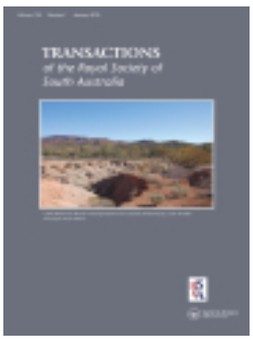

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Which species of Tortricidae leafrollers are key insect pests in South Australian vineyards?

Mary J. Retallack [a], Duncan Mackay [b], Linda J. Thomson [c] and Michael A. Keller [a]

[a]School of Agriculture, Food and Wine, The University of Adelaide, Glen Osmond, SA, Australia; [b]College of Science and Engineering, Flinders University, Bedford Park, SA, Australia; [c]School of BioSciences, The University of Melbourne, Parkville, Victoria, Australia

ABSTRACT

Light brown apple moth, *Epiphyas postvittana* (Lepidoptera: Tortricidae) is regarded as the key insect pest in Australian vineyards and it is also an important pest of apples and citrus. *E. postvittana* is indigenous to Australia and has a wide geographical distribution. Recent observations suggest that leafroller species other than *E. postvittana* may be causing damage in grapevine canopies. A study of tortricids was undertaken in Adelaide Hills and McLaren Vale vineyards, South Australia. A total of 407 specimens of Tortricidae were collected from grapevine canopies. Molecular techniques were used to identify species. The mean prevalence of *E. postvittana* per sample was 91.0% in 2014/15 and 96.2% in 2015/16. Larval *Acropolitis rudisana*, lucerne leafroller, *Merophyas divulsana* and cotton tipworm, *Crocidosema plebejana* were also found on the grapevine canopy at much lower densities for the first time. The presence of leafroller species *A. rudisana, M. divulsana* and *C. plebejana* on grapevines confirms these species of Tortricidae may also be present in South Australian vineyards. This study confirms that *E. postvittana* is the most common tortricid pest in Adelaide Hills and McLaren Vale vineyards and also illustrates the utility of molecular methods in determining with confidence the species identity of larval Tortricidae.

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Introduction

Light brown apple moth (LBAM), *Epiphyas postvittana* (Lepidoptera: Tortricidae) is the key insect pest that causes economic damage in Australian vineyards and it is also an important pest of apples and citrus (Johnston, 1963; Mo et al., 2006). *E. postvittana* is indigenous to Australia and has a wide geographical distribution including New Zealand, USA, UK, Ireland and parts of Europe (Suckling & Brockerhoff, 2010). Larval *E. postvittana* damage leaves, flower clusters and berry skins. Damaged skins provide infection sites for *Botrytis cinerea* and other bunch moulds, which result in a reduction in fruit quality and yield losses (Ferguson, 1995). Bunch rots can be caused by filamentous fungi, yeast and bacteria (Steel et al., 2013). Annual national losses from *E.*

CONTACT Mary J. Retallack mary.retallack@adelaide.edu.au; mary@viti.com.au

postvittana and related bunch rots were estimated to be $70 million per year in Australia (Scholefield & Morison, 2010).

Recent observations by Feng et al. (2016) suggest that species of Tortricidae other than *E. postvittana* may be present in Australian vineyards. They found *Acropolitis rudisana* present in woody habitats adjacent to vineyards and *Merophyas divulsana* present on cover plants in vineyards.

Given the cryptic nature of the larvae of Tortricidae having no defining morphological features; this raises the question, are other species of previously unnoticed tortricids present in grapevine canopies? If so, what is the likely impact on wine producing grapevines and/or existing integrated pest management (IPM) practices?

The role of Tortricidae has not been fully elucidated in Australian vineyards. Better species identification will provide a better understanding of tortricid activity and an improved understanding of the horticultural risk posed by each species to ensure effective IPM control strategies for all species present (Bernard et al., 2007).

Lepidoptera: Tortricidae

Tortricidae is a diverse family of moths, which includes more than 10,000 described species worldwide (Gilligan et al., 2014) and at least 249 named species in Australia (Horak, 2006). Larval Tortricidae are called leafrollers because they commonly build protective feeding shelters, by folding leaves over their bodies and use webbing to secure these structures. Tortricidae have a wide host range of woody and herbaceous plants (Brown et al., 2010). The larvae of Tortricidae have a similar appearance, which makes it impossible to identify species without a microscope or other laboratory technique such as DNA analysis (Barr et al., 2011; Feng et al., 2016).

