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Contrasting the population genetic structure of two velvet worm taxa (Onychophora : Peripatopsidae : *Peripatopsis*) in forest fragments along the south-eastern Cape, South Africa

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Abstract. During the present study, we examined the phylogeography and systematics of two species of velvet worm (*Peripatopsis* Pocock, 1894) in the forested region of the southern Cape of South Africa. A total of 89 *P. moseleyi* (Wood-Mason, 1879) and 65 *P. sedgwicki* (Purcell, 1899) specimens were collected and sequenced for the cytochrome *c* oxidase subunit I mtDNA (COI). In addition, a single *P. sedgwicki* specimen per sample locality was sequenced for the 18S rRNA locus. Furthermore, morphological variation among *P. sedgwicki* sample localities were explored using traditional alpha taxonomic characters. DNA sequence data were subjected to phylogenetic analyses using Bayesian inference and population genetic analyses using haplotype networks and analyses of molecular variance (AMOVAs). Phylogenetic results revealed the presence of four and three clades within *P. moseleyi* and *P. sedgwicki* respectively. Haplotype networks were characterised by the absence of shared haplotypes between clades, suggesting genetic isolation, a result corroborated by the AMOVA and highly significant F_{ST} values. Specimens from Fort Fordyce Nature Reserve were both genetically and morphologically distinct from the two remaining *P. sedgwicki* clades. The latter result suggests the presence of a novel lineage nested within *P. sedgwicki* and suggests that species boundaries within this taxon require re-examination.

Additional keywords: conservation, forest fragmentation, speciation.

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Introduction

South African forests comprise <0.5% of the total land surface of the country and are thought to represent an ancient biome, rich in biodiversity (Eeley *et al.* 1999; Mucina and Rutherford 2006; McDonald and Daniels 2012). Forests are thought to have had a continuous distribution throughout South Africa during mesic periods such as the Miocene epoch (Kotze and Samways 1999; Mucina and Rutherford 2006), although more recent research suggests that forests are ancient and were widely distributed during the Oligocene (Daniels *et al.* 2016). Climatic ameliorations during the Miocene coupled with several marine transgression and regressions and orographic events (Lindesay 1998; Partridge and Maud 2000) resulted in a dramatic reduction and fragmentation of forest cover, a pattern that continued throughout the Plio/Pleistocene (McDonald and Daniels 2012; Huntley *et al.* 2014). Currently, forests in South Africa are restricted to areas of high rainfall and high altitude as well as deep valleys and gorges along mountainous and coastal regions (Eeley *et al.* 1999; Mucina and Geldenhuys 2006; McDonald and Daniels 2012). The contractions and expansions of forest habitat likely resulted in the formation of complex phylogeographic patterning, potentially promoting cladogenesis among forest-dwelling habitat specialists (McDonald and Daniels 2012; Daniels *et al.* 2016).

The forest biome in South Africa is thought to harbour significant levels of α diversity and endemism for both fauna and flora (Eeley *et al.* 1999; Lawes *et al.* 2000). However, taxonomic diversity in most forest-dwelling groups, particularly invertebrates, remain poorly studied (Eeley *et al.* 1999; Lawes *et al.* 2000). Consequently, the National Research Foundation under the auspices of the Foundational Biodiversity Information Program (FBIP) launched a program to examine biodiversity in neglected megadiverse areas within South Africa. One of the neglected biomes is the forested regions of the Eastern Cape Province that is rich in biodiversity, yet poorly studied. Hence, large-scale targeted sampling of selected faunal groups was initiated under the auspice of a FBIP grant focussed on forest habitats in the Eastern Cape Province. Among the focal invertebrate taxa targeted were velvet worms.

