltd) modified with lateral holes covered with voile mesh for ventilation. Crosses of single mating pairs were performed on laboratory benches at $26 \pm 2.0\,^{\circ}\mathrm{C}$ and 14:10 (L:D) photoperiod using 3-week old *D. tenuifolia* seedlings as the host plant. After seven days, adults were collected into a 1.5 mL tube and fresh frozen at $-80\,^{\circ}\mathrm{C}$ for species confirmation using PCR-RFLP. Seedlings were examined and eggs counted under a stereo microscope, then returned to enclosures to allow egg hatch. Larvae were provided with fresh 3–4 week old seedlings until pupation, then pupae were individually collected into 5 mL tubes. Hybrid F1xF1 crosses and back-crosses were then performed as above. The presence of egg and adult offspring was recorded for all replicates, and for the majority of replicates ($>$80 %), the numbers of offspring were counted and used to calculate a mean.

Insecticide bioassays

Insecticide susceptibility of field-collected *Plutella* strains was compared to the susceptible *P. xylostella* (S) reference in dose-response assays using four commercial insecticides: Dominex ($100\,\mathrm{g\,L^{-1}}$ alpha-cypermethrin), Proclaim ($44\,\mathrm{g\,kg^{-1}}$ emamectin benzoate), Coragen ($200\,\mathrm{g\,L^{-1}}$ chlorantraniliprole) and Success Neo ($120\,\mathrm{g\,L^{-1}}$ spinetoram). Bioassays

were performed by placing 3^{rd} instar larvae onto inverted leaf discs embedded in 1 % agar in 90 mm Petri dishes. Cabbage leaves, *Brassica oleracea* var. *capitata* were used for *P. xylostella* and canola leaves, *B. napus* var. "ATR Stingray", were used for *P. australiana*. Eight concentrations and a water-only control were evaluated for each insecticide using four replicates of ten larvae. A 4 mL aliquot of test solution was applied directly to leaves using a Potter Spray Tower (Burkard Manufacturing Co. Ltd.) calibrated to deliver an aliquot of $3.52 \pm 0.09\,\mathrm{mg\,cm^{-2}}$. After application, each dish was placed in a controlled temperature room at $25 \pm 0.5\,^{\circ}\mathrm{C}$, then mortality was assessed after 48 h (Dominex, Success Neo) or 72 h (Proclaim, Coragen). Dose-response analysis was performed using log-logistic regression in R package drc [187] and the fitted models were used to estimate the lethal concentration predicted to cause 50 % (LC_{50}) and 99 % (LC_{99}) mortality of the test population. Resistance ratios were calculated by dividing the LC_{50} and LC_{99} estimates for field strains by the corresponding *LC* estimates for the *P. xylostella* (S) reference strain.

3.3 Results

Geographic distribution and host associations

Plutella larvae were collected from brassicaceous plants at 75 locations in Australia and 1477 individuals were genotyped at the COI locus using PCR-RFLP to identify species. Of these, 88 % ($n = 1300$) were genotyped as *P. xylostella*, 10 % ($n = 147$) as *P. australiana* and 2 % ($n = 30$) were unresolved (Table 3.1). *Plutella australiana* was identified in around one quarter ($n = 20/75$) of collections distributed across southern Australia, while *P. xylostella* occurred at all locations except Cunnamulla, Queensland, in a collection from wild African mustard, *Brassica tournefortii* (Table 3.1). The sex ratio was not different from 1:1 for *P. xylostella* (481 females, 517 males, $\chi^2 = 1.2986$, $p = 0.2545$) or *P. australiana* (63 females, 55 males, $\chi^2 = 0.5424$, $p = 0.4615$). The relative incidence and abundance of *P. australiana* was >2-fold higher in the eastern state of New South Wales than in other states (Figure 3.1). *Plutella australiana* larvae were detected in 29 % ($n = 5/17$) of collections from wild brassicas and from species including wild radish, *Raphanus raphanistrum*, wild turnip, *Rapistrum rugosum*, African mustard, *B. tournefortii*, and mixed stands of sand rocket, *D. tenuifolia* and wall rocket, *D. muralis* (Table 3.2). Among cultivated crops, *P. australiana* larvae occurred in 36 % ($n = 14/39$) of samples from canola, consisting of 11 % of total *Plutella* individuals from those crops, but were not identified from commercial *Brassica* vegetable farms (Table 3.2). However, *P. australiana* eggs were collected from kale at one farm.

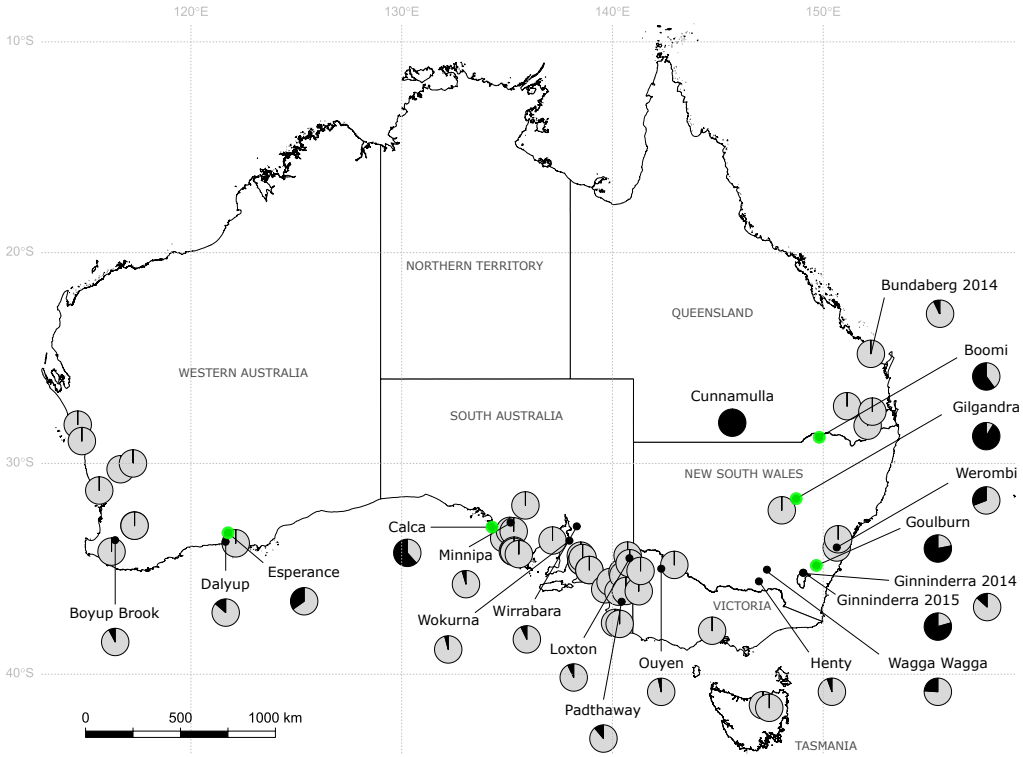

Fig. 3.1 The geographic distribution of *P. xylostella* (light grey) and *P. australiana* individuals (black) in larval collections from brassicaceous plants in Australia during 2014 and 2015. Pie diagrams show the relative proportion of each species at each location. Overlapped pies represent locations with 100 % *P. xylostella*. Green highlighted circles indicate five locations from which individuals of each species were RAD sequenced.

Table 3.2 Frequency of *P. australiana* in *Plutella* collections from different *Brassica* host types.

Host	No. locations	No. *P.aus* locations	No. genotyped	No. *P.aus*
Wild brassicas	17	5 (0.29)	268	50 (0.19)
Canola crops	39	14 (0.36)	848	93 (0.11)
Vegetable crops	16	1 (0.06)	287	4 (0.01)
Forage brassicas	3	0 (0.00)	44	0 (0.00)

Presented are the numbers and proportion in parentheses of *P. australiana* across collection locations and individuals genotyped.

Wolbachia infections

Plutella individuals ($n = 1447$) were screened for *Wolbachia* infection using 16S rRNA PCR assays. Only 1.5 % ($n = 19/1300$) of *P. xylostella* collected from eight different locations were infected (Table 3.1). In contrast, all 147 *P. australiana* individuals were infected with *Wolbachia* across the 20 locations where this species occurred. To identify *Wolbachia* strains, a *Wolbachia surface protein* (*wsp*) amplicon was sequenced from 14 *P. xylostella* and 30 *P. australiana* individuals. Each species was infected with a different strain. The *wsp* sequence for Australian *P. xylostella* showed 100 % identity to a *Wolbachia* supergroup A isolate infecting *P. xylostella* from Malaysia, *plutWA1* [52]. For *P. australiana*, the *wsp* sequence showed 100 % identity to a *Wolbachia* supergroup B isolate infecting a mosquito, *Culex pipiens*, from Turkey and the winter moth, *Operophtera brumata*, from the Netherlands (Figure 3.2).

Crossing experiments

Inter-species single pair mating experiments showed that hybridization between *P. australiana* and *P. xylostella* was possible, yet less successful than intra-species crosses. While most intra-species crosses produced adult offspring, the fecundity of *P. xylostella* was >2-fold higher than *P. australiana* (Table 3.3). Both reciprocal inter-species crosses produced F1 adult offspring, but success was asymmetric and notably higher in the pairs with *P. australiana* females. In this direction, there was a strong male bias in the F1 progeny: from 76 cross replicates, 16 collectively produced 9 female and 80 male adults, a ratio of 8.9. Hybrid F1xF1 crosses for both parental lines produced F2 adult offspring (Table 3.4). For the *P. australiana* maternal line, parental back-crosses using F1 hybrid males successfully produced offspring, while parental back-crosses with F1 hybrid females were sterile. For the *P. xylostella* maternal line, low fitness allowed only a single parental back-cross replicate, which involved a hybrid female and was sterile.

Mitochondrial haplotype diversity

Mitochondrial haplotype networks of Australian *Plutella* were constructed using a 613 bp COI alignment that included 81 sequences from this study and 108 from Landry and Hebert [131]. We found low haplotype diversity within Australian *P. xylostella*, consistent with previous reports [202, 52, 118]. Only five haplotypes were identified among 102 individuals, including three identified by Saw et al. [202] and three occurring in single individuals (Figure 3.3a). The most common haplotype, PxCOI01, occurred at high frequency and differed

Table 3.3 Fecundity of intra-species and reciprocal inter-species single pair crosses of *P. australiana* (*P.aus*) and *P. xylostella* (*P.x*).

Cross (♀ x ♂)	No. replicates	No. reps eggs	No. reps adults	Mean ± SEM no. eggs	Mean ± SEM no. adults
P.aus♀ x *P.aus*♂	42	37 (0.88)	34 (0.81)	40.86 ± 5.33	9.66 ± 1.70
P.x♀ x *P.x*♂	63	59 (0.94)	59 (0.94)	83.82 ± 10.61	24.28 ± 3.27
P.aus♀ x *P.x*♂	76	49 (0.65)	16 (0.21)	18.43 ± 3.02	1.17 ± 0.33
P.x♀ x *P.aus*♂	85	62 (0.73)	3 (0.04)	15.16 ± 2.37	0.06 ± 0.03

Presented are the number and proportion in parentheses of replicates (reps) that produced eggs and adult offspring, and the mean ± standard error of the mean number of eggs and adult offspring per replicate.

Table 3.4 Fecundity of hybrid F1 crosses and back-crosses.

Cross (♀ x ♂)	No. replicates	No. reps eggs	No. reps adults	Mean ± SEM no. eggs	Mean ± SEM no. adults
F0 *P.aus*♀ source					
(*P.aus*♀ x *P.x*♂)♀ x (*P.aus*♀ x *P.x*♂)♂	4	4 (1.00)	2 (0.50)	66.00 ± 60.00	–
(*P.aus*♀ x *P.x*♂)♀ x *P.aus*♂	7	7 (1.00)	0 (0.00)	20.33 ± 11.86	0.00 ± 0.00
P.aus♀ x (*P.aus*♀ x *P.x*♂)♂	9	5 (0.56)	2 (0.22)	6.38 ± 3.54	0.22 ± 0.44
(*P.aus*♀ x *P.x*♂)♀ x *P.x*♂	4	4 (1.00)	0 (0.00)	39.00 ± 19.00	0.00 ± 0.00
P.x♀ x (*P.aus*♀ x *P.x*♂)♂	15	15 (1.00)	4 (0.27)	36.75 ± 3.21	0.33 ± 0.62
F0 *P.x*♀ source					
(*P.x*♀ x *P.aus*♂)♀ x (*P.x*♀ x *P.aus*♂)♂	6	5 (0.83)	4 (0.67)	74.50 ± 22.79	6.17 ± 5.27
(*P.x*♀ x *P.aus*♂)♀ x *P.aus*♂	1	0 (0.00)	0 (0.00)	0.00	0.00

Presented are the number and proportion in parentheses of replicates (reps) producing eggs and adult offspring, and the mean ± standard error of the mean numbers of eggs and adults offspring per replicate. A dash denotes an absence of count data.

by a single base mutation from other haplotypes (Figure 3.3a, Table B.1). Nine closely related haplotypes were identified in 87 *P. australiana* individuals with seven occurring in single individuals (Figure 3.3b). The most common haplotype, PaCOI01, occurred at high frequency and differed by 1-2 base mutations from other haplotypes (Figure 3.3a, Table B.2).

Nuclear diversity and population structure

At five collection locations, *P. australiana* co-occurred with *P. xylostella* in sufficient numbers to enable comparison of nuclear genomes, though the relative abundance of species varied between locations. To ensure representation from the south-west region of Australia, the Esperance population ($n = 5$) was formed by including one *P. australiana* individual from Boyup Brook. Despite only two *P. xylostella* individuals at Gilgandra, this population had 17 *P. australiana* individuals and was included. To generate nuclear SNP markers, we performed RADseq for a total of 52 *P. australiana* and 47 *P. xylostella* individuals. Illumina sequencing and demultiplexing using RADtools [21] yielded 276.8 million raw sequence reads. Following read quality filtering and mapping, genotypes were called for 99 individuals from the two species. Hard filtering retained 300 241 confidently-called variant and invariant nuclear sites at a mean depth > 36 per individual, and a subset of 689 widely-dispersed nuclear SNP variants (to avoid linkage bias) at a mean depth > 36 per individual, for comparative analyses of genetic diversity and population structure. The dataset with all confidently-called sites was used to estimate population-level genetic diversity. Estimates of nuclear genetic diversity across 300 241 variant and invariant sites revealed a striking contrast between *Plutella* species, with notably higher diversity within populations of *P. australiana* than co-occurring populations of *P. xylostella* (Table 3.5). The mean observed heterozygosity within populations ranged from 0.013–0.016 for *P. australiana* and 0.009–0.010 for *P. xylostella*. Similarly, the average numbers of SNPs, indels and private alleles were considerably higher within *P. australiana* populations. As *P. australiana* may have fixed nucleotide differences relative to the *P. xylostella* reference genome that may affect population level statistics, we also removed indels from this dataset and directly compared the heterozygosity among individuals using 289 347 sites. *Plutella australiana* individuals had on average a 1.5-fold higher proportion of heterozygous sites than *P. xylostella* individuals (Figure 3.4).

Genetic structure among co-occurring populations of *Plutella* species was investigated using 689 widely-dispersed nuclear SNPs in the program STRUCTURE. The delta K method predicted a strong optimal at $K = 2$ genotypic clusters. *Plutella australiana* and *P. xylostella* individuals were clearly separated into distinct genotypic clusters in accordance to their species identified through mtDNA genotypes regardless of geographic location (Figure 3.5,

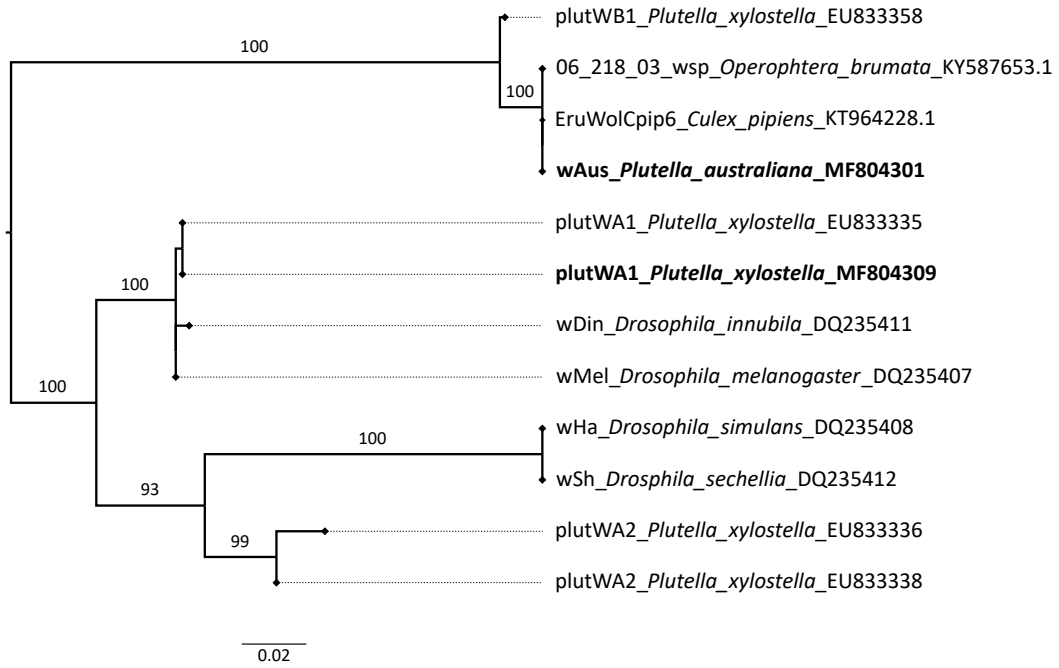

Fig. 3.2 Maximum likelihood phylogeny of 493 bp of *Wolbachia wsp* amplicons for *Plutella* and other arthropods. The strain infecting *P. australiana* (*wAus*) was identical to a *Wolbachia* supergroup B strain reported from *Culex pipiens* and *Operophtera brumata*. The strain infecting Australian *P. xylostella* was identical to a supergroup A strain (*plutWA1*) reported from Malaysian *P. xylostella*. Labels include the *Wolbachia* strain, host species and GenBank accession number. Labels in bold denote strains sequenced in this study. The scale bar shows the mean number of nucleotide substitutions per site.

left panel). Five individuals across four locations showed greater than 1 % admixture, as shown by sharing of colored bars. Assessing population structure from datasets with multiple species can mask heirachical structure [120]. To address this, genotypes were separately called for 52 *P. australiana* individuals, and hard-filtering retained a set of 974 widely-dispersed SNP variants at a mean depth > 33 per individual for examination of finer scale structure among five populations. The delta K method predicted a weak modal signal at $K = 3$, but the highest likelihood value occurred at $K = 1$. Bar plots for $K = 3$ showed a high degree of admixture among individuals across the five populations, consistent with high levels of gene flow across Australia (Figure 3.5, right panel). Pairwise F_{ST} was then calculated for the five *P. australiana* populations using 974 SNPs. The global estimate of F_{ST} was not significantly different from zero, indicating the populations are not differentiated ($F_{\mathrm{ST}} = 0.0002$, 99 % CI $= -0.0274, 0.0387$). Further, pairwise F_{ST} values were low, ranging from -0.0041 to 0.0038, suggesting substantial gene flow among populations separated by distances of between 341 and 2700 kilometres (Table 3.6).

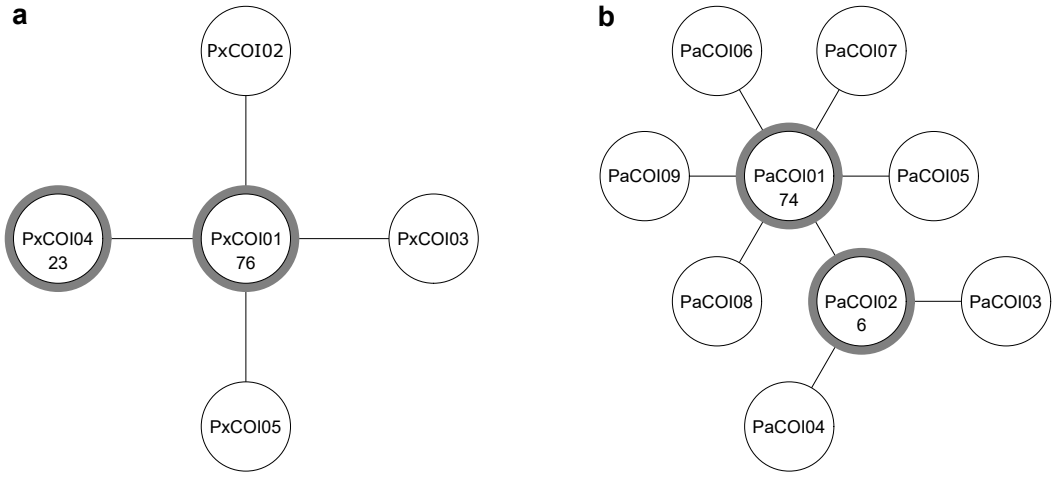

Fig. 3.3 Mitochondrial DNA haplotype network for **a** *P. xylostella* ($n = 102$, 44 from this study, 58 from [131]) and **b** *P. australiana* ($n = 87$, 37 from this study, 50 from [131]) individuals from Australia based on a 613 bp COI sequence alignment. Haplotypes shared by more than one individual are shown in circles with a grey border with the number of individuals indicated inside the circle. Haplotypes connected by a line differ by a single mutation.

Table 3.5 Population statistics for variant and invariant sites for sympatric populations of *P. australiana* (*P. aus*) and *P. xylostella* (*P. x*) from five locations.

Population	Species	n	Sites	Site depth	SNPs	Indels	Private sites	H_O	H_S	F_{IS}
Boomi NSW	*P. aus*	11.1	276 939	40	7198	1112	212	0.013	0.015	0.089
	P. x	9.4	282 989	42	4316	549	30	0.009	0.010	0.039
Calca SA	*P. aus*	8.7	261 496	30	6629	989	210	0.014	0.015	0.059
	P. x	8.2	274 973	42	4126	538	40	0.009	0.010	0.050
Esperance WA	*P. aus*	4.5	269 268	28	6543	998	210	0.016	0.015	–0.032
	P. x	11.0	275 299	35	4046	520	23	0.010	0.010	0.019
Gilgandra NSW	*P. aus*	15.7	277 136	39	7154	1088	212	0.014	0.015	0.079
	P. x	1.9	277 846	42	4149	505	28	0.009	0.009	–0.056
Goulburn NSW	*P. aus*	6.8	256 343	29	6471	968	190	0.013	0.015	0.058
	P. x	12.8	274 700	36	4052	513	26	0.009	0.010	0.052

n, number of individuals genotyped per locus; H_O, observed heterozygosity; H_S, gene diversity; F_{IS}, Nei's inbreeding coefficient.

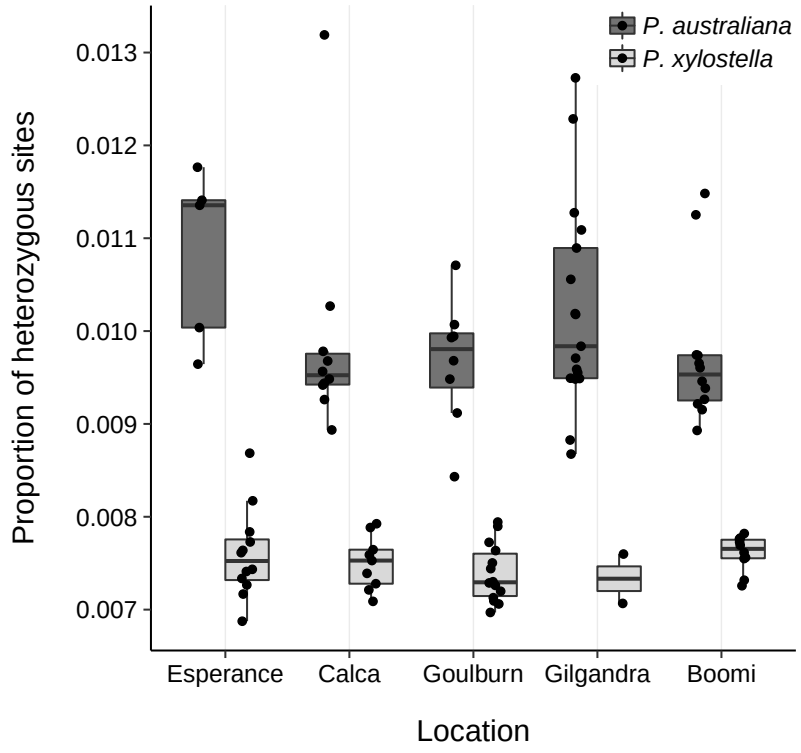

Fig. 3.4 Boxplot showing the proportion of heterozygous sites across 289 347 confidently-called nuclear sites for individuals of *P. xylostella* (light grey boxes, $n = 47$) and *P. australiana* (dark grey boxes, $n = 52$) from five locations. Heterozygosity was consistently higher in *P. australiana*.

Insecticide susceptibility

Bioassays revealed highly contrasting responses to insecticide exposure in *P. xylostella* and *P. australiana* field strains (Figure 3.6). *Plutella australiana* showed extremely high susceptibility to all four insecticides evaluated: resistance ratios at the LC_{50} and LC_{99} estimates were less than 1.0 and showed that this strain was 1.5-fold to 7.4-fold more susceptible than the laboratory *P. xylostella* (S) reference (Table B.3). In contrast, resistance ratios at the LC_{50} for the field *P. xylostella* strain ranged from 2.9 for Success Neo to 41.4 for Dominex, indicating increased tolerance to all insecticides. Comparison of the LC_{99} estimates with commercial field doses for each insecticide implies differences in field efficacy between species. The commercial field rate of Dominex was $>$ 8-fold lower than the LC_{99} for *P. xylostella*, suggesting likely poor field control of this strain, but was $>$ 17-fold higher than the LC_{99} for *P. australiana* (Figure 3.6). Control mortality was similar for the field and reference strains, averaging 3.1 to 4.4 % across all bioassays.

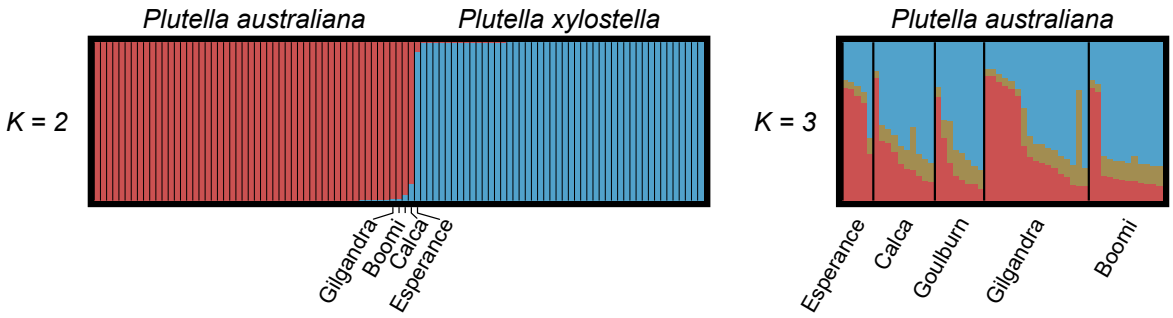

Fig. 3.5 Proportional assignment of *Plutella* individuals to genotypic clusters, K, based on STRUCTURE analysis. Individuals are represented by vertical bars and genotypic clusters are represented by different colors. Left panel: Analysis at $K = 2$ for 52 *P. australiana* and 47 *P. xylostella* individuals sorted left-to right by proportion of cluster membership. The predominantly red bars correspond to *P. australiana* individuals and the predominantly blue bars correspond to *P. xylostella* individuals identified through mtDNA genotypes. Locations are labelled for five individuals showing >1 % genotypic admixture. Right panel: Analysis at $K = 3$ for 52 *P. australiana* individuals sorted left-to-right by proportion of cluster membership within geographic locations, showing a high degree of genotypic admixture among individuals across locations.

Table 3.6 Pairwise comparisons[1] of Weir and Cockerham's [245] F_{ST} (below diagonal) and geographic distance in kilometres (above diagonal) among populations of *P. australiana* from five locations.

	Boo	Cal	Esp	Gil	Gou
Boomi	–	1555	2714	341	677
Calca	–0.0041	–	1167	1365	1434
Esperance	0.0038	0.0014	–	2531	2572
Gilgandra	0.0000	0.0036	–0.0005	–	364
Goulburn	–0.0015	–0.0014	0.0034	0.0005	–

[1] Exact G tests were non-significant for all population pairs ($p > 0.05$).

3.4 Discussion

Cryptic species arise when divergence does not lead to morphological change [25]. The recent discovery of a cryptic ally, *P. australiana*, to the diamondback moth, *P. xylostella*, was unexpected given the breadth of previous molecular studies of this insect. Several factors may have contributed to this discovery, including the use of light traps for specimen collection, rather than limiting sampling to *Brassica* vegetable farms. Landry and Hebert [131] also isolated DNA from legs, keeping most of each specimen intact and providing a morphological reference for examining unexpected genotypes. It is also possible that *P. australiana* was previously overlooked from nuclear DNA studies due to biases in amplification of divergent alleles. Here, we sought to determine whether *P. australiana* is an agricultural pest, and to understand its ecological and genetic differences from *P. xylostella*.

Extensive larval sampling from wild and cultivated brassicaceous plants revealed that *P. australiana* co-occurs widely with *P. xylostella* throughout southern Australia and utilizes some of the same host plants. The relative abundance of *P. australiana* was on average 9-fold lower than *P. xylostella*. We observed higher proportions of *P. australiana* in larval collections from the eastern state of New South Wales, similar to the light trap samples from Landry and Hebert [131], possibly reflecting habitat suitability. Although we did not detect *P. australiana* in limited sampling from the island state of Tasmania, the presence of brassicas in the region and evidence from light traps that wind currents can transport *Plutella* moths across Bass Strait (Lionel Hill, Pers. Comm.) suggest it is likely to occur there.