E. postvittana

Epiphyas postvittana is an Australian native leafroller which was first described in 1863 (Geier & Briese, 1981) (Figure 1(a)). It is a damaging pest of grapevines in Australia (Buchanan et al., 1991; Glenn and Hoffmann, 1997). It has been recorded from more than 500 plant species in 121 families and 363 genera (Brown et al., 2010), including a range of broad leaved weeds often found in vineyards, such as capeweed, *Arctotheca calendula* and plantain, *Plantago lanceolata*.

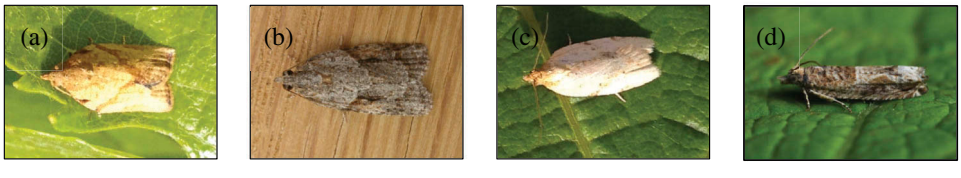

Figure 1. (a) LBAM, *E. posvittana*, (b) *A. rudisana*, (c) lucerne leafroller, *M. divulsana*, (d) cotton tipworm, *C. plebejana*.

Images 1(a) and 1(c) by Mary Retallack. Image 1(b) Acropolitis rudisana by Hobern (2008) is licensed under the Creative Commons Attribution 2.0 Generic license. Image 1(d) by uncredited at http://revtangen.blogspot.com.au/2016/09/

The larva passes through six instars (Danthanarayana, 1975) and grows up to 20 mm in length. In the field, early instars of *E. postvittana* selectively feed on the undersides of grapevines leaves within a silk refuge. They are often found in the developing leaves at the apical meristem. Older larvae can be found on older leaves, or within the developing inflorescences, or bunches of grapes (Brown et al., 2010). *E. postvittana* typically completes three to four generations annually in Australia (Magarey et al., 1994).

A. rudisana

Acropolitis rudisana (Lepidoptera: Tortricidae) is a native leafroller and is widespread in eastern Australia (Figure 1(b)). Hosts of *A. rudisana* include weed species often found in Australian vineyards such as clover, *Trifolium* sp., capeweed, *A. calendula* and grapevines, *Vitis* sp., but not specifically *Vitis vinifera* L. (Brown et al., 2008). There is a scarcity of published information about the biology of *A. rudisana*.

M. divulsana

The lucerne leafroller, *M. divulsana* (Lepidoptera: Tortricidae), is a significant pest of cultivated lucerne, *Medicago sativa* (Allsopp et al., 1983; Whittle et al., 1991), and is a native Australian species (Figure 1(c)). Hosts of *M. divulsana* include weed species often found in Australian vineyards such as plantain, *Plantago* sp., clover, *Trifolium* sp. and capeweed, *A. calendula* (Brown et al., 2008). Little is known about the presence of *M. divulsana* in perennial horticultural crops and *V. vinifera* has not previously been regarded as a host species. When field conditions are conducive, successive discrete generations of *M. divulsana* occur during summer and autumn approximately 5 weeks apart (Whittle et al., 1991).

Crocidosema plebejana

The cotton tipworm, *C. plebejana* (Lepidoptera: Tortricidae) is an introduced pest of cotton in Australia (Bishop & Blood, 1978) (Figure 1(d)). Outbreaks are associated with the growth of its main host marshmallow, *Malva parviflora* (Hamilton & Zalucki, 1993; Williams et al., 2011) which is often found in vineyards. *C. plebejana* has not been found previously on *V. vinifera*.

Aims

We sought to ask which tortricids are present in South Australian vineyards, and does the diversity of tortricids vary significantly among vineyards? If species of tortricids other than *E. postvittana* are present and have different behavioural characteristics, then this may change the management approaches adopted for leafroller control both in vineyards and other horticultural crops such as apples and citrus. To answer these questions, we used molecular methods to determine the species of Tortricidae present on the canopies of *V. vinifera* in Adelaide Hills and McLaren Vale vineyards in the 2014/15 and 2015/16 growing seasons.

