Biological Control 132 (2019) 8–15

Contents lists available at ScienceDirect

Biological Control

journal homepage: www.elsevier.com/locate/ybcon

Molecular characterization of *Hydrellia lagarosiphon*, a leaf mining biological control agent for *Lagarosiphon major*, reveals weak variance across large geographic areas in South Africa

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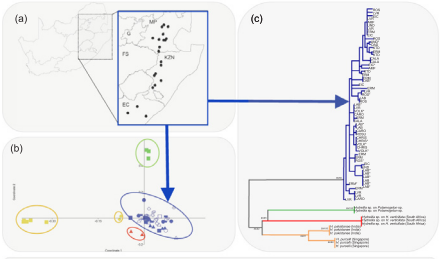
GRAPHICAL ABSTRACT



South African *Hydrellia lagarosiphon* populations sampled in this study (a), reveal weak variance across large geographic areas, using Inter-Simple Sequence Repeats (b) and mtDNA COI sequences (c)

1. Introduction

Native range exploration to identify new biological control agents is technically difficult and time-consuming, yet it is the foundation on which all other elements of a successful biological research programme ultimately depend (Goolsby et al., 2006). During field surveys agents demonstrating a high degree of host specificity and damage efficacy are prioritized. However, due to logistical and technical constraints potentially favourable natural enemies are usually only collected from one or a small number of source populations. Selecting the natural enemy population for host range testing and eventual release is important, as it has become increasingly evident that insect species occurring across a wide geographical range are generally structured into genetically differentiated populations (Madeira et al., 2001; Rauth et al., 2011). Highlighting these biogeographical population level differences can help anticipate potential variation in an agent's climatic adaptations or host-related traits (Paterson et al., 2016), and this information can in turn guide the selection of a more effective and safe agent.

The aquatic plant African curly leaved waterweed, *Lagarosiphon major* (Ridl.) Moss ex Wager (Hydrocharitaceae) is a non-native, canopy forming macrophyte found in several European countries (Preston et al., 2002; Reynolds, 2002; Symoens and Triest, 1983; van Valkenburg and Pot, 2008) as well as New Zealand (McGregor and Gourlay, 2002) and Australia (Bowmer et al., 1995). In an attempt to establish a long-term control strategy for this vigorous and highly invasive plant, a biological control programme was initiated with surveys for natural enemies of *L. major* undertaken in South Africa between 2008 and 2011 (Baars et al., 2010; Earle et al., 2013). These field surveys revealed a suite of phytophagous natural enemies associated with *L. major*, mostly new to science, and include ephydrid flies, pyralid moths, and stem-mining midges (Baars et al., 2010; Earle et al., 2013; Mangan and Baars, 2013). Of those found, *Hydrellia lagarosiphon* Deeming has been identified as the most promising candidate agent (Baars et al., 2010; Van Achterberg and Prinsloo, 2012). The fly is widely distributed under a range of climatic conditions throughout South Africa, indicating that if released the fly may establish well in new areas within the geographic

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https://doi.org/10.1016/j.biocontrol.2019.01.017
Received 10 November 2018; Received in revised form 17 January 2019; Accepted 29 January 2019
Available online 30 January 2019
1049-9644/

Table 1
Sampling sites with geographical data and the hosts of 14 ephydrid fly populations in South Africa.

Site (code)	Altitude (m, a.s.L.)	Latitude	Longitude	*n*	Host plant(s)	Province
Amersfort (AMF)	1706	27°00′37.01	29°50′27.66	2	*L. muscoides*	MP
Cala (CALA)	1187	31°31′26.90	27°41′48.91	3	*L. major*	EC
Carolina (CARO)	1696	26°06′19.82	30°07′30.27	4	*L. major*	MP
Chrissiesmeer* (CHRIS)	1655	26°32′15.02	30°14′48.90	3	*L. major*	MP
David Aucamp Dam* (DAD)	1405	31°04′19.90	28°19′40.45	2	*L. major*	EC
Kubusi farm dam* (LAB)	605	32°33′52.01	22°29′22.42	10	*L. major*	EC
Elands irrigation canal & river* (EIC)	1083	25°35′48.98	30°27′54.32	5	*L. major*	KZN
Ermelo dam & wetlands (ERM)	1690	26°32′39.37	30°07′09.38	11	*L. major* & *L. muscoides*	MP
Khotso dam (KTD)	1262	28°58′01.58	29°30′05.23	4	*L. major* & *L. muscoides*	KZN
Lakenvlei (LVR)	1876	25°36′23.60	30°01′57.32	8	*L. muscoides*	MP
Rosetta* (ROS)	1459	29°18′18.92	29°58′28.95	6	*L. major*	KZN
Roadway (POT)	1432	28°15′42.15	29°58′28.88	2	*Potamogeton* spp.	KZN
Underberg* (UND)	1628	29°48′09.72	29°23′34 43	3	*L. major* & *L. muscoides*	MP
Volksrust farm dam* (VOLK)	1660	27°16′56.95	29°47′33.06	4	*L. major*	KZN

Prov: Province.
EC, Eastern Cape; MP, Mpumalanga; KZN, KwaZulu-Natal.
* Indicates sites where adult flies were also reared from pupae within infested plant materia.

range of the weed. *Hydrellia lagarosiphon* frequently attains high population densities and causes severe damage despite notable parasitism by braconid wasps in its native range (Baars et al., 2010; Van Achterberg and Prinsloo, 2012).