Our study confirms that the host range of *P. australiana* includes canola crops and wild brassicaceous species. In laboratory rearing, *P. australiana* completed development on sand rocket, *D. tenuifolia*, and canola, *B. napus*, and was also collected from several other wild species, though without rearing to confirm host status. Our sampling focused on relatively few introduced brassicaceous species common in agricultural areas, yet the Australian Brassicales is represented by 11 plant families [10], including several non-Brassicaceae on which *P. xylostella* and its allies have been documented feeding, such as Capparaceae [189], Cleomaceae [131] and Tropaeolaceae [200]. The Australian Brassicaceae has records for 61 genera and 205 species [10], including many introduced species but also a diversity of native genera, such as *Lepidium*, *Blennodium*, and *Arabidella*, that occur over vast areas of Australia. Wider sampling of native Brassicales may identify other suitable hosts for *P. australiana*.

Plutella australiana larvae were not identified among samples from sixteen commercial *Brassica* vegetable crops despite the high suitability of these crops for *P. xylostella* [228], however eggs were collected from kale. It is possible that extreme insecticide susceptibility prevents juvenile *P. australiana* populations from establishing, as commercial *Brassica*

vegetable crops are typically sprayed multiple times per crop cycle [15]. Our data show that *P. australiana* is far more susceptible than *P. xylostella* to four commonly used insecticides. At commercial application rates, these insecticides are likely to provide high-level control of *P. australiana* in Australian *Brassica* crops, but some products may provide marginal or poor control against *P. xylostella* due to insecticide resistance (Figure 3.6) [15, 66]. Alternatively, some vegetable cultivars may not be attractive for oviposition or suitable for larval survival in *P. australiana*. We noted that *P. australiana* cultures provided with cabbage seedlings failed to produce viable eggs over seven days, but after replacing cabbage with *Diplotaxis* seedlings, egg-laying then occurred within 24 h. Exposure to host plants stimulates reproductive behaviour in *P. xylostella* [175], but olfactory cues for host recognition or oviposition [223, 184, 119] may differ between these *Plutella* species. Host preference and performance studies are required to test these hypotheses.

Insecticide bioassays have been conducted routinely on Australian *P. xylostella* to monitor levels of insecticide resistance in field populations [15, 66]. This method appears unlikely to be affected by the presence of *P. australiana* under typical conditions, as a period of laboratory rearing is usually necessary to multiply individuals prior to screening. In our experience, laboratory rearing of the two *Plutella* species on cabbage plants selects against *P. australiana* individuals when competing with *P. xylostella* in cages, causing the complete loss of *P. australiana* within a few generations. The reasons for this are unknown but may include differences in host preference or development rate, or direct competition.

Crossing experiments revealed that hybridization can occur between *P. australiana* and *P. xylostella* under controlled conditions and is most likely to occur in crosses involving *Wolbachia*-infected *P. australiana* females. Hybridization occurs in around 10 % of animal species, particularly in captivity [143], but asymmetric reproductive isolation is commonly observed in reciprocal crosses between taxa [234]. In our experiments, a strong male bias in the offspring of interspecific crosses and failure to back-cross hybrid females both follow Haldane's rule [92], which predicts greater hybrid inviability or sterility in the heterogametic sex (female, in Lepidoptera). This pattern can arise from epistatic interactions between sex-linked and/or autosomal genes that result in genetic incompatibilities [113, 235]. Although the back-crosses with F1 hybrid females were sterile, the back-crosses with hybrid males (to both species) were viable, which could enable the transfer of genes between hybrid and/or parental species. However, it is unclear whether hybridization occurs in the wild.

Although *P. australiana* and *P. xylostella* show deep divergence (8.6 %) in mtDNA [131], the sole use of mtDNA can be unreliable for inference of evolutionary history and should be corroborated using evidence from nuclear markers [106]. Our analysis revealed striking differences in nuclear diversity across the genome between co-existing populations of each

Plutella species collected at the same locations and times, and from the same host plant species. *Plutella xylostella* populations from Australia and New Zealand have low levels of genetic diversity compared with populations from other continents, thought to reflect the recent introduction of this species from a small founding population [65, 202, 118]. Consistent with this view, we found a remarkable 1.5-fold reduction in heterozygosity across $> 300\,000$ sites in *P. xylostella* compared with sympatric *P. australiana* populations. However, both species showed limited mtDNA diversity with a single predominant haplotype. While outgroups from other continents were not available, comparative analysis of these closely-related Australian *Plutella* species suggested that patterns of mitochondrial and nuclear diversity are concordant in *P. xylostella* and consistent with a demographic bottleneck [202, 52], but discordant in *P. australiana*.

Sequence variation in mitochondrial DNA can be strongly influenced by *Wolbachia* infection [211]. Extensive *Wolbachia* screening showed that each *Plutella* species was infected with a different strain at contrasting frequencies, and fit a 'most-or-few' pattern whereby species infection rates are often very low ($< 10\,\%$) or very high ($> 90\,\%$) [102]. Infection incidence in *P. xylostella* was lower in Australia (1 %) than previously reported across global samples (5 %) [52]. Our finding of a single supergroup A strain showing 100 % sequence similarity to a strain reported in *P. xylostella* from Malaysia, *plutWA1* [52], provides some support of an Asian origin for Australian *P. xylostella* [202], though does not preclude this strain also occurring elsewhere.

Fixation of infection in *P. australiana* suggests that *Wolbachia* manipulates the reproductive biology of this species. We found no evidence of sex-ratio distortion, which has been associated with a *Wolbachia* strain, *plutWB1*, in *P. xylostella* [52]. High infection can be driven by cytoplasmic incompatibility (CI) [114]. The high frequency (87 %) of a single mtDNA haplotype among *P. australiana* individuals implies that the spread of *Wolbachia* infection has driven a selective sweep of coinherited mtDNA through the population, causing a loss of mtDNA diversity [211]. High nuclear diversity (relative to sympatric *P. xylostella*) supports this hypothesis, because a demographic bottleneck should reduce diversity across the entire genome [106].

Plutella australiana and *P. xylostella* have co-existed in Australia for at least 125 years ($\approx$ 1300 generations), yet have strongly divergent mitochondrial and nuclear genomes, *Wolbachia* infections and insecticide susceptibility phenotypes. Our observations during laboratory rearing and crossing experiments also suggested that interspecific differences in host plant use may exist. What explains such strong divergence between the two *Plutella* species, given sympatry and the capacity to hybridize? Endemism of *P. australiana* [131] implies an ancient evolutionary history in Australia, and our data provide support for existing

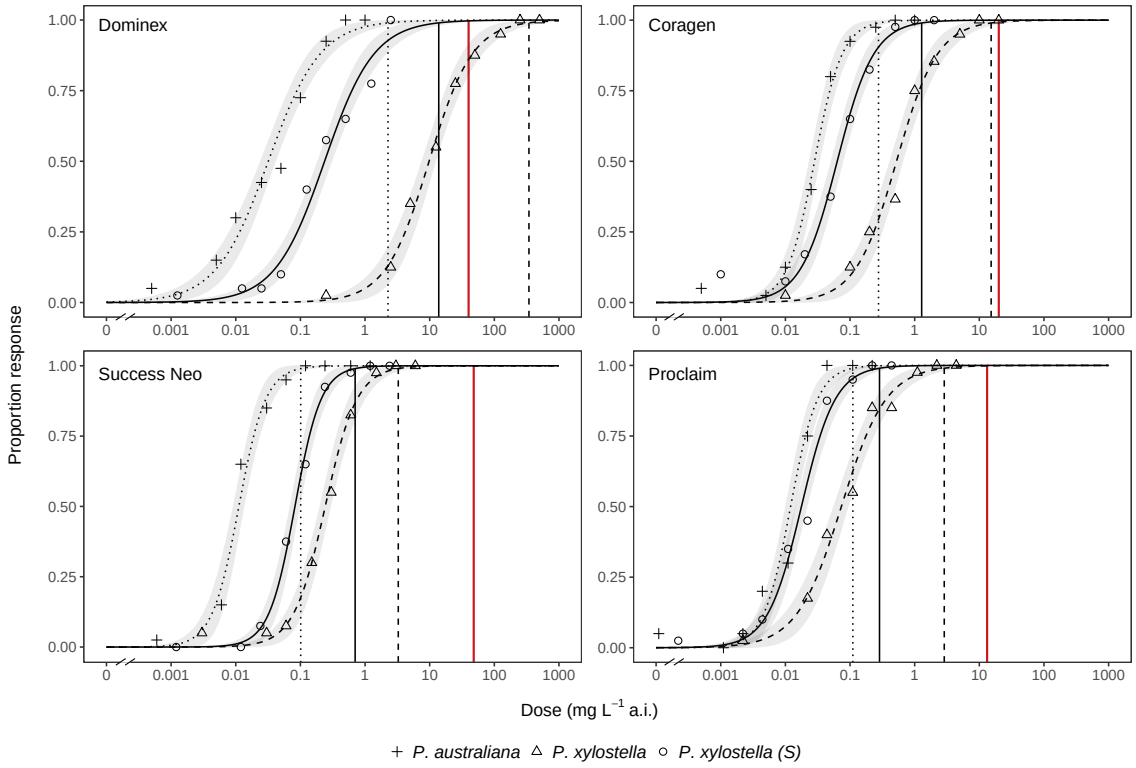

Fig. 3.6 Insecticide bioassay dose-response curves for *P. australiana* (dotted line) and *P. xylostella* (dashed line) field strains collected from Angle Vale and Urrbrae, South Australia, and a susceptible *P. xylostella* (S) reference strain (solid line), exposed to four commercial insecticides: Dominex, Coragen, Proclaim and Success Neo. Points are the mean observed response across 4 bioassay replicates of 10 larvae each and lines are the fitted log-logistic response curves with 95 % confidence intervals shown in grey shading. The vertical red line represents the approximate commercial field dose for each insecticide and vertical black lines represent the estimated LC_{99} for the corresponding *Plutella* strain.

views that Australian *P. xylostella* were recently introduced from a small ancestral source population, possibly from Asia [202, 52, 118]. Therefore, the two *Plutella* species may have diverged in allopatry and recently come into secondary contact. Maintenance of divergence suggests strong continuing reproductive isolation, which can evolve as a side-effect of allopatric divergence [232]. All 99 individuals that were RAD sequenced showed concordance in nuclear multilocus genotypes and mtDNA genotypes identified through PCR-RFLP regardless of geographic location, as shown by STRUCTURE analysis. Cryptic species in sympatry provides strong evidence of limited genetic exchange [25]. A small degree of genotypic admixture evident for a few individuals in the STRUCTURE plots might be explained by ancestral polymorphism or introgressive hybridization [97], or alternatively, could be an artefact if our dataset is not representative of the entire genetic background [61]. The level of hybridization that may be occurring between these species is unknown. Isolation may not be uniform across the genome [93, 94], and scans of larger genomic regions may be required to identify introgression and detect hybrids.

The factors leading to reproductive isolation between the two *Plutella* species in nature are unknown but could include a range of pre- or post-mating isolation mechanisms, such as assortive mating or hybrid fitness costs. Behavioural mating choices are often the main isolating factor in sympatric animals [143]. Does *Wolbachia* cause a reproductive barrier? The contrast in infection status creates the potential for cytoplasmic incompatibility between species [109]. Interspecific crosses showed a pattern of asymmetric isolation consistent with the expected effects of unidirectional CI, where 21 % crosses involving infected *P. australiana* females produced viable offspring, while the reciprocal CI-cross direction (uninfected *P. xylostella* females crossed with infected *P. australiana* males) was nearly sterile. However, this pattern was not continued in the F1 generation: infected hybrid males (derived from the *P. australiana* maternal line) produced offspring at comparable rates when back-crossed to either uninfected *P. xylostella* or infected *P. australiana* female parents. The role of *Wolbachia*-induced postzygotic isolation between the two *Plutella* species requires further study, though our results suggest it could be more important in the F0 generation. *Wolbachia* can contribute to post-zygotic genetic isolation after speciation by complementing hybrid incompatibilities [109, 79]. Symbiont infections could also influence mating behaviour and contribute to pre-mating isolation [212].

Conclusions

The discovery of cryptic pest species introduces complexities for their management and also exciting opportunities for understanding ecological traits. We found strong genomic

and phenotypic divergence in two cryptic mitochondrial *Plutella* lineages co-existing in nature, supporting their status as distinct species [131] despite the capacity to hybridize. Reproductive isolation is likely to have evolved during allopatric speciation, and genome-wide sequence data suggest it has been maintained following secondary contact. Variation in *Wolbachia* infections might be one factor reinforcing reproductive barriers.

Plutella australiana co-occurs with *P. xylostella* throughout agricultural regions of southern Australia, but made up only 10 % of *Plutella* juveniles collected from cultivated and wild brassicaceous plants. A lack of population structure across neutral SNP markers suggests that *P. australiana* populations are linked by high levels of gene flow, and also that *P. australiana* is a highly mobile species, which is supported by light trap collections [131] and seasonal colonization of canola crops. Future molecular analysis of Australian *Plutella* should include a species identification step using a molecular diagnostic assay. For ecological studies, it may be possible to perform molecular species identification to confidently distinguish a representative sub-sample of individuals or pooled samples. Our study has shown that while *P. australiana* can attack canola crops, there is no evidence of pest status in commercial *Brassica* vegetables crops, and bioassays suggested that field populations should be easily controlled with insecticides. Though *P. australiana* is a potential pest of some Australian *Brassica* crops, it is of secondary importance to the diamondback moth, *P. xylostella*.

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Availability of data and materials

The DNA sequence data are available from GenBank under the accessions MF804301-MF804314 (*Wolbachia wsp*) and MF151826-MF151906 (mitochondrial COI). The raw RAD-seq fastq files will be submitted to the NCBI Sequence Read Archive.

Authors' contributions

All authors conceived and designed experiments. KDP collected *Plutella* samples, and KDP and SWB performed RAD-seq, COI genotyping, data analysis and wrote the manuscript. SWB and CMW genotyped *Wolbachia* and sequenced *wsp*. KJP, JKK and GJB cultured *Plutella* strains and performed insecticide bioassays and crossing experiments. All authors read and approved the final manuscript.

Competing interests

The authors declare that they have no competing interests.

3.5 Addendum

This section contains explanatory commentary in response to the following points raised by Examiner 1 during thesis examination with respect to Chapter 3.

Examiner comments

Sampling methods and subsequent rearing

In the methods, it is reported that at least 25 individuals from each location were collected, but the actual number collected from each location appears not to have been recorded. The collected insects were then reared on the host plant on which they were collected for 2 days before being transferred onto cabbage to continue development. This raises two concerns:
1. Without some measure of relative abundance of immature insects collected at the different sites, how is it possible to use genotyping of what appears to be only a fraction of the individuals collected at a site (see Table 3.1; at 60 of the 75 sites < 25 individuals were genotyped) to make statements about the relative abundance of the two *Plutella* species overall? In the results, statements about relative abundance are made based on the number of individuals of each species identified based on those insects that were subject to genotyping. Unless the number of individuals genotyped for a site represents the abundance of insects collected at that site (nowhere in the methods is this suggested) then the overall assessment of relative abundance of the two species in the field is not valid. Further explanation of the relationship between number genotyped and estimated relative abundance at a site is required. Presumably numbers < 25 represent post-collection mortality (see below).
2. Was there an attempt to investigate if switching insects from the host plants that they were collected on in the field to cabbage to complete development in the laboratory had an effect on larval mortality? It is well known that *P. xylostella* survival and development is affected by host plants [e.g. 83] and that switching host plants during pre-imaginal development can be detrimental to survival. Further, differences in response to the rearing regime (e.g. greater mortality of one species) between *P. australiana* and *P. xylostella* could bias estimates of the incidence of each species in the original samples.

Laboratory cultures and insecticide bioassays

1. Why were different host plants used for rearing the different *Plutella* species? It would have made sense to rear both on the same host - maybe canola? This would have facilitated running the insecticide bioassays on the same host plants, which would have allowed for better relative comparisons of the toxicities of the different insecticides to be made.

2. Why was canola used for *P. australiana* assays and cabbage for the *P. xylostella* assays? The latter has waxy surfaces and aqueous insecticide applications often run off or collect in large droplets, leading to reduced retention of the insecticide and uneven distributions on the leaf surface respectively. How can you be sure that such phenomena did not contribute to the different responses to the different insecticides that you report? Further, no surfactant or "sticker" was used to aid leaf coverage. Use of a common host would have removed these problems with experimental design (further there is also evidence that host plant identity can affect susceptibility to insecticides even if acquired dose is controlled for). This requires explanation/ justification and should be considered when interpreting the results.

Explanatory comments

Sampling methods and subsequent rearing

It was not possible within project resources to genotype all individuals collected, hence sub-sampling was required. A random sub-sample of immatures could have been preserved immediately after collection from each location for subsequent genotyping, thereby avoiding any biases introduced by rearing. However, to avoid costly DNA extraction (and downstream library preparation and sequencing) of parasitised individuals, which would later need to be excluded from the analysis due to contamination with parasitoid DNA (parasitoids would also soak up sequence reads), samples were reared to the pupal stage when parasitised individuals could be readily identified and removed. Maximising the efficiency of genotyping effort increased the number and geographic spread of samples that could be screened, allowing a wider assessment of the distribution and host plant associations of *P. australiana*, which were the main aims of this part of the study.

Plutella sample collection and rearing was performed before any *P. australiana* host plants were known – it was this genotyping study that discovered the host plant associations of *P. australiana* and subsequently led to an ability to successfully culture it. Because three *P. australiana* individuals had earlier been discovered on a wild brassicaceous species, *Diplotaxis tenuifolia* (Chapter 2), it was assumed *Brassica* species may be suitable hosts and cabbage was provided as supplementary food for insects to complete development to the pupal stage.

The number of individuals collected at each location was not recorded, as counting immatures among foliage and debris in sample containers was prohibitively time-consuming given the large number of samples collected in 2014 and 2015. It was estimated that ≈25–60 immatures were collected at most sites, though several population samples had fewer and several had many hundreds of individuals. Most population samples were received in the post

after several days developing in the collection container on leaves of the original host plant. Samples consisted mostly of mid–late stage larvae and pupae by the time the samples were received. After 1-2 days additional rearing on the original host material, by which time leaves were drying out, freshly cut 6–8 week old cabbage leaves were added to rearing containers as required without (to avoid handling damage) physically handling or transferring larvae. Each sample was further reared for up to ≈one week if necessary until sufficient numbers of pupae (≥25–40 non-parasitised pupae where possible) were obtained for preservation. This process likely selected for older immature stages among the cohort, which are less likely than younger stages to be subject to species differences in survivorship on different host plants [e.g. 107].

Post-collection mortality did occur in most samples due to parasitism by *Diadegma* spp. and *Ippeus appanteles*. The incidence of parasitised pupae often exceeded 10 %, sometimes exceeded 50 % and reached 100 % in one sample collected from *Brassica* forage crops, resulting in many populations with < 25 non-parasitised individuals. The possibility that *Plutella* species may be subject to different rates of parasitism could also introduce bias. A recent laboratory study showed that *D. semiclausum* readily parasitised *P. australiana* in no-choice and choice situations at comparable rates to *P. xylostella* [19], but rates of parasitism of *P. australiana* by other parasitoid species remain to be investigated. In future, finding ways to remove parasitised individuals at younger stages, such as by dissecting larvae, could reduce the need for rearing.

The genotyped samples represent a random sample of male and female individuals that successfully developed to the pupal stage. Were there differences in larval survival between *Plutella* species reared on cabbage? *Plutella australiana* immatures were able to complete development to the pupal stage as shown by its presence among genotyped individuals from 20 locations (Tables 3.1, 3.2). Because many immatures were at later developmental stages prior to rearing, it is likely that minimal (or no) feeding on cabbage was required for most individuals to reach pupal stage, and that any feeding on cabbage at a late developmental stage led to minimal difference in survivorship [e.g. 107]. Laboratory work has shown that *P. australiana* can successfully develop from egg to adult exclusively on a diet of cabbage leaves [19].

We might expect that greater mortality of *P. australiana* than *P. xylostella* could occur through rearing on cabbage given our observations (see Discussion, section 3.4). Among genotyped samples, we found a 9-fold lower frequency of *P. australiana* than *P. xylostella*. If this reflected reduced larval survival of *P. australiana*, we might expect to find this species at a relatively lower frequency across all sites. Yet, at some locations we recorded *P. australiana* at relatively high frequency among immature *Plutella* collected from different host plants,

including canola at Ginninderra (78 %) and Goulburn (79 %), wild turnip at Gilgandra (91 %) and African mustard at Cunnamulla (100 %). Furthermore, we detected a relatively high frequency of *P. australiana* among *Plutella* larvae collected from eastern Australia, which accorded with a relatively high frequency of *P. australiana* among *Plutella* moths collected in light traps from similar areas [131].

Rearing on different host plants can influence various metrics of *P. xylostella* performance including pupal weight, fecundity, longevity, developmental time and survival [22, 107, 83, 201], as pointed out by Examiner 1. Several studies have shown reduced larval survival of *P. xylostella* reared on sub-optimal host plants [e.g. 107, 201]. However, in many cases larval survival is not significantly different between host plants tested, even where other performance metrics are reduced [e.g. 83]. In the paper cited by Examiner 1 [83], only development time and body mass of *P. xylostella* differed among cohorts reared on cultivated, feral or wild *Brassica* populations. The different plant populations "did not affect larval survival to the adult stage ($\chi^2 = 0.96$, $df = 2$, $p = 0.62$)", and greater than 70 % *P. xylostella* survival was reported across all hosts. Whether switching larvae during pre-imaginal development may have caused mortality was not assessed in our study.

A recent laboratory study compared egg-to-adult survival of both *Plutella* species reared on seven different host plant species [19]. When reared on cabbage, egg-to-adult survival was estimated at 54 % for *P. australiana* ($n = 439$ eggs) and 79 % for *P. xylostella* ($n = 1486$ eggs). Although a 25 % difference between species, this represent a worst-case scenario as potential differences in egg hatch rate were not measured. Hence, any differences in larval-to-adult survival between *Plutella* species are likely to be less than 25 %, and possibly negligible due to the late developmental stages of most reared individuals. Future detailed studies of host plant preference-performance between the two *Plutella* species will shed more light on whether our rearing and genotyping may have biased our estimates of the relative frequencies of these *Plutella* species in the field.

Laboratory cultures and insecticide bioassays

We were unable to successfully culture *P. australiana* on cabbage alone. It was found that paired male and female moths often failed to mate in the presence of cabbage plants, and choice experiments in the laboratory showed that cabbage is not a preferred oviposition host [19]. The *P. australiana* culture was successful using a wild host species, *Diplotaxis tenuifolia*, and subsequently found to be more productive on canola. The Waite Susceptible strain has been maintained on cabbage for > 25 years, hence the *P. xylostella* field strain was similarly reared on cabbage. There were insufficient project resources or laboratory space to

establish separate cultures of the field and susceptible *P. xylostella* strains on canola for the bioassays.

Did performing bioassays on different host plants for the two *Plutella* species influence the results? If differences in host suitability introduced any bias, the same issue applies if canola was used for all three *Plutella* strains. When the bioassays were performed, whether canola was an equally suitable host for the two *Plutella* species was not known, just as the relative suitability of cabbage or canola as hosts for the respective *Plutella* species are not fully understood. Recently, it was shown that *P. australiana* was 50 % less fecund, had lower pupal weights and a significantly longer development time than *P. xylostella* when both species were reared on canola [19], implying that canola is not equally suitable. Canola is the most suitable host for *P. australiana* that has yet been identified [19], and it is standard practice to perform bioassays on a suitable host plant substrate. Control mortality was low across all experiments (0–4 %) and did not differ between strains or bioassays, suggesting that host substrate did not affect comparisons between species.

Difference in leaf surface properties had no influence on the dose the insects were exposed to given the bioassay method used. The Potter Tower vertically applied a known aliquot of insecticide spray directly onto each Petri dish containing a horizontal leaf disc. The chemical was allowed to dry, leaving (unlike leaf-dip bioassay methods) a quantified residue of active ingredient, and the insects were allowed to move freely on the treated leaf for 48 or 72 hours exposure time. The bioassay results clearly demonstrated major differences in insecticide susceptibility between these *Plutella* species and that *P. australiana* can be easily controlled with insecticide.

Statement of Authorship - Chapter 4

Principal Author

Title of Paper:	Australian populations of the diamondback moth, *Plutella xylostella* L., are genetically homogeneous across neutral SNPs
Publication Status:	Unpublished and Unsubmitted work written in a manuscript style.
Publication Details:	Perry KD, Keller MA, Baxter SW. Australian populations of the diamondback moth, *Plutella xylostella* L., are genetically homogeneous across neutral SNPs. Unpublished and Unsubmitted.
Name of Principle Author (Candidate):	Kym D Perry
Contribution to the Paper:	Organised collection of *Plutella* samples across Australia, performed RAD-seq and bioinformatics analysis, wrote the manuscript.
Overall percentage (%):	90 %

Signature: _____ Date: _____

Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

i the candidate's stated contribution to the publication is accurate (as detailed above);
ii permission is granted for the candidate to include the publication in the thesis; and
iii the sum of all co-author contributions is equal to 100 % less the candidate's stated contribution.

Name of Co-Author:	Michael A Keller
Contribution to the Paper:	Contributed to experimental design and editing the manuscript.

Signature: _____ Date: _____

Name of Co-Author:	Simon W Baxter
Contribution to the Paper:	Designed RAD-seq primers and library pooling strategy, edited the manuscript.

Signature: _____ Date: _____

Chapter 4

Australian populations of the diamondback moth, *Plutella xylostella* L., are genetically homogeneous across neutral SNPs

Abstract

Estimating population structure in insect pests can provide valuable insights into patterns of gene flow and dispersal in agricultural systems, with important consequences for management. The diamondback moth, *Plutella xylostella* L., threatens *Brassica* vegetable and canola crops throughout the world owing to its high dispersal ability and propensity to evolve insecticide resistance. In southern Australia, *P. xylostella* seasonally colonises winter canola crops leading to periodic outbreaks but movement patterns among *Brassica* host types and the origins of seasonal populations are uncertain. A microsatellite study using six loci suggested that Australian *P. xylostella* may form a panmictic population, but resolving movement patterns requires greater resolution which may be available using massively parallel sequencing methods. Here, we re-examined population genetic structure in Australian *P. xylostella* using genome-wide SNPs. A total of 59 populations were collected from canola crops, *Brassica* vegetable and forage crops and wild brassicaceous plants throughout southern Australia in two consecutive years and 833 individuals were genotyped using RAD-seq. Despite a geographic sampling scale >3500 km and high statistical power in 1032 SNP markers, we found no genetic differentiation among any *P. xylostella* populations regardless of location, host plant species or sampling year, and no evidence for isolation by distance.

Hierarchical STRUCTURE analysis at $K = 2 - 5$ showed nearly uniform ancestry across the major genotypic cluster in most samples for 2014 and 2015. A small number of divergent samples were associated with specific geographic locations and may reflect an artefact of sampling related individuals. Given low genetic diversity in Australian *P. xylostella*, neutral genetic homogeneity may reflect either high gene flow or recent ancestry, alternative hypotheses which could not be distinguished from our data. Our data support existing views that neutral genetic markers cannot distinguish the movements of *P. xylostella* in Australia, but methods employed here may be more informative in *P. xylostella* from other regions with higher genetic diversity.

4.1 Introduction

Highly mobile insect pests have the capacity to exploit ephemeral host resources and colonise annual crops [253, 148, 63]. They are difficult pests to manage due to the unpredictable nature of seasonal outbreaks, particularly when populations carry insecticide-resistant genotypes. Movement in agricultural systems affects seasonal dynamics but also leads to gene flow, with consequences for local adaptation [30, 167] and the spread of insecticide resistance [66]. Molecular studies of population structure can provide insights into patterns of gene flow and dispersal of insect pests in agricultural landscapes [66, 57].

The diamondback moth, *Plutella xylostella* L., is the most widespread and destructive pest of brassicaceous crops worldwide [257, 134]. It attacks *Brassica* vegetable crops throughout tropical and temperate regions [228], but more recently has become a sporadic pest of canola crops grown over wide areas in temperate regions [153, 55, 56, 77, 78]. Insecticides remain the main form of control, but the propensity of *P. xylostella* to rapidly evolve insecticide resistance and a lack of alternative control options has led it to evolve resistance to most pesticides [78]. In Australia, *P. xylostella* has been the key pest in *Brassica* vegetable crops since the late 1800s [65] and canola crops since expanded production in the 1990s [77]. Canola crops are grown over 3 million hectares annually [1], providing a vast resources for *P. xylostella* during winter. Sporadic outbreaks in Australian canola crops during spring cause substantial crop losses [89, 77]. In *Brassica* vegetables, limited movement [152] and frequent insecticide use creates high selection pressure for *P. xylotella* to evolve insecticide resistance [66, 15]. In canola-growing areas, the seasonal availability of host plants forces *P. xylostella* to regularly move among crops and weeds, which can homogenise insecticide resistance [17]. The sources of populations that seasonally colonise canola need to be identified to improve capacity to forecast outbreak risk and manage insecticide resistance at appropriate scales [65, 77, 134].

A variety of molecular markers have been used to investigate population structure in *P. xylostella*, including allozymes, ISSRs, microsatellites and mtDNA. Populations from different continents are clearly differentiated [65, 174, 118]. At smaller scales, a lack of genetic differentiation among *P. xylostella* populations within parts of Asia [125, 243, 250], the USA [34, 37] and Australasia [65, 202] suggest that regional populations frequently intermix. Because migration homogenises allele frequencies, knowledge of movement patterns has been elusive. Many population genetic studies in this species have been inconclusive due to limited sampling [reviewed in 64], and few studies have sampled at a sufficient scale or resolution to investigate movement at the landscape scale. However, successful studies have recently emerged in China. Wei et al. [243] found that 27 geographically distinct *P. xylostella* populations across China were not differentiated across nine microsatellites, but the geographic distribution of mtDNA haplotypes revealed seasonal migrations into northern China, since corroborated by Yang et al. [250] and Fu et al. [76].

In Australia, Endersby et al. [65], using six microsatellites, found no differentiation among 17 populations from across Australia and one from New Zealand, despite sampling $>$5000 km. The Australian and New Zealand populations were clearly differentiated from samples collected in Asia and Africa. However, Australian *P. xylostella* has low genetic diversity and may have been recently introduced [65, 202, 52, 169]. Levels of gene flow remain to be resolved, because genetic homogeneity across neutral markers could reflect recent ancestry [77] and because the statistical power of six microsatellites to detect weak structure is uncertain. The recent discovery of a cryptic species, *Plutella australiana* [131, 169], may explain some inconsistent patterns of population structure identified in previous studies [174, 196].

