DNA from 33-year-old dried moth specimens help confirm larva as the elusive *Wiseana fuliginea*

N.K. Richards¹, H. Ehau-Taumaunu^{1,2,3} and C.M. Ferguson⁴

¹AgResearch, Lincoln Science Centre, Private Bag 4749, Christchurch 8140, New Zealand

²School of Biological Sciences, University of Auckland, Private Bag 92019, Auckland 1142, New Zealand

³Bio-Protection Research Centre, Lincoln University, PO Box 85084, Canterbury 7647, New Zealand

⁴AgResearch, Invermay Agricultural Centre, Private Bag 50034, Mosgiel 9053, New Zealand

Corresponding author: nicola.richards@agresearch.co.nz

Abstract Caterpillars of the genus *Wiseana*, commonly known as porina, are pests of improved pastures in New Zealand. Seven species are currently recognised but morphological identification of individual species is extremely difficult. Therefore, two new molecular-based identification methods have recently been developed. However, analysis of an adult *W. fuliginea* specimen was required to confirm the tentative identification of a *W. fuliginea* larva collected from Southland. No adult *W. fuliginea* have been collected in the last twenty years so DNA was extracted from voucher specimens of 33-year-old dried *W. fuliginea* adults held by the New Zealand Arthropod Collection. A 1,035 bp sequence of the cytochrome oxidase I gene for each of the two museum *W. fuliginea* voucher moths was generated and proved identical to the sequence from the Southland larva. A new method for confirming the identification of porina specimens is available as a result of this work.

Keywords Porina, museum specimen, insect identification.

INTRODUCTION

Caterpillars of the endemic genus *Wiseana* Viette (Lepidoptera: Hepialidae), commonly known as porina, occupy niches from alpine regions to lowland plains (Brown et al. 2000) and have been shown to feed on a variety of plants (Atijegbie et al. 2016, Ehau-Taumanu et al. 2016). They are significant pests of improved pasture, in which both ryegrass (*Lolium* spp.) and white clover (*Trifolium repens*) are attacked, throughout much of New Zealand (Barratt et al. 1990) costing farmers up to \$500M p.a. (C. M. Ferguson, unpublished). With taxonomic expertise, moths may be identified to species level using morphological criteria such

as wing-scale shape, antennal-segment shape and examination of dissected genitalia (Dugdale 1994). However, identification of the pasture-defoliating caterpillars to species level using morphological features is impossible. As a result, porina have been historically treated as a single pest complex yet inter-specific differences among porina are known to impact on the efficacy of some porina management strategies. The inability to distinguish between porina species from larval specimens has impeded the development and optimisation of sustainable management options (Ferguson 2000).

Seven *Wiseana* species are currently recognised based on adult morphology: *W. cervinata* (Walker), *W. copularis* (Meyrick), *W. fuliginea* (Butler), *W. jocosa* (Meyrick), *W. mimica* (Philpott), *W. signata* (Walker) and *W. umbraculata* (Guenée) (Dugdale 1994; Nielsen et al. 2000). Allozyme and phylogenetic studies have already revealed sub-populations or new haplotypes within three of the species (*W. cervinata*, *W. copularis*, *W. signata*), tentatively associated with geographic boundaries (MacArthur 1986; Herbert 1995; Brown et al. 1999a). A recent study showed this geographic association held true for haplotypes of *W. cervinata* and *W. signata*, however the supposed 'northern' haplotype of *W. copularis* was found not only in the North Island but also the South Island and Chatham Islands of New Zealand (Richards et al. 2017).