Following its discovery, the fly was taxonomically described and is morphologically distinguishable from other *Hydrellia* species based on variation in the male genitalia (Deeming, 2012). The emergence of molecular genetic fingerprinting techniques as a means of assisting the identification of insect species has highlighted that phenotypic differentiation does not always correlate with genetic diversification and a considerable proportion of biological diversity can be morphologically hidden (Herbert et al., 2004; Smith et al., 2006; Van Driesche and Bellows, 1996). Distribution records from the South African National Biodiversity Institute and published literature indicate *L. major* displays patchy distribution across a wide geographical range, with sites frequently isolated in terms of elevation and eco-region. The Drakensberg Mountains are the highest part of the Highveld in South Africa (~3500 m a.s.L.) dividing the Mpumalanga Province into two parts; the Highveld, north of Johannesburg (~1700 m a.s.L.) consisting mainly of grassland and the lower altitude subtropical Lowveld consisting of mostly savanna habitat. The mountain range also isolates the KwaZulu-Natal Province from Lesotho and the Free State Province. Given the large spatial scale covered during these field studies, as well as the range of eco-regions encountered, it is possible that genetic divergence will occur in fly populations that are completely isolated, or have restricted gene flow (Slatkin, 1985).

While the characterisation of morphological traits for the taxonomic description of new biological control agents is essential, obtaining a genetic perspective on population structure and inter- and intra-specific diversity is now widely practiced. Genetic markers can assist with taxonomic circumscription, the identification of species, subspecies and races and provide unambiguous taxonomic resolution to help interpret local biological adaptations on a population by population level (Moffat and Smith, 2015; Mound et al., 2010; Rauth et al., 2011; Vorsino et al., 2014). More significantly the presence of cryptic species which are difficult to resolve using morphological characteristics alone, highlights the potential benefits of employing genetic assessment prior to the selection of a potential biocontrol agent for both testing and release (Paterson et al., 2016; Toševski et al., 2013). In addition, the application of genetic marker based methods to problematic taxonomic groups, not only identifies cryptic species but can also resolve favourable trait distribution across populations (e.g. host specificity or climatic adaptations) and assist in the monitoring of biological agent control releases (Behura, 2006; Gariepy et al., 2007; Hufbauer et al., 2004; Moffat and Smith, 2015; Mound et al., 2010; Olivieri et al., 2008; Rauth et al., 2011; Vorsino et al., 2014). This precautionary, but informed approach is essential in reducing unanticipated non-target effects and inefficient biocontrol and highlights the importance of testing agents on a population by population basis given that population specific biological traits and adaptations may exist (Paterson et al., 2016; Toševski et al., 2013). Therefore, obtaining genetic level insight is particularly relevant for biological control and the lack of genetic information prior to the field release of biological control agents has been implicated in the failure of some of these programmes (Hopper and Roush, 1993; Hufbauer and Roderick, 2005; Roderick and Navajas, 2003). It is also noteworthy that stringent species identification is now a requirement of numerous national biocontrol programmes (Goolsby et al., 2006) and that biocontrol release in Europe now requires genetic barcoding to underpin species identification (Bigler et al., 2005).

In this study, we investigated patterns of genetic diversity in *H. lagarosiphon* across South Africa using both nuclear microsatellite and mitochondrial DNA (mtDNA) markers to establish the levels of genetic variance across the large geographic area surveyed. This information will guide the selection of source populations for laboratory screening, indicating potential variation in an agent's climatic adaptation or host-related traits, and demonstrate the potential for effective dispersal into new environments.

