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BIODIVERSITY RESEARCH

Genetic analysis along an invasion pathway reveals endemic cryptic taxa, but a single species with little population structure in the introduced range

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ABSTRACT

Aim The invasion pathways of pest arthropods can be traced using genetic tools to develop an understanding of the processes that have shaped successful invasions and to inform both pest management and conservation strategies in their non-native and native ranges, respectively. The redlegged earth mite, *Halotydeus destructor,* is a major economic pest in Australia, successfully establishing and spreading after arrival from South Africa more than 100 years ago. *Halotydeus destructor* has recently expanded its range and evolved resistance to numerous pesticides in Australia, raising questions around its origin and spread.

Location South Africa and Australia.

Methods We sampled *H. destructor* populations in South Africa and Australia and developed a microsatellite marker library. We then examined genetic variation using mtDNA and microsatellite markers across both native and invasive ranges to determine endemic genetic diversity within South Africa, identify the likely origin of invasive populations and test genetic divergence across Australia.

Results The data show that *H. destructor* comprises a cryptic species complex in South Africa, with putative climatic/host plant associations that may correspond to regional variation. A lineage similar to that found near Cape Town has spread throughout Western and eastern Australia, where populations remain genetically similar.

Main conclusions Tracing the invasion pathway of this economically important pest revealed cryptic lineages in South Africa which points to the need for a taxonomic revision. The absence of significant genetic structure across the wide invasive range of *H. destructor* within Australia has implications for the development (and spread) of pesticide resistance and also points to recent local adaptation in physiological traits.

Keywords
biogeography, biological invasions, cryptic species, *Halotydeus destructor*, population genetics.

INTRODUCTION

Invasive terrestrial arthropods include some of the most important global pest species that threaten agricultural production and challenge food security (Armstrong & Ball, 2005; Bebber *et al.*, 2014). With global trade and tourism driving movement of such species (Banks *et al.*, 2015), it is important to understand patterns of invasion so that risks

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and barriers can be identified (Blackburn *et al.*, 2011). Genetic markers are particularly useful in this context because they can capture historical invasion processes, patterns of admixture following invasion and adaptive changes that may have occurred in populations subsequent to invasions taking place (Armstrong & Ball, 2005; Handley *et al.*, 2011; Chown *et al.*, 2015).

Patterns of neutral genetic variation across populations within both native and invasive ranges are commonly used to gain insights into processes such as invasion route (Estoup & Guillemaud, 2010), number of invasion events (Ciosi *et al.*, 2008; Yang *et al.*, 2012b) and demographic history (Yang *et al.*, 2012a; Zhang *et al.*, 2012). Importantly, this information can help determine breeding systems, dispersal patterns and spatial connectivity of populations among invasive pest populations (e.g. Endersby *et al.*, 2006; Ciosi *et al.*, 2008; Miller *et al.*, 2012; Yang *et al.*, 2012a; Zhang *et al.*, 2012). Understanding the dynamics of pest populations is important when assessing risks associated with range expansions and the evolution and spread of pesticide resistance (Nahrung & Allen, 2003; Endersby *et al.*, 2006, 2007). However, for population genetic research to be useful, species boundaries need to be defined so that genetic tools and inference are applied correctly.

Historically, invasive invertebrate species have been defined morphologically and considered as single entities, complete with their own suite of biological, physiological and ecological traits. However, morphologically based definitions can be challenged by genetic studies revealing cryptic species through high levels of genetic differentiation among groups (Bickford *et al.*, 2007; Mathews *et al.*, 2008). Genetic data have been used to identify morphologically cryptic species that were once reported as a single species (e.g. *Bemisia tabaci,* De Barro *et al.*, 2011; Boykin *et al.*, 2012), while conversely, other species that first appeared to be separate have been resolved as conspecific (e.g. *Bactrocera dorsalis/invadens,* Boykin *et al.*, 2015). Such findings have implications for global trade regulations (Schutze *et al.*, 2015) and management practices being applied across different regions (Perring, 2001; Boykin *et al.*, 2012). Further, cryptic pest species raise questions about ecological and evolutionary processes for both endemic and invasive diversity (Mathews *et al.*, 2008) and the drivers of invasive success. Such information may help reveal why a certain lineage has become a successful invasive, aid in the search for more appropriate biocontrol agents, and improve understanding of the ecology of the species in its native range. By understanding genetic diversity and species boundaries across both native and invasive ranges, better conservation and management strategies may thus be constructed (Bickford *et al.*, 2007).

The redlegged earth mite, *Halotydeus destructor* Tucker (Acari: Penthaleidae), is a polyphagous mite native to the Western and Northern Cape provinces of South Africa. The species was introduced to Australia over 100 years ago, likely due to the movement of ship ballast (soil), where it subsequently established as a serious economic pest (Wallace & Mahon, 1971; Ridsdill-Smith, 1997; Ridsdill-Smith & Pavri, 2000). *Halotydeus destructor* occurs mainly in regions with cool wet winters (Ridsdill-Smith & Annells, 1997) and undergoes at least three generations a year before entering into facultative summer diapause (Ridsdill-Smith *et al.*, 2005). The mites are particularly damaging at the establishment phase of crops (Robinson & Hoffmann, 2001) and reduce the productivity of established pastures (James & O'Malley, 1991). The feeding damage caused to Australian grain crops alone currently stands at >$40 million per annum, with another $20.5 million spent on pesticide treatments each year (Murray *et al.*, 2013). Due to its economic importance, *H. destructor* has received considerable research attention, mainly focusing on pesticide efficacy, competitive interactions and potential biological control (e.g. Umina & Hoffmann, 1999; Weeks & Hoffmann, 2000; Halliday & Paull, 2004; Ridsdill-Smith *et al.*, 2008). More recent studies have focused on the ecological niche of *H. destructor,* and how this differs between native and invasive populations (Hill *et al.*, 2012b, 2013), and detecting evolved pesticide resistance in some populations (Umina, 2007; Umina *et al.*, 2012). However, despite evidence of local physiological adaptation (Hill *et al.*, 2013), information on patterns of genetic differentiation across native and invasive ranges is limited.

Two previous studies have investigated genetic differentiation in *H. destructor* from South Africa and Australia based on allozyme markers (Weeks *et al.*, 1995; Qin, 1997). These studies were based on very few markers but nevertheless suggested that Australian populations of *H. destructor* are likely to be conspecific and panmictic (Weeks *et al.*, 1995) and derived from an area near Cape Town, South Africa (Qin, 1997). The lack of genetic differentiation across Australian populations suggests colonization by large populations and moderate gene flow among populations, perhaps assisted by human activities. In contrast, South African populations appear to be geographically structured between the Northern Cape Province and Cape Town (Qin, 1997). However, the low number of allozyme markers available had limited power in resolving genetic differentiation.

In this study, we use a combination of microsatellite and mtDNA variation to determine the extent of genetic differentiation among *H. destructor* across its native and invasive ranges. First, we undertake a phylogenetic analysis to examine the relationship between Australian and South African populations and identify potential invasion sources, as well as testing for the possible existence of a cryptic species complex. Secondly, we test whether Australian populations exhibit less genetic diversity than South African populations, congruent with patterns observed in previous allozyme studies (Qin, 1997) and thermal tolerance data (Hill *et al.*, 2013). Thirdly, we attempt to retrace the invasion history of Australian *H. destructor* populations, to see whether they represent a single introduction. Finally, we assess the extent of gene flow between populations in eastern Australia and

Western Australia to understand population processes and the potential for ongoing gene flow that might impact the spread of pesticide resistance and other adaptive shifts.