Materials and methods

Arthropod collection in the field

Lepidopteran larvae were collected from grapevine canopies during periods of peak activity from mid- to late-October until mid-December, over two successive seasons. Samples were collected weekly from 30 October 2014 to 11 December 2014 (season 2014/15) and from 16 October 2015 to 9 December 2015 (season 2015/16).

A total of 18 sample sites were assessed during 2014/15 and 2015/16. Larval samples were collected from seven vineyards in the Adelaide Hills near Mount Torrens (Site 1: 34°53′38.23″S 138°55′55.45″E), Mount Barker (Site 2: 35°4′11.46″S 138°54′15.18″E, Site 3: 35°4′13.50″S 138°5414.68"E), Nairne (Site 9: 35°3'9.55"S 138°54'48.54"E), Lenswood (Site 10: 34°53'31.56"S 138°50'5.01"E), Ashton (Site 17: 34°56′54.93″S 138°43′45.70″E), The Range (Site 18: 35°14′34.34″S 138°38′29.03″E); and 11 in the McLaren Vale wine region near McLaren Vale (Site 4: 35°11′18.58″S 138°31′0.72″E, Site 5: 35°11′18.30″S 138°31′4.38″E, Site 6: 35°11′25.21″S 138°30′54.28″E, Site 8: 35°12′28.58″S 138°32′47.70″ E, Site 11: 35°12′31.80″S 138°31′47.20″E, Site 13: 35°17′9.54″S 138°31′20.94″E, Site 14: 35°17′12.54″S 138°31′21.25″E), McLaren Flat (Site 7: 35°11′48.23″S 138°34′21.87″E, Site 12: 35°13′9.85″S 138°33′27.03″E), Chapel Hill (Site 15: 35°10′18.23″S 138°33′0.98″E, Site 16: 35°10′6.38″S 138°33′58.33″E), where tortricids were reported to be present by local vignerons and via CropWatch bulletins (Hamilton, 2014).

Typically, two to four pairs of rows were assessed per site. These vineyards grew a range of varieties including Chardonnay (Sites 2 and 10), Viognier (Site 9), Pinot Noir (Sites 1 and 17), Grenache (Site 7), Shiraz (Sites 3, 5, 8, 11, 12, 14, 15, 16 and 18), Cabernet Franc (Site 4), Sangiovese (Site 6) and Mataro (Site 13). The sampling techniques used did not lend themselves to making extensive comparisons between the sample sites.

Season 2014/15

A random sampling technique was used in 2014/15. Each sub-sample was collected by firmly striking the grapevine cordon five times with a rubber mallet, over a beat net measuring 700 mm × 700 mm that held a 250 ml collection container. This process was repeated five times for each composite sample, alternating between each side of a pair of vine rows. A total of 10 composite samples (replicates) were collected from each vineyard per sampling date.

Arthropods were killed in the field using ethyl acetate vapour. The larvae of Tortricidae were removed and placed in 95% ethyl alcohol (EtOH) and stored in a refrigerator at 4°C. At the end of the season, larval samples were stored at −80°C prior to processing in April 2015. A total of 64 specimens comprising 6 pupae, 1 moth and 57 larvae were collected in season 2014/15.

Season 2015/16

Due to the low number of larval samples collected in 2014/15 as a result of the method employed, a targeted sampling technique was used in season 2015/16 to ensure maximal capture. Grapevine shoots were systematically scanned over a

30 min period to find larvae, which were deposited in a 10 ml tube containing 95% EtOH in the field. Samples were stored in a refrigerator at 4°C, prior to PCR-based analysis of DNA gene barcodes in January 2016. A total of 369 larvae were collected in season 2015/16.

Molecular analysis

DNA extraction

The DNA extraction protocol followed the DNeasy Blood and Tissue Kit (Qiagen, Hilden, Germany). The samples were placed in tubes, macerated using individual grinding sticks and left to incubate for 2 h at 56°C following the manufacturer's protocol. The concentrations of DNA samples were estimated using a NanoDrop® ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE). Extracted DNA was stored at −20°C.

PCR protocol and Sanger sequencing

The specimens were characterised through amplification of the mitochondrial cytochrome oxidase 1 (MT-CO1) gene using a PCR-based protocol to determine each species. The selected larvae were PCR amplified and sequenced in both directions for the barcode region of CO1 using the universal primer pairs LepF (5′-ATT CAACCAATCATAAAGATATTGG-3′) and LepR (5′-TAAACTTCTGGATGTCC AAAAAATCA-3′) which targeted the full-length 658 bp DNA barcode fragment (Hajibabaei et al., 2006; Rougerie et al., 2011).