2. Materials and methods

2.1. Insect collection

Hydrellia lagarosiphon populations were sampled from South Africa during field surveys and included sites from some of the most southerly records in Eastern Cape Province (~750 m a.s.L.) to high altitude sites (1400–2000 m a.s.L.) in Mpumalanga Province (Table 1). Adult flies were collected at 14 sites where leaf-mining damage was evident. Host plants included the target plant *L. major*, as well as a species in the same genus *Lagarosiphon muscoides* Harv., and a *Potomageton* species on which ephydrid fly damage was also evident. These included individuals from three laboratory cultures (originating from Kubusi farm dam in the Eastern Cape, Chrissiesmeer in Mpumalanga, and Volksrust farm dam near the Mpumalanga-KwaZulu-Natal border) under evaluation to assess potential performance differences and preliminary host specificity between populations from different eco-regions. In addition, pupae were sampled directly from both *L. major* and *L. mucsoides*, reared to the adult stage and included in the analysis. Additional specimens were included in the analysis as outgroups; *Hydrellia* sp. indet. (reared on *Hydrilla verticillata* in South Africa), *Hydrellia pakistanae* Deonier originally collected in India and *Hydrellia purcelli* Deeming

originally collected in Singapore (supplied by Angela Bownes, Agricultural Research Council, Plant Protection Research Institute, South Africa).

2.2. DNA extraction

All individuals were stored individually in 100% ethanol. DNA was extracted from 100 adult *Hydrellia* collected on *Lagarosiphon* species, *Potamogeton* sp., and *Hydrilla verticillata* using the Qiagen DNeasy Blood and Tissue kit (Qiagen GmbH, Hilden, Germany) following the manufacturer's protocol. DNA quality and concentration was measured using a NanoDrop® ND-1000 Spectrophotometer (Labtech Int., UK). These extractions were then stored at −20 °C until required.

2.3. mtDNA sequencing

The cytochrome oxidase I (COI) region of the mitochondrial genome (mtDNA) was amplified for all samples. PCR reactions were carried out in 20 µl volumes under the following conditions; 4 µl of genomic DNA, 8.48 µl of ddH_20, 1.8 µl of $MgCl_2$ (25 mM), 0.6 µl of each primer (10 µM each; Nancy S (5′-CCCGGTAAAATTAAAATATAAAC-3′) and LCO_Hym (5′-TATCAACCAATCATAAAGATATTGG-3′) (Simon et al., 1994), 0.4 µl dNTPs (10 mM each), 4.0 µl 10X buffer (Promega) and 0.12 µl of *Taq* polymerase. Thermal profiles started with an initial denaturing @ 94 °C for 60 s, followed by 32 cycles of 94 °C for 60 s, 50 °C for 45 s and 72 °C for 60 s. The cycle ended with one final extension of 240 s at 72 °C. The PCR products were purified using the Novagen® Spinprep™ PCR Clean-up Kit according to the manufacturer's protocol. The sequencing reactions were conducted at Macrogen Inc. (South Korea). Sequences were deposited in GenBank (Accessions MK164650 to MK164651).

2.4. mtDNA sequences analyses

Chromatogram contigs were assembled in Sequencher 4.2 (GeneCodes) and sequence alignments were proof read manually and aligned using Se-Al 2.0 (Rambaut, 2001). Phylogenetic analyses were conducted using MEGA 5 including the neighbor joining and maximum likelihood methods (Tamura et al., 2011). The haplotype diversity (*h*), mean pairwise differences (MPD) and nucleotide diversity (π) for each population were estimated using DnaSp. Ver. 5 (Librado and Rozas, 2009). Population structure was analysed using the Analysis of Molecular Variance (AMOVA) (Excoffier et al., 1992) and by calculating the F_{ST} values (Hudson et al., 1992) between populations, using the Kimura two-parameter distance method (Kimura, 1980). The statistical significance was determined by performing 1000 permutations of the original data set using Arlequin 3.0 (Excoffier et al., 2005).

2.5. Inter-simple sequence repeat PCR amplification

ISSRs were conducted on 11 of the 14 populations using the universal primers 809 and 826 from University of British Columbia Nucleic Acid Protein Service Unit Primer set #9 (Abbot, 2001), fluorescently labeled with 6-FAM dye. PCR reactions were carried out in 20 µl volumes with primer concentration 0.8 µM and 10 µl of Promega Master Mix (Madison, WI, USA) (reaction concentration of 1 U of Taq, 1.5 mM $MgCl_2$, and 0.2 µM dNTPs) per reaction. $MgCl_2$ was added to a final concentration of 2.5 mM. Thermal profiles started with an initial denaturing @ 94 °C for 2 min, followed by 35 cycles of 94 °C for 30 s, 44 °C for 45 s and 72 °C for 90 s. The cycle ended with one final extension of 20 min at 72 °C. Banding patterns generated by the labeled primers were visualized using capillary electrophoresis on an ABI 3130 genetic analyser at Stellenbosch University, Stellenbosch, South Africa. Every sample was replicated from the PCR step so there were two sets of binary data for each sample. Only peaks that were present in both replicates were used for further analyses. Bands that were only present in one of the replicates were treated as missing data.