METHODS

Population sampling

Halotydeus destructor populations were sampled in Australia and South Africa between 2009 and 2011 (Fig. 1). South Africa populations were selected from three geographical regions based on groupings identified by Qin (1997) and to reflect populations that may occupy different niches based on species distribution modelling (Hill *et al.*, 2012b). A population from Stellenbosch (near Cape Town) representing the putative source of Australian populations was also included. In Australia, three geographical regions were sampled: Western Australia, eastern Australia (South Australia, Victoria and New South Wales) and Tasmania. We also included populations in inland eastern Australia that appear to have undergone recent range expansion (Hill *et al.*, 2012b, 2013). Samples were collected by vacuum using a Stihl blower-vac (Model SH 55; Andreas Stihl AG & Co. KG, Waiblingen, Germany) with a fine gauze sieve (200 μm) attached. Samples were returned to the laboratory, identified and placed directly into ethanol, and then frozen at −80 °C. In South Africa, *H. destructor* occurs sympatrically with at least one other described congeneric species, *Halotydeus anthropus* Qin & Halliday, and is not readily distinguishable in the field. The key character used to differentiate *Halotydeus* spp. is that *H. destructor* possess two setae on each of coxae III and IV, while *H. anthropus* only possesses one (Qin & Halliday, 1996a). South African mites were identified to species under light microscope at 100× magnification (B. Halliday, pers. comm.).

DNA extraction, amplification and sequencing

Total genomic DNA was extracted with a modified Chelex extraction protocol (Walsh *et al.*, 1991). Individual whole mite specimens were placed in a 1.5-mL tube with 100 μL of 5% Chelex (Roche, Mannheim, Germany) solution, 3 μL Proteinase K (10 mg mL^{-1}) and two glass beads (2 mm), with tubes then vigorously shaken by Mixer mill at 25 times per second for 2 min to crush the mites. Then, samples were incubated at 56 °C for 1 h with periodic vortexing, followed by further digestion at 95 °C for 8 min. DNA extractions were cooled on ice for 20 min and stored at −20 °C until analysis. Prior to PCR, Chelex extractions were homogenized by inversion and centrifuged at 13,000 rpm for 2 min. Supernatant was subsequently taken for PCR from the bottom half of the supernatant, above the Chelex resin precipitate.

DNA sequence data from the mitochondrial cytochrome oxidase I (*COI*) gene were analysed to assess the species status of *H. destructor*. Approximately 580 bp of the *COI* gene was amplified by PCR using primers HCO2198 and LCO1490 (Folmer *et al.*, 1994). PCRs were prepared in 30 μL volumes including 25 μL mastermix and 5 μL DNA extraction. If the $MgCl_2$ is 2 mM, master mix includes 16.1 μL ddH2O, 3 μL (1×) NEB buffer with 20 mM $MgCl_2$, 0.3 μL BSA (10 mg mL^{-1}), 2.4 μL dNTPs (2.5), 1.5 μL HCO primer (10 μM), 1.5 μL LCO primer (10 μM), 0.2 μL NEB taq (1 unit). PCRs were conducted using an Eppendorf Gradient S Master Cycler with cycling conditions consisting of an initial denaturing step of 95 °C for 2 min followed by 10 cycles of touch down, 95 °C for 20 s, 55 °C decreasing to 46 °C for 45 s (decreasing by 1 °C each cycle), 72 °C for 30 s followed by 28 cycles of 95 °C for 20 s, 45 °C for 45 s (increasing the annealing time by 2 s each cycle), 72 °C for 45 s and a final elongation step of 72 °C for 5 min. PCR products were directly sequenced in both directions using

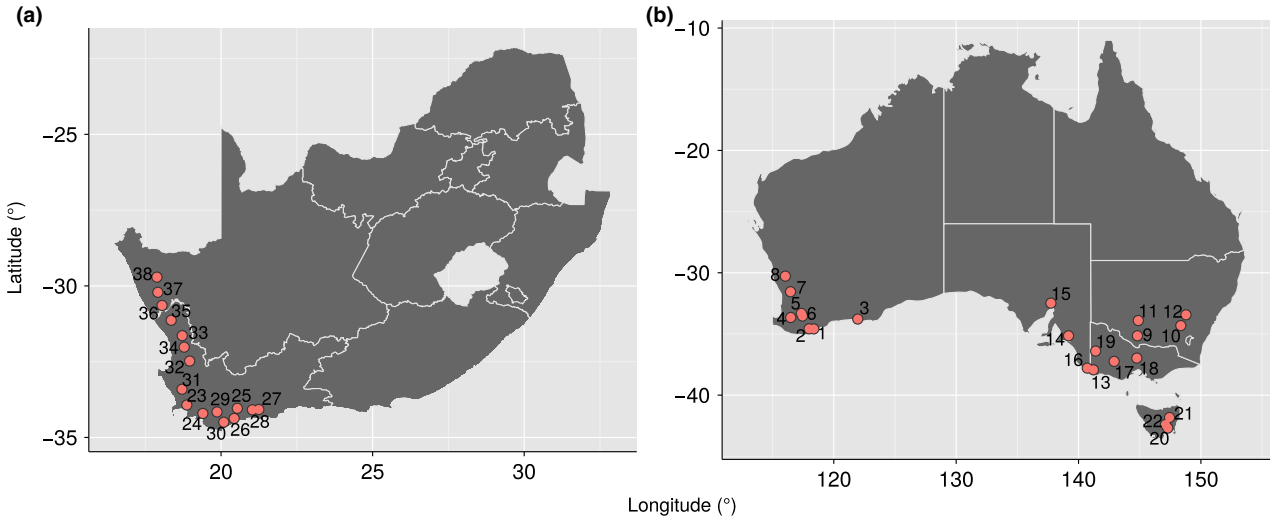

Figure 1 Sample localities of *Halotydeus* spp. in (a) South Africa and (b) Australia. Numbers correspond to sample locations are listed in Table 1.

the primers described above on an ABI 3730 DNA Analyzer, Applied-Biosystems, Foster City, CA. Forward and reverse sequences were aligned and manually edited in GENEIOUS 5.6.5 (Biomatters 2012).

Phylogenetic analysis

Phylogenetic reconstructions were performed using unique haplotypes and Bayesian inference (BI) methods implemented in BEAST 2.1.3 (Bouckaert *et al.*, 2014). We ran JMODELTEST v.0.1.1 (Darriba *et al.*, 2012) to select the best-fit model of evolution, based on Akaike information criteria (AIC) (Akaike, 1981) (GTR + G in each case). Operators were auto-optimized, and five independent Markov chain Monte Carlo (MCMC) runs were performed with a Yule (speciation) tree-prior, each running for 5×10^6 generations, sampling every 10,000 states. Log files were examined with TRACER v.1.5 (Drummond & Rambaut, 2007) to ensure that runs were sampling from the same posterior distribution, to determine appropriate burn-in and to ensure that effective sample sizes of parameters of interest were > 1000. Tree files of independent runs were then combined with LOGCOMBINER v.2.1.3, discarding the first 20% and re-sampling at lower frequency of 15,000. The maximum clade credibility tree was recovered from a sample of 10,000 posterior trees, and branch support was annotated, using TREEANNOTATOR v.2.1.3. All analyses started with a random starting tree and seed with no root specified. The root was estimated with sequence data from two trombidiform mite species, including one from the family Rhagidiidae and an unclassified Trombidiformes species (GenBank Accession Numbers JX837057 and JX838738). These were selected based on BLAST analyses that revealed they shared the greatest sequence similarity to haplotypes characterized in this study. The gene tree could not be time calibrated as no reliable substitution rate estimates are currently available for Trombidiformes, and recent studies indicate that substitution rates are variable among Acari lineages (Dabert *et al.*, 2010).