Over the past decade, a revolution in massively parallel sequencing (MPS) technologies [84] and associated genotyping methods has facilitated genome-wide genetic marker sets and brought unprecedented resolution to questions of population structure [50, 156]. Restriction-site-associated DNA sequencing (RAD-seq) [13] enables sequencing of targeted short regions across the genome, allowing simultaneous SNP discovery and genotyping in model and non-model species [21, 4]. The ability to sequence orthologous regions across multiple individuals at high sequencing coverage makes it possible to confidently genotype single nucleotide polymorphisms (SNPs) and generate high density markers for population genetic studies [49, 156]. Microsatellites remain popular for population genetic studies due to high polymorphism [177], but can be outperformed by large SNP panels in resolving population structure [90, 240], including in insects [181, 182]. RAD-seq genotypes thousands of SNPs in *P. xylostella* [171] and has resolved species-level nuclear divergence among cryptic

Australian *Plutella* species [169], suggesting potential to provide new insights into the movement patterns of Australian *P. xylostella*.

Here, we re-examined whether geographic, host plant-related or temporal population genetic structure exists among Australian *P. xylostella*. *Plutella* was collected from canola crops, *Brassica* vegetable crops, *Brassica* forage crops and wild brassicaceous species throughout southern Australia, and in two consecutive years to facilitate temporal comparisons. After molecular species identification, *P. xylostella* individuals were genotyped across genome-wide sites using RAD sequencing. Given the vast size of the Australian continent, we hypothesised that detectable levels of genetic structure exists.

4.2 Methods

Sample collection

Plutella larvae (rarely, eggs or pupae) were collected from canola crops, *Brassica* vegetable crops, *Brassica* forage crops and wild brassicaceous plants throughout Australia between March 2014 and December 2015. The wild species *Plutella* were collected from included wild radish, *Raphanus raphanistrum*, turnip weed, *Rapistrum rugosum*, sea rocket, *Cakile maritima*, Ward's weed, *Carrichtera annua*, and mixed stands of sand rocket, *Diplotaxis tenuifolia* and wall rocket, *D. muralis*. At each location, at least 25 individuals were collected from randomly-selected plants to achieve a representative sample. Samples were collected from *Brassica* vegetables by hand, from sea rocket by beating plants over a collection tray, and from other hosts using a sweep net. Each population sample was separately reared in ventilated plastic containers on leaves of the original host material for 1-2 days and thereafter on cabbage leaves. Non-parasitised pupae or late-instar larvae were fresh frozen at $-80\,^{\circ}\mathrm{C}$. A subset of locations was sampled in both years to allow temporal comparisons.

DNA isolation and species identification

For each population, 16 individuals were sequenced where possible after removing parasitised individuals. To avoid biases due to sex-linked markers [23], we visually determined the sex of individual pupae (but not larvae) by examining external genital morphology [188] under a dissecting microscope, then male and female individuals were selected to achieve a balanced sex ratio within each population where possible. Genomic DNA was isolated by homogenising whole individuals using a TissueLyser II (Qiagen) followed by two phenol and one chloroform extractions according to Zraket et al. [261]. DNA was treated with RNase A, then precipitated and re-suspended in TE buffer. To distinguish *P. xylostella* from the cryptic

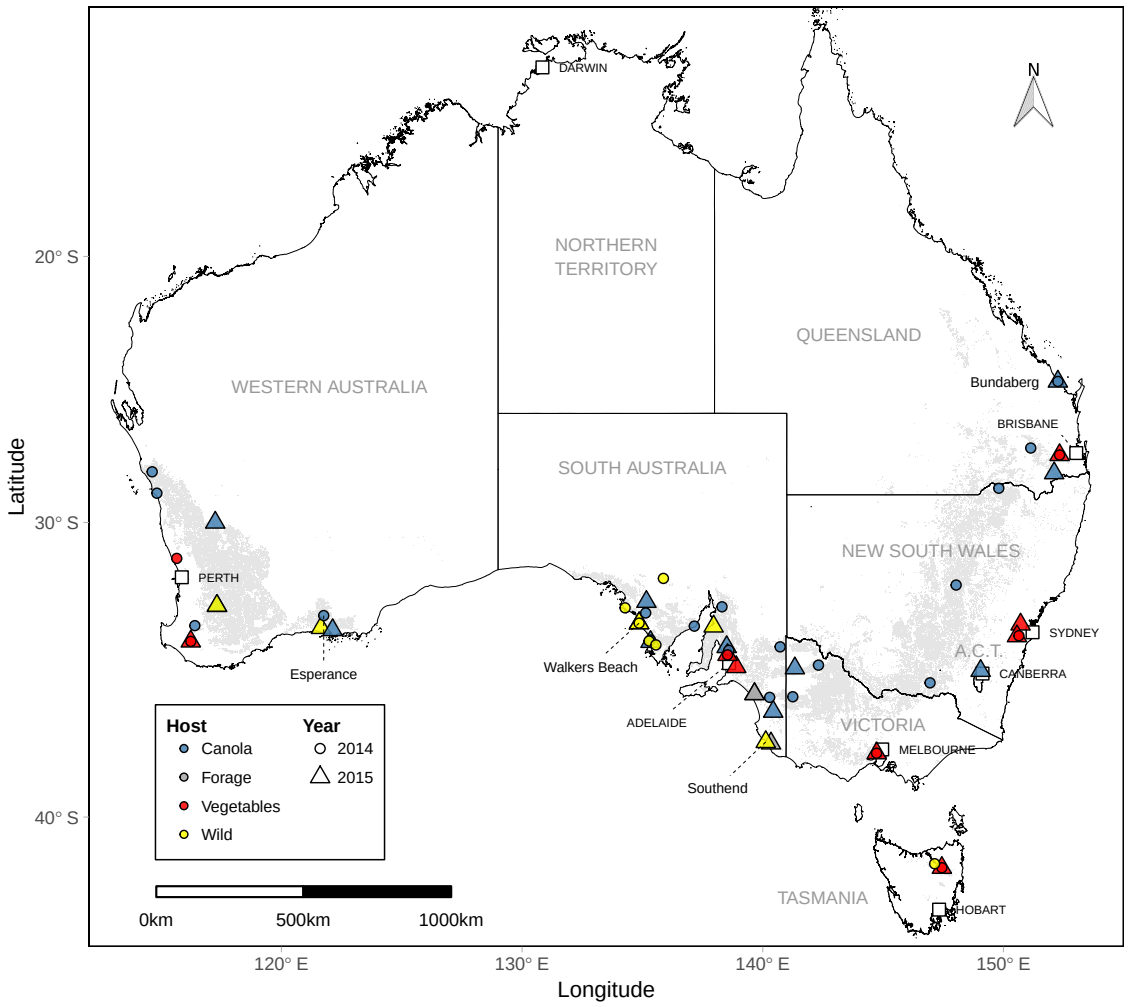

Fig. 4.1 Geographic locations of 59 *P. xylostella* populations collected in Australia in 2014 and 2015 and sequenced using RAD-seq. Collections from different *Brassica* host types are represented by different colours.

species, *P. australiana*, species identification was performed using a PCR-RFLP assay [169] and *P. xylostella* were retained for analysis.

RAD-seq library preparation and sequencing

Libraries were prepared for restriction-site-associated DNA sequencing (RAD-seq) according to a protocol modified from Baird et al. [13]. Genomic DNA was quantified using a Qubit 2.0 fluorometer (Invitrogen) and 200 ng digested with 10 units of high fidelity *Sbf1* in Cutsmart buffer (NEB) for 1 hour at 37 °C, then heat inactivated at 80 °C for 20 minutes. One microlitre of P1 adapter (100 nM) with a 6-base molecular identifier (MID) (top strand 5′-TCGTCGGC AGCGTCAGATGTGTATAAGAGACAGxxxxxxTGCA-3′, bottom strand 5′-[P]xxxxxxCT GTCTCTTATACACATCTGACGCTGCCGACGA-3′, x represents sites for MIDs) were then added using 0.5 µL T4 DNA ligase (Promega), 1 nM ATP and Cutsmart buffer. Sixteen individuals with unique P1 adapters were pooled per library. To minimise sequencing biases or batch effects, individuals from each population were randomised across 2-4 (usually 4) libraries and each library was sequenced across 2-4 sequencing lanes. Library pools were sheared using a Bioruptor sonicator (Diagenode), ends repaired using a Quick Blunting Kit (NEB), adenine overhangs added then P2 adapters (top strand 5′-[P]CTGTCTCTTATACAC ATCTCCAGAATAG-3′, bottom strand 5′-GTCTCGTGGGCTCGGAGATGTGTATAAGA GACAGT-3′) ligated, then (majority of libraries) size-selected (300-700 bp) on agarose gel to remove primer dimer. DNA purification between steps was performed using a MinElute PCR purification kit (Qiagen). Final library amplification was performed using KAPA HiFi Hotstart Readymix (Kapa Biosystems) and Nextera i7 and i5 indexed primers with PCR conditions: 95 °C for 3 minutes, two cycles of 98 °C for 20 seconds, 54 °C for 15 seconds, 72 °C for 1 minute, then 15 cycles of 98 °C for 20 seconds, 65 °C for 15 seconds, 72 °C for 1 minute followed by a final extension of 72 °C for 5 minutes. Libraries were size-selected (300-700 bp) on agarose gel and purified using a minElute Gel Extraction Kit (Qiagen). Illumina paired-end sequencing was performed across seven lanes using HiSeq2500 (100 bp) or NextSeq500 (75 bp) at the Australian Genome Research Facility (AGRF). An additional 16 individuals were included from a separate sequencing run across two lanes of Illumina HiSeq2500 at the Australian Cancer Research Facility (ACRF, Adelaide).

Read filtering and variant calling

Sequence read quality was examined using FastQC [5]. As Nextseq reads had low quality base calls within restriction sites (a common problem when using fixed-length MIDs on this platform, which cause low sequence diversity and cluster signal in this region), we

Table 4.1 Summary of *P. xylostella* collections from Australia.

Location[1]	Collection date	Coordinates	Host plant	No. sequenced[2]		
				Total	♂	♀
Boomi NSW	Sep-2014	28.76°S 149.81°E	Canola	10	7	3
Ginninderra NSW	Oct-2015	35.19°S 149.05°E	Canola	14	6	8
Henty NSW	Oct-2014	35.60°S 146.95°E	Canola	16	9	7
Narromine NSW	Sep-2014	32.22°S 148.03°E	Canola	16	7	3
Richmond NSW	Oct-2015	33.60°S 150.71°E	Cabbage	16	7	9
Werombi NSW	Nov-2014	33.99°S 150.64°E	Vegetables	10	5	4
Werombi NSW	Oct-2015	34.00°S 150.56°E	Kale	9	4	5
Bundaberg QLD	Oct-2014	24.80°S 152.26°E	Canola	12	7	3
Bundaberg QLD	Sep-2015	24.80°S 152.26°E	Canola	16	5	10
Dalby QLD	Sep-2014	27.28°S 151.13°E	Canola	14	6	8
Gatton QLD	Oct-2014	27.54°S 152.33°E	Broccoli	14	6	5
Gatton QLD	Nov-2015	27.54°S 152.33°E	Broccoli	14	7	7
Warwick QLD	Oct-2015	28.21°S 152.11°E	Canola	14	8	6
Calca SA	Apr-2014	33.02°S 134.28°E	Lincoln weed and Dog weed	9	4	1
Cocata SA	Sep-2014	33.20°S 135.13°E	Canola	16	4	7
Colebatch SA	Feb-2015	35.97°S 139.66°E	Brassica forage	12	4	5
Cowell SA	Sep-2014	33.66°S 137.16°E	Canola	16	6	0
Keith SA	Oct-2014	36.09°S 140.29°E	Canola	12	5	6
Littlehampton SA	Sep-2015	35.06°S 138.90°E	Brussels sprouts	9	3	3
Loxton SA	Sep-2014	34.37°S 140.72°E	Canola	16	8	8
Mallala SA	Sep-2015	34.38°S 138.50°E	Canola	15	9	6
Millicent SA	Apr-2015	37.61°S 140.34°E	Canola	9	2	0
Minnipa SA	Oct-2015	32.81°S 135.16°E	Canola	16	8	6
Moonaree SA	Aug-2014	31.99°S 135.87°E	Ward's weed	16	0	0
Mt Hope SA	Sep-2014	34.14°S 135.33°E	Canola	16	7	6
Mt Hope SA	Sep-2015	34.20°S 135.34°E	Canola	16	7	9
Padthaway SA	Oct-2015	36.56°S 140.43°E	Canola	14	9	5
Picnic Beach SA	Apr-2014	34.17°S 135.27°E	Sea rocket	8	0	2
Redbanks SA	Oct-2014	34.49°S 138.59°E	Canola	15	3	6
Southend SA	Apr-2015	37.57°S 140.12°E	Sea rocket	16	8	8
Tintinara SA	Oct-2015	35.97°S 139.66°E	Brassica forage	16	8	8
Virginia SA	Oct-2014	34.64°S 138.54°E	Broccoli	16	4	1
Virginia SA	Sep-2015	34.64°S 138.54°E	Cabbage	16	10	5
Walkers Beach SA	Sep-2014	33.55°S 134.86°E	Sea rocket	16	7	6
Walkers Beach SA	Mar-2015	33.55°S 134.86°E	Sea rocket	16	8	8
Walkers Beach SA	Sep-2015	33.55°S 134.86°E	Sea rocket	12	6	6
Wirrabara SA	Oct-2014	32.99°S 138.31°E	Canola	15	5	3
Wokurna SA	Sep-2015	33.67°S 137.96°E	Wild radish	16	9	4
Wurramunda SA	Apr-2014	34.30°S 135.56°E	Volunteer canola	16	9	7
Deddington TAS	Nov-2014	41.59°S 147.44°E	Kale	12	6	6
Launceston TAS	Nov-2014	41.47°S 147.14°E	Wild mustard	16	9	7
Newstead TAS	Nov-2015	41.59°S 147.44°E	Cauliflower	16	5	7
Cowangie VIC	Oct-2015	35.10°S 141.33°E	Canola	15	7	5
Ouyen VIC	Sep-2014	35.00°S 142.31°E	Canola	15	9	5
Werribee VIC	Oct-2014	37.94°S 144.73°E	Cauliflower	16	2	3
Werribee VIC	Nov-2015	37.94°S 144.73°E	Cauliflower	13	7	6
Yanac VIC	Sep-2014	36.06°S 141.25°E	Canola	12	6	6
Boyup Brook WA	Sep-2014	33.64°S 116.40°E	Canola	15	5	3
Dalyup WA	Oct-2015	33.72°S 121.64°E	Wild radish	16	9	7
Esperance WA	Sep-2014	33.29°S 121.76°E	Canola	12	2	1
Esperance WA	Oct-2015	33.79°S 122.13°E	Canola	15	7	8
Gingin WA	Dec-2014	31.28°S 115.65°E	Red cabbage	16	10	6
Kalannie WA	Sep-2015	30.00°S 117.25°E	Canola	16	8	8
Manjimup WA	Dec-2014	34.18°S 116.23°E	Chinese cabbage	9	5	4
Manjimup WA	Nov-2015	34.18°S 116.23°E	Brassica vegetables	13	3	9
Narrogin WA	Oct-2015	32.96°S 117.33°E	Canola	13	7	6
Narrogin WA	Oct-2015	32.95°S 117.32°E	Wild radish and volunteer canola	16	8	8
Northampton WA	Sep-2014	28.16°S 114.63°E	Canola	16	9	4
Walkaway WA	Sep-2014	28.94°S 114.83°E	Canola	16	3	4

[1] Australian states: NSW = New South Wales, QLD = Queensland, SA = South Australia, TAS = Tasmania, VIC = Victoria, WA = Western Australia.
[2] Total includes males, females and unknown sex

opted to remove restriction sites from all reads for downstream analysis. Sequence reads were de-multiplexed using RADtools version 1.2.4 [21] allowing one base MID mismatch, then TRIMMOMATIC v0.32 [26] was used to remove restriction sites, adapter sequences, a thymine base from reverse reads introduced by the P2 adapter, and quality filter using the ILLUMINACLIP tool with parameters: TRAILING:10 SLIDINGWINDOW:4:15 MINLEN:40. Paired reads were aligned to the *P. xylostella* reference genome (accession number: GCF_-000330985.1) using STAMPY version 1.0.21 [139] with –baq and –gatkcigarworkaround options and expected substitution rate set to 0.03 to reflect our expectations of sequence divergence from the reference strain. Duplicate reads were removed using PICARD version 1.71 [29]. Genotypes were jointly called for all individuals using the Genome Analysis Tool Kit version 3.3-0 [149, 53] HaplotypeCaller tool. We determined that base quality score recalibration using bootstrapped SNP databases was inappropriate for this dataset as it globally reduced quality scores. The variant call set was hard-filtered using VCFtools version 0.1.12a [48]. After iteratively testing multiple filtering parameter sets, we removed indels and retain confidently called bi-allelic SNPs (GQ $\geqslant$ 30) genotyped in at least 80 % of individuals with a minimum genotype depth of 5, minQ $\geqslant$ 400, average site depth of 12-100, minimum minor allele frequency of 0.01 and in Hardy-Weinberg equilibrium at an alpha level of 0.05. To avoid closely-linked sites, we retained only SNPs separated by a minimum of 2000 bp using the VCFtools –thin function. In order to estimate population-level genetic diversity, from the output of GATK HaplotypeCaller we generated a set of all confidently-called (GQ $\geqslant$ 30) variant and invariant sites and hard filtered to remove sites within repetitive regions and retain sites genotyped in at least 80 % of individuals with an average site depth of 12-100. The filtered VCF files were converted to other file formats for downstream analysis using PGDSpider version 2.1.1.2 [136] and custom R scripts [168].

Genetic diversity

The R package hierfstat [85] was used to calculate within-population gene diversity (H_S), observed heterozygosity (H_O) and the inbreeding coefficient (F_{IS}) according to Nei [160]. Population means for site depth and number of SNPs, indels and private sites were calculated using the –depth function and vcfstats module in VCFtools version 0.1.12a [48].

Population differentiation

To examine population differentiation, a global estimate of F_{ST} Weir and Cockerham [245] with bootstrapped 99 % confidence intervals (10^4 bootstrap iterations) was calculated in R package diveRsity [123]. Pairwise F_{ST} values for all population pairs were calculated

and significance of differentiation determined using exact *G* tests (10^4 MCMC burnins, 10^3 batches, 10^4 iterations per batch) in GENEPOP v4.6 [195] after correction for multiple comparisons using the Bonferroni-Holm correction method [105, 6]. Isolation by distance among populations [249] was investigated separately for 2014 and 2015 datasets. We used R to construct heat maps [180] and visually inspected the congruence between pairwise matrices of untransformed geographic distances in kilometres and genetic distances, F_{ST}, for corresponding population pairs. Significance of the regressions of pairwise linearized genetic distances [218] onto log-transformed geographic distances was determined using a Mantel test with 10^4 permutations in R package ade4 version 1.7-6 [58]. Geographic distances were calculated using R package geosphere version 1.5-7 [101].

Analysis of molecular variance (AMOVA) was performed using the pegas implementation in R package poppr version 2.7.1 [121]. The data were analysed under two hierarchical model structures. In model A, all individuals were analysed together and populations were grouped into sampling years and *Brassica* host types. In Model B, a temporal analysis was performed for locations sampled in both 2014 and 2015, to investigate whether variance was greater among years within locations or vice versa.

Population structure

Two individual-based clustering approaches were used to investigate population structure. First, Bayesian clustering was implemented in the program STRUCTURE version 2.3.4 [176]. Variant data were converted from VCF to STRUCTURE format using PDGSpider version 2.1.1.2 [136]. For all runs, we used a burnin length of 5×10^5 followed by a run length of 10^6 MCMC iterations and performed fifteen independent runs for each *K* value, where *K* is the number of genotypic clusters, using a different random seed for each run, assuming the *locprior* model with correlated allele frequencies and λ set to 1. As preliminary runs showed that most structure was identified at low *K* values, we analysed *K*-values from 1-10 in both years. The optimal value of *K* was estimated using the delta *K* method [71] implemented in STRUCTURE HARVESTER [62] and inspection of the likelihood distribution for each model. *Q*-matrices were aligned using CLUMPP version 1.1.2 [110] and visualised using DISTRUCT version 1.1 [192]. To further explore clustering, we performed individual-based principal components analysis (PCA) separately for 2014 and 2015 datasets using R package adegenet version 2.0.1 [116, 117], using scaled and centred allele frequencies and imputing missing data by taking the mean of population allele frequencies.

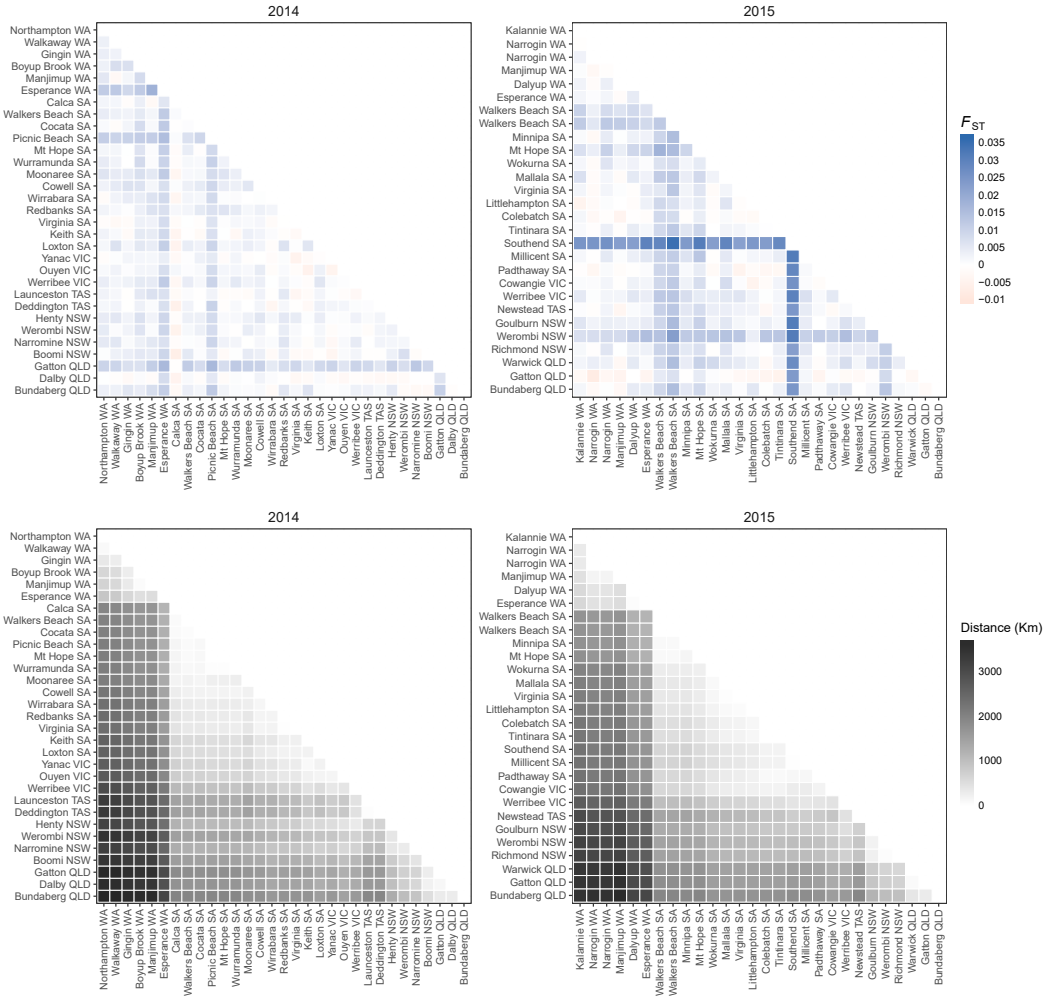

Fig. 4.2 Heat maps showing pairwise comparisons of genetic distance measured as Weir and Cockerham's (1984) F_{ST} (top panels) and geographic distance in kilometres (bottom panels) among *P. xylostella* populations collected from Australia in 2014 (left panels) and 2015 (right panels). Within each year, populations on *x* and *y*-axes are sorted geographically from north-western to north-eastern Australia in an arc following the southern coast. Visual comparison of the F_{ST} and geographic distance heat maps within each year shows no clear evidence of congruence between genetic and geographic distance among population pairs in 2014 or 2015.

Power analysis

The statistical power of the SNP marker set to detect population structure was assessing using POWSIM version 4.1 [198]. This program allows the user to test the likelihood of loci in detecting genetic differentiation for pre-defined values of F_{ST}. For the dataset, 1000 simulations were performed over a range of F_{ST} values from 0.001 to 0.01 assuming an effective population size of 5000. We used the number of subpopulations, sample sizes and allele frequencies from our data and varied the generations of drift to achieve the target F_{ST}. As POWSIM currently handles a maximum of 30 populations, for the 2014 dataset the number of subpopulations was set to this value. The null hypothesis of genetic homogeneity was tested using Fisher's exact test and a Chi-square test.

4.3 Results

Sample collection

Plutella larvae were collected from different *Brassica* host plants and locations throughout southern Australia in 2014 and 2015. After species identification using PCR-RFLP, 909 *P. xylostella* individuals from 60 locations, 32 in 2014 and 28 in 2015, were retained for analysis (Table 4.1, Figure 4.1). A total of 29 populations were collected from canola crops, 15 from *Brassica* vegetable crops, 3 from *Brassica* forage crops and 13 from brassicaceous weeds. A total of 52 populations were collected in spring and seven in autumn. Seven locations were sampled in both 2014 and 2015 to facilitate a temporal analysis, of which five locations included *Brassica* vegetable crops from the major *Brassica* vegetable production areas in each mainland Australian state (Figure 4.1). For the 82 % of individuals where sex was determined, the overall sex ratio was not different from 1 (364 males, 317 females, $\chi^2 = 3.2438$, $p = 0.0717$) and most populations had a reasonably balanced sex ratio (Table 4.1).

Read filtering and variant calling

RAD-seq was performed for 909 *P. xylostella* individuals from 60 collection locations, including 15 individuals randomly selected from different libraries and sequenced as technical duplicates to check genotypes. Illumina sequencing yielded 2.36 billion raw sequence reads after de-multiplexing. Following read trimming and filtering, mapping, genotype calling and hard-filtering, we excluded 50 individuals with greater than 60 % missing data, which was largely due to low sequencing depth (Supplementary Figure C.1), then excluded the 15 technical duplicates and a population with only two individuals remaining. Nine individuals

with unusually high levels of polypmorphism and investigated using mtDNA amplicon sequencing were found to be contaminated and were excluded. Genotyping and hard-filtering steps were then repeated for the remaining 833 individuals across 59 population samples, including 434 individuals from 31 populations collected in 2014 and 399 individuals from 28 populations collected in 2015. Hard-filtering retained 590 086 confidently-called (GQ $\geqslant 30$) variant and invariant sites at a mean depth of 33.4 per individual, and a subset of 1032 widely-dispersed (to avoid linkage bias) bi-allelic SNP variants at a mean depth of 34.0 per individual, for downstream analyses. In reference-aligned SNP datasets with read depth > 30, genotyping error rates are expected to be < 0.01 [74]. The datasets for 2014 and 2015 were analysed separately.

For the 15 technical duplicates, the VCF output from HaplotypeCaller was hard-filtered using our parameters to retain the 30 samples and a set of 1473 widely-dispersed bi-allelic SNP variants. Principal components analysis showed that sample pairs group closely together, indicating that genotype calls are highly consistent (Supplementary Figure C.2).

Genetic diversity statistics

Population-level genetic diversity was estimated using the set of 590 086 variant and invariant sites. The mean observed heterozygosity per population averaged 0.0092 ± 0.0002 SD (range $= 0.00880$, 0.0097) across the 59 populations and showed little variation when averaged across populations collected from different years (2014, $n = 31$ and 2015, $n = 28$), host plant types (canola, $n = 29$, *Brassica* vegetable crops, $n = 15$, *Brassica* forage crops, $n = 3$, and wild brassicas, $n = 15$) or seasons (autumn, $n = 7$ or spring, $n = 52$). In general, observed heterozygosity was lower than expected as shown by mostly positive F_{IS} values, suggesting some inbreeding (Tables 4.2, 4.3, Supplementary Figure C.3). The population from Southend 2015 had reduced gene diversity and fewer private sites relative to other populations. Across the 1032 SNPs, observed heterozygosity and genetic diversity within each year showed reasonable agreement (Tables 4.2, 4.3, Supplementary Figure C.3), indicating allele frequencies at these loci are in Hardy-Weinberg proportions. Again, for this marker set, the Southend 2015 population had the lowest genetic diversity among populations, contributing to a negative F_{IS} value.

Power analysis

The power analysis indicated that our SNP marker loci had a high level of statistical power to detect even weak population structure. The 1032 SNP loci had 100 % probability of

Table 4.2 Population statistics for all 590 086 confidently-called variant and invariant sites, and a subset of 1032 hard-filtered SNP loci, for 31 *P. xylostella* populations collected from Australia in 2014.