2.6. Inter-simple sequence repeat data analysis

Electropherograms were analyzed using PeakScanner v. 1.0 (Applied Biosystems, Foster City, CA) using a medium level of peak smoothing. The analyzed data set was then exported into RawGeno v. 2.0-2 (Arrigo et al., 2009) using the R© v. 3.0.1 platform (The R Foundation for Statistical Computing, Vienna, Austria). The final binary data set was then used to conduct Principal Component analysis in PAST: Paleontological Statistics Package v. 1.94 (Hammer et al., 2001) using the Jaccard's Index to convert the binary data into a distance matrix. The percentage of polymorphism and expected heterozygosity (H_E) were calculated for each population using GenAlex (Peakall and Smouse, 2006). The genetic differentiation between populations was determined using Φ_{ST}, a measure that allows intra-individual variation to be suppressed and is therefore ideal for comparing dominant binary data, with 10,000 permutations (Assoumane et al., 2013). Analysis of molecular variance (AMOVA) among and within populations and geographical regions was performed using GenAlex (Excoffier et al., 1992; Peakall and Smouse, 2006).

3. Results

3.1. Sequence data, haplotypes and genetic diversity

After excluding the ambiguously called base pairs at the beginning and ends of each sequence, a 608-bp portion of the COI mt DNA sequences was obtained for 67 *Hydrellia* collected on *Lagarosiphon* species (n = 65) and *Potamogeton* sp. (n = 2). A total of 47 different haplotypes were identified which were differentially distributed across the 14 populations sampled. Only six haplotypes were shared by at least two individuals and 41 were unique (Table 2). Of the six shared haplotypes, H_4 was found within 7 populations (CALA, CARO, CHRIS, ERM, LAB, LVR, and VOLK) and H_{10} was found in 3 populations (CARO, LVR, and VOLK). The most common haplotype (H_4) was widespread and was represented in 16.4% of the sequenced individuals, 54% of the 13 populations, and all three provinces where *Lagarosiphon* species were surveyed. Of the 47 haplotypes present within the native populations, six haplotypes were present in the laboratory culture (Table 2). The haplotypes present in the laboratory culture were present in CALA, CARO, CHRIS, ERM, LVR, and VOLK. Haplotype diversity ranged from 0.800 (LAB) to 1.00 (for 11 of the populations), with an average of 0.970 for the 13 populations (Table 3). Nucleotide diversity (π) ranged from 0.003 (VOLK) to 0.118 (DAD), with an average of 0.060.

3.2. Genetic relationships between populations

Four distinct clades were resolved by the mitochondrial CO1 sequence analysis for 76 specimens, representing four different species on a variety of host plants (Fig. 1). One clade represents specimens collected on *L. major* and *L. muscoides* during the native range exploration for an agent for *L. major*. Several specimens were also represented in a separate clade and include all individuals collected on a *Potamogeton* species. Another clade denotes *Hydrellia* sp. specimens collected on *Hydrilla verticillata* in South Africa and the final clade represents *Hydrellia pakistanae* collected on *H. verticillata* in India and *Hydrellia purcelli* collected on *H. verticillata* in Singapore. The K2P genetic distances between haplotypes within the 13 populations collected on *L. major* and *L. muscoides* in South Africa ranged from 0.006 (CHRIS) to 0.065 (LAB) (overall mean within population, 0.026). Mean distances between populations ranged from 0.004 between VOLK and CARO to 0.104 between DAD and ROS (Table 4). The Fst value for the mitochondrial sequence data was low and not statistically significant (Fst = 0.14, $P > 0.05$). An AMOVA performed on the mtDNA data revealed that 85.03% of the genetic variance was explained by intra-population variation, while the remaining 14.93% ($P < 0.01$) explained variation among populations.

Table 2
Ephydrid fly mtDNA COI haplotypes, representing the number of each haplotype (H_{1-47}) for each of the South African populations sampled.

Hp No.	AMF	CAL	CAR	CHR	DAD	LAB	EIC	ERM	KTD	LVR	ROS	POT	UND	VOL
1						X								
2						X								
3						X								
4			X	X		X		X		X				X
5										X				
6								X						X
7						X								
8								X						
9														X
10			X							X				X
11				X										
12			X			X				X				
13								X						
14									X					
15											X			
16											X			
17					X									
18		X												
19		X												
20													X	
21			X											
22										X				
23					X									
24									X					
25									X					
26									X					
27								X						
28								X						
29								X						
30													X	
31								X						
32								X						
33							X							
34							X							
35													X	
36								X						
37											X			
38							X							
39											X			
40										X				
41	X													
42	X													
43											X			
44											X			
45				X										
46												X		
47												X		

Table 3
Genetic variability within 14 ephydrid fly populations sampled in South Africa.