Mitochondrial sequences (526 bp) that spanned the 3' cytochrome oxidase I (COI) and 5' COII genes for one voucher adult specimen per species and haplotype were published by Brown et al. (1999a). The first non-sequencing based molecular tool for porina species identification used restriction fragment length polymorphisms (RFLPs) of an amplified 2,200 bp mitochondrial COI and II gene product (Brown et al. 1999b). This method proved impracticable for field studies as the restriction profiles were complex with low size variability, and the level of genetic variation within this mitochondrial region was not known. Therefore, a study was recently undertaken to develop fast and easy non-sequencing tools for porina species identification (Richards et al. 2017). This study described and validated two new, molecular-based identification methods focused on sequencing a 1,718 bp mitochondrial region that covered the whole COI gene and the 5' COII gene in porina specimens collected from broadly separated regions throughout New Zealand. As part of this study, a single larva was fortuitously collected from a pasture at Mararoa Downs, Southland, which was tentatively identified as *W. fuliginea*. This tentative identification was based on the 1,718 bp sequence for its mitochondrial COI and COII genes being more than 1% different to voucher specimens for the other known porina

species (Richards et al. 2017) and the smaller 526 bp 3' COI and 5' COII sequence being identical to the *W. fuliginea* voucher specimen described by Brown et al. (1999a). However, this 526 bp region varied by only 1 bp for *W. fuliginea* and *W. mimica* so it is not a reliable method for differentiating between these two species. Intraspecific variation is prevalent within the mitochondrial COI and COII of porina species. For example, seven *W. mimica* specimens collected from Otago and Southland varied by 1 to 2 bp within the aforementioned 526 bp region (Richards et al. 2017).

To confirm the species assignment of the unusual larva, it was necessary to source and then sequence the larger 1,718 bp COI and COII region from a voucher specimen of a *W. fuliginea* adult. Unfortunately, the voucher specimens collected by Brown et al. (1999a) could not be located. Pasture-focused porina investigations have been undertaken for the past twenty years and moths have been collected throughout the complete flying seasons by light trapping at several South and North Island sites. Collected specimens have been identified to species level using Dugdale's (1994) taxonomic key but no adults that could be definitively identified as *W. fuliginea* have been detected. In January 2017, light traps were erected on the Southland farm where the tentative *W. fuliginea* larva was collected the previous winter, but again, no *W. fuliginea* adults were captured. The authors were, therefore, delighted to be granted access to two voucher specimens of *W. fuliginea* adults collected and archived at the New Zealand Arthropod Collection (NZAC) by MacArthur (1986).

Hundsdoerfer and Kitching (2017) made use of archived voucher specimens to analyse the degree of divergence between 100-year-old specimens of a rare hawkmoth species and other readily available hawkmoth species. The current paper describes how the tentative identification of the *W. fuliginea* larva by Richards et al. (2017) was confirmed by successfully extracting DNA and sequencing 1,035 bp of the mitochondrial COI gene from museum-archived *W. fuliginea* adult voucher pin specimens. This work was conducted using established guidelines for working with historic specimens (Wandeler et al. 2007).

MATERIALS AND METHODS

Specimen collection

Two voucher moths for male *W. fuliginea* were sourced from the NZAC curated by Landcare Research (Auckland). The moths were collected by Gordon MacArthur (R177, 26 Oct 1984; R178, 29 Oct 1984) from a pasture trial site located in Invermay (Dunedin, Otago) and submitted to the NZAC as pin specimens in 1986 as part of an unpublished Master's thesis undertaken at Victoria University of Wellington.

DNA extraction

DNA was extracted from two legs for each NZAC voucher moth, using the Genomic DNA Mini Kit Tissue (Geneaid™, Taiwan) following manufacturer's specifications. Proteinase K digestion was performed for one hour at 60°C. Final elution was performed using two sequential washes of 100 µL elution buffer, yielding a final volume of 200 µL for each specimen. Genomic DNA was stored short-term in the fridge (4–8°C) or long-term in the freezer (–5 to –80°C).