Onychophora, commonly known as velvet worms, live in saproxylic environments such as decaying wood and leaf litter in the forested areas of South Africa (Daniels *et al.* 2009; Daniels and Ruhberg 2010; McDonald and Daniels 2012). Nine of the South African velvet worm species are IUCN Red–listed, rendering the fauna of conservation concern (Hamer *et al.* 1997). Two genera, *Peripatopsis* Pocock, 1894 and *Opithopatus* Purcell, 1899, occur in South Africa, and historically comprised seven and two species respectively (Hamer *et al.* 1997). Several velvet worm species were thought to have extensive geographical distribution ranges due to poorly defined morphological characters that hampered accurate species delineation (Hamer *et al.* 1997). The first DNA-based systematic study on *Peripatopsis* identified three species complexes, among widely distributed velvet worm species (*P. capensis* Grube, 1866, *P. balfouri* Sedgwick, 1885, and *P. moseleyi* Wood-Mason, 1879) (Daniels *et al.* 2009). These three velvet worm species complexes were subsequently subjected to molecular systematic scrutiny in order to resolve taxonomic uncertainties (Daniels and Ruhberg 2010; McDonald and Daniels 2012; Ruhberg and Daniels 2013; Daniels *et al.* 2013). Within the *P. capensis* species complex, two novel species were described (Daniels *et al.* 2009; McDonald and Daniels 2012), while the *P. balfouri* species complex yielded three novel species (Daniels *et al.* 2013). Finally, within the *P. moseleyi* species complex four novel species were described (Daniels and Ruhberg 2010; Ruhberg and Daniels 2013). Daniels *et al*. (2016) recently described five novel species within *Opisthopatus*. Several additional South African velvet worm species await formal description (S. R. Daniels, unpubl. data). These results demonstrate that considerable α taxonomic diversity exists within velvet worm species and that modern molecular systematic endeavours are required to recognise cryptic diversity.

Velvet worm diversity in the south-eastern Cape forests is high, and to date nine species have been recorded. *Opisthopatus cinctipes* (Purcell, 1899) is distributed around Port Elizabeth and the adjacent interior, *O. zululandi* (Daniels, Dambire, Klaus & Sharma, 2016) occurs from Port St Johns along the Indian Ocean coastal belt forests into KwaZulu-Natal, while *O. amaxhosa* (Daniels, Dambire, Klaus & Sharma, 2016) occurs in the interior of the Eastern Cape province (Daniels *et al.* 2016). *Peripatopsis clavigera* (Purcell, 1899) occurs around the Wilderness and Diepwalle area while *P. sedgwicki* (Purcell, 1899) is distributed from the Tsitsikamma forest in the southern Cape to Port Elizabeth (Hamer *et al.* 1997). *Peripatopsis moseleyi* is restricted to the Amatola Mountains, Hogsback, Seymore, Keiskammahoek, Stutterheim and Pirie forest in the Eastern Cape Province, South Africa (Ruhberg and Daniels 2013), *P. janni* (Ruhberg & Daniels, 2013) is restricted to Hogsback and the Amatola Mountains, and *P. storchi* (Ruhberg & Daniels, 2013) and *P. hamerae* (Ruhberg & Daniels, 2013) are endemic to Katberg and Groot Bruintjieshoogte (Ruhberg and Daniels 2013). Phylogenetic research on *P. clavigera* and *P. sedgwicki* has retrieved three and two clades respectively (Daniels *et al.* 2009, 2013). Apart from the initial study by Daniels *et al*. (2009) no further systematic research has been undertaken on *P. sedgwicki*. Considering the increased taxonomic diversity observed among other South African velvet worm species it would be reasonable to assume that additional α taxonomic diversity is concealed within *P. sedgwicki*. Similarly, it is fair to assume that *P. moseleyi* populations distributed among the fragmented forest patches in the Eastern Cape Province should exhibit mark genetic population differentiation. The forest habitat in the Eastern Cape Province has been subjected to considerable ancient and recent fragmentation events (Daniels *et al.* 2016). However, the impact of the forest fragmentation on the population genetic structure and taxonomy of velvet worms remains limited. Myburgh and Daniels (2015) investigated the impact of forest fragmentation on the Overberg velvet worm, *Peripatopsis overbergiensis* (McDonald & Daniels, 2012), and observed four clades and marked genetic population structuring (Myburgh and Daniels 2015). The latter study suggests that forest fragmentation may impede gene flow among conspecific populations, suggesting that small discontinuous forest patches are important because they harbour high level of genetic diversity for forest specialist invertebrates.