Population	*n*	Hp	*h* ± SD	π ± SD
AMF	2	2	1.000 ± 0.500	0.008 ± 0.003
CALA	3	3	1.000 ± 0.272	0.009 ± 0.003
CARO	4	4	1.000 ± 0.177	0.006 ± 0.002
CHRIS	3	3	1.000 ± 0.272	0.025 ± 0.015
DAD	2	2	1.000 ± 0.500	0.118 ± 0.014
LAB	10	5	0.800 ± 0.100	0.016 ± 0.003
EIC	5	5	1.000 ± 0.126	0.011 ± 0.003
ERM	11	9	0.964 ± 0.051	0.007 ± 0.003
KTD	4	4	1.000 ± 0.177	0.006 ± 0.002
LVR	8	6	0.929 ± 0.084	0.016 ± 0.004
ROS	6	6	1.000 ± 0.096	0.057 ± 0.007
POT	2	2	1.000 ± 0.500	0.016 ± 0.008
UND	3	3	1.000 ± 0.172	0.020 ± 0.004
VOLK	4	4	1.000 ± 0.177	0.003 ± 0.002

Hp, number of haplotypes; h, haplotype diversity; π, nucleotide diversity.

3.3. Inter-simple sequence repeats

The final dataset consisted of 237 fragments for 11 of the 14 locations sampled in South Africa. Fragments ranged in size from 100 to 650 bp. All populations had low levels of polymorphism and expected heterozygosity (HE, Nei's gene diversity), with CALA, CARO, KTD and POT considerably lower for both (Table 5). AMOVA of the microsatellite data (for the 10 populations collected on *Lagarosiphon* species) revealed that 16.55% ($\Phi_{ST} = 0.165$, $P < 0.01$) of the genetic variation was explained by intra-population variation, while the remaining 83.45% ($P < 0.01$) explained variation between populations (Table 6). Another AMOVA was conducted using the three provinces as groups, however very low differentiation was found among populations, 4.69% ($P < 0.01$). The *H. lagarosiphon* individuals collected on *Lagarosiphon* species are clearly distinguished by PCA, while individuals collected from *Potamogeton* sp. clustered separately (Fig. 2). *Hydrellia* sp. on *Hydrilla verticillata* in South Africa, *Hydrellia pakistanae* collected on *H. verticillata* in India *and Hydrellia purcelli* collected on *H. verticillata* in Singapore also reveal three distinct clusters. This is supported by the AMOVA which indicated that 43% of genetic diversity occurred within populations while the variability among populations collected on different plant families was 57% ($\Phi_{ST} = 0.566$, $P < 0.01$) (Table 6).