	All variant and invariant sites									1032 SNP variants				
Population	N	Sites	Site depth	SNPs	Indels	Private sites	H_O	H_S	F_{IS}	N	Site depth	H_O	H_S	F_{IS}
Boomi NSW	9.5	562586	38	8590	1653	16	0.0090	0.0095	0.0398	9.3	38	0.2096	0.2057	−0.0204
Henty NSW	15.3	564870	33	8418	1618	14	0.0092	0.0096	0.0496	14.5	33	0.2042	0.2052	0.0048
Narromine NSW	15.0	553119	30	8216	1558	18	0.0093	0.0097	0.0382	13.8	31	0.2077	0.2055	−0.0081
Werombi NSW	9.3	550438	26	8086	1518	16	0.0095	0.0097	0.0179	8.4	28	0.2120	0.2074	−0.0244
Bundaberg QLD	11.3	557174	38	8338	1578	16	0.0091	0.0096	0.0451	10.7	38	0.2050	0.2030	−0.0105
Dalby QLD	13.5	567483	36	8495	1630	16	0.0093	0.0096	0.0402	12.9	36	0.2095	0.2086	−0.0020
Gatton QLD	12.9	543911	28	7938	1491	12	0.0095	0.0096	0.0152	12.0	29	0.2160	0.2030	−0.0459
Calca SA	8.3	546958	40	8250	1588	30	0.0095	0.0099	0.0354	7.5	40	0.2208	0.2205	−0.0076
Cocata SA	15.0	553050	37	8119	1560	13	0.0093	0.0097	0.0367	13.9	37	0.2014	0.2031	0.0040
Cowell SA	15.1	557172	32	8276	1578	17	0.0094	0.0098	0.0378	13.8	32	0.2112	0.2094	−0.0077
Keith SA	10.8	532878	24	7599	1434	18	0.0097	0.0098	0.0104	9.3	26	0.2172	0.2065	−0.0385
Loxton SA	15.3	564013	42	8590	1639	22	0.0091	0.0096	0.0540	15.0	42	0.1965	0.2022	0.0182
Moonaree SA	15.2	560304	33	8354	1595	17	0.0094	0.0097	0.0385	14.0	34	0.2142	0.2082	−0.0207
Mt Hope SA	15.2	560623	37	8262	1593	14	0.0092	0.0096	0.0459	14.3	37	0.1986	0.2014	0.0067
Picnic Beach SA	7.5	550986	44	8125	1561	33	0.0097	0.0099	0.0128	6.4	44	0.2233	0.2144	−0.0400
Redbanks SA	13.2	519055	36	7591	1417	17	0.0091	0.0096	0.0536	12.9	36	0.2084	0.2056	−0.0106
Virginia SA	15.3	564927	32	8437	1620	16	0.0092	0.0097	0.0467	14.5	33	0.2087	0.2063	−0.0072
Walkers Beach SA	15.2	560602	35	8371	1599	21	0.0091	0.0097	0.0518	14.8	35	0.2002	0.2018	0.0013
Wirrabara SA	13.6	536022	38	7888	1512	13	0.0091	0.0096	0.0541	13.1	38	0.2031	0.2021	−0.0032
Wurramunda SA	15.3	565796	41	8630	1651	20	0.0091	0.0095	0.0427	15.1	41	0.2001	0.2030	0.0117
Deddington TAS	11.0	539076	25	7792	1454	17	0.0097	0.0098	0.0171	9.6	26	0.2182	0.2109	−0.0292
Launceston TAS	15.1	557084	33	8318	1602	15	0.0093	0.0097	0.0406	14.1	34	0.2110	0.2072	−0.0120
Ouyen VIC	14.2	557715	34	8246	1589	18	0.0094	0.0097	0.0350	13.2	34	0.2082	0.2061	−0.0072
Werribee VIC	15.2	560377	35	8411	1599	17	0.0092	0.0097	0.0507	14.4	35	0.2093	0.2096	0.0024
Yanac VIC	11.6	569684	39	8534	1638	17	0.0090	0.0096	0.0520	11.2	39	0.1984	0.2044	0.0168
Boyup Brook WA	14.4	566791	35	8510	1630	14	0.0092	0.0096	0.0365	13.8	35	0.2089	0.2031	−0.0219
Esperance WA	11.2	550595	33	8156	1551	30	0.0095	0.0097	0.0182	10.1	34	0.2128	0.2069	−0.0289
Gingin WA	15.2	559983	35	8353	1590	14	0.0089	0.0096	0.0710	14.8	35	0.1959	0.2014	0.0160
Manjimup WA	8.4	553540	27	8188	1551	14	0.0095	0.0096	0.0107	7.7	28	0.2061	0.2043	−0.0173
Northampton WA	15.4	568041	38	8558	1646	16	0.0090	0.0095	0.0543	15.0	38	0.1949	0.2007	0.0158
Walkaway WA	15.2	560808	38	8311	1591	16	0.0090	0.0096	0.0619	14.9	39	0.1978	0.2026	0.0207

n, number of individuals genotyped per locus; H_O, observed heterozygosity; H_S, gene diversity; F_{IS}, Nei's inbreeding coefficient.

detecting true F_{ST} values of 0.0027 or 0.0056 (Supplementary Table C.1), corresponding to the estimated global F_{ST} values for the 2014 and 2015 datasets.

Population differentiation

The global estimates of F_{ST} calculated using 1032 SNPs were not significantly different from zero in either 2014 ($F_{ST} = 0.0027$, 99 % CL −0.0043–0.0107) or 2015 ($F_{ST} = 0.0056$, 99 % CL −0.0019–0.0138), indicating a lack of genetic differentiation among populations within years. Pairwise F_{ST} values were generally very low, ranging from 0.0065 to 0.0178 (mean 0.0029 ± 0.0040 SD) in 2014 and −0.0077 to 0.0344 (mean 0.0054 ± 0.0075 SD) in 2015 (Figure 4.2, top panels). After correction for multiple comparisons (2014: $n = 465$ comparisons, 2015: $n = 365$ comparisons), no pairwise F_{ST} values were significant at the target $\alpha = 0.05$ level, indicating a lack of genetic differentiation among *P. xylostella* populations collected within a single year. The highest pairwise F_{ST} values were associated with the Southend 2015 population, ranging from 0.0221 to 0.0344 (mean 0.0265 ± 0.0035 SD, $n = 27$ comparisons), indicating allele frequencies in this population were the most

Table 4.3 Population statistics for all 590 086 confidently-called variant and invariant sites, and a subset of 1032 hard-filtered SNP loci, for 28 *P. xylostella* populations collected from Australia in 2015.

	All variant and invariant sites									1032 SNP variants				
Population	N	Sites	Site depth	SNPs	Indels	Private sites	H_O	H_S	F_{IS}	N	Site depth	H_O	H_S	F_{IS}
Goulburn NSW	13.3	559574	33	8318	1574	15	0.0090	0.0096	0.0545	12.9	34	0.1948	0.1985	0.0153
Richmond NSW	15.2	560841	37	8349	1595	14	0.0089	0.0096	0.0634	14.9	38	0.1983	0.1994	0.0005
Werombi NSW	8.5	556788	42	8473	1605	13	0.0088	0.0094	0.0438	8.3	42	0.2033	0.2008	−0.0127
Bundaberg QLD	14.5	536071	25	7777	1472	16	0.0095	0.0097	0.0371	13.3	26	0.2131	0.2065	−0.0247
Gatton QLD	12.7	533431	27	7674	1438	18	0.0095	0.0098	0.0365	11.4	28	0.2129	0.2116	−0.0019
Warwick QLD	13.2	555382	33	8328	1590	20	0.0092	0.0097	0.0461	12.3	33	0.2012	0.2020	−0.0015
Colebatch SA	11.5	565070	31	8449	1612	17	0.0091	0.0096	0.0445	11.0	32	0.2023	0.2036	0.0019
Littlehampton SA	8.4	551419	38	8463	1616	19	0.0091	0.0097	0.0495	8.2	38	0.2039	0.2068	0.0005
Mallala SA	13.9	545678	28	8012	1526	20	0.0093	0.0096	0.0350	13.1	29	0.2090	0.2060	−0.0133
Millicent SA	8.1	532566	32	8054	1539	15	0.0089	0.0096	0.0607	7.8	32	0.2020	0.2033	0.0010
Minnipa SA	15.3	563734	34	8433	1608	17	0.0091	0.0095	0.0506	14.6	34	0.2063	0.2061	−0.0004
Mt Hope SA	14.9	551211	30	8117	1542	18	0.0093	0.0097	0.0417	13.4	31	0.2164	0.2101	−0.0211
Padthaway SA	12.6	529583	30	7804	1488	16	0.0091	0.0096	0.0513	12.0	30	0.2042	0.2061	0.0005
Southend SA	15.3	563465	34	8365	1597	6	0.0093	0.0091	−0.0131	14.4	34	0.2099	0.1953	−0.0598
Tintinara SA	14.6	539118	25	7853	1499	14	0.0096	0.0097	0.0278	13.4	26	0.2093	0.2026	−0.0202
Virginia SA	15.4	567767	35	8548	1648	15	0.0092	0.0096	0.0485	14.9	35	0.2053	0.2058	−0.0023
Walkers Beach SA	15.3	564509	36	8455	1627	15	0.0091	0.0094	0.0407	14.8	36	0.1968	0.1958	−0.0067
Walkers Beach SA	11.3	557146	26	8246	1564	15	0.0095	0.0098	0.0276	10.4	27	0.2099	0.2064	−0.0137
Wokurna SA	15.1	558036	33	8246	1579	19	0.0094	0.0097	0.0379	14.0	34	0.2120	0.2105	−0.0098
Newstead TAS	15.3	565674	40	8492	1630	16	0.0091	0.0096	0.0544	15.0	40	0.2022	0.2044	0.0054
Cowangie VIC	14.4	565260	35	8457	1612	19	0.0092	0.0096	0.0445	13.7	35	0.2100	0.2083	−0.0051
Werribee VIC	11.9	538661	25	7878	1469	16	0.0093	0.0097	0.0416	11.0	27	0.2091	0.2050	−0.0176
Dalyup WA	15.3	562709	32	8452	1624	17	0.0092	0.0097	0.0459	14.4	32	0.2027	0.2048	0.0017
Esperance WA	13.5	532826	27	7718	1460	20	0.0096	0.0098	0.0239	12.2	28	0.2109	0.2043	−0.0262
Kalannie WA	15.3	564388	33	8410	1614	17	0.0091	0.0096	0.0496	14.6	34	0.2046	0.2065	0.0009
Manjimup WA	12.3	556387	36	8274	1567	18	0.0091	0.0096	0.0517	12.0	37	0.2007	0.2032	0.0095
Narrogin WA	11.9	541517	29	7947	1512	16	0.0094	0.0097	0.0376	11.1	30	0.2115	0.2104	−0.0048
Narrogin WA	15.1	557879	34	8284	1582	18	0.0090	0.0096	0.0579	14.7	35	0.1969	0.2011	0.0125

n, number of individuals genotyped per locus; H_O, observed heterozygosity; H_S, gene diversity; F_{IS}, Nei's inbreeding coefficient.

divergent from other populations (Figure 4.2, top right panel). AMOVA analysis using the set of 1032 SNPs indicated a lack of any spatial, temporal or host-plant related genetic structure among populations (Table 4.4). In model A, where populations were divided into years and *Brassica* host types, over 99 % of variance was found within populations, with negligible variance among populations or explained by year or host type. Similarly, in model B, where seven locations were sampled in both years, over 99 % of variance was found within populations. These results precluded interpretation of whether there was more spatial or temporal variance among populations.

Under isolation by distance, geographic and genetic distances should be positively correlated [249]. Populations were collected across geographic distances of up to 3756 kilometres (Northampton WA/Bundaberg QLD) in 2014 (mean distance 1323 ± 960 SD km) and 3624 kilometres (Manjimup WA/Bundaberg QLD) in 2015 (mean distance 1263 ± 917 SD km). At this large sampling scale, we expected higher F_{ST} values at higher geographic distances between population pairs, however heat maps revealed no such pattern (Figure 4.2). Mantel's tests confirmed a lack of genetic isolation by distance in 2014 (Mantel's $r = 0.1136$, $p = 0.1316$) and 2015 (Mantel's $r = -0.0901$, $p = 0.8222$) datasets, indicating that *P.*

xylostella populations in close proximity or separated by thousands of kilometres were equally differentiated.

Population structure was explored using two different individual-based clustering approaches. First, STRUCTURE analysis was performed using the widely-dispersed 1032 SNPs and analysing 2014 and 2015 populations separately. We first determined the predicted optimal values for K, then examined bar plots for several K values to assess hierarchical population structure. In 2014, the data most likely formed two genotypic clusters, with the delta K method and mean likelihood value both producing an optimal at $K = 2$ (Supplemementary Figure C.4). At this K value, bar plots showed that most individuals shared nearly uniform ancestry across the major genotypic cluster regardless of geographic location (Figure 4.3). A second genotypic cluster was largely associated with three individuals from Esperance, which showed 98.7 %, 98.7 % and 56.5 % cluster assignment, while of the remaining 396 individuals, only 17 individuals were greater than 1 % (1.0 to 9.3 %) admixed across this cluster. At $K = 3$ and $K = 4$, no significant additional population structure was detected, with the additional genotypic clusters associated with two individuals from Boyup Brook and two individuals from Cocata (Supplementary Figure C.5).

In 2015, the delta K method produced an optimal at $K = 2$ and weaker secondary modes at $K = 3$ and $K = 5$, while the highest log-likelihood occurred at $K = 5$ (Supplementary Figure C.4). The modes at $K = 3$ and $K = 5$ indicate sub-structure in the data. At $K = 2$, bar plots showed that most individuals shared nearly uniform ancestry across the major genotypic cluster regardless of geographic location (Figure 4.3). The second genotypic cluster was predominantly associated with individuals from Southend, where 10 individuals showed 31.7 to 99.4 % cluster assignment. At higher K values, some further geographic structuring was identified. At $K = 3$, two clusters were mainly associated with Southend (cluster A, 7 individuals with 26.1 % to 98.6 % assignment; cluster B: 10 individuals with 33.2 % to 99.5 % assignment) (Supplementary Figure C.5). At $K = 4$, the additional cluster was mainly associated with individuals collected from Walkers Beach in both autumn and spring 2015, showing a consistent pattern at both time points. At $K = 5$, the additional cluster was mostly represented by three individuals from Werombi. To further examine hierarchical structure, we reanalysed the 2015 data after removing Southend. This resulted in a weak delta K optimal at $K = 3$, but showed the same clustering pattern as the full 2015 dataset at $K = 5$ and is not presented.

PCA identified clustering patterns consistent with the STRUCTURE analysis. In both years, eigenvalues for the first principle component (PC) were not strongly different from other PCs, indicating no clear axis of variance in the data, and individuals across different geographic populations clustered together to a high degree (Figure 4.4). In both years, PCA

Table 4.4 Analysis of molecular variance under two hierarchical model structures. In Model A, all 59 populations collected from four *Brassica* host types in 2014 and 2015 were analyzed and variance was partitioned among years, among host within years and among populations within host. In Model B, populations from 7 locations sampled in both 2014 and 2015 were analyzed and variance was partitioned among years and among locations within years.

AMOVA summary					
MODEL A					
Source	df	SS	MS	Est. var.	%
Year	1	141.844	141.844	0.015	0.01
Host	5	651.478	130.296	0.049	0.04
Population	52	6487.275	124.755	0.902	0.80
Error	774	86712.706	112.032	112.032	99.15
Total	832	93993.302		112.998	100.00
MODEL B					
Source	df	SS	MS	Est. var.	%
Year	1	102.038	102.038	−0.162	−0.15
Location	12	1409.908	117.492	0.923	0.88
Error	181	18947.900	104.685	104.684	99.28
Total	194	20459.846		105.445	100.00

identified the most divergent individuals consistent with those in the STRUCTURE analysis. In 2014, three individuals from Esperance WA and two individuals from Cocata SA clustered distinctly along separate PC axes. In 2015, two groups of individuals from Southend (SA) clustered distinctly along the two PCs axes, and three individuals from Werombi NSW formed an identifiable cluster along the vertical PC axis.

4.4 Discussion

Successfully invading species can often have low genetic diversity compared to their population of origin [86]. Although six microsatellites revealed a lack of population structure among *P. xylostella* in Australia [65], low genetic diversity raised the possibility the species has undergone a recent range expansion [202, 258]. Whether this marker set had adequate power to detect weak population structure was also uncertain. Further, the discovery of a cryptic species, *P. australiana*, [131] explained some inconsistencies between two other studies [174, 196]. Here, we-examined population genetic structure in Australian *P. xylostella* through extensive field sampling from brassicaceous hosts throughout southern Australia, molecular species identification, and a powerful genome-wide SNPs market set derived from RAD-seq.

We found a clear lack of genetic structure among Australian *P. xylostella* populations regardless of geographic location, *Brassica* host type or sampling year. This pattern was temporally stable over two years at seven re-sampled locations. Spatial and temporal sampling of populations, and high statistical power of SNP marker datasets, provided results that are highly consistent with six microsatellites previously documented by Endersby et al. [65]. In our study, a thorough spatial and temporal sampling scale, and a high degree of statistical power in our SNP marker set, allow us to conclude with confidence that Australian *P. xylostella* forms a single homogeneous population, based on genome-wide bi-allelic neutral markers.

Genetic variation and population structure can reflect a combination a historical processes and current gene flow patterns [30]. Several lines of evidence suggest the high level of genetic heterogeneity in our results are not entirely representative of current gene flow. We found no evidence of isolation by distance, despite a sampling scale $>$3000 km. This implies populations separated by thousands of km, or $<$10 km are equally likely to interbreed, which seems unlikely. Even if larvae were frequently transported long distances transport on infested host material [e.g. 209], this seems unlikely to explain genetic homogeneity across vast areas regardless of geographic proximity. A more plausible explanation is that our results largely reflect the recent demographic history of *P. xylostella* in Australia, characterised by

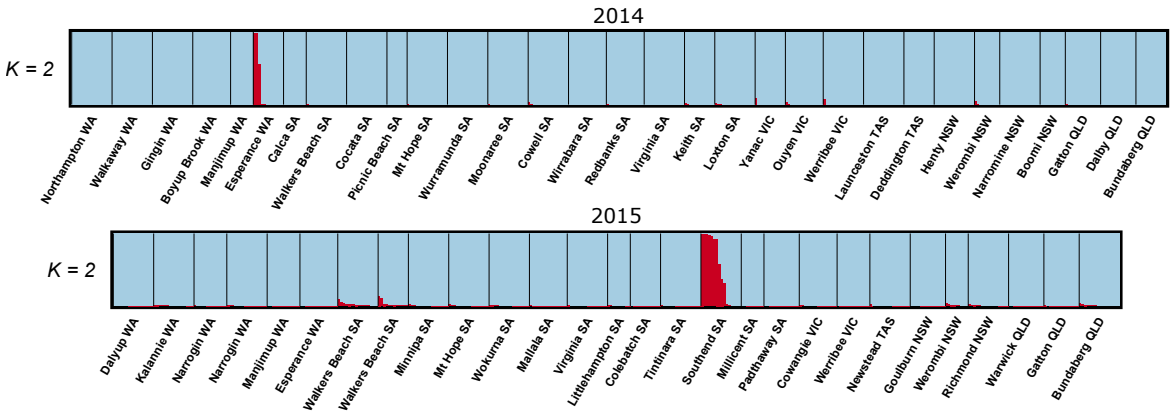

Fig. 4.3 Proportional assignment to genotypic clusters, K, based on STRUCTURE analysis of *P. xylostella* individuals from Australia in 2014 and 2015. Individuals are represented by vertical bars and genotypic clusters are represented by different colours. The individuals collected from each year were analysed separately and in both years the data most likely formed two genotypic clusters. Top panel: Analysis at $K = 2$ for 434 individuals collected from 31 locations in 2014. Bottom panel: Analysis at $K = 2$ for 399 individuals collected from 28 locations in 2015. Within each year, bar plots show a high degree of genotypic admixture across individuals regardless of geographic location, as shown by sharing of blue-coloured bars, with a second genotypic cluster represented by red-coloured bars shared predominantly by several individuals within a single sampled location.

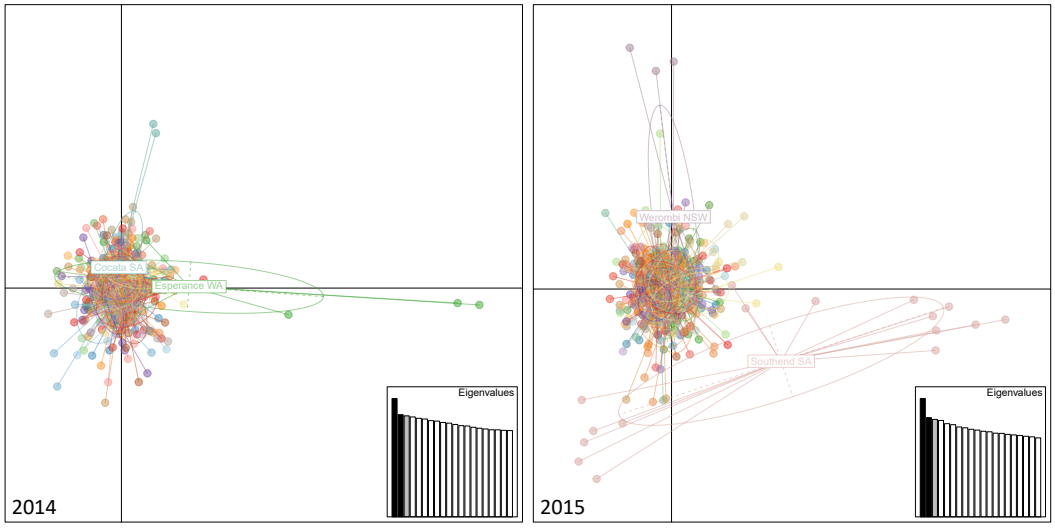

Fig. 4.4 Principal components analysis of *P. xylostella* individuals collected from Australia in 2014 and 2015. Individuals are represented by small circles colour-coded by geographic population. Populations with some divergent individuals are labelled.

a founder effect [202, 65, 52, 169]. In interpreting our results, the term "panmixia" may not be entirely appropriate as it implies random inter-breeding of *P. xylostella* throughout Australia, whereas our data cannot distinguish gene flow from recent ancestry as pointed out by several authors previously [65, 77]. Gene flow undoubtedly occurs, however, as shown by seasonal colonisation of canola crops over large areas [77], collection of moths in light traps [131, 65] and pheromone traps [95, 185], and detection of insecticide resistance in *P. xylostella* collected from unsprayed weeds [66, 17].

Gene flow creates the potential for the spatial spread of insecticide resistant genotypes, which has consequences for managing resistance in Australia. Within Australian canola-growing areas, and among crops and weeds, *P. xylostella* has largely homogeneous resistance profiles, implying regular intermixing [17]. For certain insecticides, resistance ratios are higher in *Brassica* vegetable production areas [66, 17]. *Plutella xlyostella* movement in these crops is limited [152], leading to a local build-up of resistance over successive generations. Spring flights of moths into *Brassica* vegetable crops are thought to occur seasonally in spring [95, 185], which could dilute resistance.

Individual-based clustering analyses clearly showed the overall lack of genetic divergence among populations. In both years, STRUCTURE analysis identified $K = 2$ as the optimal number of genotypic clusters among the range of K values tested ($K = 1 - 10$). This is a common result among studies employing the delta K method [111], partly because it is not possible to obtain an optimal at $K = 1$, and partly because $K = 2$ often represents the top level of hierarchical population structure. However, in both years, a small number of divergent samples were identified in specific geographic locations. This was particularly evident in the Southend population in 2015 where, at values of $K > 3$ values, two genotypic clusters occurred predominantly in this population. Do these patterns reflect genetic isolation? STRUCTURE sorts groups into Hardy-Weinberg/linkage populations [120] but under the assumptions of independent loci [176]. The Southend population had the lowest gene diversity and number of private sites, and the highest pairwise F_{ST} values in population comparisons. This population was collected from an isolated patch of few, large sea rocket plants. Therefore, cluster patterns are likely to reflect an artefact of sampling of related individuals at Southend [191, 242]. In our hierarchical STRUCTURE analysis in 2014 and 2015, additional clusters at successively higher K values tended to occur in a small number of individuals at single locations, suggesting the possibility that STRUCTURE simply grouped the next most related samples at each hierarchical level. These results highlight the need for caution when related samples are present to avoid false inferences of population structure.

Conclusions

Neutral markers cannot distinguish *P. xylostella* dispersal patterns in Australia due to low genetic diversity. The methods we employed here might be more successful overseas outside Australia, where *P. xylostella* has higher genetic diversity. Incorporating independent genome-wide nuclear markers and mtDNA markers has the potential to provide more resolution in questions of population structure, as mtDNA haplotypes that are shared or unique can provide insights into the relative frequency of long range and localised movement. To identify sources of canola colonisation, other approaches will be needed to help elucidate seasonal movements, such as analysis of seasonal changes in climate suitability to understand seasonal population fluctuations and identify potential source areas suitable for population growth at key times.

Statement of Authorship - Chapter 5

Principal Author

Title of Paper:	Seasonal colonisation of canola crops by *Plutella xylostella* in South Australia originates from localised source populations.
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Name of Principle Author (Candidate):	Kym D Perry
Contribution to the Paper:	Conceived experiments, performed autumn surveys, coordinated trapping networks, constructed light traps, performed CLIMEX modelling, developed R package dbmdev, performed *Plutella* developmental modelling, data analysis and wrote the manuscript.
Overall percentage (%):	95 %
Signature:	_____ Date: _____

Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

i the candidate's stated contribution to the publication is accurate (as detailed above);
ii permission is granted for the candidate to include the publication in the thesis; and
iii the sum of all co-author contributions is equal to 100 % less the candidate's stated contribution.

Name of Co-Author:	Michael A Keller
Contribution to the Paper:	Fitted *Plutella* developmental model parameters, contributed to experimental design, edited the manuscript.
Signature:	_____ Date: _____

Chapter 5

Colonisation of canola crops by *P. xylostella* in southern Australia originates from local population sources

Abstract

The invasion of annual crops by migratory insect pests causes substantial damage worldwide. Yet, patterns of dispersal and crop colonisation at landscape and regional scales are often poorly characterised. The diamondback moth, *Plutella xylostella* L., a notorious global *Brassica* pest, seasonally colonises winter canola crops in southern and western Australia leading to intermittent outbreaks, but the source populations are uncertain. Here, we used a variety of methods to investigate the colonisation process over three years. Potential wild brassicaceous hosts were sampled in autumn, then subsequent crop colonisation was measured across a regional network of sentinel canola crops in South Australia. We derived a temperature-development model for *P. xylostella* and used it to infer the timing of oviposition in each crop, and compare between sites and years. Each year, wild brassicas harboured *P. xylostella* in cropping areas prior to sowing, then most canola crops were colonised soon after germination, earlier than previously assumed. The autumn abundance of the insect and its hosts and the subsequent colonisation pattern in canola varied widely between years, driven by pre-season rainfall. Elevated pre-season rainfall in 2014 and 2016 was followed by earlier colonisation of canola than in 2015, which followed dry pre-season conditions when hosts were locally scarce. We used a CLIMEX model to explore spatio-temporal fluctuations in the potential distribution and abundance of *P. xylostella* during the study based on climate. CLIMEX predictions and continuous light trapping at four locations

suggested that *P. xylostella* persisted in canola production areas throughout the year. The concordance of *P. xylostella* seasonal dynamics with observed local weather and CLIMEX modelling suggested that local *P. xylostella* over-summering on wild brassicaceous plants are the primary source of seasonal infestation of winter canola crops in South Australia. In this region, local factors may contribute to seasonal outbreaks and colonising populations are likely to be insecticide-resistant.

5.1 Introduction

The seasonal colonisation of annual crops by herbivorous insects inflicts substantial damage on a global scale [163]. Successfully predicting and managing pest outbreaks requires an understanding of their capacity to colonise crops from alternate host refuges in the surrounding landscape or more distant regions [166], and of their genetic background and insecticide resistance status based on population sources [241, 63].

The diamondback moth is a ubiquitous specialist of wild and cultivated brassicas. It is the most destructive pest of *Brassica* vegetable crops throughout tropical and temperate horticultural regions [228, 78, 134], where host plants are continuously available and it is essentially a resident pest. More recently, its pest status has increased in canola crops grown in temperate regions of North America [55, 56], southern Africa [153] and Australia [77], which are colonised on an annual basis. The migratory ability of *P. xylostella*, sporadic nature of outbreaks and its capacity to rapidly evolve resistance to insecticides [78] create significant management challenges in canola. This has generated an ongoing interest in better characterising dispersal to aid in forecasting outbreaks [228, 77, 134].

Intermittent outbreaks of *P. xylostella* can occur in Australian canola crops during spring and cause substantial crop losses [89, 77]. Canola is an important break crop in rotations with cereal and pulse crops in Australia [43]. Approximately 3 million hectares of canola are grown annually between autumn and spring in southern and western Australia [1] under a Mediterranean climate characterised by cool, wet winters and hot, dry summers. Management is reliant on insecticides but often difficult due to widespread insecticide resistance [66, 67, 15] and ineffective spray application.

The seasonal dynamics of *P. xylostella* in Australian canola are incompletely understood, as few ecological studies exist [77]. The recent discovery of a cryptic species, *Plutella australiana*, adds further uncertainty [131, 169]. Vast areas of canola are seasonally colonised from unknown source areas, but populations vary widely between regions and years. In some years, larval populations build rapidly to extremely high densities in spring and destroy maturing plants [89]. Seasonal climatic conditions during the growing season are believed

to be primarily responsible for outbreaks, but clear relationships between *P. xylostella* abundance, rainfall and temperature have not been demonstrated [185, 77]. *Plutella xylostella* breeds continuously and thermal conditions allow the potential for ≈ 6-12 generations per year in southern Australia [15] and ≈ 3.5-5.3 generations per canola cropping cycle [185], depending on region and arrival timing. Field studies, genetic studies and insecticide resistance data suggest that *P. xylostella* disperses regularly in Australia [95, 65, 66, 185]. However, little is known about movement among wild and cultivated *Brassica* host plants and the source populations that seasonally colonise Australian canola crops. The relative contributions of wide scale immigration and local population increase to spring outbreaks is also uncertain [77].

The movement of *P. xylostella* has been studied at local or large geographic scales. Mark-recapture studies have focused on local scale movement ($\leqslant$1 km) within *Brassica* vegetable fields and surrounding areas [152, 203]. Long distance migration (≈hundreds of km) has been inferred mostly indirectly [40, 44] or observed using radars [38, 132], sometimes coupled with wind trajectory analysis [75, 55, 38]. Seasonal migrations occur regularly in the northern Hemisphere. In Canada, where cold extremes limit overwintering, canola crops are colonised following long distance migration from southern USA [55, 56, 47]. Across neutral genetic markers, *P. xylostella* generally shows little genetic differentiation among regional populations, precluding identification of movement pathways [but see 243, 250], including in Australia [65, 169]. Surprisingly little information exists on dispersal patterns at intermediate landscape scales (*c.* $\geqslant$ one to tens of km) relevant to the colonisation of *Brassica* crops [77, 78].

In Australia, *P. xylostella* survives between canola-growing seasons on alternate brassicaceous hosts. Potential non-canola hosts in summer include restricted areas of *Brassica* vegetable crops near metropolitan centres and forage crops in higher rainfall zones [185, 15]. Brassicaceous weeds are widespread and maintain *P. xylostella* during non-cropping periods [95, 89, 88], however the role of weeds in *P. xylostella* dynamics in canola has not been investigated [77]. Summer heat extremes and a scarcity of hosts makes the summer survival of *P. xylostella* in canola growing areas uncertain [254, 255]. Local persistence is important to establish because these populations are potential sources of colonisation and resistant genotypes [228].