Molecular-based species delineation

We implemented a DNA taxonomy approach to evaluate the presence of cryptic species (Modica *et al.*, 2014; Lang *et al.*, 2015; Toussaint *et al.*, 2015). We applied the combination of the Poisson tree processes model for species delimitation (PTP), and a Bayesian implementation of PTP (bPTP) to infer putative species boundaries on a given phylogenetic input tree (Zhang *et al.*, 2013). The PTP/bPTP model requires a bifurcated phylogenetic tree and model speciations or branching events as the number of substitutions. We used the following parameters: MCMC, 500,000 generations; thinning, 100; burn-in, 0.1; seed, 123, and assessed convergence in each case to ensure reliability of results. As the tree was not time calibrated, the alternative general mixed Yule coalescent approach (Pons *et al.*, 2006; Fujisawa & Barraclough, 2013) could not be applied.

Microsatellite marker development

There are only a limited number of mite genetic studies that have worked with microsatellite markers, and their characterization has generally been limited to traditional enrichment protocols with varying success (Osakabe *et al.*, 2000; Evans *et al.*, 2003; Miller *et al.*, 2012). More recently, next generation sequencing technologies have been used successfully for the rapid identification and characterization of polymorphic microsatellite loci across a wide range of taxonomic groups (Miller *et al.*, 2013a; Berman *et al.*, 2014; Weeks *et al.*, 2014). Consequently, we followed the methods presented in Miller *et al.* (2013a) and Blacket *et al.* (2012). Briefly, a single DNA extraction was performed on approximately 400 individual mites (to yield at least 10 μg of DNA), using a Qiagen DNeasy kit following the manufacturers specifications. DNA was subsequently processed by the Australian Genome Research Facility (AGRF, Melbourne, Australia) where it was nebulized, ligated with 454 sequencing primers and tagged with a unique oligo sequence allowing sequences to be separated from pooled species DNA sequences using post-run bioinformatic tools. The DNA sample was analysed with high-throughput DNA sequencing on 1/16th of a 70 × 75 mm PicoTiterPlate on a Roche 454 Genome Sequencer FLX with the Titanium Sequencing kit XLR 70 (Margulies *et al.*, 2005). Using the software QDD (Meglécz *et al.*, 2010), 667 unique sequence contigs possessing microsatellite motifs were identified. Primer3 (Rozen & Skaletsky, 2000) was used to design optimal primer sets for each unique contig where possible, and we finalized a set of 40 contigs for analysis, all of which contained di-nucleotide repeats.

Loci were screened for polymorphism with template DNA from eight individuals, including four from Booligal, New South Wales and four from Toodyay, Western Australia. Loci were pooled into ten groups of four, labelled with unique fluorophores (FAM, NED, VIC, PET) and co-amplified by multiplex PCR using a Qiagen multiplex kit (Qiagen, Hilden, Germany) and an Eppendorf Mastercycler S gradient PCR machine following the protocol described by Blacket *et al.* (2012). PCRs were performed in 11 μL reactions containing 5 μL Qiagen multiplex mix, 0.125 μM each forward tailed primer and 0.125 μM corresponding fluorescently labelled primer, 0.25 μM pigtailed reverse primer (Brownstein *et al.*, 1996) and 2 μL template DNA. PCR cycling conditions consisted of an initial denaturation at 95 °C for 15 min, followed by 40 cycles of 95 °C for 20 s, 59 °C for 90 s and 69 °C for 60 s. A final extension step of 69 °C for 30 min preceded an indefinite hold period at 20 °C.

Genotyping was performed using an Applied Biosystems 3730 capillary analyser, and product lengths were determined relative to a GS500LIZ_3730 size standard (AGRF). Genotypes and product lengths were scored manually and assessed for polymorphisms with GENEMAPPER v.4.0 (Applied-Biosystems, Foster City, CA). From a total of 40 loci, 14 were found to be polymorphic and the remaining 26 were either

monomorphic or failed to amplify. The subset of 14 polymorphic loci were pooled into three groups for multiplexing, based on observed locus-specific allele size ranges and further characterized in 30 individuals from both the Booligal (BOO) and Toodyay (TOO) populations. After successful amplification and genotyping of these populations, analysis was extended to 19 further populations from Australia and five from South Africa. Thirty mites were analysed per population.

Population genetic analysis

Descriptive statistics were calculated for the microsatellite data with FSTAT v.2.9.3 (Goudet, 1995). We calculated allelic richness per population averaged over loci, Weir and Cockerham's measure of F_{IS}, a global estimate of F_{ST} (with 95% confidence limits) (Weir & Cockerham, 1984), population pairwise measures of F_{ST} and their significance determined with permutations (1000), and pairs of loci tested for linkage disequilibrium using a log-likelihood ratio test. The software MICRO-CHECKER (Van Oosterhout *et al.*, 2004) was used to assess microsatellite loci for null alleles and scoring errors as outlined by Brookfield (1996).

Estimates of observed (H_O) and expected (H_E) heterozygosity were determined using the Excel Microsatellite Toolkit (Park, 2001) and deviations from Hardy–Weinberg equilibrium (HWE) were tested with GENEPOP v.3.4 (Raymond & Rousset, 1995). An AMOVA was performed in GENALEX (Peakall & Smouse, 2006) with pairwise F_{ST} as the distance measure, 10,000 permutations and missing data for loci set at 10%. The model for analysis partitioned variation among regions (South Africa, Western Australia, eastern Australia and Tasmania), among sample sites within regions and within sample sites. A factorial correspondence analysis (FCA), implemented in GENETIX v.4.05 (Belkhir *et al.*, 2004), was used to summarize patterns of genetic differentiation. The first two underlying factors explaining the majority of variation in multilocus genotypes across loci were plotted.

Bayesian analyses were conducted with three software packages to estimate the number of populations within the sample data. The program STRUCTURE (Pritchard *et al.*, 2000) was used to identify the number of distinct clusters/populations, assign individuals to clusters and identify migrants and admixed individuals based on genetic data only. To determine the number of populations (K), three independent simulations for $K = 1$–10 with 10,000 burn-in and 100,000 data iterations were run. Analyses were performed with the admixture model of population structure (i.e. each individual draws some fraction of their genome from each of the K populations), and allele frequencies were set as independent among populations. Results of all simulations were analysed with Structure Harvester (Earl & vonHoldt, 2012) to examine delta K using the Evanno method (Evanno *et al.*, 2005). Analysis of population genetic structure in a geographically constrained Bayesian model was performed using GENELAND (Guillot *et al.*, 2005). The inference algorithm was launched with a single step approach (Guillot, 2008) with the Dirichlet distribution as prior for allele frequencies and where K was allowed to vary from 1 to 22. Ten independent runs of 100,000 MCMC iterations were performed.

Environmental relationships

Finally, we examined differences in the species-environment relationships across mite populations. Such information is useful to determine patterns of distribution and identify environmental gradients that may underpin biodiversity. Previous studies using niche-based analyses revealed that invasive populations of *H. destructor* have shifted in response to key environmental variables, and that distribution models built on the Australian range do not predict the northern parts of the South African distribution (Hill *et al.*, 2012b). To test whether our *COI*-based species identification may align with environmental variation, we looked at environmental similarity of all sites employing the four bioclimatic variables correlated with *H. destructor* distribution limits identified in Hill *et al.* (2012b). We employed principal component analysis to reduce the variation across these sites to the first two components and examined clustering of the putative species on a biplot.