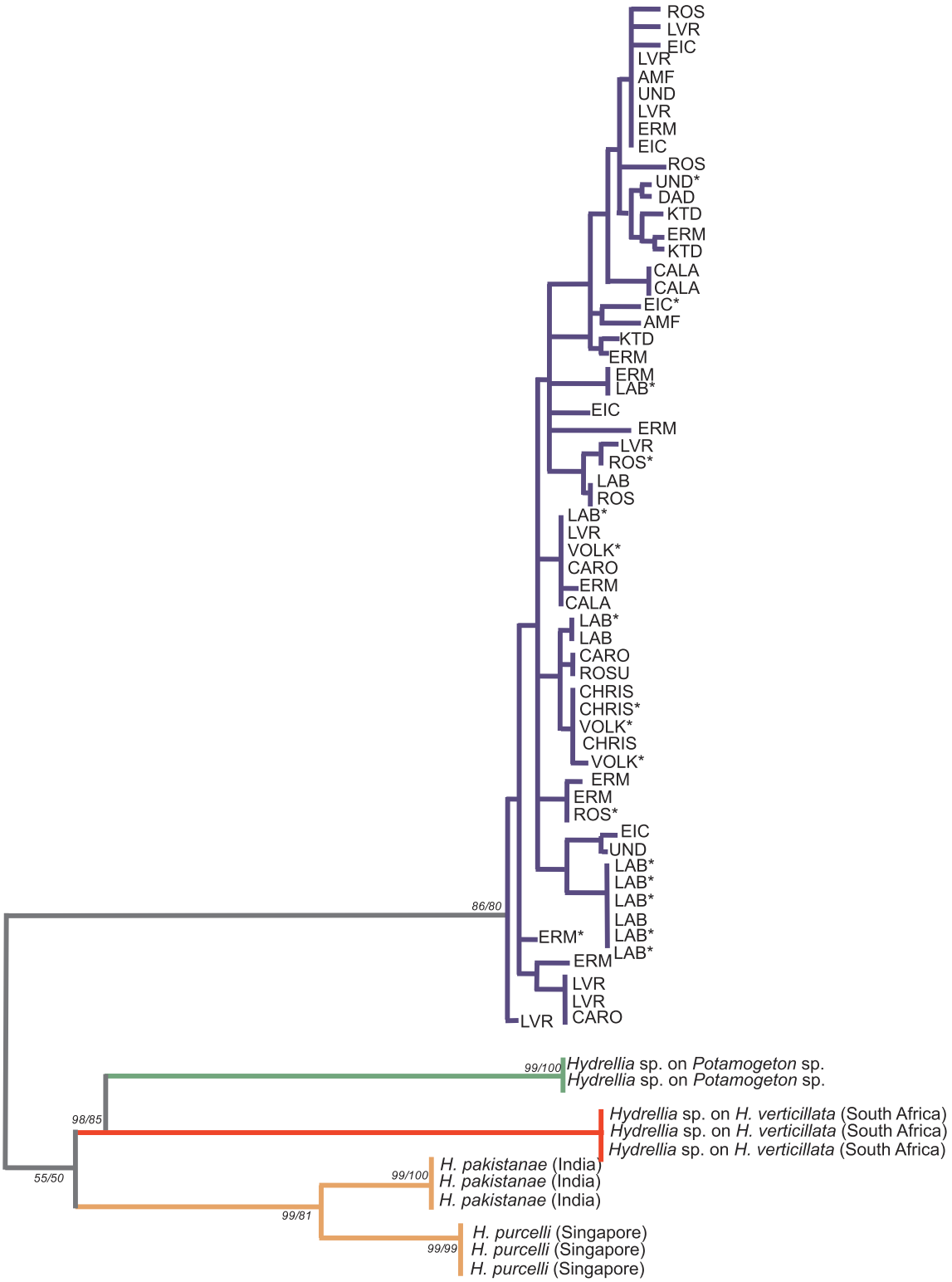

Fig. 1. Maximum likelihood tree using the GTR + G model of substitution of 47 cytochrome oxidase 1 region haplotypes (608 bp) resolving four major lineages; South African *H. lagarosiphon* samples are indicated in blue, *Hydrellia* sp. on *Potamogeton* sp. samples are indicated in green, South African *Hydrellia* sp. on *Hydrilla verticillata* are indicated in red and *H. pakistanae* on *H. verticillata* from India and *H. purcelli* on *H. verticillata* Singapore are indicated in yellow. Bootstrap values obtained for major lineages by neighbor joining and maximum likelihood analyses are shown above/below nodes. * Indicates sites where adult flies were also reared from pupae within infested plant material. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

4. Discussion

This study reveals high levels of genetic diversity and low levels of genetic differentiation across the individuals collected on *Lagarosiphon* for both mitochondrial and nuclear markers despite the large geographic distances separating the populations. The high levels of genetic diversity observed appear to be correlated with low Fst values and Φ_{ST}, suggesting low genetic differentiation between populations of *H.*

Table 4
Pairwise F_{ST} values between (below diagonal) and within (diagonal) *H. lagarosiphon* populations based on the Kimura two parameter distance between mtDNA haplotypes.

Site code	AMF	CALA	CARO	CHRIS	DAD	EIC	ERM	KTD	LAB	LVR	ROS	UND	VOLK
AMF	0.016												
CALA	0.009	0.019											
CARO	0.010	0.008	0.007										
CHRIS	0.021	0.019	0.015	0.002									
DAD	0.075	0.076	0.076	0.083	0.011								
EIC	0.008	0.011	0.011	0.022	0.077	0.026							
ERM	0.013	0.011	0.006	0.016	0.077	0.014	0.006						
KTD	0.007	0.009	0.009	0.022	0.076	0.008	0.013	0.006					
LAB	0.015	0.016	0.013	0.023	0.085	0.017	0.015	0.016	0.065				
LVR	0.016	0.015	0.012	0.019	0.080	0.017	0.013	0.016	0.019	0.141			
ROS	0.039	0.038	0.035	0.046	0.104	0.040	0.037	0.038	0.043	0.042	0.016		
UND	0.011	0.013	0.014	0.026	0.079	0.012	0.018	0.011	0.020	0.020	0.043	0.008	
VOLK	0.012	0.010	0.004	0.014	0.076	0.013	0.005	0.012	0.014	0.011	0.035	0.017	0.024

F_{ST} values range from 0.0 (no differentiation) to 1.0 (complete differentiation).

Table 5
Population genetic parameter estimates for 11 *Hydrellia* populations sampled in South Africa using the ISSR data set.