The present study has two objectives. First, to explore the impact of forest fragmentation on the population genetic structure of *P. moseleyi* and, second, to examine species boundaries within *P. sedgwicki* by undertaking more extensive geographic sampling using both mtDNA and nuDNA sequence data. We advance two hypotheses: first, that *P. moseleyi* populations among isolated forest fragments of the Eastern Cape Province will demonstrate high levels of population differentiation and, second, that extensive sampling of *P. sedgwicki* will result in increased α taxonomic diversity.

Materials and methods

Taxon sampling

Velvet worms were hand-collected from saproxylic environments, beneath or inside decaying logs, or under stones and leaf litter in forested regions in the south-eastern Cape, South Africa. Locality coordinates were recorded with a hand-held GPS (Garmin-Trek Summit). We aimed to collect a minimum of five animals per locality; however, this was not always possible because velvet worms frequently occur in low numbers (Daniels, pers. obs.). Specimens were frozen, and placed in labelled honey jars filled with absolute ethanol. During the present study *P. moseleyi* specimens were collected from six localities in the Eastern Cape Province, South Africa (Fig. 1*A*; Table 1). These samples were combined with 47 *P. moseleyi* specimens (Daniels and Ruhberg 2010) to yield a total of 89 specimens (Table 1). In addition, 27 *P. sedgwicki* specimens were collected from Fort Fordyce Nature Reserve in the Eastern Cape Province, South Africa (Fig. 1*B*; Table 1) and combined with 31 *P. sedgwicki* specimens from eight localities that were previously generated by Daniels *et al*. (2009). The *P. sedgwicki* specimens from Rivendell Farm (n = 6) and Zuurberg (n = 1) were collected before the initiation of the present study but have not been included in any previous analyses. Hence, a total of 65 *P. sedgwicki* specimens were used during the present study.

DNA extraction, PCR and sequencing

Genomic DNA was extracted from velvet worm specimen tissue biopsies using a Macherey-Nagel DNA extraction kit