Amplification and primer design

PCR reactions contained *i-StarTaq*™ DNA polymerase (iNtRON Biotechnology, South Korea) at approx. 1 unit/20 µL PCR, 2 mM MgCl₂, 0.2 mM dNTPs, 0.2 µM primers (Integrated DNA Technologies, Singapore) and 1 µL template DNA at ca. 1 ng per 20 µL of PCR reaction. Cycling conditions depended on the primer pair, but in general started at 95°C for 2 min, followed by 40 cycles of 95°C for 30 sec, 45 to 50°C for 30 sec and 72°C for 30 to 45 sec; the final step was 5 min at 72°C. All PCR runs included a template-free reaction as a negative control. The primers used to amplify the 5' COI gene barcode region (Folmer et al. 1994), mid COI (Richards et al. 2017) and 3' COI/ 5' COII gene regions (Brown et al. 1994) are listed in Table 1. Mitochondrial sequences, spanning the COI and COII genes (Richards et al. 2017 KY353012–KY353086), for all porina species including the tentative *W. fuliginea* larva (KY353038) were aligned using the programme MUSCLE (Edgar 2004). A range of primers were then

designed to border short regions (ca. 100–400 bp) containing species-specific polymorphisms. Only sequences for the primer pairs that worked for the NZAC voucher specimens are listed in Table 1. PCR products and 100 bp ladder (DNA Marker 1, A&A Biotechnology, Poland) were run on 1% agarose gels containing RedSafe™ (iNtRON Biotechnology, South Korea), in 0.5 × UltraPure™ TBE Buffer (Invitrogen, USA).

Sequencing and alignment

Sanger sequencing (BigDye®v 3.1, Applied Biosystems) was conducted at MacroGen (Korea). For each NZAC voucher moth, the overlapping short mitochondrial sequences were trimmed and aligned using Geneious version 8.1.5 (<http://www.geneious.com>, Kearse et al. 2012) to create one sequence and then compared with previously published sequences (Brown et al. 1999a AF098332–AF098359; Richards et al. 2017 KY353012–KY353086). Multiple alignments of the nucleotide sequences were generated using the program MUSCLE (Edgar 2004).

RESULTS

No products were amplified from the 33-year-old NZAC *W. fuliginea* voucher specimens using the previously described mitochondrial COI and COII primer sets (Folmer et al. 1994, Brown et al. 1994; Richards et al. 2017). However, amplification of shorter regions within the COI gene using primers designed in this study (Table 1) was successful, and the resulting sequences were aligned to produce a 1,035 bp sequence that was identical to the sequence from the 'tentative' *W. fuliginea* Southland larva (KY353038, Richards et al. 2017). The sequences from the *W. fuliginea* museum voucher specimens, InvR177_Otago and InvR178_Otago, were deposited in Genbank under accession numbers MF069505 and MF069506, respectively.

DISCUSSION

Light trapping at several South and North Island sites over twenty years has not yielded a single adult identified as *W. fuliginea*. This result may reflect that the morphological characters

Table 1 Details for PCR primer pairs based in the porina mitochondrial cytochrome oxidase I (COI) gene

Primer name ¹	Primer sequence (5' to 3')	Product size (bp)	Target region	Reference
LCO1490	GGTCAACAAATCATAAAGATATTGG			Folmer et al. 1994
HCO1764	GCATTTCCCTCGTTTAAATA	274	5' COI	this study
LCO1635	GTAATTGTAACAGCACATGC			this study
HCO1879	CCTTTATCYTCTAATATTGC	244	5' COI	this study
LCO1812	AGAAGAATTGTAGAAAATGG			this study
HCO2198	TAAACTTCAGGGTGACCAAAAAATCA	386	5' COI	Folmer et al. 1994
LCO2184	GGTCATCCTGAAGTATATAT			this study
HCO2374	GGTATAGATGTAGATACYCG	190	5' COI	this study
LCO2355	GGTATAGATGTAGATACYCG			Richards et al. 2017
HCO2563	GCTAACTCTTCAATTGATAT	208	5' COI	this study
LCO1490	GGTCAACAAATCATAAAGATATTGG			Folmer et al. 1994
HCO2198	TAAACTTCAGGGTGACCAAAAAATCA	708	Barcode	Folmer et al. 1994
LCO2069	ACCTGTATTAGCRGGWGCTA			Richards et al. 2017
HCO2915	CGTTTCTAATTATTGATTCTC	846	mid COI	Richards et al. 2017
S2792	ATACCTCGACGTTATTCAGA			Brown et al. 1994
HCO3306	GGTAATTGCAGGTAAGATTGT	528	3' COI to	Brown et al. 1994
A3389	TCATAAGTTCARTATCATTG	597	5' COII	Richards et al. 2017