PCR was carried out in a 50 µL reaction volume, containing 5 µL of 10× PCR buffer minus Mg, 1.5 µL 50 mM $MgCl_2$, 1 µL of primer mix (10 µM each), 1 µL of 10 mM dNTP mixture, 2 µL of template DNA, 0.2 µL of Taq DNA polymerase (Platinum™ Taq DNA polymerase; Invitrogen™) and 38.3 µL nuclease-free water up to 50 µL volume.

When sequence results were inconclusive or the sample of Tortricidae had been parasitised by a braconid wasp, *Dolichogenidea* sp. in the field prior to collection and its DNA dominated the sequence, then Lepidoptera-specific primers LepF (5′-ATTCAACCAATCATAAAGATATTGG-3′) and MH-MR1 (5′-CCTGTTCCAG CTCCATTTTC-3′) were used to sequence the partial DNA barcode fragment of 311 bp (Hajibabaei et al., 2006; Rougerie et al., 2011) to confirm the species.

The thermal profile used for both barcoding reactions consisted of an initial denaturing step of 1 min at 94°C, followed by five cycles of 40 s at 94°C, 40 s at 45°C and 1 min at 72°C, followed by 35 cycles of 40 s at 94°C, 40 s at 51°C and 1 min at 72°C, with a final extension step at 72°C for 5 min (Hajibabaei et al., 2006; Rougerie et al., 2011).

The PCR products were run in 2% agarose (LE Analytical grade, Promega) via electrophoresis at 120 V for 30 min to check for single amplicons of the expected size and visualised in UV light. Samples of unpurified PCR product showing strong bands were sent to the Australian Genomic Research Facility, Adelaide, South Australia for Sanger sequencing. Dual-direction sequencing using the LepF and LepR (or subsequently LepF and MH-MR1) primers was carried out in 20 µL reaction volumes.

Data analysis

CO1 DNA sequences were obtained from the PCR amplicons. The quality of the forward and reverse sequences was confirmed by the number of Q20 bases detected. These sequences were trimmed and aligned using the program Geneious® then matched with partial CO1 sequences in the GenBank public database (https://blast.ncbi.nlm.nih.gov/Blast.cgi) via a BLAST search. Key GenBank accession numbers used to confirm the identity of each species of tortricid, included HM346472.1 (*E. postvittana*), KF402639.1 (*A. rudisana*), KF153775.1 (*M. divulsana*) and KC315445.1 (*C. plebejana*). The corresponding GenBank accession numbers for isolates of Tortricidae generated in this study are MG851725–MG851793.

At one site *A. rudisana* was apparently found more frequently than at the other sites. The Fisher Exact Test (http://www.quantitativeskills.com/sisa/statistics/fisher.htm) was used to test if this was an exceptionally high incidence (=occurrence in a sample). The incidence frequency at this site and at all other sites were cast in a contingency table. The probability for this table and all others more extreme were calculated and the sum indicated the overall probability of this observation.

Results and discussion

Prevalence of E. postvittana

Epiphyas postvittana was consistently the dominant species of Tortricidae found in Adelaide Hills and McLaren Vale vineyards. A total of 433 larval Lepidoptera was collected from grapevine canopies and identified using PCR-based analysis of DNA gene barcodes. Of these, 407 were larval Tortricidae (n = 43 in 2014/15 and n = 364 in 2015/16). The difference in the number of larvae collected was a result of the two different collection methods (random versus targeted). The mean prevalence of *E. postvittana* per sample of moth larvae was 91.0% in season 2014/15 and 96.2% in season 2015/16 (Table 1).

These results confirm *E. postvittana* (n = 389 specimens) is the most common tortricid pest in Adelaide Hills and McLaren Vale vineyards. *A. rudisana* (n = 16 specimens), *M. divulsana* (n = 1 specimen) and *C. plebejana* (n = 1 specimen) larvae were found for the first time in a grapevine canopy, but at much lower densities.