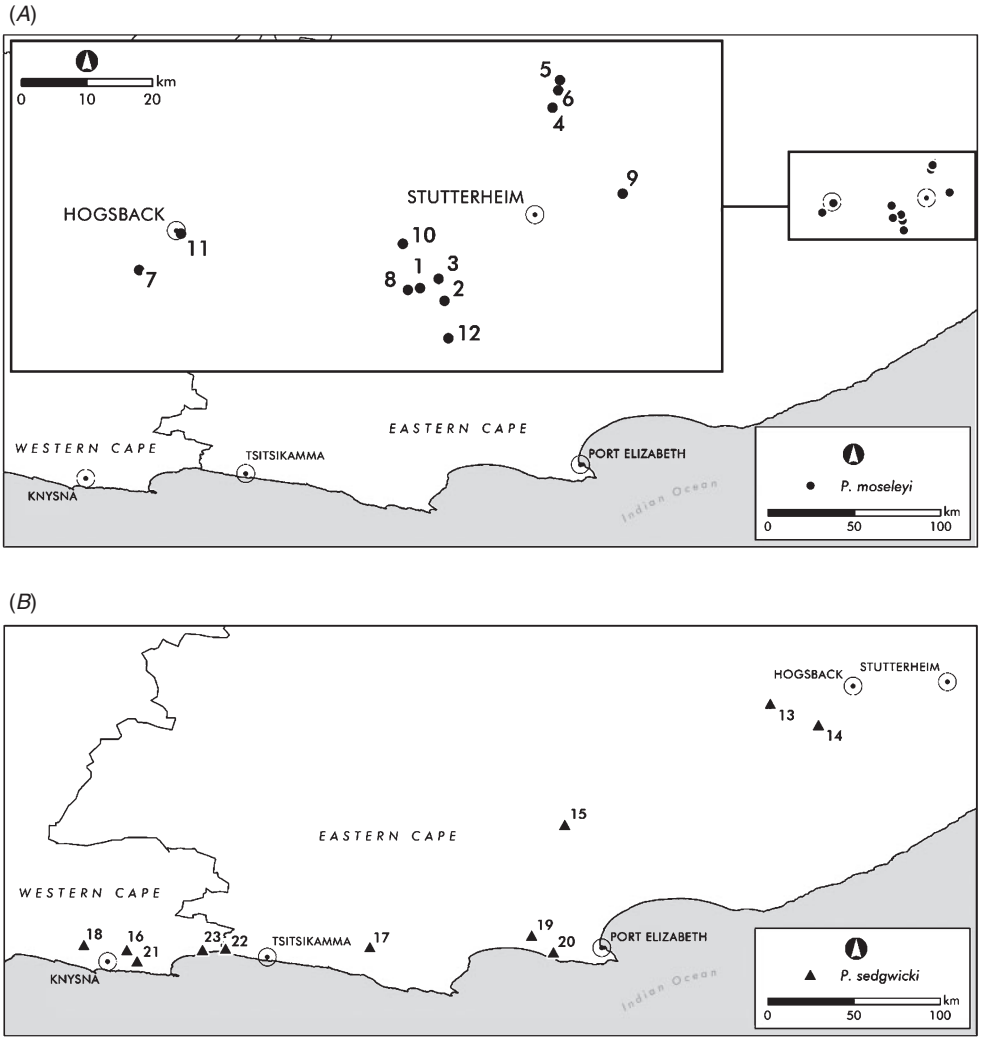

Fig. 1. (*A*) The sample localities (1–12) for *Peripatopsis moseleyi* in the Eastern Cape Province of South Africa. (*B*) The sample localities (13–23) for *P. sedgwicki* in the south-eastern Cape of South Africa. The sample localities on each map correspond to those in Table 1.

following the manufacturer's protocol. Extracted DNA was stored at 4°C and a dilution of 1 µL DNA and 19 µL deionised water was prepared before use in PCR. Two DNA loci, the mitochondrial cytochrome *c* oxidase subunit I (COI) and the nuclear 18S rDNA (18S rRNA), were targeted. All samples were sequenced for the rapidly evolving COI locus. The COI locus has been used extensively in phylogeographic studies among South African velvet worms (McDonald and Daniels 2012; Myburgh and Daniels 2015; Daniels *et al.* 2016), allowing us to combine data from two earlier studies with those COI sequences generated during the present study. For *P. sedgwicki* a single specimen per locality was also sequenced for the small ribosomal subunit (18S rRNA) nuclear locus. The 18S rRNA locus has also been used in phylogenetic studies in velvet worms (Daniels *et al.* 2009; McDonald and Daniels 2012; Daniels *et al.* 2013, 2016; Myburgh and Daniels 2015).

The primer pair LCO-1490 and HCO-2198 (Folmer *et al.* 1994) was used to amplify the COI locus, while the primer pair 18S-5F and 18S-7R (Giribet *et al.* 1996) was used to amplify a fragment of the 18S locus. PCRs consisted of 25 µL reactions comprising 14.9 µL distilled H_2O, 3.5 µL 25 mM $MgCl_2$, 2.5 µL Mg^{2+} free buffer, 0.5 µL of each 10 mM oligonucleotide primer, 0.5 µL 10 mM dNTPs, 0.1 µL Taq polymerase and 2 µL of diluted template DNA. PCR cycling conditions were as follows: denaturation at 94°C for 4 min, followed by 36 cycles of denaturation at 94°C for 30 s, annealing at 42°C for 35 s, and extension at 72°C for 35 s. A final extension occurred at 72°C for 10 min. PCR products were electrophoresed on a 1% agarose gel for 120 min, at 90 V. PCR products were visualised using UV light and PCR bands were isolated using a Biospin Gel Extraction Kit, following the manufacturer's protocol. Purified PCR products were sequenced at the Central Analytical Facilities of Stellenbosch University using an ABI 3730 XL automated machine.