Population	*n*	No. of polymorphic bands	% polymorphic bands;	H_E, expected heterozygosity (Nei's gene diversity) (SE)
AMF	12	46	19.41%	0.041 (0.006)
CALA	6	15	6.33%	0.019 (0.005)
CARO	4	8	3.38%	0.014 (0.005)
CHRIS	–	–	–	–
DAD	10	34	14.35%	0.036 (0.006)
LAB	–	–	–	–
EIC	8	29	12.24%	0.037 (0.007)
ERM	10	39	16.46%	0.037 (0.006)
KTD	4	12	5.06%	0.021 (0.006)
LVR	8	39	16.46%	0.043 (0.007)
ROS	12	38	16.03%	0.036 (0.006)
POT	3	29	2.53%	0.011 (0.004)
UND	10	37	15.61%	0.040 (0.007)
VOLK	–	–	–	–

lagarosiphon collected on *L. major* and *L. muscoides*. This is further supported by the AMOVA which identified that most of the genetic diversity is within and not between populations. Until recently, genetic screening of biological control agents was not common practice (Paterson et al., 2016; Toševski et al., 2013) and the information presented here provides a valuable insight into the genetic structure of *H. lagarosiphon*, and highlights the apparent absence of cryptic species in South African *H. lagarosiphon*. In addition, the low levels of divergence between the populations suggest there is a higher likelihood that the host ranges of the populations will be similar. However, Paterson et al. (2016) recommend that for potential biological control agents multiple populations of the same species are screened separately to avoid unpredicted non-target effects. Host specificity testing, carried out on the Kubusi farm dam population, show that *H. lagarosiphon* is specific to *Lagarosiphon* and poses no threat to either exotic or native plants in the order Alismatales in Ireland and New Zealand (Mangan, 2013; Baars and Paynter, 2014). However, further host testing will be required should the release of any additional populations of *H. lagarosiphon* be pursued.

Fry and Zink (1998) suggest that a lack of geographic structure in rapidly evolving molecular markers, such as mtDNA, implies high levels of gene flow. However, it is also possible that low Fst values (as observed here) may be indicative of high levels of polymorphism even where gene flow is reduced (Jin and Chakraborty, 1995). The large distance (> 1000 km) between the Eastern Cape and Mpumalanga sites in particular, separated by mountain ranges (Drakensberg Mountains) which form natural geographic barriers, would potentially inhibit the natural movement of *H. lagarosiphon* between these regions. However, *L. major* and *L. muscoides* proliferate in man-made dams and impoundments throughout the country (Baars et al., 2010) and networks of impoundments, such as those found across South Africa, can drastically alter the distribution of freshwater ecosystems, even across large spatial scales (Johnson et al., 2008). These networks provide the potential for increased connectivity and mobility of *H. lagarosiphon* between sites, resulting in high levels of gene flow, which highlights the potential for quick and efficient spread of *H. lagarosiphon*, should it be released into new environments as a control measure for *L. major* (Jonsen et al., 2007; Rauth et al., 2011). Promisingly, the release of the closely related *Hydrellia pakistanae* in Florida U.S.A., resulted in wide dispersal and establishment of the fly throughout the majority of *Hydrilla*-infested water bodies in the state, which was in part attributed to the fly's natural dispersal capabilities (Center, 1997).

The high levels of genetic diversity and population admixture, combined with low levels of genetic differentiation do not support

Table 6
AMOVA output of *H. lagarosiphon* ISSR data for (A) 10 populations collected on *Lagarosiphon* species, (B) for the same populations across the three provinces and, (C) the blue and green clusters characterized by the principal component analysis (see Fig. 2).

	Source	SS	d.f.	Variance component	% variance	Φ_{ST}	P
A	Within populations	508.650	72	7.065	83.45	0.165	0.001
	Among populations	165.679	9	1.401	16.55		
	Total	674.329	81	8.466			
B	Within populations	638.666	79	8.084	95.30	0.04	0.001
	Among populations	35.663	2	0.398	4.69		
	Total	674.329	81	8.482			
C	Within populations	678.329	83	8.173	43.36	0.566	0.000
	Among populations	69.953	1	10.673	56.64		
	Total	748.282	84	18.846			