Other species of Lepidoptera found included apple looper, *Phrissogonus laticostata* (Lepidoptera: Geometridae) (n = 18), native budworm, *Helicoverpa punctigera* (Lepidoptera: Noctuidae) (n = 2), diamondback moth, *Plutella xylostella* (Lepidoptera: Plutellidae (n = 1) and five undetermined specimens which could not be identified due to the low number of Q20 bases detected.

Prevalence of A. rudisana at key vineyard sites

Of the 15 larval *A. rudisana* collected in season 2015/16 in Adelaide Hills and McLaren Vale vineyards, nine specimens (60%) were collected from the #16 vineyard site, which is located adjacent to a large area of remnant bushland. Furthermore, of the 25 unique visits to sample sites, *A. rudisana* was present at the #16 vineyard 100% of the time, versus 23% for the remaining pooled data of the sites sampled. This indicates that *A.*

Table 1. Mean prevalence of Tortricidae per sample in 2014/15 and 2015/16.

	N		*E. postvittana*	*A. rudisana*	*M. divulsana*	*C. plebejana*
Season	(Unique visits to sample sites)	(Tortricid specimens)	Mean (±95% CI)	Mean (±95% CI)	Mean (±95% CI)	Mean (±95% CI)
2014/15	13	43	91.0% (74.2–100%)	7.7% (−9.1 to 24.5%)	0% (NA)	1.3% (−1.5 to 4.2)
2015/16	25	364	96.2% (93.5–99.0%)	3.7% (1.0–6.5%)	0.04% (0.0–0.1%)	0% (NA)

rudisana was arguably more likely to be found at the #16 vineyard site ($p = 0.055$, Fisher's Exact test). Other vineyards in South Australia may also have *A. rudisana* present and this may warrant investigation on a vineyard-by-vineyard basis.

This is the first time a complex of leafroller larvae present on grapevine canopies have been characterised using molecular biological techniques. The presence of *E. postvittana* has been well documented in vineyards. However, the presence of *A. rudisana, M. divulsana* and *C. plebejana* on *V. vinifera* canopies has not been described previously.

Pheromones for mating disruption

Pheromone infused twist ties have been used successfully in large scale mating disruption trials in south-eastern Australia (Mo et al., 2006). Pheromone traps have also been used successfully to survey the distribution of *E. postvittana* in California (Brown et al., 2010) and control *M. divulsana* in lucerne crops in Australia (Bishop, 1993; Whittle et al., 1991). However, they are not currently, widely employed by vignerons in Australia.

New specialised pheromone and lure application technology provides an alternative to existing pheromone application. This technology provides a similar efficacy to disrupt the mating of LBAM when compared to pheromone infused twist ties, while streamlining the application of pheromones, via manual or mechanical application (Suckling, Brockerhoff, et al., 2012, Suckling, Sullivan, et al., 2012). Growers may wish to try this "next generation" pheromone application method in the future. However, the use of synthetic pheromones is highly target specific (Brockerhoff et al., 2012), and the effectiveness of mating disruption will fail if non-target species of Tortricidae are present.

Similarly, if pheromone traps specific to *E. postvittana* are used to indicate leafroller activity, other species of Tortricidae such as *A. rudisana, M. divulsana* and *C. plebejana* will not be detected. This emphasises the importance of knowing the species of Lepidoptera present prior to implementing an IPM plan.

Overwintering moth larvae

Australian vineyard managers often scout broadleaf weeds in the mid-row for the presence of moth larvae, to provide an indication of leafroller activity early in the growing season (Brockerhoff et al., 2011). Given the impossibility of identifying larval tortricids in the field to species, if the larvae are all assumed to be *E. postvittana* the abundance of damaging species of tortricids may be overestimated, leading to unnecessary waste of time and resources.

Alternative prey for predator arthropods

Merophyas divulsana is found on mid-row cover plants in the vineyard (Feng et al., 2016), but has not been previously described on grapevine canopies. A single *M. divulsana* and *C. plebejana* larva was each found in a grapevine canopy over the two sampling seasons, suggesting that it is unlikely *M. divulsana* or *C. plebejana* frequently

migrates into the grapevine canopy. This may be due to *M. divulsana* and *C. plebejana* not preferring the physical cues or the foliar chemistry of grapevines (Rizvi & Raman, 2016). If *M. divulsana* or *C. plebejana* is present on grapevines, then it is likely to be in very low abundance and of insignificant impact and risk.