Table 1. List of sample sites for the two velvet worm species (*Peripatopsis*) from forested regions in the south-eastern Cape of South Africa

Locality no.	Sample site	Species	*N*	GPS coordinates	Reference
1	Isidenge A	*P. moseleyi*	3	32°40′31″S, 27°29′07″E	Present study
2	Isidenge B	*P. moseleyi*	19	32°41′34″S, 27°16′68″E	Present study
3	Sandiles Rest	*P. moseleyi*	5	32°23′25″S, 27°26′99″E	Present study
4	Qacu A	*P. moseleyi*	4	32°25′40″S, 27°26′62″E	Daniels and Ruhberg (2010)
5	Qacu B	*P. moseleyi*	9	32°41′34″S, 27°26′69″E	Present study
6	Qacu C	*P. moseleyi*	6	32°24′15″S, 27°26.91″E	Present study
7	Seymore	*P. moseleyi*	4	32°38′62″S, 26°52′58″E	Daniels and Ruhberg (2010)
8	Amatola	*P. moseleyi*	10	32°40′40″S, 27°15′07″E	Daniels and Ruhberg (2010)
9	Stutterheim	*P. moseleyi*	5	32°32′45″S, 27°32′49″E	Daniels and Ruhberg (2010)
10	Keiskammahoek	*P. moseleyi*	5	32°36′52″S, 27°14′42″E	Daniels and Ruhberg (2010)
11	Hogsback	*P. moseleyi*	10	32°36′03″S, 26°55′85″E	Daniels and Ruhberg (2010)
12	Pirie forest	*P. moseleyi*	9	32°44′38″S, 27°17′87″E	Daniels and Ruhberg (2010)
13	Fort Fordyce	*P. sedgwicki*	27	32°41′45″S, 27°29′09″E	Present study
14	Rivendell Farm	*P. sedgwicki*	6	32°47′60″S, 26°45′10″E	Present study
15	Zuurberg	*P. sedgwicki*	1	33°19′12″S, 25°25′48″E	Present study
16	Diepwalle	*P. sedgwicki*	4	33°58′29″S, 23°08′37″E	Daniels *et al.* (2009)
17	Essenbos	*P. sedgwicki*	5	33°57′32″S, 24°24′47″E	Daniels *et al.* (2009)
18	Homtini	*P. sedgwicki*	3	33°56′51″S, 22°55′12″E	Daniels *et al.* (2009)
19	Ladyslipper	*P. sedgwicki*	1	33°98′00″S, 25°27′04″E	Daniels *et al.* (2009)
20	Port Elizabeth	*P. sedgwicki*	4	33°98′51″S, 25°36′59″E	Daniels *et al.* (2009)
21	Garden of Eden	*P. sedgwicki*	4	34°01′58″S, 23°11′52″E	Daniels *et al.* (2009)
22	Khoisan Village	*P. sedgwicki*	5	33°57′60″S, 23°39′31″E	Daniels *et al.* (2009)
23	Natures Valley	*P. sedgwicki*	5	33°97′26″S, 23°53′93″E	Daniels *et al.* (2009)

Phylogenetic analysis

Sequences were manually checked for base ambiguities using Sequence Navigator (Applied Biosystems, Foster City, CA, USA) and were aligned using CLUSTAL X (Thompson *et al.* 1997). Bayesian inference was conducted using MrBayes 3.1.2. (Ronquist *et al.* 2012) and model selection for Bayesian inference (BI) was determined using jModelTest 2 (Darriba *et al.* 2012). For *P. sedgwicki* DNA substitution models were recalculated for the combined analyses (COI and 18S rDNA) since we used only a single COI sequence per sample locality. Ten Monte Carlo Markov Chains were run with 10 million generations sampling every 10 000th tree. This was done for each analysis including the combined COI and 18S rDNA analysis. The program AWYT (Nylander *et al.* 2008) was used to summarise the post-burn-in. Posterior probabilities (PP) for each node were then estimated and PP < 0.95 were regarded as unsupported. Uncorrected 'p' distance values were calculated for the COI locus for both velvet worm species using PAUP 4.10 (Swofford, 2002). Sister species were used as outgroups in the phylogenetic analyses. For *P. moseleyi*, *P. birgeri* was used as an outgroup, while for *P. sedgwicki*, *P. moseleyi* and *P. janni* were used as outgroups (Daniels *et al.* 2009; Daniels and Ruhberg 2010; Ruhberg and Daniels 2013).