¹New primers were named using the system described by Folmer et al. (1994) where L and H refer to light and heavy DNA strands, CO refers to cytochrome oxidase, and the numbers refer to the position of the *Drosophila yakuba* 5' nucleotide.

described by Dugdale (1994) to distinguish *W. fuliginea* from other porina species are unreliable and/or that this species is rare in pasture environments. Identifying porina species using morphological characters alone is extremely difficult even for an experienced taxonomist. For example, moths collected by B. Brown (23 Oct 1997, Birdling's Flat, Canterbury) and C. Ferguson (22 Nov 1997, Waimahaka, Southland) and determined by J. Dugdale to be *W. fuliginea*, have been subsequently shown to be *W. cervinata* and *W. jocosa*, respectively using molecular technologies (N. Richards unpublished). Barratt et al. (1990) reported *W. fuliginea* as being associated with wet lowlands, and it appears to be the least widespread of the porina species being collected from only three locations nationwide (Dugdale 1994). The pasture from which our tentative *W. fuliginea* larva specimen was collected was adjacent to a

native bush margin and associated patches of grassland in a high rainfall area. It is possible that such a habitat is a natural environment for *W. fuliginea* and that its apparent rarity in pastoral environments may be due to our inability to identify larvae until now.

Amplification of the DNA from the museum voucher moths with the same primers used by Brown et al. (1999a) and Richards et al. (2017) was unsuccessful, most likely due to degradation of the DNA by endogenous nuclease activity and hydrolytic damage (Wandeler et al. 2007). This was overcome by designing a series of primers that amplified smaller overlapping regions of the porina mitochondrial COI gene (< 400 bp, Table 1), similar to the method used by Hundsdoerfer and Kitching (2017) to successfully retrieve mitochondrial COI and COII sequences from 100-year-old museum hawkmoth specimens.

This study successfully confirmed that the larva collected from Te Anau and tentatively identified as *W. fuliginea* was the elusive *W. fuliginea* species. More importantly, a 1,718 bp region of the mitochondrial COI and COII genes is now available for all porina species and haplotypes thereby completing the Richards et al. (2017) study that developed a molecular identification technique for accurate determination of porina moths and larvae to species and sub species level. Indeed, these new molecular porina identification tools have already been used in several field-based studies. Ferguson et al. (2016) reported that the main flight time in the Manawatu for a *W. copularis* northern haplotype occurred during March/April, much later in the southern hemisphere growing season than traditional flights times of October and January displayed in other regions. Further, it was shown that although other porina species were collected during these flights, only *W. copularis* northern haplotype larvae were found in pastures (Richards et al. 2017). This result highlights the fact that generalised porina flight periods are not accurate indicators of localised activity, and that species composition, regional differences in species composition and timing of caterpillar development need to be considered for successful porina management strategies. A related three-year study is the first to present data on porina flight activity followed up with the species composition of porina larvae collected from the same locations (Mansfield et al. 2017). Again, the results of that study illustrated that the species composition of porina moth flights, does not necessarily match the composition of larval populations in pasture. The work conducted by Ehau-Taumaunu (2017) investigated the species distribution and bacterial microbiota of porina larvae collected from five pasture and three native habitats across New Zealand was able to determine the species of larvae collected from both habitats and showed that the composition varied with habitat.

It is now possible to accurately determine which porina species are pests of agricultural significance and to overcome the restrictions of dealing with a pest complex. Ecological studies can be carried

out with confidence and porina research can be brought into the twenty-first century.

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