Studying the colonisation process has particular advantages in identifying pest movement in agro-ecosystems. In insect-free habitats, such as newly planted sentinel crops, colonisation can be directly measured [98, 80]. The difficulty distinguishing locally established individuals from migrants does not arise, as all colonising adults and their progeny are immigrants. The unique opportunity to identify migrants and their arrival timing enables inference of their

most likely origins, using additional information such as host plant distribution, climatically suitable areas and wind patterns.

Measuring dispersal and colonisation of small insects at landscape scale is inherently difficult [159, 140, 129]. Studying dispersal using a variety of methods can enhance understanding of movement. Both pheromone and light traps have been used to measure temporal changes in moth activity. Pheromone traps capture male moths and are efficient in detecting *P. xylostella* in an area [260]. Light traps can be more reliable [14] and capture both sexes, which is an advantage in studying colonisation. Networks of traps and widespread sampling can provide insights into the spatial dynamics of insect pest populations [231, 12, 206, 215].

Bioclimatic models, such as CLIMEX [225, 226], can be useful to infer changes in the potential distribution and abundance of a species based on climate. A CLIMEX model, developed by Zalucki and Furlong [254, 255] and updated by Li et al. [135], reasonably predicts the core and seasonal global distribution of *P. xylostella* and its seasonal abundance in parts of Asia and the UK. Annualised model predictions, based on long-term average data, suggest most of Australia is unsuitable for *P. xylostella* and year-round persistence is most likely restricted to coastal regions in the southern half of the continent. Examining how climate suitability changes seasonally can provide insights into potential re-distribution patterns and when areas become suitable for establishment. Recent enhancements to the CLIMEX software [130] facilitate spatio-temporal predictions at weekly intervals, providing an opportunity to examine how the distribution of *P. xylostella* may change seasonally [see 46]. While CLIMEX is generally used with long-term average data, using actual seasonal climate data can aid the interpretation of field abundance data.

Here, we investigated the seasonal colonisation of canola crops by *P. xylostella* at a regional scale in southern Australia over three years using a variety of techniques. Potential wild host plants for *P. xylostella* were sampled during the autumn pre-cropping period, then subsequent crop colonisation was measured across a regional network of sentinel canola crops. We derived a temperature-dependent development model for *P. xylostella*, then used it with local temperature data to predict initial oviposition in each crop and infer the timing of colonisation. To identify potential source areas, we used a CLIMEX model to explore spatio-temporal changes in the potential distribution and abundance of the insect using seasonal climate observations. The flight dynamics of *P. xylostella* was measured through continuous light trapping at four locations. Together, the results reveal the seasonal pattern of colonisation of canola by this species.

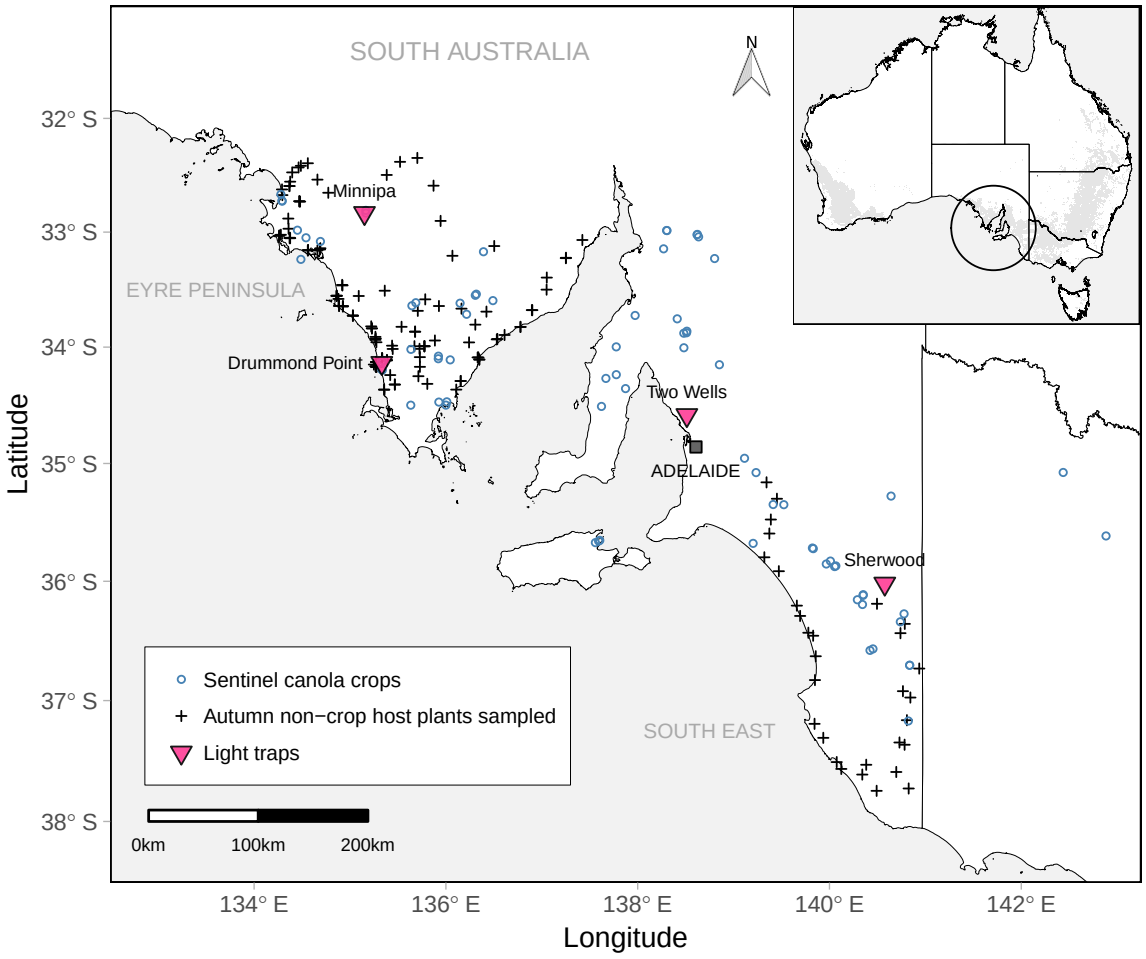

Fig. 5.1 Geographic map showing field locations in southern Australia sampled for *P. xylostella* between 2014–2016. Symbols show non-crop brassicaceous host plants sampled during pre-cropping season autumn surveys in the Eyre Peninsula and South East regions of South Australia, sentinel canola crops, and light traps operated continuously. Light trapping locations are labelled. In the inset map of Australia, grey shading indicates dryland winter cropping areas.

5.2 Methods

Climate data

Daily and monthly gridded climate data for Australia covering the temporal period of the study were obtained from SILO [213] at a spatial resolution of 0.05°. Time series climate data for sampled field locations were extracted from corresponding grid cells using a custom R script [168]. To examine pre-cropping season rainfall, we calculated an average of cumulative daily rainfall across all field sampling locations (Figure 5.1) for February to April from 2014–2016.

CLIMEX modelling

We used CLIMEX version 4.0.2 [130] using *P. xylostella* parameters from Li et al. [135] and monthly gridded climate data for Australia from 2014 to 2016 to predict spatio-temporal climate suitability for *P. xylostella* during the study. Monthly gridded climate data were converted to CLIMEX format using a custom R script [168], then the CLIMEX Compare Locations Years model was run inputting the following variables: monthly total precipitation, monthly mean minimum temperature, monthly mean maximum temperature and monthly mean vapour pressure. Modelling output (netCDF format) was imported to R [180] for plotting spatial maps of the Annual Growth Index (GI_A), Ecoclimatic Index (EI) and stress indices (cold, wet, dry, heat) and constructing time series map sequences of the Weekly Growth Index (GI_W). Temporal variation in climate suitability for *P. xylostella* was examined for individual sampling locations using the CLIMEX Compare Years model and inputting the following variables from corresponding grid cells: daily rainfall, daily maximum (T_{max}) and minimum (T_{min}) temperature, daily relative humidity at T_{max} and T_{min}. For all CLIMEX models, no constraints were imposed such that modelling output reflected the expected species response to climate alone [130].

Autumn sampling of non-crop brassiceous plants

Field surveys were conducted annually between 2014 and 2016 to assess the abundance of potential non-crop hosts and *P. xylostella* during the autumn pre-cropping season period (Figure 5.1). The Eyre Peninsula region was sampled in all years and the South East region was sampled in 2015. The Eyre Peninsula is the state's principle canola production region. It is a low-medium rainfall zone ($200-500$ mm per annum [11]) and has relatively frequent *P. xylostella* infestations requiring management. The South East region experiences higher

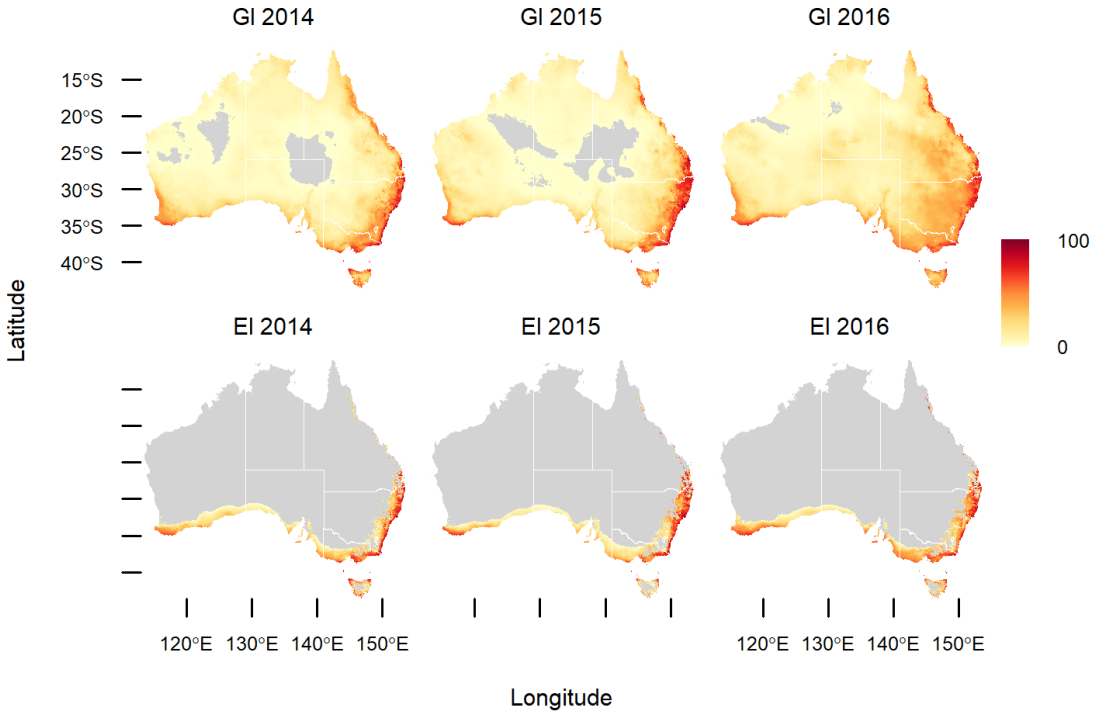

Fig. 5.2 Climate suitability of Australian locations for *P. xylostella* predicted using a CLIMEX model with parameters from Li et al. [135]. The Annual Growth Index (GI_A) indicates suitability of locations for population growth during favourable seasons and the Ecoclimatic Index (EI) indicates the overall suitability of locations for year-round persistence, taking into account growth and stress indices. Indices are scaled from 0 (grey, unsuitable) to 100 (extremely suitable).

rainfall (300 – 600 mm per annum) [11]. Both regions are remote from commercial *Brassica* vegetable farms, and wild brassicas available sporadically are potential hosts between growing seasons. In the South East, small areas of spring-sown canola and *Brassica* forage crops are additional summer hosts ([185]. Geographic distances between sampling locations were calculated using R package geosphere version 1.5.7 [101].

Surveys were conducted each year in early March and again four weeks later in early April by traversing roads and sampling stands of brassicaceous plants for *P. xylostella*. The aim was to cover as much ground as possible and deliberately bias sampling to maximise the chance of detecting *P. xylostella*. At intervals of approximately 20 km, depending on plant abundance, the largest available stand of brassicaceous plants was sampled. Coastal sand dunes were visited to enable sampling of sea rocket, *Cakile maritima*, on which *P. xylostella* has been observed (MA Keller). In March, at each site a delta-type pheromone trap (Desire, ETEC New Zealand) baited with female sex pheromone lure (Suterra) was positioned adjacent to brassicaceous plants at canopy height. Brassicaceous plants were sampled for immature stages using a 38 cm diameter sweep net, taking the plant area equivalent of 20 x 1 m sweeps across at least 20 individual plants and recording the total number of *P. xylostella* larvae. *Cakile maritima* was sampled by beating plants over a 420 x 300 mm collection tray for 5 seconds, taking a plant area equivalent of 20 x 1 m samples across at least 20 plants. In April, plants at the same locations were sampled for larvae using the same methods and the numbers of male *P. xylostella* moths in pheromone traps were recorded. Digital photographs of plant stands were taken to record plant abundance and condition.

Measuring colonisation of canola

The colonisation of canola crops by *P. xylostella* was measured at a regional scale in South Australia annually between 2014 and 2016. Each year, a network of sentinel canola crops was established in South Australia and Western Victoria between 134.3°E and 142.9°E and 32.7°S 37.2°S (Figure 5.1). The geographic scale of the networks was similar between years but the locations of individual sites varied. Respectively in 2014, 2015 and 2016 the mean distance between sites was 310, 282 and 303 km and the maximum distance between any two sites was 779, 815 and 797 km.

Sentinel crops were monitored by field agronomists experienced in insect management who each monitored 1–2 crops. At each site, two delta-type pheromone traps containing a female sex pheromone lure were placed 200 m apart and 10 m into the crop. Crops were sampled at weekly to fortnightly intervals continuing until immature stages of *P. xylostella* were first detected. On each sampling date, the number of moths trapped were recorded and canola plants were sampled directly for immature *P. xylostella* (eggs, larvae

or pupae). Crops younger than 6-leaf stage were sampled by walking a 100-200 m transect and spending 15 minutes visually searching plants and leaves. More advanced crops were sampled using a 38 cm diameter sweep net and taking 100 x 1 m sweeps. Immatures from the first cohort detected were collected into 100 % USP-grade propylene glycol and submitted to our laboratory for confirmation of species identification and determination of developmental stages for modelling biofixes (see below). The crop was again sampled ≈one week later and a second sample of immatures similarly collected and submitted.

Modelling temperature-dependent *P. xylostella* development

We derived a stage-specific development model for *P. xylostella* and back-predicted the date of initial oviposition in each crop to determine colonisation timing. Briere's model function II [28] was parameterised by fitting non-linear regressions to temperature-development data for *P. xylostella* [137] using R package nlreg version 1.2.2.1 [27]. The four model parameters, a, T_{min}, T_{max} and m were estimated separately for the egg, each instar, pre-pupal and pupal stages (Table D.1). As individual models for 1^{st} and 2^{nd} instars did not converge, we modelled these stages together. Model fits for all life stage showed a good fit to observed data (Supplementary Figure D.5).

The samples of immature *P. xylostella* provided by agronomists were examined to determine developmental stages. The head capsule width of each larva was measured at the widest point under a dissecting microscope fitted with a micrometer, then the plotted frequency distribution of widths was examined and visible peaks assumed to correspond to larval instars. The size range of head capsules for each instar was visually inferred from the frequency distribution and used to determine the developmental stage of each larval individual. For each site, the collection date and most advanced developmental stage present in the sample were used as the modelling biofix. For sites where samples were unavailable, we assumed a 4^{th} instar biofix, as this was the most common stage collected. Temperature data for modelling were obtained by extracting daily maximum and minimum temperature data for each site from corresponding grid cells, then interpolating to hourly temperatures using two sine functions and a quadratic function according to Cesaraccio et al. [36]. We used our model to back-predict *P. xylostella* development at each site using an R package dbmdev [170] we developed for this purpose. As immature stages could have been collected between early or late stage-development, three back-predictions were performed per site using the specified biofixes but varying the "starting development" parameter for the starting lifestage from 0 (no development), 0.5 (median-development) and 1 (completed development) to give a predicted date range for oviposition.

Light trapping

Light traps were maintained continuously at four locations in South Australia (Figure 5.1) between August 2014 and December 2016 to measure the flight dynamics of male and female *P. xylostella* . Traps were deployed in canola production areas but locally remote from canola crops or significant *Brassica* stands to increase the likelihood of trapping dispersing individuals. Traps were located at Drummond Point (−34.137°N, 135.331°E) and Minnipa (−32.833°N, 135.151°E) in the Eyre Peninsula region, at Two Wells (−34.586°N, 138.513°E) on the Northern Adelaide Plains and at Sherwood (−36.020°N, 140.577°E) in the South East. The Two Wells trap was $\leqslant$ 10 km from commercial *Brassica* vegetable production farms but other traps were more than $\leqslant$ 250 km away.

Light traps were constructed using a 60 L plastic bin and an inverted polished fibreglass cone (collection funnel) mounted inside the opening (Supplementary Figure D.4). A 250 mL polypropylene collection jar was mounted below the cone, leaving a 2 mm gap around the perimeter to facilitate water run-off during rain events. Three acrylic trap vanes were mounted to protrude 60 cm above the trap. Lighting consisted of 1 m UV-LED light strip with 60 m^{-1} lights (model: SMD5050, Volka Lighting, Melbourne, Australia) fixed vertically to the trap vanes to emit light in all directions and powered using an 18 amp-hour rechargeable 12 V battery connected to a 20 W solar panel and solar regulator. The light was programmed using a digital timer to switch on for 12 h each night between 1800 and 600 h.

Trap samples were collected into 100 % USP-grade propylene glycol. Collection jars were changed at intervals of one to several days and stored at −20 °C. *Plutella* individuals were morphologically identified from light trap samples under a dissecting microscope and sex determined from external genitalia [9]. The mean nightly number of male and female moths captured was calculated and plotted on a phenological timescale, using our development model and local temperature data to predict *P. xylostella* generation times across the trapping period, and re-tracing one generation to assess the correspondence of flight peaks with prior population peaks.

5.3 Results

Climate suitability for the insect

An an annual basis, overall climate suitability for *P. xylostella* across its Australian range was similar between years (Figure 5.2). Large areas of Australia experienced favourable seasons for population growth ($GI_A > 0$) but most of the continent was unsuitable for permanent persistence (EI $\approx$ 0). Of stress indices examined, heat stress accumulation was the main

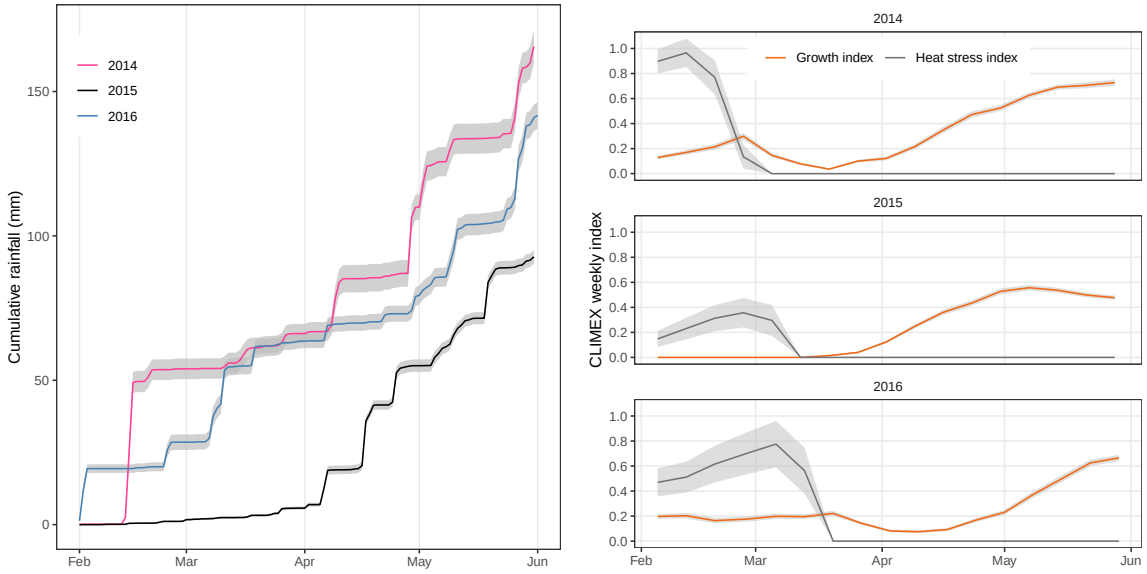

Fig. 5.3 Cumulative rainfall (left panels) and temporal changes in the CLIMEX Weekly Growth Index (GI_W) and Heat Stress Index (right panels) for *P. xylostella* across field sites during the three-month pre-cropping period (February to April) and the early part of the winter cropping season (≈May onward) (right panels). Data shown are the mean ± SEM (grey-shading) values based on gridded climate data across 181 grid cells corresponding to 231 distinct field locations sampled in our study.

population-limiting factor (not shown). Coastal regions in the southern half of the Australian continent proximate to canola production areas were most likely to support *P. xylostella* throughout the year.

Examining seasonal variation revealed large spatio-temporal fluctuations in GI_W within years and between years, reflecting a highly seasonal environment and inter-annual climate variability (Supplementary Movie 1: https://doi.org/10.25909/5bec00c181a02). Consistently each year, canola production areas in southern Australia became generally favourable for *P. xylostella* during the cropping phase (≈May to November). Between growing seasons (December to April), high inter-annual variation in GI_W across south-eastern and south-western Australia suggested that the seasonal distribution and abundance of *P. xylostella* during the non-cropping period could vary widely between years. Part of northern Australia became more suitable for *P. xylostella* at this time.

Table 5.1 The incidence and abundance of *Plutella* at sites sampled during field surveys in South Australia during March and April in each year from 2014-2016. Presented are the numbers and proportion in parentheses of: Sites sampled (N), sites (N_{POSITIVE}) where any lifestage was detected, positive sites (where moths (N_{MOTHS}) or larvae (N_{LARVAE} were detected, and the mean ± SEM numbers of larvae or moths collected at positive sites (where lifestage was present). Moths or larvae were not sampled at all sites, hence, for example, $N_{\text{MOTHS}} = 24/25$ indicates that moths were detected at 24 sites of 25 sampled. A dash indicates no sampling.

			Plutella incidence			*Plutella* abundance		
Host	Year	N	N_{POSITIVE}	N_{MOTHS}	N_{LARVAE}	Mean ± SEM larvae per site (March)	Mean ± SEM larvae per site (April)	Mean ± SEM moths per site (March-April)
Lincoln weed, *Diplotaxis tenuifolia* L.DC	2014	29	26 (0.90)	24/25 (0.96)	15/29 (0.52)	2.62 ± 0.99	5.00 ± 3.58	5.20 ± 0.92
	2015	28	6 (0.21)	6/28 (0.21)	0/28 (0.00)	–	–	1.33 ± 0.21
	2016	21	21 (1.00)	21/21 (1.00)	12/21 (0.57)	2.44 ± 0.71	3.83 ± 1.28	27.52 ± 6.98
Sea rocket, *Cakile maritima* Scop.,	2014	3	2 (0.67)	0/0 –	2/3 (0.67)	12.00 ± 2.00	150.50 ± 99.50	–
	2015	16	9 (0.56)	1/6 (0.17)	9/16 (0.56)	14.67 ± 10.17	21.43 ± 14.22	7.00
	2016	7	7 (1.00)	1/3 (0.33)	7/7 (1.00)	68.00 ± 36.88	25.40 ± 13.69	1.00
Weedy canola, *Brassica napus*, L.	2015	4	0 (0.00)	0/4 (0.00)	0/4 (0.00)	–	–	–
	2016	1	1 (1.00)	1/1 (1.00)	0/1 (0.00)	–	–	2.00
Brassica forage crops	2015	10	5 (0.50)	5/9 (0.56)	4/10 (0.40)	23.00 ± 13.34	1.50 ± 0.50	32.60 ± 12.74
Buchan weed, *Hirschfeldia incana* L.,	2015	5	0 (0.00)	0/5 (0.00)	0/5 (0.00)	–	–	–
Nil host	2015	21	0 (0.00)	0/21 (0.00)	0/0 –	–	–	–
	2016	7	4 (0.57)	4/7 (0.57)	0/0 –	–	–	1.25 ± 0.25

Pre-season weather patterns

Pre season rainfall patterns varied widely between years (Figure 5.3). In 2014, substantial rain fell in February (52 ± 29mm, mean $\pm$ SD) and April (40 ± 20 mm), and in 2016, rain fell in February (35 ± 20 mm) and March (35 ± 18 mm). By contrast, in 2015 very little rain fell during February (1.1 ± 1.7 mm) and March (5.4 ± 6.2 mm) before season-opening rains in April (47 ± 18mm).

At the same locations, the favourability of pre-season weather for *P. xylostella* predicted using CLIMEX showed a clear seasonal pattern (Figure 5.3). Heat stress accumulation during summer was the major population-limiting factor while other stress indices were negligible (not shown). Climatic conditions became increasingly favourable for populations growth from approximately April onward coinciding with increased rainfall and the cessation of heat stress. The potential for local population growth during February and March varied between years, as shown by a positive values for GI_W in 2014 and 2016 but zero GI_W throughout these two months in 2015.

Pre-season climate suitability for *P. xylostella* was examined on the Eyre Peninsula, where annual data on *P. xylostella* seasonal abundance on wild hosts were available from autumn surveys, and highlighted a striking contrast between years. Areas potentially suitable for population growth ($GI_W > 0$) appeared during February and March in 2014 and 2016 but there were no suitable areas during the same period in 2015 (Supplementary Movie 2: https://doi.org/10.25909/5bec01d93d72f).

The abundance of *P. xylostella* and potential hosts during autumn

Field surveys each year identified wild potential brassicaceous hosts of *P. xylostella* during March and April (Table 5.1, Figure 5.4). On Eyre Peninsula, two *Diplotaxis* species, Lincoln weed, *D. tenuifolia*, and wall rocket, *D. muralis*, were widespread over thousands of hectares in pasture paddocks and along roadsides interspersed among cropping paddocks. Sea rocket, *Cakile maritima*, occurred commonly along the foreshore of coastal sand dunes, consistent with its widespread distribution in southern coastal regions [45]. Small areas of weedy canola were observed in fallow cropping paddocks during March, but these were usually removed using using herbicides by April. In parts of the South East region surveyed in 2015, *C. maritima* was common in sand dunes but brassicaceous weeds were otherwise scarce and limited to small patches of *D. tenuifolia*, and Buchan weed, *Hirschfeldia incana*, occurring along roadsides. Isolated dryland and irrigated *Brassica* forage crops and paddocks of self-sown canola were observed during March and April (Supplementary Figure D.3).

Overall, there was a striking contrast in the availability of potential autumn hosts for *P. xylostella* on Eyre Peninsula across three years. Rainfall patterns strongly influenced the seasonal phenology and abundance of brassicaceous species during autumn, and their availability for *P. xylostella*. In 2014 and 2016, *Diplotaxis* spp. plants were green, flowering and abundant during March in response to February rainfall (Supplementary Figure D.1). By April, these plants had largely senesced. In 2015, *Diplotaxis* plants were highly moisture-stressed and stunted in response to very dry conditions, resulting in a distinct lack of *P. xylostella* hosts. Green *C. maritima* plants were present in sand dunes in all 3 years, though at lower abundance in 2015 (Supplementary Figure D.2).

Pheromone trapping and sampling of larval host plants detected *P. xylostella* across wide areas of the Eyre Peninsula and South East regions during autumn in each year sampled (Figure 5.4, Supplementary Figure D.3). Male moths were trapped at sites with *D. tenuifolia*, *D. muralis*, *Brassica* forage crops and weedy canola, indicating the presence of a local adult population (Table 5.1). Moth counts were not available at *C. maritima* sites due to sand clogging traps. In 2016, a small number of moths were trapped at sites with apparently nil host plants, suggesting that local brassicas were not identified or that male moths were dispersing widely across the landscape.

Larvae of all sizes were found on *D. tenuifolia*, *D. muralis*, *C. maritima* and *Brassica* forage crops, indicating a local breeding population at numerous sites. Across all years, larvae were detected at 35 % ($n = 27/78$) of sampled *Diplotaxis* spp. sites, 40 % ($n = 4/10$) of sites with *Brassica* forage crops and 69 % ($n = 18/26$) of sites with *C. maritima* (Table 5.1). No larvae were detected on sites with weedy canola ($n = 5$), and no moths or larvae were detected at sites with *H. incana* ($n = 5$), which was only sampled in 2015.

Diplotaxis spp. and *C. maritima* were sampled for *P. xylostella* in all three years to allow temporal comparisons. Overall, inter-seasonal patterns of *P. xylostella* abundance during autumn reflected the seasonal abundance of its brassicaceous hosts and the favourability of local climatic conditions for this species. The incidence and abundance of *P. xylostella* across sites was highest in 2014 and lowest in 2015. At *Diplotaxis* sites, larvae were found at about half of sites in 2014 and 2016 at similar abundance, but were not detected at any sites in 2015 (Table 5.1). On *C. maritima*, larvae were detected at the majority of sites (56 % to 100 %) in all three years, implying this species may provide a refuge for *P. xylostella* even in dry years.