Population genetic analyses using COI

Using statistical parsimony (Templeton *et al.* 1992) in TCS 1.06 (Clement *et al.* 2000) a COI haplotype network was constructed for both *Peripatopsis* species. The population genetic structure for *P. moseleyi* and *P. sedgwicki* was determined by using a hierarchical analysis of molecular variance (AMOVA) in Arlequin 3.01 (Schneider *et al.* 2000) since the preliminary analyses demonstrated the presence of four clades and three clades respectively. To assess for variation within and among populations various standard diversity indices as well as pairwise differences between populations were determined in Arlequin 3.01 (Schneider *et al.* 2000). Indices such as number of haplotypes (*Nh*), number of polymorphic sites (*Np*), haplotypic diversity (*h*) and nucleotide diversity (π) were calculated to assess population structure and genetic diversity. The history of demographic stability and recent population expansion was determined within populations using Fu's F_s (Fu 1997) calculated in Arlequin 3.01 (Schneider *et al.* 2000). F_{ST} values were also calculated in Arlequin 3.01 (Schneider *et al.* 2000).

Species delimitation with Bayesian GMYC

A Bayesian implementation of the generalised mixed Yule-coalescent model (GMYC) was used to infer species limits for the mitochondrial dataset, using the R package bGMYC (Reid and Carstens 2012). To account for error in phylogenetic estimation, 100 post-burn-in trees were randomly selected for analysis. A Markov chain was run for 10^6 generations and 10^3 generations were discarded as burn-in, sampling the chain every 1000th generation. A uniform prior for the number of species was applied, with a lower bound of 3 and an upper bound of 73 (the total number of terminals in the analysis). Convergence was assessed visually by examining the performance of the chain. The 'check rates' function was used to determine the rate of branching of the coalescent model to that of the Yule model.

Morphological examination

In order to delineate possible evolutionary lineages within *P. sedgwicki* a morphological examination of specimens was undertaken and combined with data collected for specimens used by Daniels *et al.* (2009). The following morphological characters were assessed: the number of leg pairs, the presence or absence of a claw on the genital leg pair, the papillar rows

between leg pairs, and dorsal body colour. These characters have previously been used to varying degrees of success to describe velvet worm species (Daniels *et al.* 2013, 2016; Ruhberg and Daniels 2013).

Results

Phylogenetic analysis for P. moseleyi

mtDNA tree topology (COI)

The partial COI fragment for *P. moseleyi* comprised 631 base pairs. The 42 new sequences generated during the present study were deposited in GenBank (accession numbers MF327423–MF327464). The substitution model TIM+I+G (ln (L) = 1744.65, AIC = 3505.30) was selected. The BI analyses retrieved a monophyletic *P. moseleyi* (Fig. 2) and comprised four statistically well supported clades (PP > 0.95). Clade 1 comprised specimens from Amatola, Isidenge A and B and Pirie forest. Clade 2 comprised specimens from Keiskammahoek exclusively while its sister clade, Clade 3, comprised specimens from Hogsback and Seymore. Clade 4 comprised specimens from Qacu A, B and C, Sandiles Rest and Stutterheim. Uncorrected 'p' sequence divergence of Clade 1 from Clade 4 was 7.5%, Clade 1 was 6.5% divergent from Clade 3, while Clade 1 was 6% divergent from Clade 2 (Table 2).

Population genetic analyses for P. moseleyi

A total of 21 COI haplotypes in four haploclusters were retrieved among the 89 *P. moseleyi* specimens (Fig. 3, Table 3). The AMOVA results revealed that 97.61% variation exists among *P. moseleyi* populations (Va = 13.76, d.f. = 11, s.s. = 1072.71, $P < 0.01$, and 2.39% variation occurred within populations (Vb = 0.33, d.f. = 76, s.s. = 25.56, $P < 0.01$).