RESULTS

Population sampling

We included 22 Australian populations of *H. destructor* and 14 populations from South Africa, with an additional two South African populations determined to be *H. anthropus* based on morphological characters (primarily the number of coxal setae, Qin & Halliday, 1996; B. Halliday, pers comm.) (Fig. 1; Table 1). The remainder of the South African populations had no obvious morphological characters to separate them into morphospecies beyond *H. destructor*.

Many of the same host plants were present in both South Africa and Australia, with clovers (*Trifolium* spp.) and *Oxalis* sp. being common across our samples, and cape weed (*Arctotheca calendula*) and Paterson's curse (*Echium plantagineum*) also present across much of the native and invasive ranges. Populations in the northern parts of South Africa mostly came from *Oxalis* sp. (which is native in South Africa) as opposed to *Trifolium* spp. in the southern parts of the range (Table 1).

Lineages and species identification

We successfully amplified *COI* mitochondrial DNA data for all 14 sample locations in South Africa, eight sample locations from Australia (Fig. 1; Table 1), and two additional sample locations of *H. anthropus* (HDB and RDL) from South Africa as identified through morphological characters (Fig. 1; Table 1). Bayesian inference analyses of the

Table 1 Sampling locations targeting *Halotydeus destructor* across South Africa and Australia and number of individuals included in genotyping and population genetic analysis. Australian sample locations are from the states Western Australia and Tasmania, and the region eastern Australia (South Australia, Victoria and New South Wales). South African sample locations are from the Western Cape and Northern Cape provinces.

	Species ID	Population	Locality	Latitude	Longitude	*n* (microsats)	Haplotype (*n*)	Host
Western Australia								
1	*Halotydeus destructor*	WEL	Wellstead	−34.5863	118.3643	25	1 (2)	*Trifolium* sp., *Plantago* sp., *Lolium* sp.
2	*H. destructor*	KAM	Kamballup	−34.579	117.979	27		*Arctotheca calendula, Plantago* sp.
3	*H. destructor*	ESP	Esperence	−33.78188	121.9565	28		*Arctotheca calendula, Plantago* sp.
4	*H. destructor*	BLE	Bleechmore	−33.657	116.477	28		*Trifolium* sp., *Plantago* sp., *Lolium* sp.
5	*H. destructor*	BAX	Baxter	−33.53743	117.44051	30		*Arctotheca calendula, Plantago* sp.
6	*H. destructor*	WAG	Wagin	−33.3182833	117.34985	30		*Arctotheca calendula*
7	*H. destructor*	TOO	Toodyay	−31.55085	116.462067	30		*Arctotheca calendula*
8	*H. destructor*	WAT	Watheroo	−30.2761833	116.040567	30	6 (2)	*Arctotheca calendula*
Eastern Australia								
9	*H. destructor*	WAN	Wanganella	−35.1483333	144.810833	29	4 (1), 6 (1), 35 (1)	*Trifolium* sp. Adj. salt bush
10	*H. destructor*	YOU	Young	−34.31905	148.35695	30	1 (2), 2 (1)	*Echium plantagineum* & *Poa* sp., adj. pasture.
11	*H. destructor*	BOO	Booligal	−33.9038889	144.884444	30	1 (1)	*Trifolium* sp., *Arctotheca calendula*
12	*H. destructor*	CAN	Canowindra	−33.43552	148.77982	30		*Arctotheca calendula*, *Echium plantagineum*
13	*H. destructor*	MGR	Mount Gambier	−37.792	140.713	30		*Trifolium* sp. adj pasture
14	*H. destructor*	MBE	Murray Bridge	−35.1498333	139.180528	30		*Hordeum vulgare*, *Poa* spp.
15	*H. destructor*	PAA	Port Augusta	−32.4825556	137.743139	30		*Hordeum vulgare*, *Arctotheca calendula*
16	*H. destructor*	DRT	Dartmoor	−37.931	141.216		1 (2)	*Trifolium* sp. adj pasture
17	*H. destructor*	ARA	Ararat	−37.245	142.912	29		*Arctotheca calendula*, adj. pasture
18	*H. destructor*	HEA	Heathcote	−36.9725	144.763056	30	6 (2)	*Malva* sp.
19	*H. destructor*	NHI	Nhill	−36.399	141.396	30		*Oxalis* sp. and *Arctotheca calendula* adj wheat field
Tasmania								
20	*H. destructor*	BRI	Brighton	−42.6930833	147.294133	30		*Trifolium* sp., *Plantago* sp., *Poa* sp.
21	*H. destructor*	BOT	Bothwell	−42.4035333	147.104	30		*Trifolium* sp., *Plantago* sp.
22	*H. destructor*	CON	Conara	−41.82985	147.43615	30	1 (1), 2 (1)	*Arctotheca calendula*
Western Cape								
23	*H. destructor*	STE	Stellenbosch	−33.928593	18.869861	30	3 (2)	*Trifolium* sp.
24	*Halotydeus* spp.	GRY	Greyton	−34.20878	19.39912		29 (1), 30 (1), 31 (1)	*Trifolium* sp., *Echium plantagineum*, *Fumaria* sp.
25	*H. destructor*	BFR	Buffeljadsrivier	−34.039515	20.5369		8 (1), 9 (2)	*Trifolium* sp. and *Poa* spp.
26	*H. destructor*	EBD	East of Bredasdorp	−34.37308	20.43224		5 (1), 33 (1)	*Oxalis* sp., *Poa* spp., *Sinapis arvensis*, *Plantago* sp.
27	*H. anthropus*	HDB*	Heidelberg	−34.0872	21.03158		23 (1), 24 (1)	*Trifolium* sp. and *Plantago* sp.

Table 1 Continued.

	Species ID	Population	Locality	Latitude	Longitude	*n* (microsats)	Haplotype (*n*)	Host
	28 *H. anthropus*	RDL*	Riversdale	−34.07784	21.24533		25 (2)	*Plantago* sp., *Trifolium* sp., *Helminthotheca echioides*, *Oxalis* sp., *Sinapis arvensis*
	29 *Halotydeus* spp.	RSD	Riviersonderend	−34.158	19.86683		22 (2)	*Trifolium* sp.
	30 *Halotydeus* spp.	BDP	Bredasdorp	−34.49661	20.08819		18 (1), 19 (1), 20 (1), 21 (1)	*Trifolium* sp. and *Poa* spp.
	31 *H. destructor*	MLM	Malmesbury	−33.40916	18.71113		7 (1), 26 (1)	*Oxalis* sp., *Plantago* sp., *Poa* spp.
	32 *Halotydeus* spp.	NCD	North of Citrusdal	−32.47917	18.96356		27 (1), 28 (1)	*Echium plantagineum* & *Trifolium* sp.
	33 *Halotydeus* spp.	NCW	North of Clanwilliam	−32.02033	18.78299		10 (1), 11 (1)	*Oxalis* sp.
	34 *Halotydeus* spp.	VRD	Vanrhynsdorp	−31.64277	18.71912		13 (2)	*Oxalis* sp.
	35 *Halotydeus* spp.	NWR	Nuwerus	−31.13376	18.35381		15 (2)	*Oxalis* sp.
	36 *Halotydeus* spp.	SGR	South of Garies	−30.64446	18.05325		16 (1), 17 (1), 32 (1)	*Oxalis* sp.
Northern Cape								
	39 *Halotydeus* spp.	KMK	Kamieskroon	−30.21109	17.91422		12 (2)	*Oxalis* sp.
	38 *Halotydeus* spp.	SBK	Springbok	−29.71148	17.88412		12 (1), 14 (1)	*Arctotheca calendula*, *Oxalis* sp.