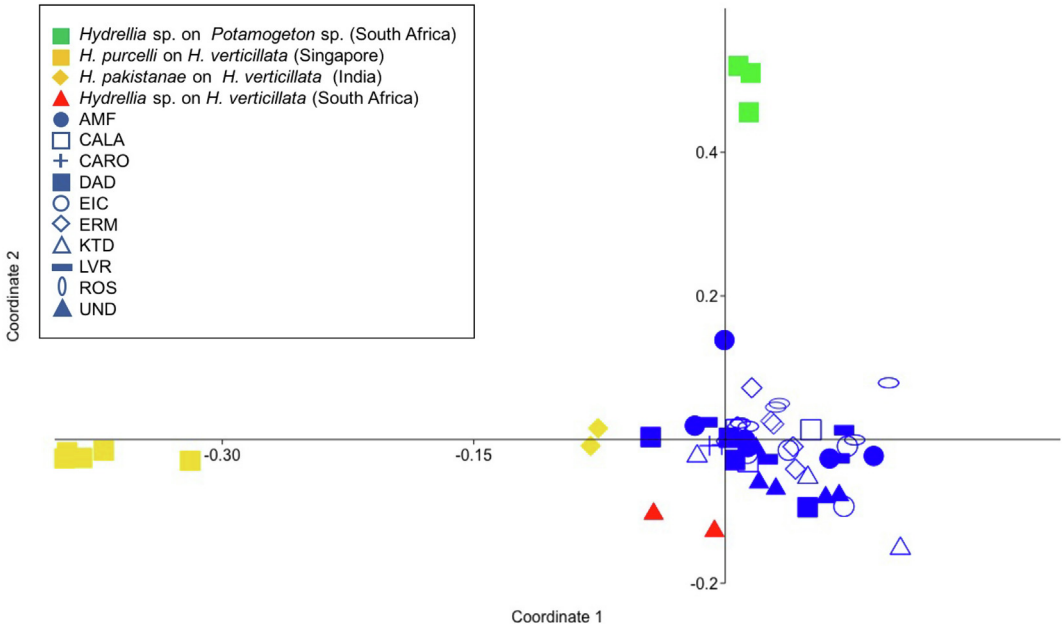

Fig. 2. Principal component analysis (PCA) resolving clusters of *Hydrellia lagarosiphon* individuals collected on *L. major* and *L. muscoides*, *Hydrellia* sp. individuals collected on *Potamogeton* sp. and *H. pakistanae* and *H. purcelli* collected on *H. verticillata.*

genetic divergence by isolation. As a result, evidence of local adaptation and potential performance differences between populations originating from different parts of South Africa are unlikely. Preliminary data collected on the performance of three populations from different eco-regions (Kubusi farm dam in the Eastern Cape, Chrissiesmeer in Mpumalanga, and Volksrust farm dam near the Mpumalanga-KwaZulu-Natal border) support these findings, with no significant differences evident for the reproductive development or thermal tolerances of *H. lagarosiphon* (Earle and Baars, 2011). These data suggest that none of the ecoregion-specific populations are favorably adapted to low temperature ranges and the selection of a particular population as the founder as a biocontrol agent stock will not confer any developmental performance benefit. Encouragingly, the colonization potential of the Kubusi farm dam (LAB) population has been evaluated, and results indicate that although climate may be a potential limiting factor affecting the colonization success of *H. lagarosiphon* in certain areas, most biogeographical regions in Europe appeared suitable for the establishment of permanent populations of the fly (Mangan and Baars, 2013).

Maintaining a high level of genetic diversity within a field-released population is also an important step in successful biocontrol establishment, as it is likely to increase the probability of survival, local adaptation, and breeding after establishment in the field (Angalet et al., 1979; Lloyd et al., 2005; Reed and Frankham, 2001). One method of facilitating establishment success of an introduced species is to cross genetically differentiated populations (Rius and Darling, 2014). This approach can increase genetic diversity, create new genotypes and mask recessive deleterious mutations (Frankham, 2005; Li et al., 2018), and was common practice in the early 1990s (Paynter et al., 2008). However, it is no longer a recommended policy as the prediction of the long-term dynamics under field conditions, in particular host range evolution, can be extremely difficult (Futuyma et al., 1995). Large founder populations of a single consignment are therefore preferable, which reduce the probability of a genetic bottleneck (Taylor et al., 2011), and increase the likelihood of sampling a genetically diverse stock population during host specificity testing (Rauth et al., 2011). The rearing technique adopted by our group, of frequently transferring individuals between culture cages to avoid inbreeding and to maximise genetic diversity, has resulted in vigorous population growth since the establishment of laboratory cultures in 2009 and this population has maintained substantial genetic variation over this time.

The results of our study highlight the benefits of using molecular genetics to obtain a better understanding of population-level genetic structure and variation in potential biocontrol agents. In addition to identifying cryptic species, genetic analysis, as shown here, can identify potential variation (or lack thereof) in an agent's climatic adaptation or host-related traits and can predict the potential for effective dispersal into new environments. Such information is essential to the selection and efficient use of any potential biological control agent and we support the recommendation of adopting molecular genetic analyses as a prerequisite for any future biocontrol assessment programmes that wish to maximise the efficiency and potential success of newly released agents.

Author statement

R.M., J.C.C., and J.-R.B. conceived the experiments. R.M. and J.C.C. contributed to sample preparation and planned the experiments. R.M. carried out the experiments, conducted the analysis and prepared the manuscript. All authors provided critical feedback and helped shape the final manuscript.

Acknowledgements

We gratefully acknowledge the ongoing help we have received from the staff of Inland Fisheries Ireland and the assistance provided by colleagues in the Department of Zoology and Entomology, Rhodes University, South Africa. Funding was provided by the Irish Research Council, the CAISIE Project EU Life+ Programme, and the South African Research Chairs Initiative of the Department of Science and Technology and the National Research Foundation of South Africa.

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