Colonisation of canola by *P. xylostella*

Networks of sentinel canola crops established each year for monitoring *P. xylostella* consisted of 41 sites in 2014, 43 in 2015 and 18 in 2016. We later excluded data from 25 sites with

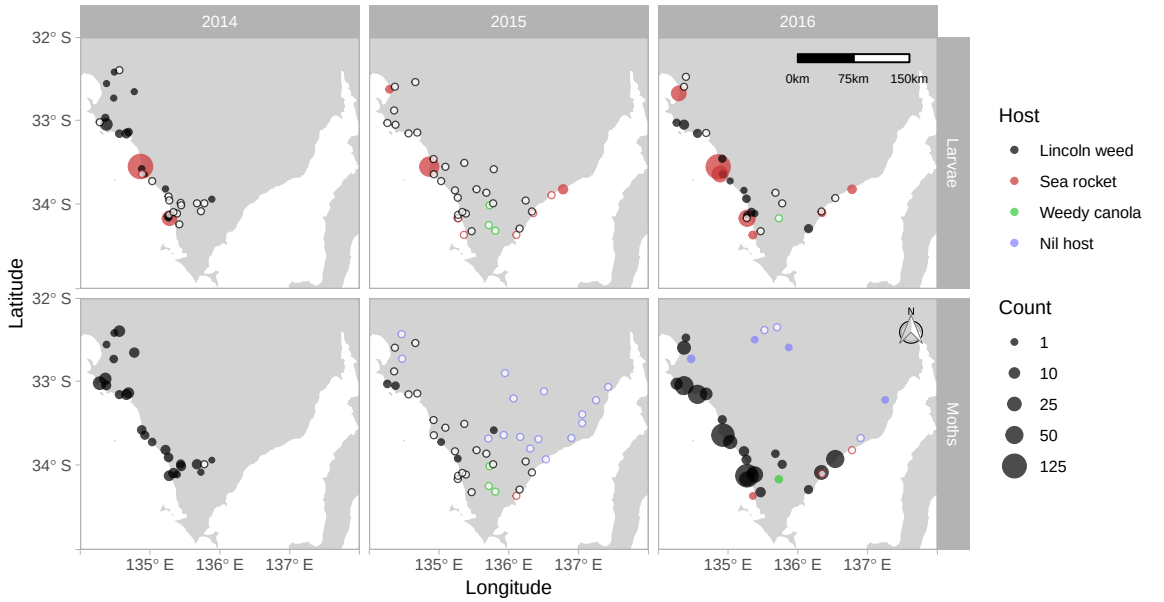

Fig. 5.4 Abundance of *P. xylostella* moths (lower panels) or larvae (upper panels) associated with wild brassicaceous plants during annual surveys on the Eyre Peninsula in South Australia during March and April between 2014-2016. Larval data are site means of total *P. xylostella* larvae per 20 sweep net samples (or for sea rocket, per 20 counts after beating into a tray) across March and April. Moth data are the number of male moths trapped in pheromone traps over a 4-week period to early April. Circle size is scaled to the magnitude of counts and open circles represent the absence of *P. xylostella* at a sampled site. Filled and open circles are colour-coded by host plant.

insufficient sampling to accurately detect *P. xylostella* arrival and for analysis retained data from 77 sites including 30 in 2014, 30 in 2015 and 17 in 2016 (Figure 5.1).

Each year, pheromone trapping detected male moths dispersing from alternative natal hosts into canola during germination. Average weekly trap catches of male moths across seedling canola crops during May and June was highest in 2014 (40.1 ± 55.0, mean $\pm$ SD)), lowest in 2015 (4.7 ± 11) and intermediate in 2016 (14.4 ± 23.2), following a similar pattern to the seasonal abundance of *P. xylostella* on wild hosts during each autumn pre-cropping season period.

Immature *P. xylostella* were ultimately detected in 93.5 % ($n = 72/77$) of crops, showing that *P. xylostella* colonised most canola crops each season. From 58 of the 72 colonised crops, samples of the first immature cohort were collected for species identification and measurement of larval head capsules. In total, 439 immature *P. xylostella* were collected, including 430 larvae and 9 pupae, of which 390 were from the first cohort while additional samples were collected later. *Plutella* was morphologically identified in all samples, indicating no misidentifications.

The frequency distribution of head capsule widths for 430 larvae showed four clear peaks corresponding to the four larval instars (Supplementary Figure D.6). The size range of head widths showed some overlap, but was visually estimated from the frequency distribution of head width and used to determine the instar of each larval individual. Of the 390 individuals, 55 % ($n = 216$) were fourth instars, 25 % ($n = 94$) were second instars, 15 % ($n = 57$) were third instars, 4 % ($n = 15$) were first instars and 2 % ($n = 8$) were pupae.

For each colonised site, we used our development model with local temperature data to back-predict the date of first oviposition using the collection date and most advanced developmental stage (instar or pupa) as the modelling biofix. Where immature samples were unavailable, we assumed a fourth instar biofix as this was the most common stage detected. Across the study, most canola crops (71 %, $n = 55/77$) were colonised by *P. xylostella* during April, May or June, soon after germination (Figure 5.5, Supplementary Movie https://doi.org/10.25909/5bebc11b1f1d4). Of remaining crops, 4 % were colonised in July ($n = 5/77$), 13 % ($n = 10/77$) in August and 6 % ($n = 5/77$) in September. The pattern of colonisation varied between years, though median sowing dates for each year were similar (1-7 May). Average colonisation dates were 27 May $\pm$ 18 (SD) days (25 ± 17 days after sowing (DAS)) in 2014, 10 July $\pm$ 39 days (70 ± 43 DAS) in 2015 and 11 June $\pm$36 days (35 ± 26 DAS) in 2016. Colonisation occurred earliest and most synchronously in 2014. In 2015, there a pause in colonisation occurred during mid-winter (July) when cold and wet conditions typically limit *P. xylostella* flight activity.

For each sentinel crop, we assessed the suitability of local weather conditions for *P. xylostella* prior to colonisation by back-predicting generation times from the colonisation date and visualising the CLIMEX GI_W and pheromone trap counts (Supplementary Figures D.9, D.10, D.11). Across all sites and years, with the exception of a single site (NA-Bordertown-2014), a positive GI_W for several generations prior to colonisation across suggested local *P. xylostella* population growth was possible. Climate suitability is a leading indicator of *P. xylostella* abundance. At numerous sites, pheromone trap counts showed good agreement with GI_W lagged (shifted right) by one generation (e.g. NM-MtHope-2014, PA-Arthurton-2015, TR-Karkoo-2016), implying moths may have been derived locally.

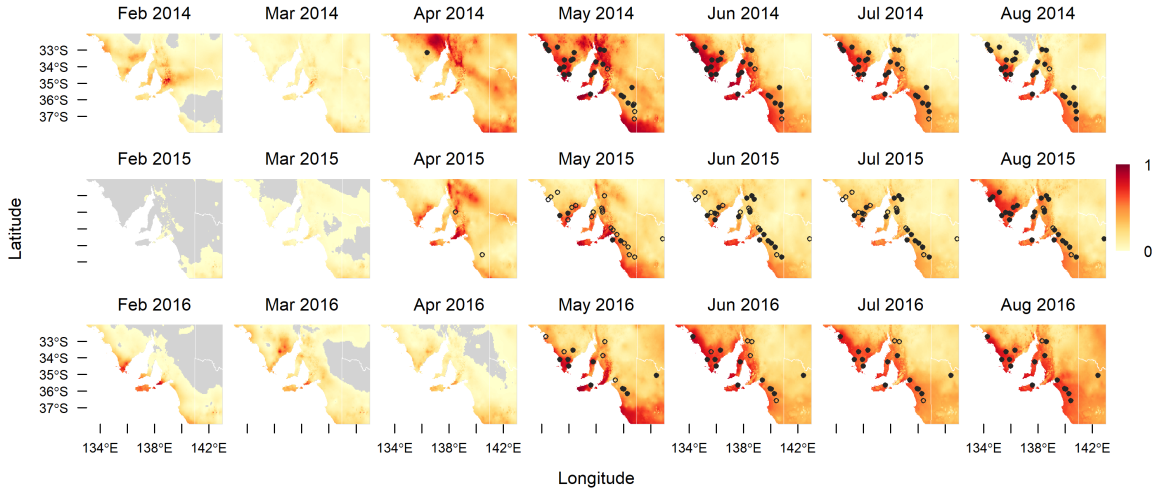

Fig. 5.5 Spatio-temporal maps of South Australia at monthly intervals showing the CLIMEX GI_W and pattern of colonisation of sentinel canola crops by *P. xylostella* over three years. Circles represent sentinel canola crops and show the presence (filled circles) or absence (open circles) of immature *P. xylostella* life-stages. Map colours represent monthly mean of the CLIMEX Weekly Growth Index GI_W for *P. xylostella* scaled from 0 (grey, unsuitable) to 1 (red, extremely suitable) for population growth.

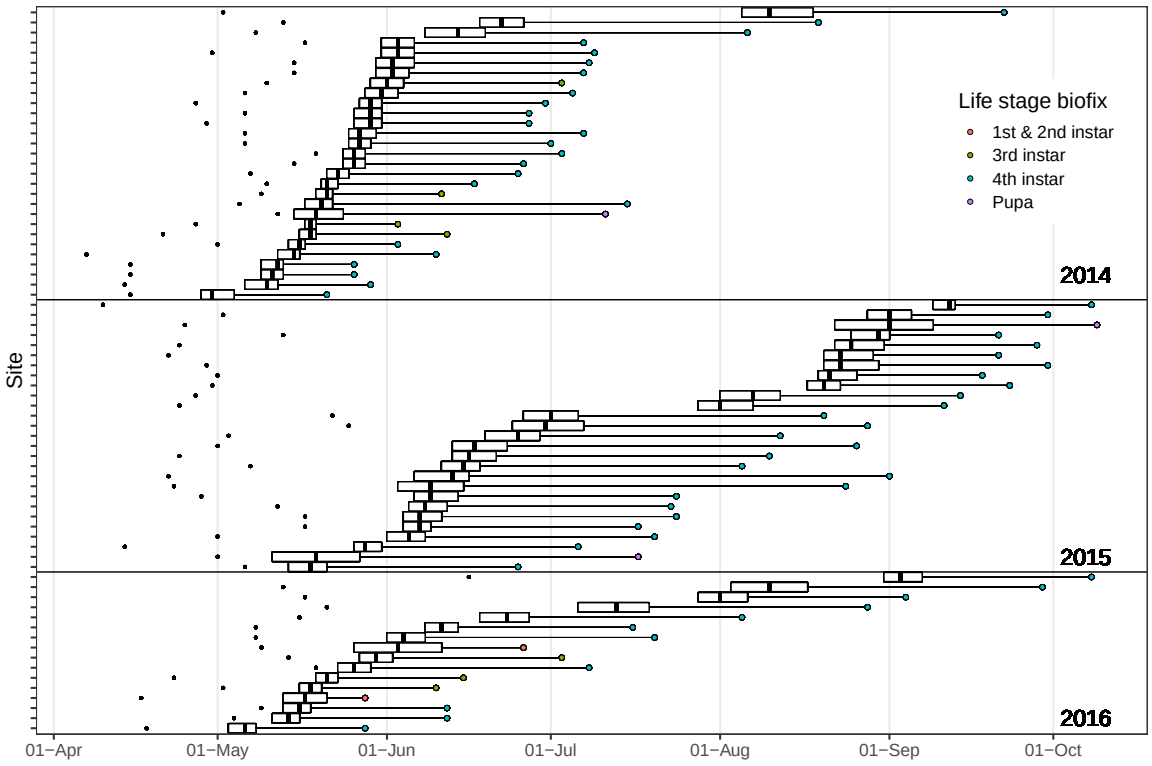

Fig. 5.6 Temporal pattern of colonisation by *P. xylostella* across spatial networks of sentinel canola crops in South Australia in three consecutive years, sorted in ascending date order within year. Small white boxes represent the median date (inner vertical black line) and date range (left and right box margins) of crop colonisation for a site predicted using a temperature-dependent development model for *P. xylostella*. Small coloured circles to the right of white boxes, joined by a line, indicate the modelling biofix based on the detection date and *P. xylostella* developmental stage. Small black circles to the left of white boxes mark the crop sowing date.

Light trapping

Light trap catches of male and female moths peaked during September to November and declined to low levels from December to August or September (Supplementary Figure D.7). At Drummond Point, small flight peaks occurred in May 2014 and April to June in 2015, as crops emerged. Overall, light traps caught more males ($n = 9113$) than females ($n = 6025$), a sex ratio of $>$1.5:1. There was no evidence that sex ratios changed during the season or with the magnitude of flights (Supplementary Figure D.7).

The relative proximity of traps to canola crops varied between trap locations and years, which explains some variation in overall moth abundance. Higher abundance in the Drummond Point trap reflects close proximity to canola and abundant *Diplotaxis*, and proximity ($<$ 1 km) to coastal sand dunes with *C. maritima*. The Drummond Point light trap caught moths in all months except April 2015, suggesting *P. xylostella* persists throughout the year (Supplementary Figure D.8). At this site, moths were more abundant during the non-cropping period (December to May) in 2016 than 2015, in accordance with pre-season rainfall patterns and abundance of brassicaceous vegetation. At Minnipa, Sherwood and Two Wells, where canola or wild brassicas were locally scarce, very few moths were trapped between December and August or September.

Plotting trap catches on a phenological timescale showed that most flight peaks corresponded with peaks one generation earlier, suggesting moths were derived from a local population [185]. At Drummond Point, two peaks in early May 2015 did not correspond with prior peaks in April, implying possible dispersal events (Figure 5.7). Sampling of wild hosts during April 2015 detected larvae on *C. maritima* 24 km away and moths trapped adjacent *Diplotaxis* 78 km away, however abundant nearby hosts not sampled, including *C. maritima*, could have harboured the insect. Interestingly, the Drummond Point trap detected small flights peaks at intervals of ≈one generation from December 2015 to May 2016, a non-cropping period. Given that harvest of canola in spring 2015 could lead to a relatively synchronous emigration, it is possible that a local *P. xylostella* cohort developed through several generation on weeds near the trap.

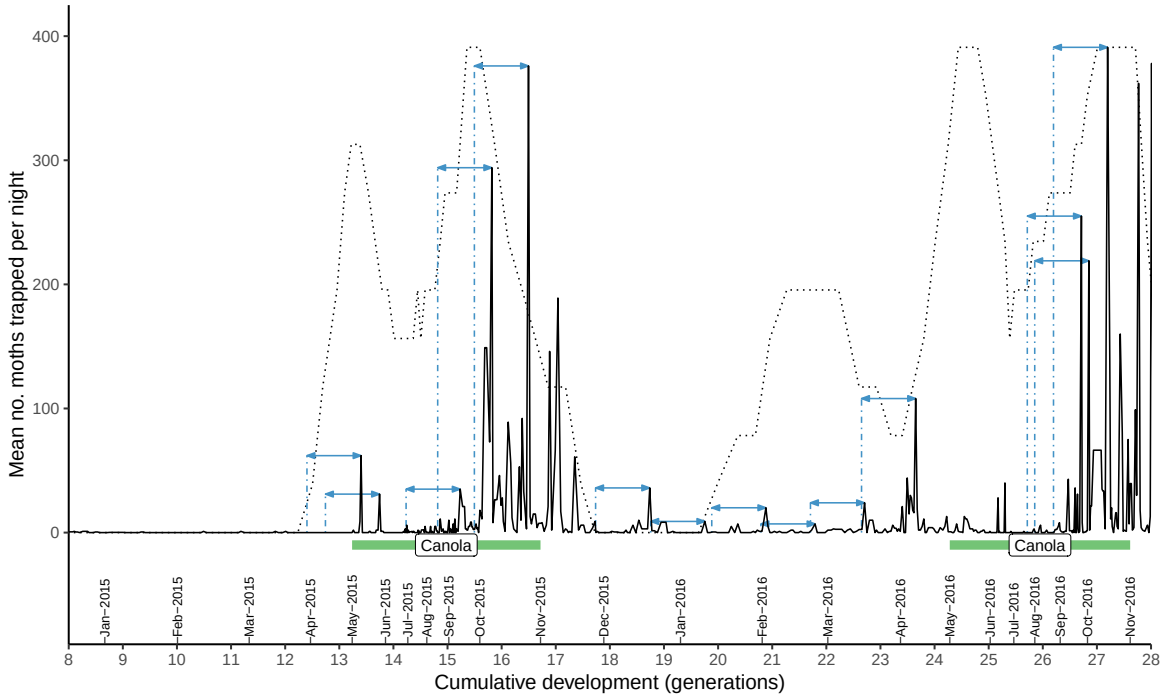

Fig. 5.7 Nightly light trap catch of *P. xylostella* at Drummond Point on the Eyre Peninsula in South Australia plotted on a phenological timescale. Re-tracing flight peaks by one generation (marked with blue dotted lines and arrows) shows the correspondence of flight peaks with prior peaks. The CLIMEX Weekly Growth Index (shown as a black dotted line) is scaled as a proportion of the maximum moth counts.

5.4 Discussion

The dynamics of pests in agricultural landscapes depends on complex interactions between climate, host resources, biotic regulation and dispersal [231, 225, 24]. Although diamondback moth movement has been studied extensively, understanding of movement at landscape scales has been elusive [78]. In investigating the role of movement in the seasonal dynamics of *P. xylostella*, our field sampling was geographically broad and focused specifically on the colonisation process. We aimed to identify local *P. xylostella* populations and potential hosts during the autumn pre-cropping period, weather effects and the timing of canola colonisation to determine whether local or distant sources best explained the observed pattern.

The presence of the cryptic moth, *Plutella australiana* [131, 169], raises the question of species identity in our study. Molecular species identification is required to distinguish these Australian species [171, 169] but was not feasible for most of our samples. In our analysis, we assumed our field data relate to *P. xylostella*. In *Plutella* populations collected throughout southern Australia during 2014 and 2015 overlapping our study, both species co-occurred widely on canola and brassicaceous weeds but 90 % were *P. xylostella*, which occurred at all but one location while *P. australiana* occurred at only one-third of locations [169]. This indicates our assumption is reasonable. Molecular identification of moths from selected light trap samples mostly failed due to low DNA quality and was uninformative.

Regional canola crops were consistently colonised soon after germination each year, allowing *P. xylostella* to develop through multiple generations during each crop cycle. The relative contribution of migration and local build-up to spring populations has been uncertain [77]. Later synchronous colonisation would have suggested a major role for widespread migration [89]. However, gradual population increases through autumn and winter clearly contributes to spring dynamics and damaging outbreaks. Increased movement among crops and weeds in spring may also supplement local populations [95, 89, 185].

The phenology of colonisation varied between years, being earliest in 2014 and latest in 2015. Based on average colonisation dates and seasonal temperatures, our development model predicts that *P. xylostella* developed through 3.27 (2014), 2.45 (2015), 2.46 (2016) generations on canola crops during each cropping cycle (1 May – 31 October). This implied that earlier colonisation (in 2014) could lead to higher populations in spring. *Plutella xylostella* outbreaks occurred in parts of South Australia during spring in 2014, but not in 2015 or 2016. Although based on only three seasons, our findings suggest that early season dynamics may be one indicator of outbreak risk. Whether this pattern is consistent in other regions requires investigation. *Plutella xylostella* outbreaks occur more frequently in parts of Western Australia where thermal conditions allow up to 5 generations per crop [185] and colonisation timing may be even more important. Longer term population datasets are

required to link colonisation with spring dynamics. An ability to forecast outbreak risk in autumn would provide pest managers information far enough in advance to be useful for management [142, 253].

Engaging widely with agronomists allowed us to collect field observational data at a regional geographic scale [215]. The consultants were experienced with insect pest management and no cases of mis-identifications occurred. The colonisation data showed patterns consistent with observed weather and suggested that data quality was high. Our stage-specific modelling approach to identify oviposition timing should have improved the precision of our estimates of colonisation timing. Sampling error caused by delayed detection of immature *P. xylostella* (e.g. weekly sampling) or variation in sampling efficiency between sites was accounted for by incorporating lifestage in the biofix for each model. Effectively then, the total sampling effort per crop accumulated across each week, which was an advantage. Although our *P. xylostella* model is yet to be validated in the field, and we used derived temperatures to run the models, any limitations associated with our assumptions were consistent. Combined sampling and modelling facilitated useful comparisons of *P. xylostella* phenology across sites and years.

Non-crop hosts are central to the ecology of insect pests in agricultural landscapes [256]. *Plutella xylostella* exploits a diversity of brassicaceous plants in Australia [95, 77, 66, 88] and their role in recorded *P. xylostella* outbreaks in canola has been implied but never investigated [77]. We found that two summer-growing taxa, *Diplotaxis* species and *C. maritima*, were of particular importance as autumn hosts for *P. xylostella* in the areas sampled. Our observations and data suggested that *Diplotaxis* is a sub-optimal host while *C. maritima* may be highly suitable, which could be further investigated in preference-performance studies. Despite potential low suitability, *Diplotaxis* species occurred across thousands of hectares and could produce millions of *P. xylostella* individuals [see 87, for an analogy]. *Cakile maritima* provided a consistent refuge and may be important in *P. xylostella* ecology in Australia. *Diplotaxis* was largely absent in the sampled areas of the South East region, highlighting spatial variation in the availability of host plants.

On the Eyre Peninsula, pre-season abundance of host plants showed striking temporal variability within and between years, driven by pre-season rainfall. Elevated pre-season rainfall has preceded some recorded outbreaks but not others, making relationships with rainfall unclear [185, 77]. We observed rapid senescence of wild brassicas over four weeks during autumn, reducing host plant suitability for *P. xylostella* [32], which is likely to trigger dispersal [115, 54, 33]. Given the marginality of summer conditions for weeds and *P. xylostella*, the ecosystem is sensitive to rapidly changing seasonal conditions. Rainfall that promotes weed growth in summer may be a "dead end" for the insect in many years unless

follow-up rain provides a green bridge of suitable plants until canola crops become available. Rainfall also needs to fall where *Brassica* plants occur. The timing and location of rainfall, not just magnitude, and the vegetation response must be important in determining pre-season *P. xylostella* abundance and may explain variability in previous broad analyses of climate effects [77].

Multiple lines of evidence suggest that localised origins, rather than longer distance immigration, explain the observed colonisation pattern. Consistently each season, a widespread *P. xylostella* population was present during March and April, moths dispersed into canola crops during germination in May, and most crops were colonised soon afterwards. Climate conditions were suitable for *P. xylostella* population growth for several generations preceding colonisation. Across regional crops, there was no synchronous pulse in colonisation in any year to suggest a large-scale immigration, and light trapping during May to July (colonisation period) did not detect widespread flights.

Our findings do not preclude immigrations occurring later in the season. CLIMEX modelling predicted that arid pastoral areas to the north of agricultural zones become climatically suitable for *P. xylostella* establishment at certain times of the year. Our sampling focused in agricultural zones, but a diversity of introduced and native Brassicaceae occurs across vast inland areas following winter rainfall [10]. In late winter in 2014 and 2015, we found *P. xylostella* on Ward's weed, *Carrichtera annua*, an annual herbaceous plant, in non-cropping zones >100 km to the north of the Eyre Peninsula. The senescence of *C. annua* and other brassicas in spring could potentially trigger widespread dispersal flights. Northerly winds seasonally transport *Helicoverpa* species from winter breeding areas in the inland into southern cropping zones in spring [142], and could also transport *P. xylostella*. A better understanding could be gained by monitoring *P. xylostella* dynamics in non-cropping areas.

Local survival in an area could enable insecticide resistant genotypes to persist [228]. Survival of *P. xylostella* throughout summer in canola-growing areas was not explicitly assessed in our study but seems likely. CLIMEX predicted that coastal areas proximate to canola production should support permanent *P. xylostella* populations, and light traps detected moths in most months, even though brassicas were locally scarce in the vicinity of most traps. Periods of zero GI_W do not preclude local survival of *P. xylostella* in favourable microclimates [130], as demonstrated by *P. xylostella* occurring on *C. maritima* during the particularly dry autumn in 2015. Insecticide bioassays showed that *P. xylostella* populations collected from *Diplotaxis* and *C. maritima* during April 2014 had near-identical insecticide resistance profiles to populations collected from canola crops in South Australia between 2012 and 2013 [17]. This indicates frequent movement between canola and unsprayed weeds, as reported previously [66].

Chapter 6

General synthesis and conclusions

Dispersal is central to the ecology and evolution of species and populations [158]. It drives colonisation and extinction in local areas and leads to genomic exchange with consequences for local adaptation and speciation [30]. Mobile insect pests are a global problem for agriculture and knowledge of their movement patterns in annual cropping systems is essential for tactical management.

The aim of this thesis was to provide new understanding of the dispersal of *P. xylostella* at a landscape and regional scale and the process of its seasonal colonisation of winter canola crops in southern Australia. Prior to this work, ecological datasets describing the seasonal dynamics of this species in Australian canola did not exist [77]. It was envisaged that deeper knowledge of the role of dispersal and the source areas of immigrant populations could provide a basis for future ecological studies to build on, and thereby contribute to the future development of forecasting capacity and insecticide resistance management strategies for the Australian canola industry.

The movement of *P. xylostella* was investigated from multiple angles: First, population genetic studies assessed patterns of gene flow and population structure in *P. xylostella* from different geographic locations and brassicaceous hosts throughout the Australian cropping zone (Chapters 2, 3, 4). Second, extensive *Plutella* collections were used to identify the geographic distribution, host associations and genetic structure of the cryptic species, *Plutella australiana* (Chapter 3). Third, the seasonal dynamics of *P. xylostella* and its colonisation patterns in canola crops were assessed in South Australia (Chapter 5). In this section, the key findings of the research are synthesised and the implications discussed.

6.1 Research overview

Cryptic *Plutella* species

The reporting in 2013 of the discovery of *Plutella australiana* [131] took diamondback researchers by surprise, and in the absence of any knowledge of its biology, raised questions for pest management in *Brassica* crops. Using diverse datasets, it was shown that *P. australiana* is widely distributed in Australia but is not a significant pest threat. For Australian *Brassica* growers, current pest management tactics targeting *P. xylostella* do not need to account for the possible presence of *P. australiana*. Insecticides should provide high-level control of this species and there is presently no evidence to suggest it may evolve insecticide resistance. For researchers, undetected cryptic diversity can confound results [96, 51, 205]. Now that *P. australiana* is known, molecular studies will benefit from the ability to readily distinguish the two *Plutella* species using a simple molecular assay, which will need to be a routine step in Australia (Chapters 2, 3). For ecological studies, resolving species identity is more difficult, and projects may require additional resources to perform molecular species identification for a subset of samples where possible.

The findings across different experimental datasets provided a coherent hypothesis about how divergence between the two Australian *Plutella* species arose. Although reproductive isolation is incomplete in laboratory studies, contrasting genomic structure, *Wolbachia* infections, and phenotypes suggested the two species have come into secondary contact following allopatric divergence (Chapter 3). The existence of two cryptic *Plutella* species provides a model system for evolutionary biologists interested in hybridisation, genetic and behavioural mechanisms of reproductive isolation and interactions with endosymbionts. *Plutella australiana* can also provide a useful outgroup for rooting molecular phylogenies. Further exploring the apparent differences in host use between the *Plutella* species could provide insights into mechanisms of host adaptation in *P. xylostella*. As the work focused in cropping zones, the host plants utilized by both *Plutella* species in inland areas was not explored. Future sampling in non-cropping zones would be useful in identify additional host plants *of Plutella australiana* and informing the extent to which these areas may harbour populations of both *Plutella* species during favourable seasons.

Population structure and gene flow

Studying population structure can provide insights in patterns of gene flow among geographically distinct populations [190]. This approach essentially estimates dispersal at an evolutionary scale through its effects on allele frequencies [30]. In this work, population

genetic studies of *P. xylostella* were conducted at an Australia-wide scale to infer dispersal patterns among geographic areas and brassicaceous host plant species.

A previous assessment using six microsatellites found little structure in Australian *P. xylostella*, but clear differentiation from populations in Asia and Africa [65]. The large size of the Australian continent suggested some structure should exist, and modest number of markers used left open the possibility that weak structure had not been detected. The recent advent of massively parallel sequencing (MPS) platforms and associated genotyping methods provided the opportunity to re-assess this question using a more powerful marker set [84, 4].

Although most population genetic studies of *P. xylostella* still use microsatellites [243, 250], RAD-seq [13] generated thousands of useful genome-wide markers (Chapter 2). The detection of weak population structure among some laboratory-reared populations, even though it was the result of inbreeding, provided the impetus for a comprehensive assessment of Australian *P. xylostella* field populations (Chapter 4). Despite extensive collections spanning several thousand kilometres, genome-wide SNPs from a powerful genome-wide marker set confirmed that Australian *P. xylostella* forms a homogenous population across neutral markers. This agreed with Endersby et al. [65], and the high statistical power of SNP markers used confidently resolves the nature of population structure. Endersby et al. [65] argued that low genetic diversity in Australian *P. xylostella* precludes the use of neutral markers to identify movement patterns, which is supported by this work. It remains an open question whether MPS methods employed here might be more informative in other countries where *P. xylostella* has higher genetic diversity. This question might have been answered if outgroups from other regions had been included.

Gene flow has implications for insecticide resistance management [34, 66]. The RAD-seq data did not allow us to conclude that the Australian *P. xylostella* population is panmictic, as this would imply random interbreeding among populations across the continent regardless of geographic proximity. *Plutella xylostella* shows no geographic population structure across genome-wide neutral SNP markers, but this might reflect recent ancestry rather than current patterns of gene flow [202, 77]. However, if this species did arrive in Australia through a small founding population [202, 52, 169], then its current widespread distribution itself demonstrates a capacity for geographic gene flow. Rare long distance dispersal events could explain genetic homogeneity. At evolutionary scales, very few migrants per generation effectively homogenise allele frequencies [217, 151], particularly in species with large effective population sizes, such as *P. xylostella*, or where genetic diversity is limited as observed in Australian populations [202]. It is also possible that longer distance dispersal occurs relatively frequently, either through long distance flight or population redistribution across successive generations. Light trapping data from northern Tasmania provide circumstantial

evidence that the magnitude and frequency of *P. xylostella* flights into the region from the Australian mainland in late spring and early summer may have increased since the advent of massively increased canola production [204], though other factors (e.g. local weather, wind patterns, landscape composition) could also explain this pattern. Evidence for gene flow suggests that resistance alleles arising in one area could quickly spread to other areas, where they have the potential to be locally selected to a high frequency through insecticide use [193].

The seasonal colonisation pattern in canola

In investigating the role of dispersal in the seasonal dynamics of *P. xylostella*, there were two reasons that the work focused particularly on colonisation. First, the arrival timing of *P. xylostella* has a significant bearing on the potential for population increase within crops. Second, the problem of distinguishing immigrant from local moths does not arise during colonisation. Identify the arrival of immigrants provided a unique chance to infer their origins.

In South Australia, combined observational and modelling datasets revealed the seasonal invasion of winter canola by *P. xylostella* (Chapter 5). Networks of sentinel canola fields were successful in measuring colonisation of canola across a large area. A key finding was that most crops were colonised soon after crop emergence, earlier than previously assumed [89]. This occurred each year despite a wide contrast in pre-season populations in local areas, which suggests that this is a consistent pattern in this region. Local *P. xylostella* population increase within canola crops clearly contributes to population size in spring, though does not preclude a role for migrations to contribute to spring population recruitment. In late winter, non-cropping areas to the north of cropping zones contain suitable brassicaceous host plants that support *P. xylostella*. Movement among infested fields is likely to increase in spring in association with rising temperatures and *P. xylostella* populations, and the senescence of weeds [95, 185].