However, larval *M. divulsana* and *C. plebejana* may provide a source of alternative prey or hosts, to boost the presence of predators and parasitoids of *E. postvittana* when insectary food (nectar and pollen) sources are low early in the growing season (Barnes et al., 2010; Gurr et al., 2004; Hassell & May, 1986). This decoupling of reliance on early-flowering insectary plants, potentially allows predators of *E. postvittana* to colonise and provide natural biological control in vineyards more quickly. Similarly, larval *A. rudisana, M. divulsana* and *C. plebejana* provide diversified host options for parasitoids of *E. postvittana*, such as *Dolichogenidea tasmanica, Therophilus unimaculatus* and the commercially available *Trichogramma carverae* in vineyards (Feng et al., 2016; Yazdani et al., 2015).

Biosecurity

This research provides a benchmark for four species of Tortricidae and provides a possible methodology for avoiding the challenge of identifying species of Lepidoptera from immature life stages correctly in the field, if species are represented in reference databases. These findings also reinforce the need for robust molecular-based protocols for the rapid identification of exotic pests, to enable the deployment of early intervention management options following a pest incursion.

Critically, accurate identifications would enable a thorough understanding of a pests' host preferences and distribution which amongst other things is needed to determine the capacity of an introduced pest species to displace current species populations.

If there was an incursion of omnivorous leafroller, *Platynota stultana*, European grapevine moth, *Lobesia botrana* or American berry moth, *Polychrosis viteana* into Australia, it is conceivable that they could invade and remain undetected in vineyards for a prolonged period, as has been the case with identifying the presence of *A. rudisana, M. divulsana* and *C. plebejana* on *V. vinifera*.

Conclusion

This research has demonstrated that LBAM, *E. postvittana* is a key tortricid pest of South Australian vineyards. Low densities of *A. rudisana, M. divulsana* and *C. plebejana* have been found on the canopies of *V. vinifera* for the first time. As they are closely related to *E. postvittana*, it is anticipated *A. rudisana, M. divulsana* and *C. plebejana* can be managed through existing IPM strategies.

Acropolitis rudisana, M. divulsana and *C. plebejana* may also provide a valuable source of alternative hosts for parasitoids and alternative prey for predators, when they are located in vineyard mid-rows. This is especially important during the winter period and early in the growing season, when alternative prey is needed to boost the presence of key predators of *E. postvittana*, so they can provide natural biological control before LBAM populations reach damaging levels in grapevine canopies. This study highlights

the importance of using molecular methods to determine the species of Tortricidae at the larval stage with confidence.

The role of Tortricidae should be elucidated in Australian vineyards. First, the level of damage that *A. rudisana, M. divulsana* and *C. plebejana* can make should be studied. Then, if these species aren't important economically, they might be used as an alternative host for *D. tasmanica* the key parasitoid of *E. postvittana. Trichogramma carverae* is available commercially as a biological control option for *E. postvittana*. It is not known if they will also parasitise *A. rudisana, M. divulsana* and *C. plebejana*.

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Disclosure statement

The authors declare there are no conflicts of interest.

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Key messages

- Light brown apple moth, *Epiphyas postvittana* (Lepidoptera: Tortricidae) is regarded as the key insect pest in Australian vineyards.
- However, larvae of Tortricidae have no defining morphological features and molecular methods are required to determine with confidence the species identity of larval Tortricidae.
- A study of tortricids was undertaken in South Australian vineyards.
- In addition to *E. postvittana*, larval tortricids *Acropolitis rudisana, Merophyas divulsana* and *Crocidosema plebejana* were also found on the grapevine canopy at much lower densities for the first time.

Authorship declaration

MR collected samples, conducted molecular laboratory analysis, interpreted data and wrote the manuscript. DM, LT and MK contributed to the manuscript production. MK helped in developing the idea, provided guidance throughout and assisted with statistical analysis. All authors have contributed significantly and agree with the manuscript.

Data availability

The datasets generated and analysed during the current study are available in the GenBank repository (Accession Numbers: MG851725–MG851793) and in The University of Adelaide Figshare online digital repository (DOI 10.25909/5b67e3522d9bd).

ORCID

Mary J. Retallack http://orcid.org/0000-0003-4442-1408
Duncan Mackay http://orcid.org/0000-0001-6065-8531
Linda J. Thomson http://orcid.org/0000-0001-7049-0744
Michael A. Keller http://orcid.org/0000-0003-0721-9753

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