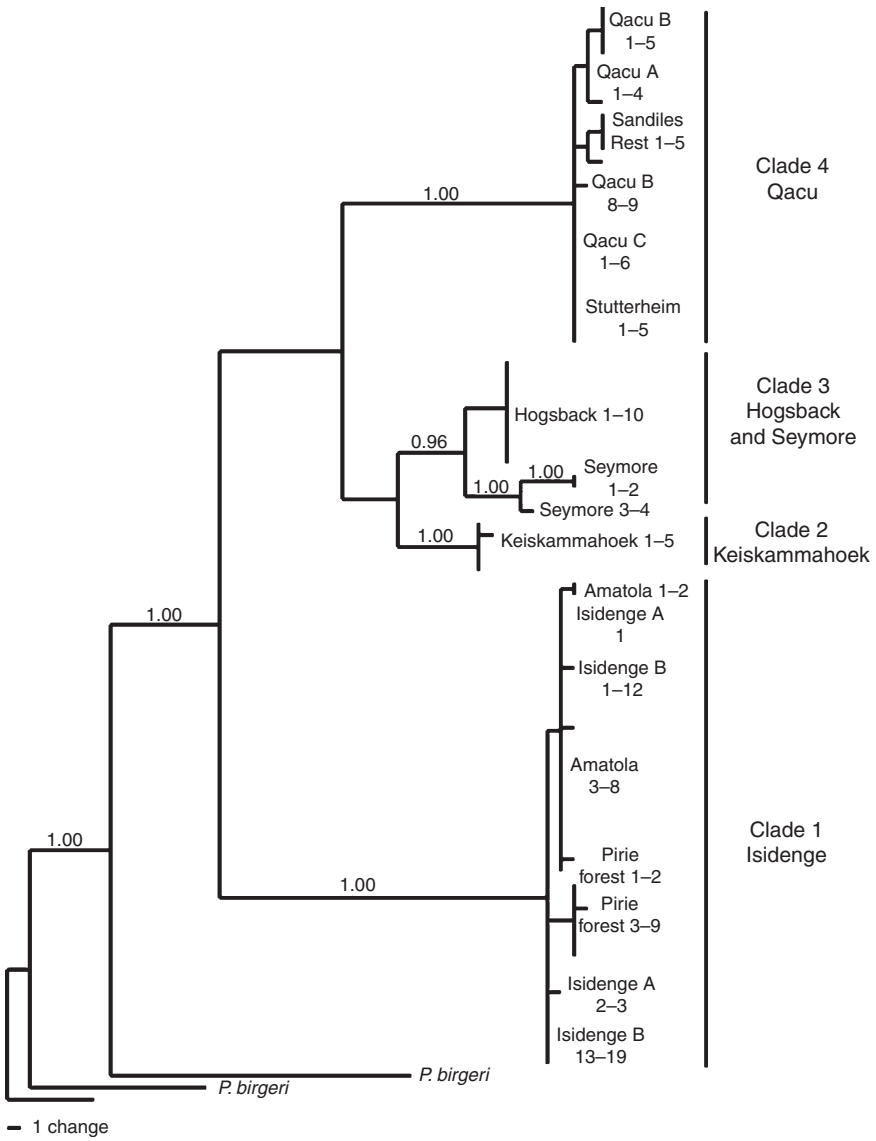

Fig. 2. A Bayesian Inference topology of *Peripatopsis moseleyi* based on the COI mtDNA sequence data demonstrating the presence of four clades. Only PP values >0.95 are shown.

Table 2. Uncorrected 'p' sequence divergence among *Peripatopsis moseleyi* sample sites for the COI locus
Sample sites correspond to Fig. 1*A* and Table 1. *$P < 0.05$

Sample site	1	2	3	4	5	6	7	8	9	10	11
1. Isidenge A*	–										
2. Isidenge B*	0.00–0.32	–									
3. Sandiles Rest*	6.83–7.14	6.82–7.14	–								
4. Qacu A	7.14–7.46	7.14–7.46	0.48–0.64	–							
5. Qacu B*	6.83–7.46	6.98–7.46	0.32–0.64	0.16–0.32	–						
6. Qacu C*	6.98–7.14	6.98–7.14	0.32–0.32	0.16–0.32	0.00–0.32	–					
7. Seymore	5.88–6.35	5.88–6.35	3.98–4.77	4.12–4.75	3.82–4.77	3.98–4.45	–				
8. Amatola	0.00–0.32	0.00–0.31	6.84–7.15	7.13–7.45	6.99–7.47	6.99–7.15	5.86–6.34	–			
9. Stutterheim	6.98–7.14	6.99–7.14	0.32–0.32	0.16–0.32	0.00–0.32	0.00	3.96–4.44	6.97–7.13	–		
10. Keiskammahoek	5.72–6.04	5.72–6.03	4.30–4.61	4.28–4.6	3.98–4.61	4.14–4.29	2.38–3.01	5.86–6.02	4.12–4.28	–	
11. Hogsback	5.88–6.03	5.87–6.03	3.66–3.81	3.65–3.8	3.34–3.81	3.50	0.95–1.43	5.86–6.02	3.49	2.22–2.38	–
12. Pirie Forest	0.00–0.64	0.00–0.64	6.84–7.15	7.13–7.61	6.84–7.63	6.99–7.31	6.02–6.50	0.00–0.79	6.97–7.29	5.71–6.02	5.86–6.02