A key question was where the colonising moths originated. Field sampling and CLIMEX modelling suggested that immigrants came primarily from the surrounding landscape rather than further afield. According to our temperature-development model, dispersal of moths in mid-May would suggest development on alternative natal hosts during April. Autumn surveys confirmed that wild brassicas and *P. xylostella* were widespread at that time. Inter-seasonal differences in the timing of colonisation showed an expected pattern based on prior local weather conditions. It is noteworthy that links between *P. xylostella* abundance and seasonal weather patterns only became apparent across the three-year dataset, where a wide

inter-annual contrast facilitated comparisons. This highlights the importance of multi-year datasets in ecological studies.

The work did not explicitly demonstrate that *P. xylostella* persisted throughout summer, as sampling occurred mainly in late summer and autumn, but it seemed likely. CLIMEX modelling predicted that local populations should persist in canola cropping zones of southern Australia (Chapter 5). Although the Weekly Growth Index was zero at times, this did not preclude the insect surviving in favourable micro-climates [130]. Light traps captured moths in most months of the year, sometimes in very low numbers in summer, even though brassicas were locally scarce in the vicinity of traps. Permanent persistence of *P. xylotella* in an area means resistance alleles can persist and be passed to subsequent generations [228]. The detection of insecticide resistance in populations from *Diplotaxis* and *C. maritima* provides clear evidence that this is occurring [17].

An R package dbmdev [170] was developed to facilitate temperature-dependent modelling of *P. xylostella* using stage-specific parameters. The tool allows rapid prediction of *P. xylostella* phenology at one site or simultaneously at many sites, facilitating phenological predictions across geographic scales. Briere's model formula II [28] was selected as it performs well among non-linear models for arthropod development [178] including *P. xylostella* [e.g. 82, 145], and has biologically meaningful parameters. Although numerous temperature-development models have been developed for *P. xylostella* [137, 145], few examples exist of applying models to predict *P. xylostella* phenology in field situations [146]. This work provides a successful case study. The dbmdev package can be used by other biologists working on *P. xylostella*, or other arthropods simply by fitting and supplying the species-specific parameters for Briere's function. Future development of the package will include allowing the user to select and use any desired modelling function [e.g. 178].

6.2 Future research

This research addressed some important aspects of *P. xylostella* dispersal and ecology across Australia, and has raised a number of new questions. This section reviews the future research opportunities arising from the research.

Forecasting outbreak risk remains an important goal for *P. xylostella* management [77, 78, 134]. In Australia, insecticides currently effective for *P. xylostella* have few alternative use patterns in canola crops [17]. This minimises selection pressure, but also means that these insecticides can be in short supply during outbreaks, leading to growers' inability to prevent substantial crop losses. Early warning could aid preparedness, such as enable sourcing of insecticide stocks, industry-wide communication and earlier intervention [142]. Forecasting

capacity requires a thorough understanding of the ecological factors driving the seasonal dynamics of *P. xylostella* [77].

In South Australia, the seasonal pattern of colonisation of canola crops by *P. xylostella* was well-explained by the abundance of wild brassicas, the insect and favourable conditions for its population growth in the local area prior to sowing. Whether this pattern is consistent in other regions is not known. *Plutella xylostella* is arguably a greater problem in parts of Western Australia where thermal conditions are more suitable for its development [185], but ecological datasets do not exist [77]. In marginal low rainfall cropping zones, such as the South Australian Mallee region, canola crops can be devastated by *P. xylostella* during outbreak years, yet summer brassicas are relatively scarce. Similar methods used in this work could be employed in other regions to identify potential local source populations.

Early season colonisation means that *P. xylostella* can develop through multiple generations during each canola cropping cycle (Chapter 5). Ultimately, growers need to make decisions on *P. xylostella* control in spring. The relationships between early and late season *P. xylostella* dynamics remain to be assessed. Population densities could be measured at key times during the crop cycle, including pre-season, at colonisation, in late winter, and in late spring. To link seasonal weather to *P. xylostella* abundance patterns, multi-year datasets could be collected across networks of multiple crops and in outbreak and non-outbreak years. The collection of such data is usually impractical but could be facilitated using collaborative networks of field agronomists providing data for central coordination by experts [e.g. 231, 215], as demonstrated by aspects of this work. Emerging technologies, mobile devices and sharing platforms could assist in harvesting datasets already being collected by agronomists as part of routine crop monitoring. Also, developments in automated trapping technologies could make it possible to deploy large spatial networks of traps to capture data on *P. xylostella* spatial dynamics.

The existence of local source populations in cropping areas does not preclude immigrations from adjacent regions. This work focused on measuring *P. xylostella* seasonal dynamics in cropping zones. CLIMEX modelling showed that vast non-cropping areas become favourable at certain times. Many native and introduced brassicas inhabit inland areas of Australia and could support *P. xylostella*. Future work could assess the seasonal dynamics of *P. xylostella* in non-cropping zones through light trapping and/or sampling of potential larval host plants following rainfall events.

Hypotheses about potential source areas need to be refined. In this work, CLIMEX modelling accounted only for the effects of climate on the potential distribution of *P. xylostella*. The realised geographic niche will always be smaller and limited by the availability of host plants or other biotic regulators [130]. More sophisticated climate models could be developed,

accounting for host plant distribution and phenology. CLIMEX models for key brassicaceous host species, for example, could be overlaid with models for the insect to narrow down putative source areas that can be sampled. This information could be coupled with wind trajectory analysis [77]. Analysis of the data using geo-statistical techniques to identify spatio-temporal patterns in the data [12, 207], or using regression-based approaches to link *P. xylostella* abundance patterns with geographical subsets of weather data [142, 164, 253], could identify putative source regions for further study.

The work presented here has generated the first evidence-based working hypotheses about the movement of *P. xylostella* in Australia, which can be further investigated. As methods become more feasible, intrinsic biogeographic markers may offer an approach for testing hypotheses about *P. xylostella* movement at larger scales [104, 103].

The work showed that non-crop brassicaceous hosts support *P. xylostella* during non-cropping periods and can affect dynamics in canola crops. Preference, performace and developmental studies of *P. xylostella* on different Australian wild hosts may provide information on which species are most important as refuges, and how host ontogeny and senescence promotes emigration [32, 33]. *Cakile maritima* appeared to be a suitable wild host (Chapter 5). Most temperature-development models for *P. xylostella* are based on populations reared on cabbage or other vegetables, however developmental times can vary on different hosts [82]. Temperature-development studies on Australian wild hosts could help refine phenological predictions during non-cropping periods. Our development model for *P. xylostella* [170] could be validated in the field through monitoring cohorts through different periods of the season.

Ultimately, integrated management is dependent on effective biological control [78]. Presently, scarce information exists on the role of natural enemies in regulating *P. xylostella* in Australian canola. Future studies should consider colonisation patterns of natural enemies, their role in outbreak and non-outbreaks years, and interactions with management practices [77].

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Appendix A

Chapter 2 Supplementary Information

Table A.1 Frequency of insecticide resistance alleles for three known point mutations in the voltage-gated sodium channel [67] in nine populations of *P. xylostella*: super-kdr-like (*skdrl*), knockdown resistance (*kdr*), and crashdown (*cdr*).

Locality	Host type	Individuals genotyped	Resistance allele frequency		
		(*skdrl*/*kdr*/*cdr*)	*skdrl*	*kdr*	*cdr*
Esperance, Western Australia	Canola	7/6/8	0.36	0.58	0.06
Nundroo, South Australia	Canola	10/10/10	0.20	0.25	0.10
Calca, South Australia	Wild host	9/9/9	0.38	0.63	0.31
Picnic Beach, South Australia	Wild host	12/9/12	0.33	0.72	0.29
Mallala, South Australia	Canola	10/6/10	0.35	0.67	0.05
Virginia, South Australia	Vegetables	10/10/10	0.30	0.40	0.60
Glenore Grove, Queensland	Vegetables	8/5/8	0.25	0.60	0.19
Mt Sylvia, Queensland	Vegetables	8/6/9	0.31	0.83	0.06
Tenthill, Queensland	Vegetables	10/10/10	0.30	0.40	0.20
By Host Type					
Canola ($n = 3$)	C	27/22/28	0.30	0.45	0.07
Wild hosts ($n = 2$)	W	21/18/21	0.35	0.65	0.30
Vegetables ($n = 4$)	V	36/31/37	0.29	0.51	0.27

Appendix B

Chapter 3 Supplementary Information

Table B.1 The four variable nucleotide sites among the five *P. xylostella* 613 bp COI haplotypes identified in 102 individuals from Australia. Shown are sequences from this study and re-analysed sequences from Landry and Hebert [131] downloaded from dx.doi.org//10.5883//DS-PLUT1. Three haplotypes correspond to those reported by Saw et al. [202]: PxCOI01/PxMt01, GenBank accession: DQ394347; PxCOI02/PxMt06, GenBank accession: DQ394352; PxCOI04/PxMt02, GenBank accession: DQ394348. Nucleotide positions were determined from sequence MF151841. Only positions that differ from haplotype PxCOI01 are shown.

	Nucleotide position					
Haplotype	343	373	541	598	No. individuals	Sequence reference
PxCOI01	T	A	A	A	76	MF151841
PxCOI02	C	.	.	.	1	PHSAU1068-12
PxCOI03	.	G	.	.	1	LOQTC311-07
PxCOI04	.	.	G	.	23	MF151838
PxCOI05	.	.	.	G	1	PHLCD068-12

Table B.2 The eight variable nucleotide sites among the nine *P. australiana* 613 bp COI haplotypes identified in 87 individuals from Australia. Haplotypes PaCOI01 and PaCOI02 were identified among sequences from this study and Landry and Hebert [131], and PaCOI04, PaCOI05, PaCOI08 and PaCOI09 were identified from Landry and Hebert [131]. Nucleotide positions were determined from sequence MF151865. Only the positions that differ from haplotype PaCOI01 are shown.

	Nucleotide position									
Haplotype	202	278	316	382	400	433	481	519	No. individuals	Sequence reference
PaCOI01	T	T	A	C	C	A	G	G	74	MF151865
PaCOI02	C	.	.	.	.	.	.	.	6	MF151831
PaCOI03	C	.	.	.	.	.	A	.	1	MF151885
PaCOI04	C	.	.	.	.	.	.	A	1	MCCAA2949-12
PaCOI05	.	C	.	.	.	.	.	.	1	PHLCA920-11
PaCOI06	.	.	G	.	.	.	.	.	1	MF151836
PaCOI07	.	.	.	A	.	.	.	.	1	MF151883
PaCOI08	.	.	.	.	T	.	.	.	1	LSM1299-11
PaCOI09	.	.	.	.	.	G	.	.	1	LNSWA731-05

Table B.3 Log-logistic regression statistics for dose-response bioassays on *P. australiana* (*P. aus*) and *P. xylostella* (*P. x*) field strains and the *P. xylostella* (S) laboratory reference strain exposed to four commercial insecticides.

Product	Strain	n	Slope (SE)	LC_{50} (95% CL) [mg L^{-1} a.i.]	RR_{LC50}	LC_{99} (95% CL) [mg L^{-1} a.i.]	RR_{LC99}
Coragen	*P. aus*	320	2.016 ± 0.236	0.028 (0.023–0.034)	0.45	0.276 (0.161–0.474)	0.22
	P. x	322	1.363 ± 0.149	0.524 (0.411–0.667)	8.26	15.235 (7.374–31.479)	11.88
	P. x (S)	323	1.528 ± 0.165	0.063 (0.051–0.079)	1.00	1.282 (0.666–2.47)	1.00
Dominex	*P. aus*	320	1.078 ± 0.117	0.032 (0.024–0.042)	0.13	2.267 (0.92–5.583)	0.16
	P. x	320	1.292 ± 0.146	9.792 (7.563–12.679)	41.38	343.317 (158.25–744.816)	24.85
	P. x (S)	320	1.130 ± 0.118	0.237 (0.182–0.308)	1.00	13.815 (5.685–33.574)	1.00
Proclaim	*P. aus*	320	2.073 ± 0.235	0.012 (0.01–0.015)	0.68	0.111 (0.066–0.186)	0.39
	P. x	320	1.254 ± 0.146	0.073 (0.056–0.096)	4.15	2.868 (1.282–6.415)	10.04
	P. x (S)	320	1.652 ± 0.181	0.018 (0.014–0.022)	1.00	0.286 (0.153–0.532)	1.00
Success Neo	*P. aus*	320	2.087 ± 0.293	0.011 (0.009–0.014)	0.14	0.101 (0.056–0.184)	0.14
	P. x	320	1.766 ± 0.196	0.242 (0.197–0.297)	2.94	3.266 (1.805–5.912)	4.65
	P. x (S)	321	2.143 ± 0.255	0.082 (0.068–0.099)	1.00	0.703 (0.417–1.184)	1.00

n, number of insects tested; LC_{50}, dose predicted to cause 50% mortality with 95% confidence limits; LC_{99}, dose predicted to cause 99% mortality with 95% confidence limits; RR_{LC50}, resistance ratio at the LC_{50} dose level; RR_{LC99}, resistance ratio at the LC_{99} dose level.

Appendix C

Chapter 4 Supplementary Information

Table C.1 Power analysis for 1032 SNP marker loci identified in Australian populations of *P. xylostella*. The probability of SNP loci detecting true population differentiation at predefined F_{ST} values according to Fisher's Exact and Chi-Squared tests. Analyses were conducted in POWSIM assuming an effective population size (N_e) of 5000. Simulations for F_{ST} = 0.0027 were conducted for 2014 data only and F_{ST} = 0.0056 for 2015 data only, corresponding to the global F_{ST} values for these years.

	2014		2015	
F_{ST}	Fisher's Exact	Chi-Square	Fisher's Exact	Chi-Square
0.0010	0.9280	0.9580	0.9200	0.9490
0.0027	1.0000	1.0000	–	–
0.0050	1.0000	1.0000	1.0000	1.0000
0.0056	–	–	1.0000	1.0000
0.0100	1.0000	1.0000	1.0000	1.0000

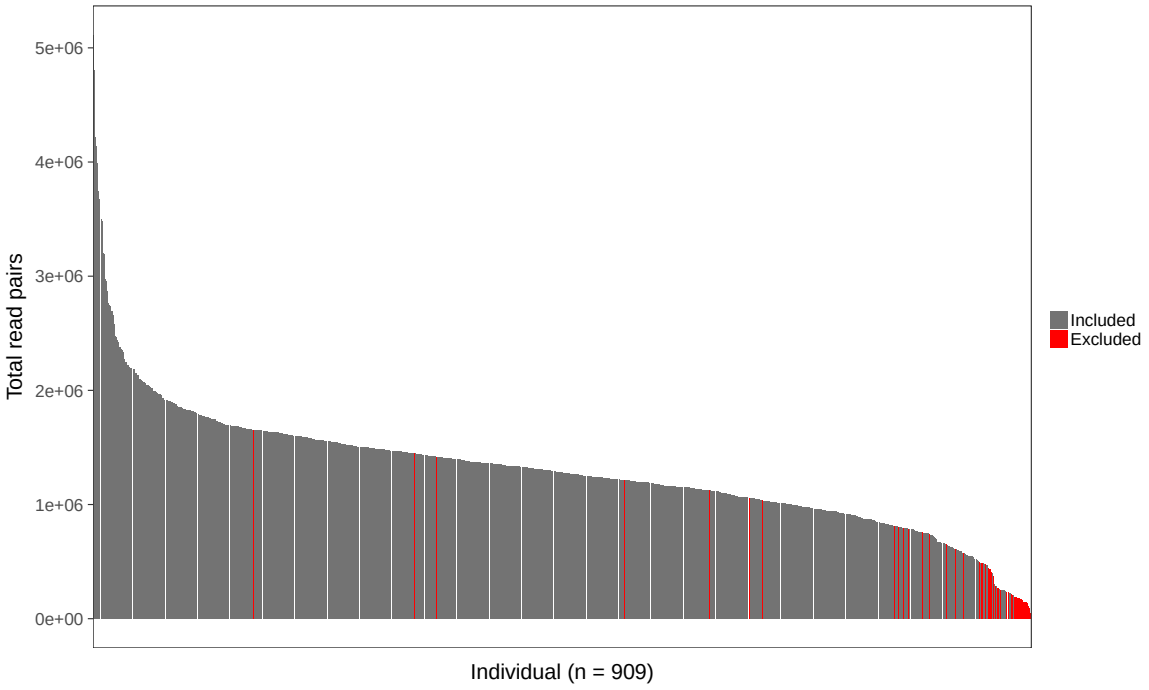

Fig. C.1 Bar plot showing the total number of sequence read pairs per *P. xylostella* individual ($n = 909$ individuals). Individuals coloured grey were included in the analysis while 50 individuals coloured in red were excluded due to greater than 60 % missing data across genotyped SNPs after initial hard filtering. For most excluded individuals, the missing data can be attributed to low sequencing depth.

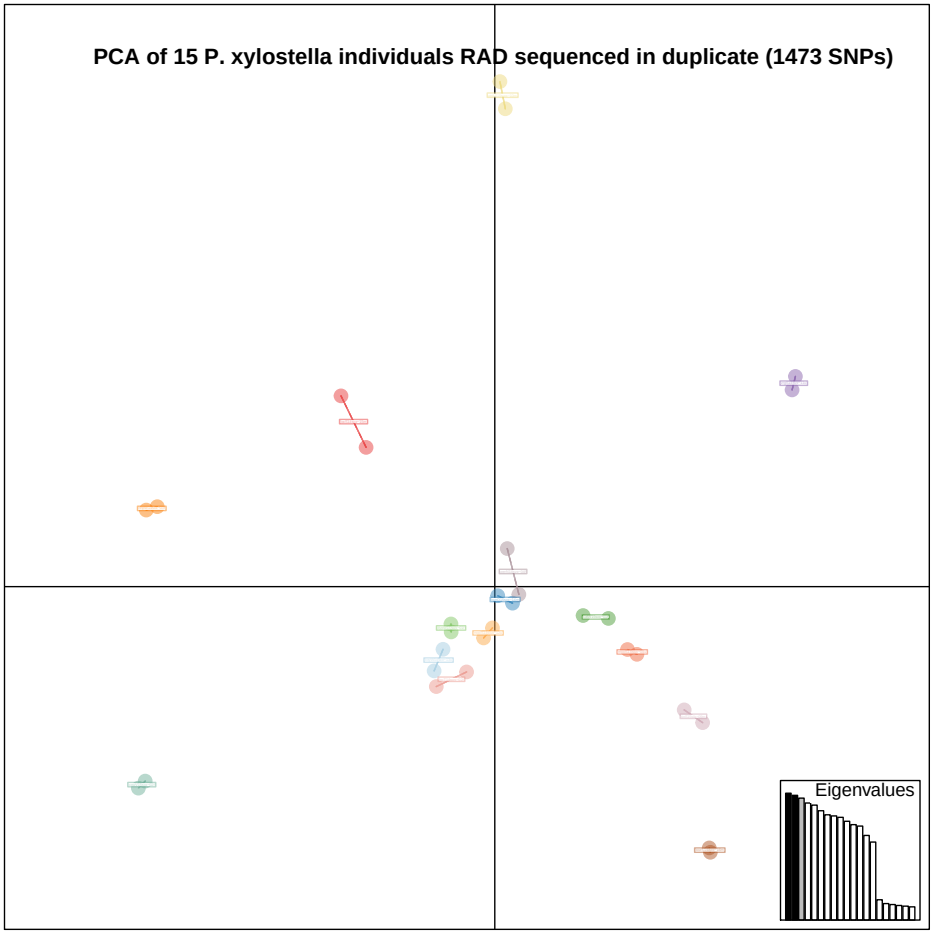

Fig. C.2 PCA for 15 individuals RAD sequenced in duplicate and genotyped using the same bioinformatics pipeline and parameters in our study. The 15 individuals were randomly selected from among the 906 individuals in our study in order to cross-check a representative sample of genotypes. A duplicate sample of each individual was prepared in a different RADseq library from the corresponding original sample for sequencing and genotyping. A total of 1473 SNPs genotyped in 80 % of individuals were used for the PCA with missing data imputed by taking the mean of allele frequencies. The 30 genotype samples are colour-coded according to their individual of origin. The duplicate sample pairs all group closely together, indicating that genotypes have been correctly assigned.

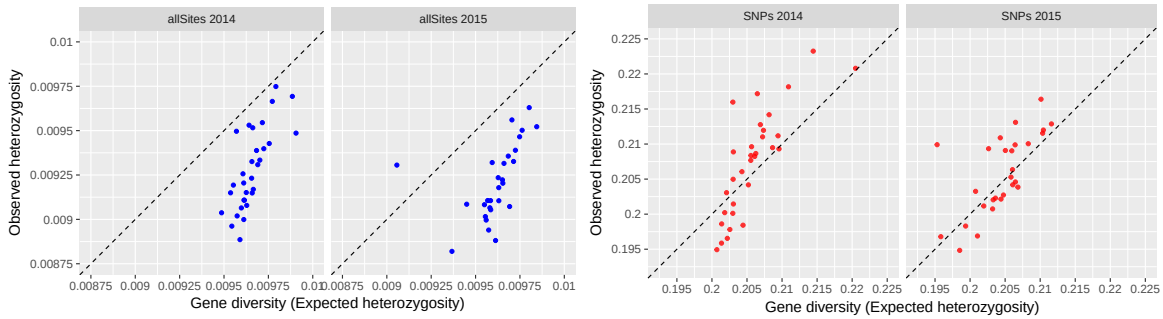

Fig. C.3 Population means for observed versus expected heterozygosity across 590 068 variant and invariant genome-wide sites (left panels, blue dots) and 1032 genome-wide SNPs (right panels, red dots) for *P. xylostella* populations collected from Australia in 2014 ($n = 31$ populations, 434 individuals) and 2015 ($n = 28$ populations, 399 individuals).

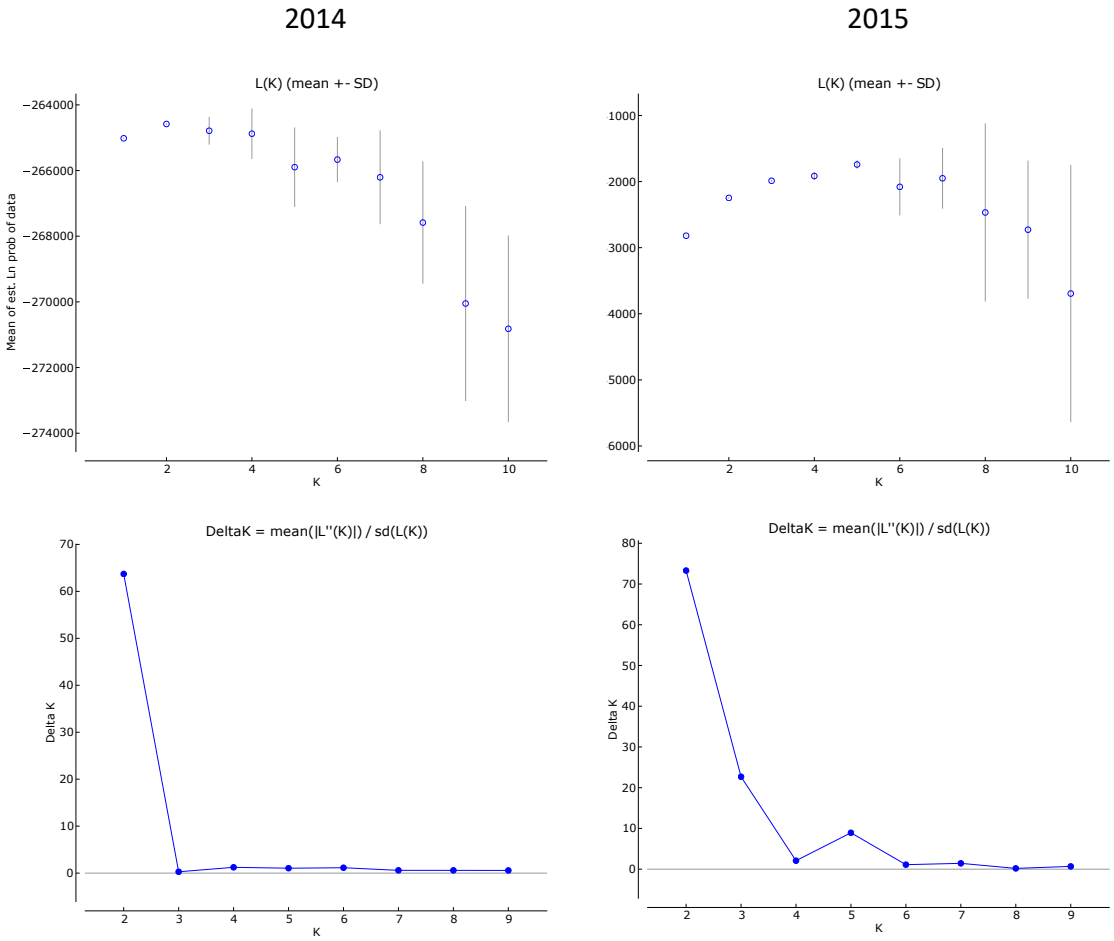

Fig. C.4 Top panels: Mean log-likelihood ($L(K)$) value over 15 STRUCTURE runs for each K value for *P. xylostella* in 2014 ($n = 31$ populations, 434 individuals) and 2015 ($n = 28$ populations, 399 individuals). Bottom panels: Plot of delta K based on the same STRUCTURE analysis according to Evanno et al. [71]. In 2014, a clear mode occurs at $K = 2$. In 2015, a mode occurs at $K = 2$ and weaker secondary modes occur at $K = 3$ and $K = 5$.

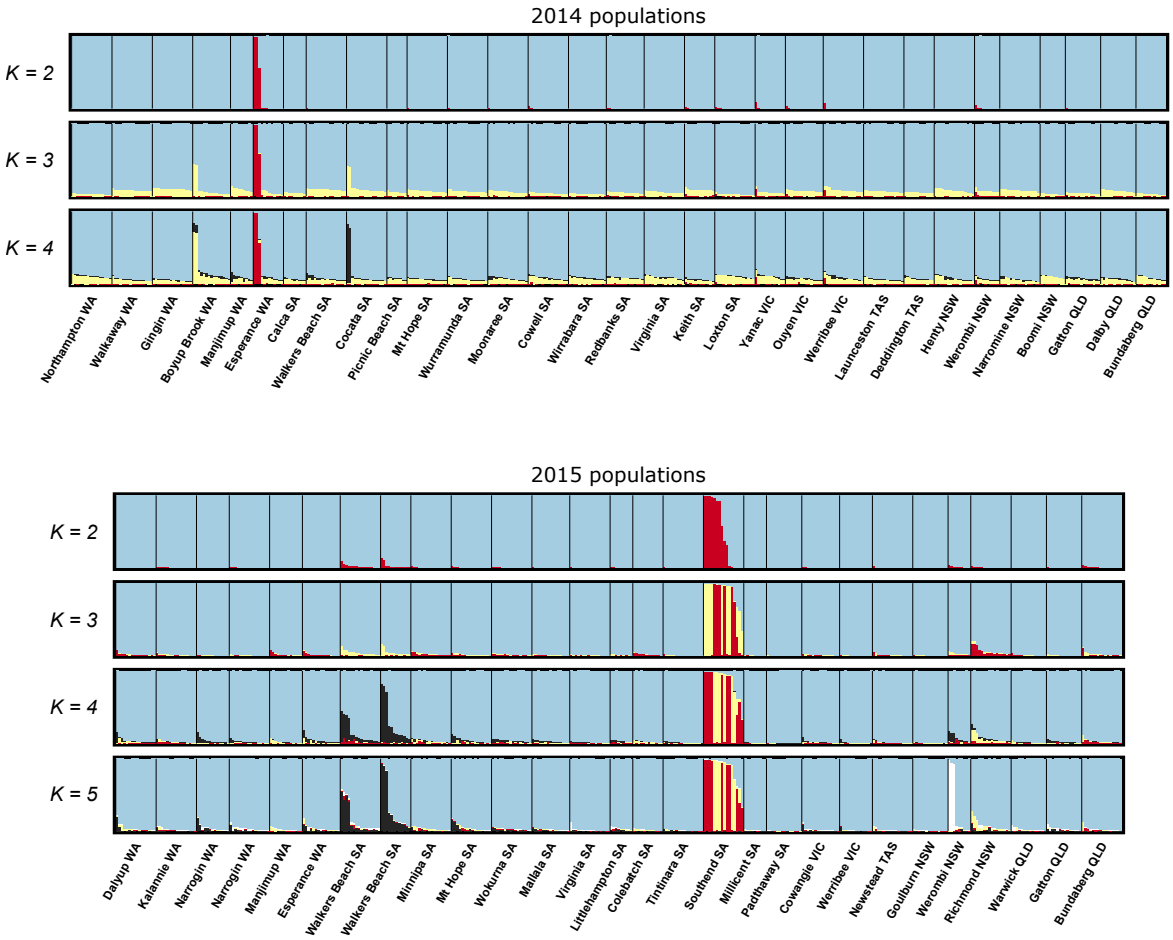

Fig. C.5 Proportional assignment to genotypic clusters, K, based on STRUCTURE analysis of *P. xylostella* individuals from Australia in 2014 and 2015. Individuals are represented by vertical bars and genotypic clusters are represented by different colours. Individuals collected from each year were analysed separately and in both years the data most likely formed two genotypic clusters. Top panel: Analysis at $K = 2-4$ for 434 individuals collected from 31 locations in 2014. Bottom panel: Analysis at $K = 2-5$ for 399 individuals collected from 28 locations in 2015.