*Sample identified as *Halotydeus anthropus* prior to molecular analysis.

mitochondrial dataset produced a generally well-resolved phylogeny composing 10 monophyletic clades (PP > 0.95) (Fig. 2). An 11th lineage was also identified and was nested within the outgroup taxa with a high level of support (PP > 0.95). In contrast, most interrelationships among lineages were generally poorly resolved (PP < 0.95). All Australian haplotypes grouped within a single well-supported clade (PP > 0.95), including shared and closely related haplotypes from South African sample locations STE, BFR, EBD and MLM (Fig. 2).

From the 34 mitochondrial haplotypes identified, the PTP/bPTP species delineation analysis identified 11 entities as separate species (acceptance rate: 0.24729; merge: 50,034; split: 49,966) corresponding with the phylogenetically distinct lineages described above (Fig. 2). Uncorrected pairwise distances between putative species groups ranged from 9.2 to 25.7% (mean = 19.0%), while distances within groups ranged from 0.2 to 2.8% (mean = 1.7%). Uncorrected pairwise distances between haplotypes and those of the outgroup taxon ranged between 17.7 and 26.6% (mean = 23.0%) (Appendix S1). Samples from South African collection sites HDB and RDL, identified as *H. anthropus*, formed a monophyletic group differing from the ingroup by an average of 20.1% sequence divergence.

Population differentiation based on microsatellites

A total of 648 individuals representing 22 *H. destructor* sample sites were successfully genotyped for 10 microsatellite loci (Fig. 1; Appendix S2). These loci were characterized by moderate to high genetic variation (8–25 alleles per locus). A total of 155 alleles were detected, with a mean of 16 alleles per locus over all sites. Allelic richness over all loci ranged between 6.33 and 7.71 (Table 2). Marker independence was confirmed across all sample sites, with linkage disequilibrium analysis indicating no significant linkage between loci. Analysis with MICROCHECKER revealed minor sporadic patterns of potential null alleles across populations; however, the only marker that appeared consistently influenced by null alleles was RL21 (four of 20 collection sites). This marker is highly polymorphic (25 alleles), and apparent null alleles may be due to mutations at the priming sites. To avoid potential biases associated with RL21, all analyses were conducted including and excluding this locus, but because these produced similar results only analyses based on all markers are presented.

Expected heterozygosities were high and ranged from 0.69 to 0.78 (mean H_E = 0.74) (Table 2). Four of the five South African populations failed to amplify for the microsatellite loci and were removed. Significant departures from HWE were observed ($P < 0.05$ across all loci) for five sites, which represented populations from each of the geographical regions (WAG, YOU, MGR, BOT and STE) (Table 2). While these departures remained significant after correction for multiple comparisons, this was driven by a different locus at each site. Overall F_{ST} was low indicating limited genetic

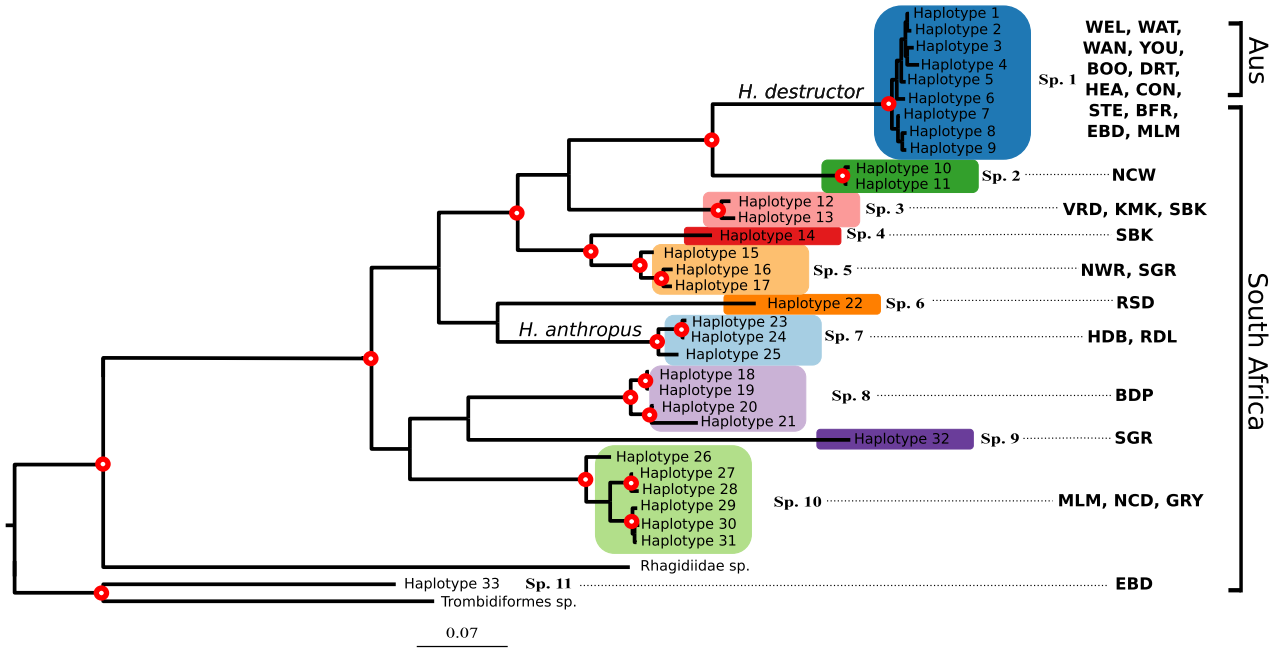

Figure 2 Bayesian inference phylogeny. Nodal support values > 95 posterior probabilities depicted by red circles. Putative species identified by bPTP numbered 1 to 11 beside terminal node. Colours assigned to each putative species correspond with Fig. 5. For haplotype association with sampling localities, refer to Table 1. Species 1 is *Halotydeus destructor*. Species 7 is *Halotydeus anthropus*. Geographical sample locations are on the right, refer to Table 1 for codes. The two outgroup samples are trombidiform mite species, including one species from the family Rhagidiidae (GenBank Accession Numbers JX837057) and an unclassified Trombidiformes species (GenBank Accession Number: JX838738).

structuring (F_{ST} = 0.05, 95% confidence intervals = 0.043–0.056). However, most pairwise comparisons of F_{ST} values were significant (although weak), suggesting some level of genetic differentiation (Table 3). This included populations collected from within the same region, such as eastern Australia. After Bonferonni correction, 12 of 231 (~5%) comparisons remained non-significant. The highest F_{ST} values were recorded in comparisons with the population from Heathcote (HEA) (F_{ST} = 0.064–0.108; Table 3), whereas there were low values among some sites from Western Australia (F_{ST} ~ 0.01). These results point to limited but significant genetic differentiation. An AMOVA showed low differentiation between regions (South Africa, Western Australia, eastern Australia and Tasmania). Variation between these regions explained 4% ($P < 0.001$) of differentiation, whereas variation within populations explained 91% ($P < 0.001$).

There was a significant association between genetic distance and geographical distance. Mantel tests showed a significant relationship between Slatkin's linearized F_{ST} and the natural log of geographical distance (Mantel r = 0.30, P = 0.05). Regression showed that this relationship was positive (Appendix S3; R^2 = 0.09); however, much of this is likely attributed to the large distance between the South African and Australia populations. When analysis was restricted to eastern Australian populations [excluding Tasmania, to compare to Weeks *et al.* (1995)], this signal was no longer significant (R^2 = 0.03, Mantel test, r = 0.17, P = 0.14), with no obvious association between geographical and genetic distance (Appendix S3).

The relationships between sample locales are depicted by the two-dimensional FCA of the microsatellite variation (Fig. 3). The two factors that explain the majority of variation had low loadings (factor 1 = 1.91%, factor 2 = 1.72%). When all multilocus genotypes are plotted against each other, there is no clear separation of samples (Fig. 3). Using the Evanno *et al.* (2005) method for determining K, STRUCTURE identified 2 clusters (K = 2) (Fig. 4). The STRUCTURE analysis indicates some divergence between eastern Australia (including Tasmania) and Western Australian population, as evidenced by the higher proportion of the ancestral genotype in Western Australia (Fig. 4). The South African sample and the Western Australian sample were in the same cluster. The GENELAND analysis was largely consistent, identifying three population clusters (K = 3), with the South African population and Western Australian populations clustering together, most eastern Australian populations clustering together, and the Heathcote (HEA) population appearing genetically distinct.

Environmental relationships

Nearly, all environmental variation found in the PCA was attributed to the first principal component axis (99.7%), with the aridity index contributing to 94.2% of this

Table 2 Site-specific statistics for *Halotydeus destructor* screened with 10 polymorphic microsatellite loci. Mean values over loci for number of alleles (*Na*), allelic richness (*a*), expected (H_E) and observed (H_O) heterozygosities, Hardy–Weinberg *P* values (HWE) and inbreeding coefficient (F_{IS}). Bold typeface indicates significance after correction for multiple comparisons. Site codes are taken from Table 1.

Site code	*a*	*r*	H_E	H_O	HWE *P*-value	F_{IS}
WEL	8.10	7.19	0.75	0.68	0.10	0.09
KAM	8.20	7.17	0.74	0.68	0.24	0.09
ESP	8.70	7.36	0.76	0.67	0.13	0.11
BLE	7.90	6.81	0.73	0.71	0.17	0.03
BAX	8.80	7.27	0.73	0.71	0.24	0.04
WAG	8.70	7.07	0.73	0.68	< **0.01**	0.07
TOO	9.20	7.79	0.78	0.72	0.14	0.07
WAT	7.70	6.45	0.70	0.62	< 0.01	0.12
WAN	8.60	7.23	0.77	0.74	0.60	0.03
YOU	8.10	6.80	0.75	0.69	< **0.01**	0.08
BOO	7.40	6.33	0.71	0.65	< 0.01	0.09
CAN	8.60	7.05	0.70	0.69	0.44	0.01
MGR	8.50	7.02	0.74	0.68	< **0.01**	0.07
MBE	8.70	7.17	0.76	0.73	0.07	0.03
PAA	8.10	6.98	0.76	0.72	0.50	0.05
ARA	9.10	7.71	0.77	0.74	0.14	0.03
HEA	6.80	5.74	0.69	0.67	0.50	0.03
NHI	8.10	6.75	0.75	0.69	0.03	0.09
BRI	7.80	6.60	0.70	0.71	0.03	−0.01
BOT	7.80	6.68	0.75	0.71	< **0.01**	0.05
CON	7.90	6.59	0.69	0.62	< 0.01	0.11
STE	8.30	6.98	0.73	0.61	< **0.01**	**0.16**

variation. The environmental variation found in *H. destructor* populations is broad overall; however, there is congruence with the *COI* results (Fig. 5a). Populations of *H. destructor* have marginal overlap with *H. anthropus* in this environmental space, and little overlap with the putative cryptic species (Fig. 5a). A number of these putative cryptic species were sampled from more arid conditions (northern South African distribution) than where either *H. destructor* or *H. anthropus* are currently found (southern South African distribution) (Fig. 5b). At least two of these cryptic species share similar environmental space with *H. destructor* and *H. anthropus* (Fig. 5a, 5b).

DISCUSSION

These findings suggest redlegged earth mites in South Africa are a complex of species. The requirements underlying the phylogenetic species concept (Wheeler & Platnick, 2000) are met by the fact that there are multiple monophyletic lineages with levels of genetic differentiation comparable to levels of interspecific divergence in other mite lineages. For example, the genetic distance between *H. destructor*, *H. anthropus* and the outgroup taxa in this study are comparable, as are interspecific *COI* divergence estimates reported for other mite groups from previous genetic studies (Roy *et al.*, 2010; Schäffer *et al.*, 2010, respectively). As *H. destructor* reproduces sexually and the different lineages often occur on sympatric hosts, it seems likely that these large genetic distances on a fine spatial scale reflect reproductive isolation. The molecular tools and approaches used here have revealed cryptic diversity that would otherwise not have been considered for this mite. Such information can now underpin a better understanding of the ecology and evolution of invasive lineages, in addition to highlighting the diversity of endemic lineages.

High levels of genetic divergence across South African populations have been previously noted based on five polymorphic allozyme loci (Qin, 1997). High genetic diversity among South Africa populations was also previously suggested by the results of physiology experiments (Hill *et al.*, 2013), which indicated higher levels of variation in thermal tolerance in South African populations of *H. destructor* when compared with Australian populations. These results likely reflect cryptic lineages in South Africa, given the high levels of mitochondrial genetic variation and failure of some microsatellites developed from Australian material to amplify across many South African populations considered in this study. Cryptic species have previously been identified in the same family as *H. destructor* (Penthaleidae), particularly in the economically important *Penthaleus* genus, which consist of morphologically cryptic species differing in host plant preference (Umina & Hoffmann, 2004), tolerance to chemicals (Umina & Hoffmann, 1999) and response to climatic factors (Hill *et al.*, 2012a). It is not yet clear whether *Halotydeus* lineages in South Africa might be constrained by host plants or climate; the status of *Halotydeus* species within South Africa warrants further research. The interaction of these cryptic lineages with vegetation outside arable farming areas (including those in South Africa) is unknown. Moreover, it is unclear which of the endemic lineages represent local pests and the extent to which different lineages are affected by biocontrol agents (which have all failed to date in the invasive Australian range; c.f. Halliday *et al.*, (2004)). Such information will aid both in managing the pest in Australia and in understanding the biodiversity value of the complex in South Africa.

Our data are consistent with an invasion pathway from South Africa into Australia, and the phylogeny supports the notion that a single lineage (consisting of several closely related mtDNA genotypes) has established successfully in Australia. The invasion route linked to ship's ballast may have led to multiple introductions from the same source population and the movement of a very large number of diapause eggs, resulting in mtDNA diversity within Australia. Given our phylogenetic findings, it is likely that the *H. destructor* lineages invading Australia occupy a much narrower distribution in South Africa than previously thought. Although we cannot determine the exact origin of the source population, the microsatellite analysis strongly suggests an origin in the Stellenbosch–Cape Town area.

Table 3 Pairwise F_{ST} (lower diagonal) and D_{est} (upper diagonal) between populations of *H. alotydeus destructor* used in this study (bold indicates non-significant after 10,000 permutations and corrections for multiple comparisons). Site codes are taken from Table 1.