Appendix D

Chapter 5 Supplementary Information

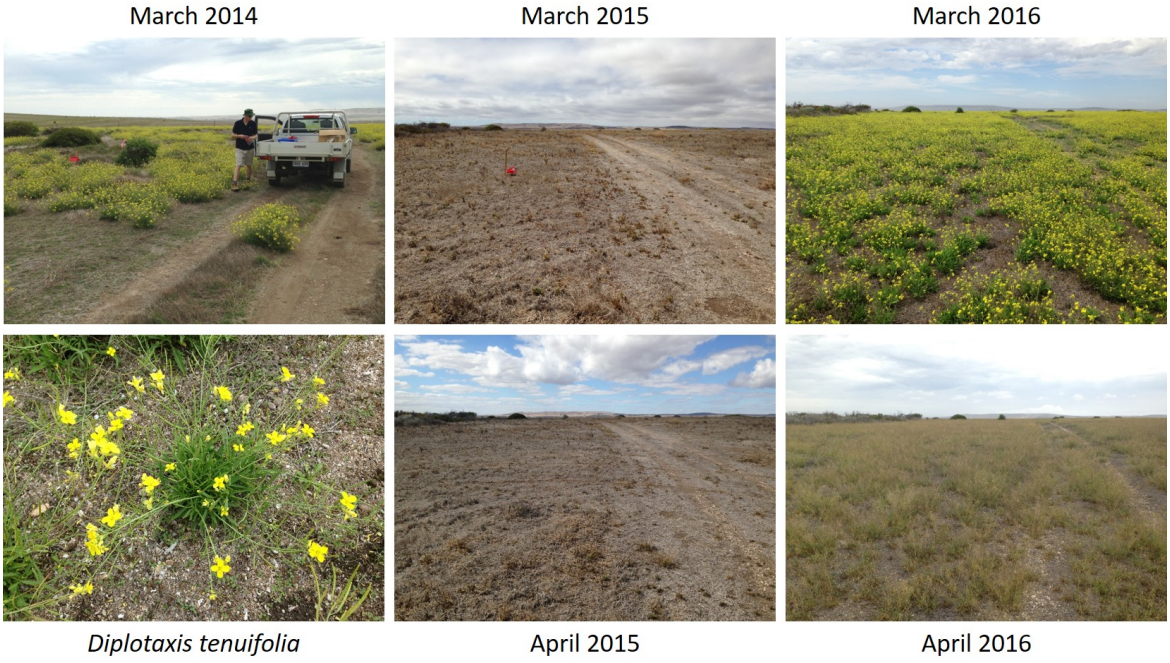

Fig. D.1 Stands of Lincoln weed, *Diplotaxis tenifolia*, photographed at the same location (Drummond Point, Eyre Peninsula, South Australia) and time of year during autumn surveys between 2014 and 2016, showing temporal variation in the abundance and condition of vegetation within and between years. Images were taken in early March and (in 2015 and 2016) four weeks later in early April.

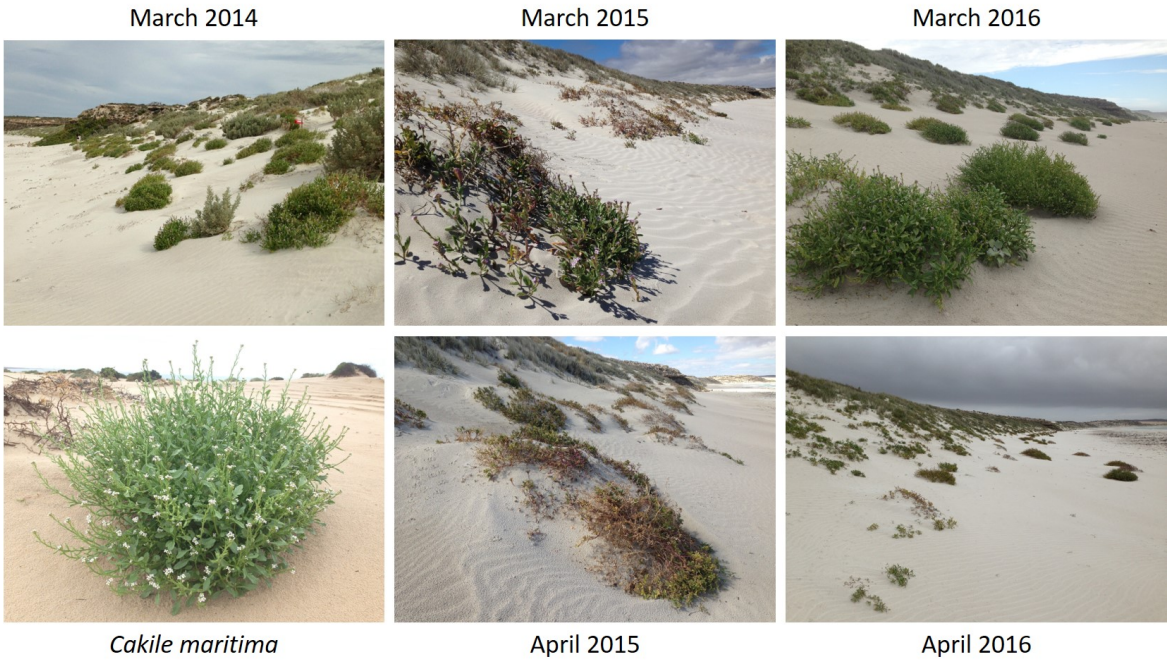

Fig. D.2 Stands of sea rocket, *Cakile maritima*, photographed at the same location (Picnic Beach, Eyre Peninsula, South Australia) and time of year during autumn surveys between 2014 and 2016, showing temporal variation in the abundance and condition of vegetation. Images were taken in early March and (in 2015 and 2016) four weeks later in early April.

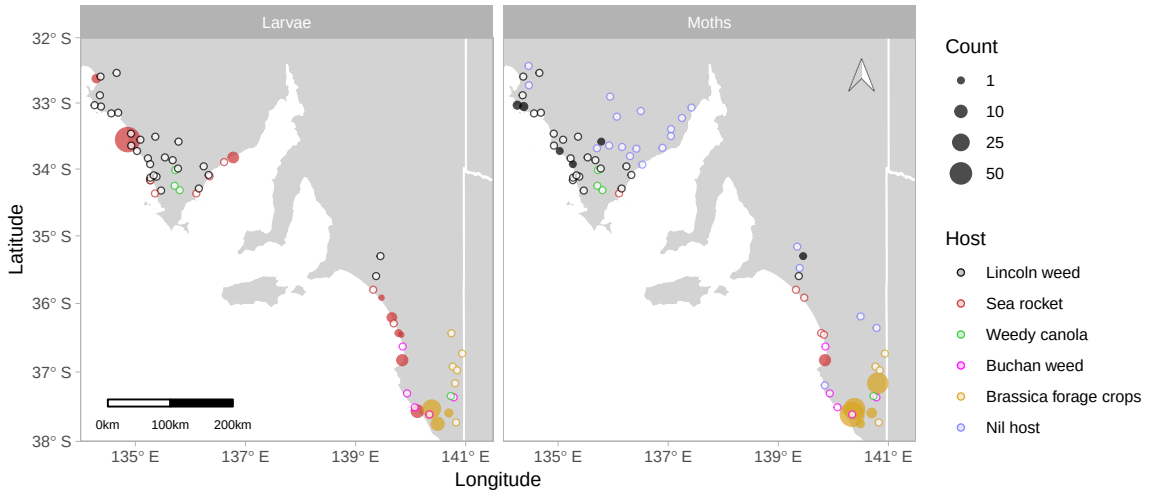

Fig. D.3 Abundance of *P. xylostella* moths (lower panels) or larvae (upper panels) associated with wild brassicaceous plants during annual surveys in the Eyre Peninsula and South East regions in South Australia during March and April between 2014-2016. Larval data are site means of total *P. xylostella* larvae per 20 sweep net samples (but for sea rocket, per 20 counts after beating into a tray) across March and April. Moth data are the number of male moths trapped in pheromone traps over a 4-week period to early April. Circle size is scaled to the magnitude of counts and open circles represent the absence of *P. xylostella* at a sampled site. Filled and open circles are colour-coded by host plant.

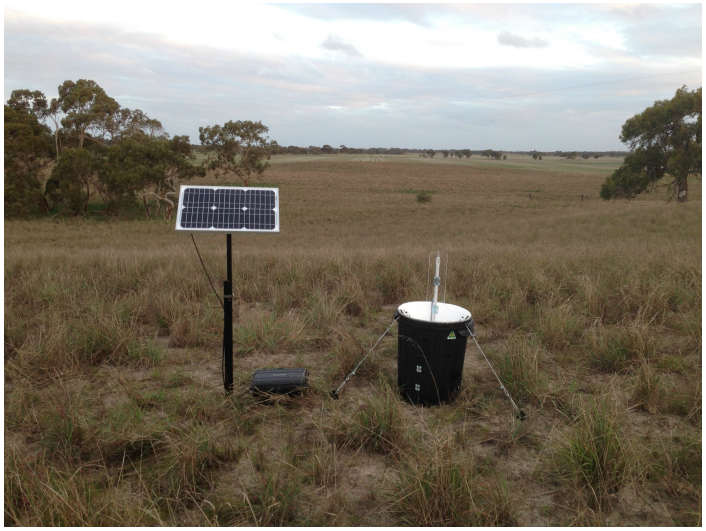

Fig. D.4 The light trap at Sherwood, South Australia. Light traps were situated in areas locally remote from brassicaceous plants in order to trap dispersing individuals.

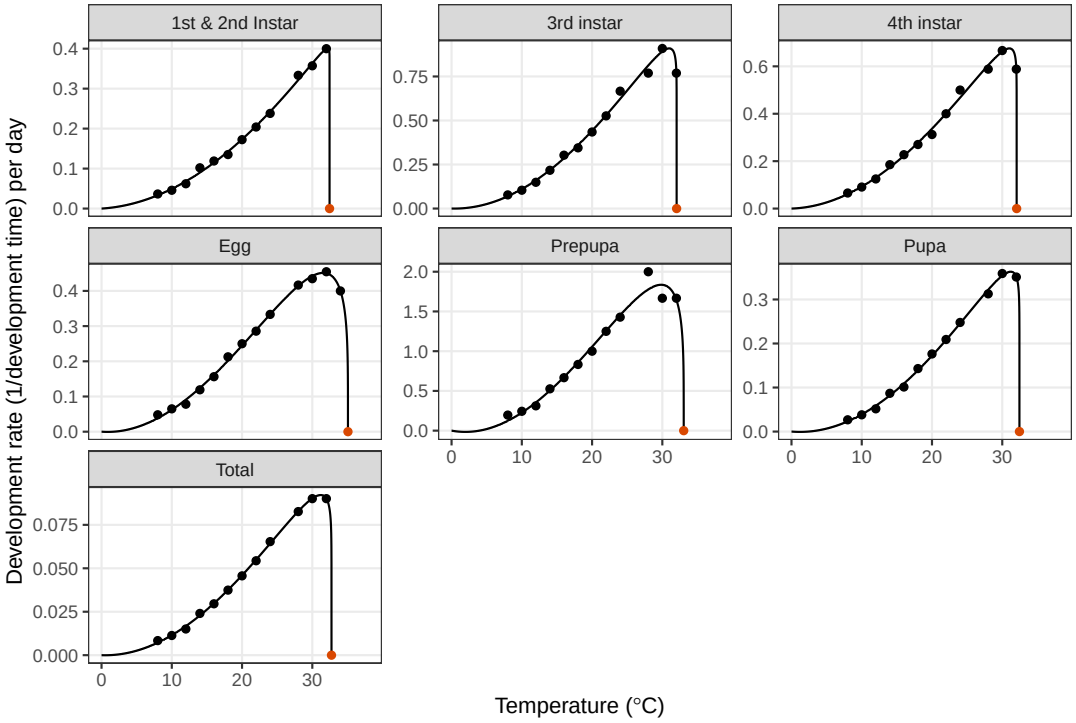

Fig. D.5 Fit of non-linear regression models (lines) to observed temperature-dependent development data (dots) [137] for life-stages of *P. xylostella*. Orange circles represent the upper temperature threshold for development for each stage estimated from Briere's model II formula [28] and were added to force the regression through a *y*-value of zero.

Table D.1 The estimated parameters of Briere's model II [28] for each life stage of *P. xylostella*.

Life stage	a	m	T_{MAX}	T_{MIN}
Egg	0.00036	4.34746	35.07536	1.75372
1^{ST} and 2^{ND} instar	0.00036	60.50944	32.45646	−2.91219
3^{RD} instar	0.00097	14.52704	32.03573	0.78935
4^{TH} instar	0.00067	15.27335	32.04873	−1.22561
Pre-pupa	0.00182	4.31778	33.04467	3.96301
Pupa	0.00040	11.99131	32.44556	2.41717
Total	0.00009	9.94308	32.73126	1.04199

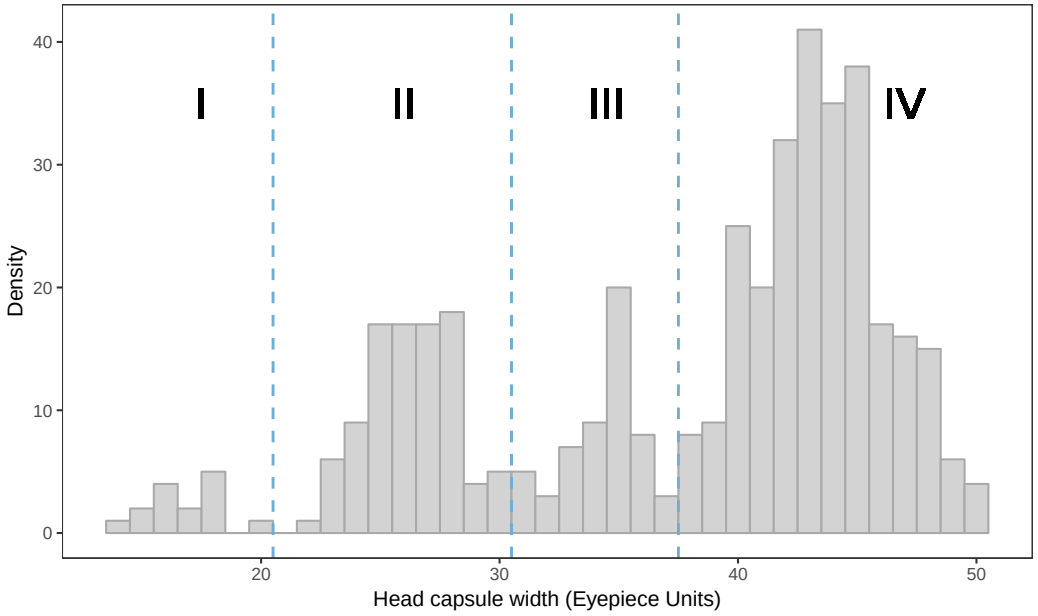

Fig. D.6 Frequency distribution of larval head capsule widths for *P. xylostella* ($n = 430$) collected in canola crops between 2014 and 2016.

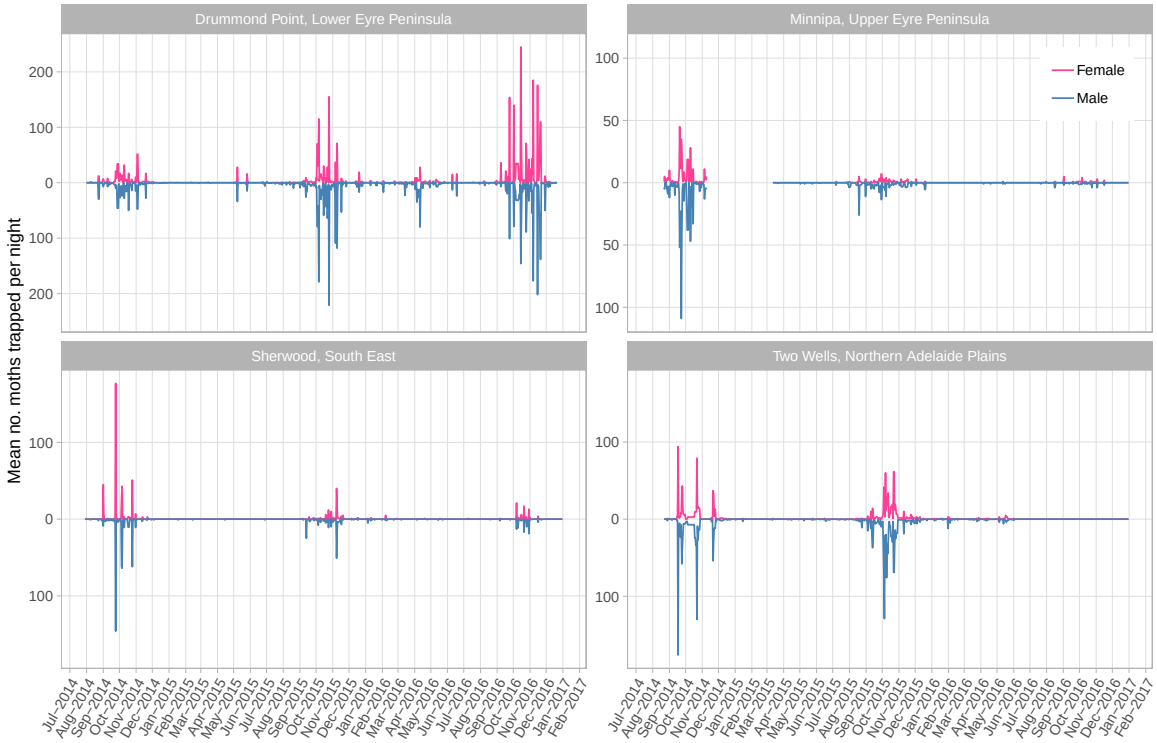

Fig. D.7 Mean nightly catch of male and female *P. xylostella* moths in light traps at four locations in South Australia over two years. At Minnipa, no trapping occurred between January to March 2015 and during January 2016.

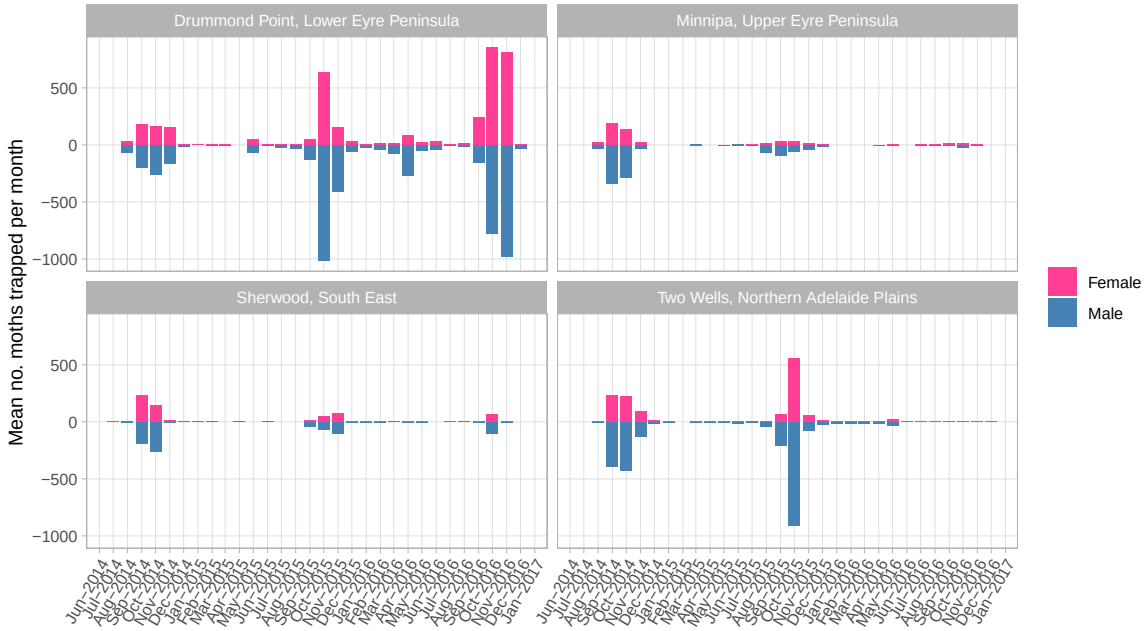

Fig. D.8 Monthly counts of male and female *P. xylostella* moths collected in light traps at four locations in South Australia. At Minnipa, no trapping occurred between January to March 2015 and during January 2016.

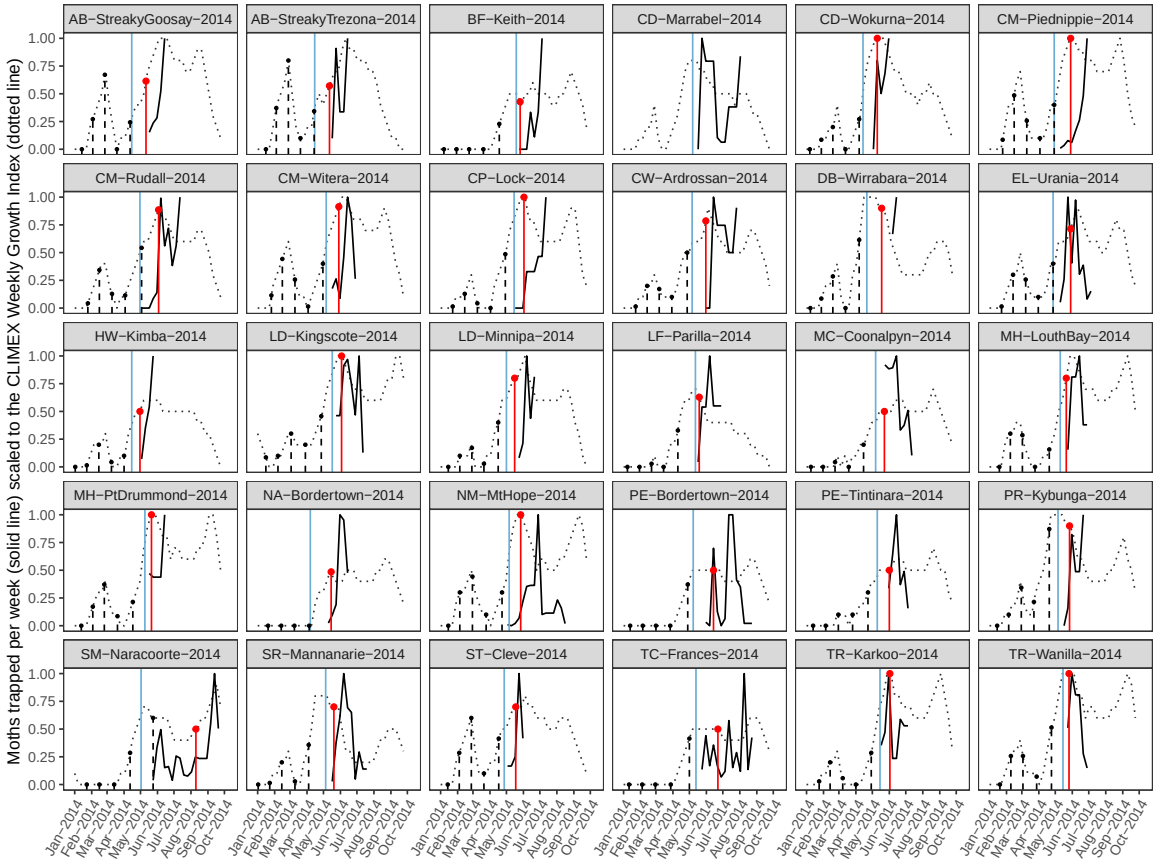

Fig. D.9 Phenology of *P. xylostella* in each sentinel canola crop in 2014. Solid black lines show counts of male moths in pheromone traps scaled as a proportion of the maximum counts (dotted black line). The dotted black line is the CLIMEX Weekly Growth Index (GI_W). The vertical blue line shows the crop sowing date and the vertical red line shows the date of colonisation by *P. xylostella*. Vertical black dotted lines represent five generations back-predicted from the colonisation date using a temperature-dependent development model for *P. xylostella* with local temperatures, allowing examination of (GI_W) prior to colonisation.

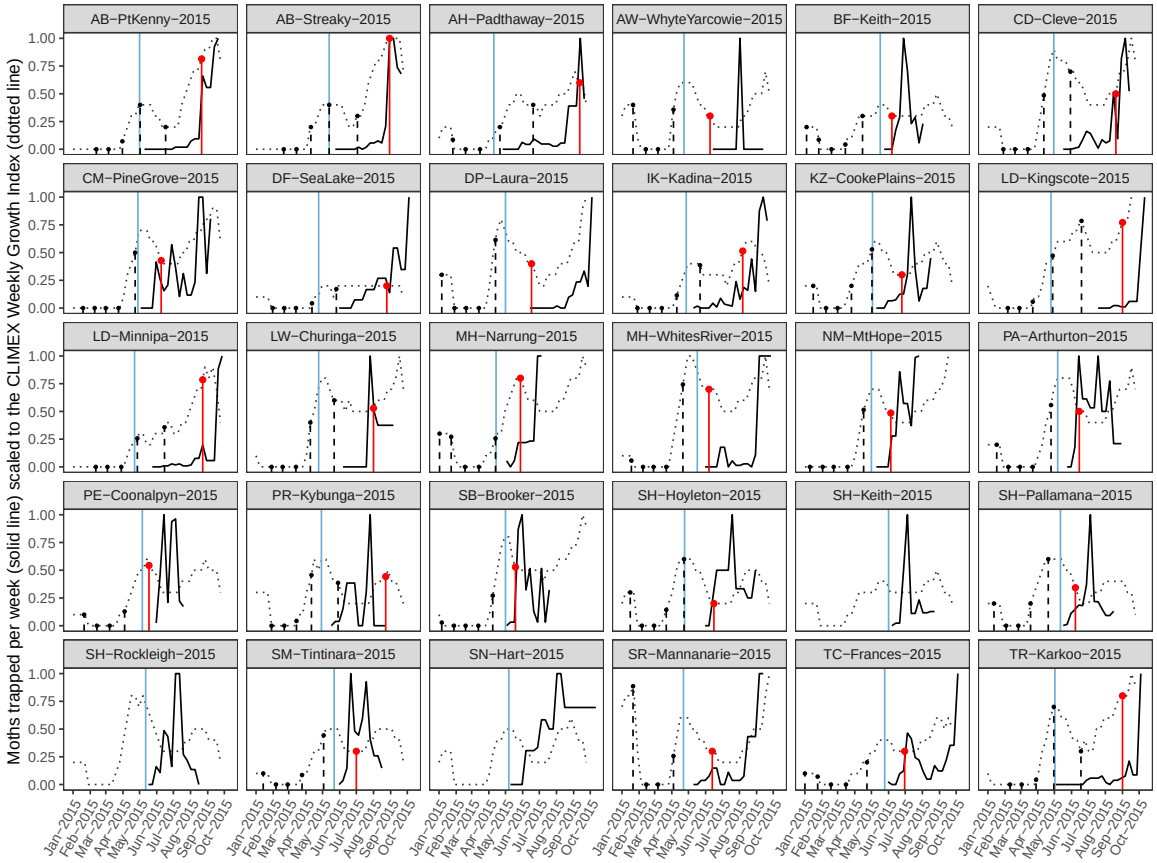

Fig. D.10 Phenology of *P. xylostella* in each sentinel canola crop in 2015. Solid black lines show counts of male moths in pheromone traps scaled as a proportion of the maximum counts (dotted black line). The dotted black line is the CLIMEX Weekly Growth Index (GI_W). The vertical blue line shows the crop sowing date and the vertical red line shows the date of colonisation by *P. xylostella*. Vertical black dotted lines represent five generations back-predicted from the colonisation date using a temperature-dependent development model for *P. xylostella* with local temperatures, allowing examination of (GI_W) prior to colonisation.

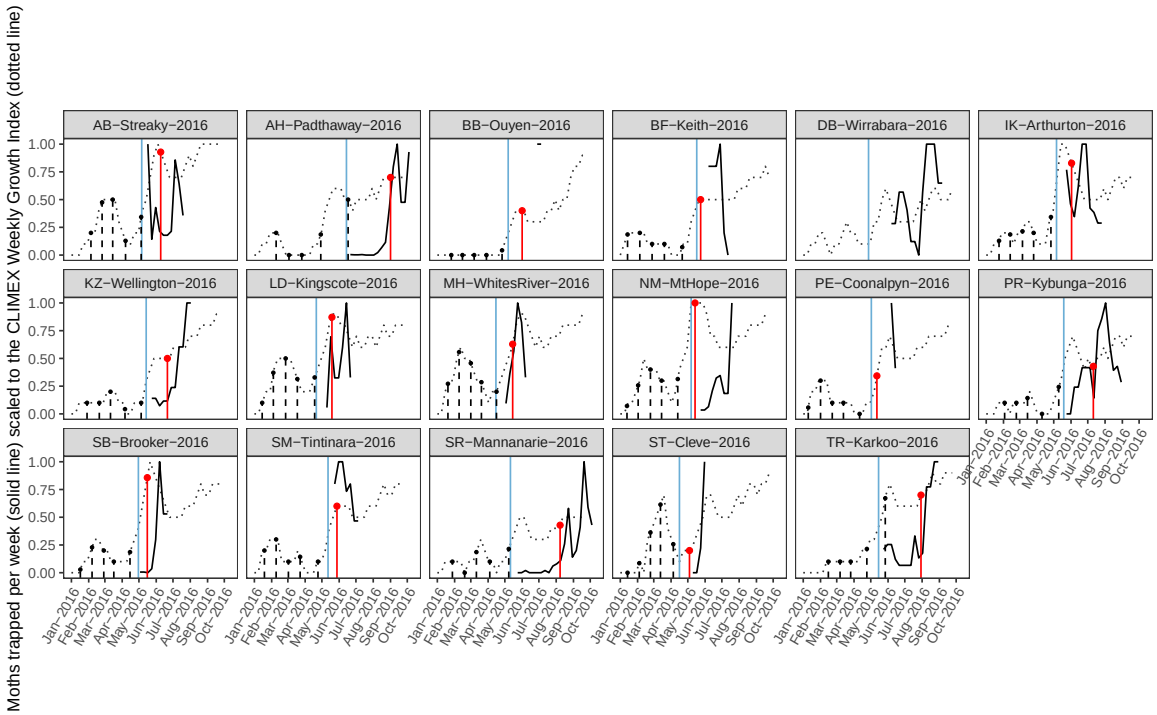

Fig. D.11 Phenology of *P. xylostella* in each sentinel canola crop in 2016. Solid black lines show counts of male moths in pheromone traps scaled as a proportion of the maximum counts (dotted black line). The dotted black line is the CLIMEX Weekly Growth Index (GI_W). The vertical blue line shows the crop sowing date and the vertical red line shows the date of colonisation by *P. xylostella*. Vertical black dotted lines represent five generations back-predicted from the colonisation date using a temperature-dependent development model for *P. xylostella* with local temperatures, allowing examination of (GI_W) prior to colonisation.