	WEL	KAM	ESP	BLE	BAX	WAG	TOO	WAT	WAN	YOU	BOO	CAN	MGR	MBE	PAA	ARA	HEA	NHI	BRI	BOT	CON	STE
WEL	*	**0.006**	**0.049**	0.113	0.056	0.080	**0.024**	0.103	0.129	0.088	0.191	0.168	0.088	0.038	0.094	0.093	0.246	0.097	0.129	0.166	0.165	0.144
KAM	**0.001**	*	0.074	0.098	0.110	0.099	**0.049**	0.096	0.153	0.099	0.159	0.130	0.131	0.098	0.089	0.109	0.329	0.120	0.122	0.156	0.192	0.207
ESP	**0.015**	0.024	*	**0.060**	0.055	0.080	0.089	0.139	0.079	0.071	0.194	0.115	0.076	0.066	0.161	0.041	0.217	0.073	0.115	0.122	0.163	0.145
BLE	0.037	0.033	0.020	*	0.070	0.169	0.150	0.110	0.120	0.065	0.189	0.119	0.084	0.100	0.126	0.079	0.301	0.096	0.110	0.152	0.163	0.213
BAX	0.019	0.038	0.018	0.025	*	0.164	0.104	0.109	0.144	0.103	0.200	0.143	0.060	0.023	0.170	0.075	0.258	0.095	0.129	0.143	0.159	0.177
WAG	**0.027**	0.034	0.026	0.058	0.057	*	0.069	0.211	0.140	0.155	0.255	0.231	0.173	0.154	0.198	0.176	0.289	0.155	0.191	0.245	0.175	0.159
TOO	**0.007**	0.015	0.026	0.046	0.032	0.022	*	0.140	0.113	0.109	0.199	0.174	0.126	0.108	0.136	0.092	0.249	0.141	0.168	0.202	0.178	0.167
WAT	0.037	0.035	0.048	0.042	0.041	0.077	0.046	*	0.182	0.096	0.177	0.134	0.159	0.122	0.101	0.141	0.299	0.152	0.165	0.141	0.142	0.150
WAN	0.039	0.047	0.024	0.038	0.046	0.045	0.032	0.061	*	0.061	0.102	0.104	0.092	0.151	0.157	**0.035**	0.186	**0.055**	0.101	0.103	0.094	0.201
YOU	0.028	0.032	0.022	0.022	0.035	0.052	0.032	0.035	0.019	*	0.105	0.088	0.072	0.105	0.117	0.061	0.217	**0.052**	0.113	0.085	0.115	0.150
BOO	0.066	0.057	0.065	0.068	0.072	0.090	0.064	0.069	0.035	0.037	*	0.105	0.189	0.194	0.202	0.118	0.276	0.116	0.157	0.101	0.089	0.282
CAN	0.060	0.048	0.040	0.045	0.053	0.084	0.057	0.054	0.036	0.032	0.042	*	0.107	0.170	0.164	0.086	0.300	0.073	0.099	0.117	0.147	0.272
MGR	0.030	0.044	0.025	0.029	0.021	0.059	0.039	0.059	0.030	0.024	0.068	0.040	*	0.094	0.186	0.067	0.200	**0.050**	0.103	0.141	0.137	0.185
MBE	**0.012**	0.031	0.020	0.033	0.008	0.051	0.032	0.043	0.045	0.033	0.066	0.060	0.031	*	0.129	0.101	0.304	0.091	0.172	0.130	0.198	0.165
PAA	0.029	0.028	0.048	0.040	0.054	0.063	0.038	0.035	0.046	0.036	0.067	0.056	0.058	0.039	*	0.150	0.321	0.150	0.203	0.178	0.211	0.250
ARA	0.028	0.034	**0.012**	0.026	0.024	0.056	0.026	0.048	**0.010**	0.019	0.040	0.030	0.022	0.031	0.044	*	0.204	0.070	0.082	0.063	0.114	0.179
HEA	0.087	0.115	0.076	0.108	0.094	0.105	0.082	0.114	0.064	0.077	0.105	0.115	0.074	0.104	0.106	0.070	*	0.261	0.290	0.261	0.225	0.211
NHI	0.031	0.039	0.023	0.032	0.032	0.052	0.042	0.054	**0.017**	0.017	0.041	0.027	0.017	0.029	0.046	0.022	0.092	*	0.115	0.132	0.127	0.232
BRI	0.047	0.045	0.041	0.042	0.048	0.071	0.055	0.065	0.035	0.041	0.061	0.040	0.039	0.060	0.068	0.029	0.112	0.041	*	0.140	0.140	0.302
BOT	0.052	0.050	0.038	0.050	0.047	0.079	0.058	0.050	0.032	0.028	0.036	0.042	0.046	0.041	0.054	0.020	0.091	0.042	0.050	*	0.129	0.174
CON	0.059	0.069	0.057	0.061	0.059	0.066	0.059	0.057	0.033	0.042	0.036	0.059	0.051	0.069	0.072	0.039	0.090	0.046	0.056	0.047	*	0.182
STE	0.047	0.068	0.047	0.072	0.061	0.055	0.051	0.055	0.063	0.049	0.098	0.097	0.063	0.054	0.077	0.056	0.078	0.075	0.106	0.057	0.067	*

* = No comparison made.

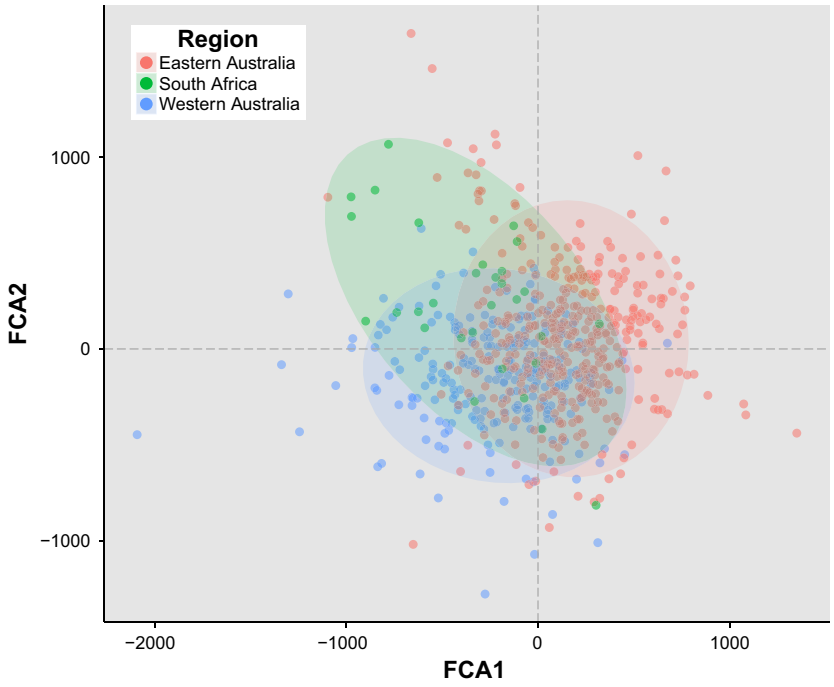

Figure 3 Two-dimensional scatter plot of the first two axes from a factorial correspondence analysis (FCA) showing the relationship among *Halotydeus destructor* sample locations for 10 polymorphic microsatellite loci. Ellipses represent 95% confidence levels.

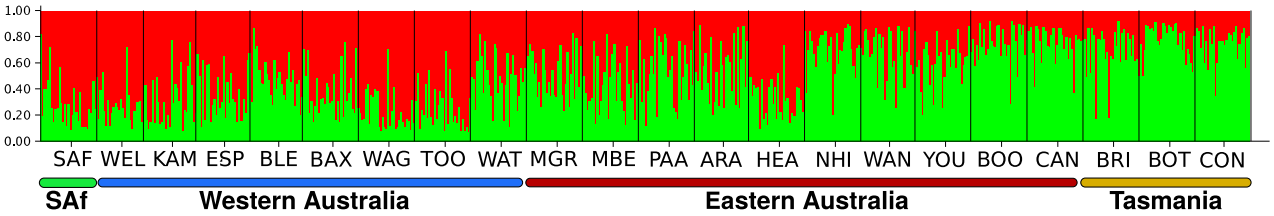

Figure 4 Membership of each *Halotydeus destructor* individual as assigned through STRUCTURE. Colour reflects population clustering (k = 2) by STRUCTURE. Single vertical lines broken into segments reflect each individual in the STRUCTURE summary plot, where segments are proportional to the membership coefficient for each of the population clusters. Site names are taken from Table 1, and sampling locations are grouped into four regions.

Low levels of genetic differentiation were observed between Australian populations of *H. destructor*, with a low F_{ST} albeit significantly different from zero. This pattern is consistent with Weeks *et al.* (1995), who estimated a weak yet significant genetic differentiation based on allozyme markers. The most likely invasion pathway suggests a predominantly west to east movement across Australia. The genetic similarity combined with a high level of allele richness has likely arisen through movements of large mite numbers (from Western Australia to eastern Australia and also into Tasmania), preventing strong genetic divergence through founder effects. The lack of differentiation between the Stellenbosch and Australian populations may be related to multiple introduction events, or may reflect large population sizes maintaining high levels of genetic diversity. The weak genetic structure across populations suggests repeated movements of mites; this has previously been noted for *Penthaleus* spp., which have overlapping ranges with *H. destructor* (Robinson *et al.*, 2002). Consistent with the findings of Weeks *et al.* (1995), we found no isolation by distance in eastern Australian populations. This may reflect ongoing gene flow, including between Tasmania and the Australian mainland, or insufficient time for substantial divergence to develop in these large populations where *H. destructor* densities are often greater than 10,000 per square metre (Ridsdill-Smith, 1997). Long-distance movement is achieved primarily through the diapausing eggs, which can be moved around by farm machinery, livestock and fodder, as well as being blown around by summer winds (Ridsdill-Smith *et al.*, 2008).

These findings have implications for the management of *H. destructor*. The lack of strong differentiation between Tasmania, the eastern region and Western Australia indicates there is an ongoing genetic exchange between these regions

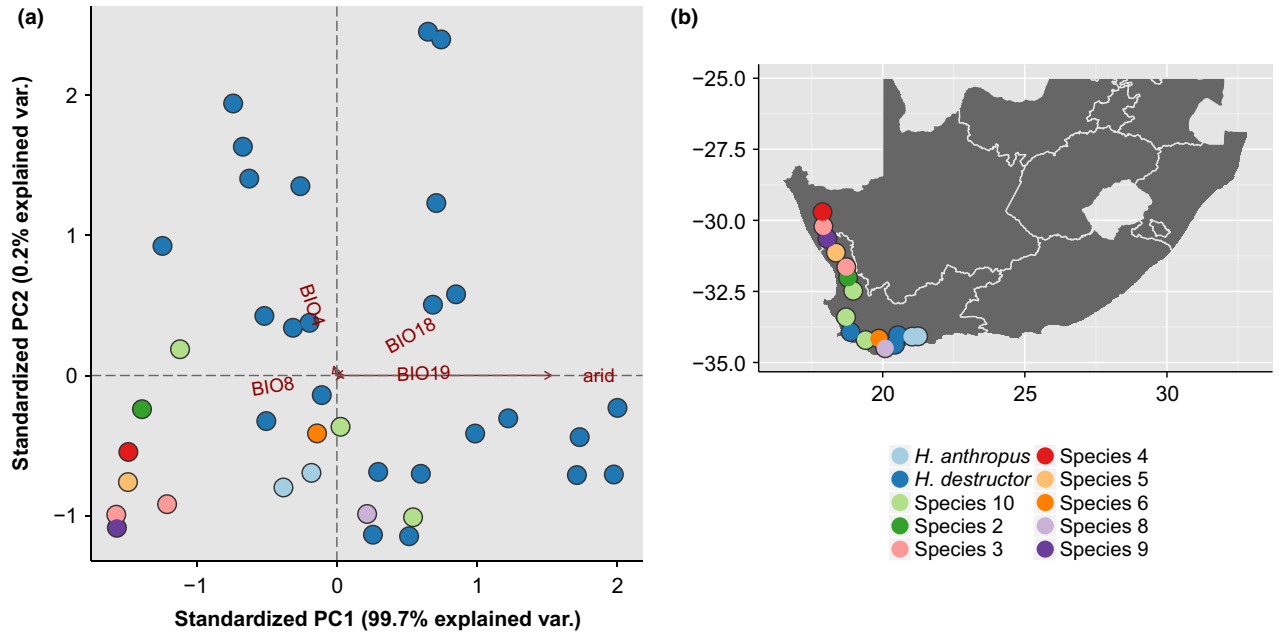

Figure 5 Geographical and environmental space occupied by putative *Halotydeus* spp. Putative species are denoted according to colour and correspond with Figure 2; legend shared between panels. (a) Principal component analysis (PCA) of environmental overlap between putative species. First principal component axis contributes 99.7% of variation with the aridity index contributing to 94.2% of this value. Second principal component axis contributes < 0.01% to overall variation, with Bio4 (isothermality index) contributing 80.8% to this value. (b) South Africa Map with putative species shown geographically, axes are as for Fig. 1. Note for site MLM both *Halotydeus destructor* and species 10 occur, but we have included this site as only species 10 for analysis.

or the movement of large numbers of colonizing mites, perhaps on multiple occasions. This means that resistance to pesticides present in Western Australia (Umina, 2007; Umina *et al.*, 2012) may already be present in eastern Australia or is likely to spread into that region in the near future. Umina *et al.* (2012) have shown resistance within Western Australia has spread quite quickly, and over large geographical distances, presumably due to a low level of long-distance movement or strong selection on low-frequency alleles. The presence of some genetic differentiation among nearby populations suggests that local management actions are unlikely to be countered through a rapid influx of migrating mites, as might be the case for more mobile species such as the aerially dispersed wheat curl mite, *Aceria tosichella* (Miller *et al.*, 2013b). Moreover, high gene flow has also not prevented the development of local variation in physiological responses (Hill *et al.*, 2013). These results highlight the importance of characterizing mite lineages with additional markers (Carew & Hoffmann 2015), in addition to benefiting biosecurity measures given that other lineages of *Halotydeus* could become pests in Australia and elsewhere if they were transferred from South Africa. Our results demonstrate that there is inherent value in applying genetic analyses across both native and invasive ranges for economic important pest species. Invasion potential can be different across genotypes (Allendorf & Lundquist, 2003) and identification of these lineages may aid in understanding invasiveness within species complexes. Further, identification of putative source populations allows hypotheses concerning the rapid evolution of populations within invasive ranges to be tested (Perdereau *et al.*, 2013). Finally, this study highlights that applied research of pest species may benefit from being framed in contexts that consider endemic diversity.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Appendix S1 Uncorrected pairwise distances between haplotypes and those of the outgroup taxon.

Appendix S2 Primer sequences of 10 microsatellite loci isolated from *Halotydeus destructor*.

Appendix S3 Regression between genetic distance and geographic distance.

BIOSKETCH

All work was undertaken at the Pest & Environmental Adaptation Research Group (www.pearg.com). The research formed a component of PhD projects undertaken by MPH and XC supervised by AAH, an entomologist and geneticist leading PEARG, and PAU, an entomologist working in the grains industry. ADM worked as a postdoc at PEARG, developing molecular markers for a variety of applications.

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