Hierarchical AMOVA results for each of the clades were as follows: for Clade 1 (Amatola, Isidenge A and B and Pirie forest), among-population variation was 59.78% (Va = 0.52, d.f. = 3, s.s. = 15.54, $P < 0.01$) and within-population variation was 40.22% (Vb = 0.35, d.f. = 37, s.s. = 13.08, $P < 0.01$). For Clade 2, no AMOVA was undertaken because it comprised samples from a single locality. For Clade 3 (Hogsback and Seymore), among-population variation was 90.20% (Va = 3.06, d.f. = 1, s.s. = 17.85, $P < 0.01$) and within-population variation was 9.8% (Vb = 0.33, d.f. = 12, s.s. = 4.00, $P < 0.01$). Finally, for Clade 4 (Qacu A, B and C, Sandiles Rest, and Stutterheim), the among-population variation was 61.42% (Va = 0.53, d.f. = 4, s.s. = 12.96, $P < 0.01$) and within-population variation was 38.58% (Vb = 0.33, d.f. = 23, s.s. = 7.68, $P < 0.01$).

F_{ST} values (Appendix 1) between populations for *P. moseleyi* were significant, and indicative of high genetic substructure. Three haplotypes were shared among some populations: Haplotype 6 was shared between Isidenge A and B, Amatola and Pirie forest whereas Haplotype 8 was shared between Isidenge A and B. Haplotype 13 was shared between specimens of Qacu B, C and Stutterheim. Shared haplotypes occurred among populations that were in close geographic proximity (Fig. 1, Table 3); most haplotypes were private. The number of haplotypes present in each population was low (Table 4), resulting in relatively low levels of polymorphic sites (Table 4). Haplotype diversity for populations was high, with the exception of Qacu C, Stutterheim and Hogsback, while nucleotide diversity was low for all populations (Table 2). This indicates that populations are most likely isolated. Eight populations had positive Fu's F_s values while one locality (Isidenge B) had a negative Fu's F_s value. Populations with positive Fu's F_s values have most likely experienced recent bottlenecks, while populations with negative Fu's F_s values could possibly be experiencing population expansion (Table 4). Notably, none of the Fu's F_s values were statistically significant ($P < 0.02$).

Phylogenetic analysis for P. sedgwicki

mtDNA tree topology (COI)

The partial COI fragment comprised 648 base pairs for 34 specimens. New sequences were deposited in GenBank (accession numbers: MF327465–MF327498). A GTR+I+G substitution model ($\ln(L) = 1919.13$, AIC = 3858.27) was selected. The BI analyses retrieved a monophyletic *P. sedgwicki* (Fig. 4) comprising three statistically well supported clades (PP > 0.95). Clade 1 comprised of all the specimens from Fort Fordyce Nature Reserve, and was sister to the remaining two clades. Clade 2, the south Western Cape clade, comprised specimens from Homtini, Garden of Eden and Diepwalle was sister to Clade 3. The latter south Eastern Cape coast and interior clade comprised specimens from Zuurberg, Rivendell Farm, Port Elizabeth, Ladyslipper, Essenbos, Natures Valley and Khoisan Village. Deeper nodal relationships for Clades 2 and 3 were poorly supported. Uncorrected 'p' sequence divergence of the (Fort Fordyce Nature Reserve) Clade 1 from the south Western Cape clade was 7.5%, while Clade 1 was 6.8% divergent from Clade 3. Uncorrected 'p' sequence divergence for Clade 3 from Clade 2 was 6% (Table 5).

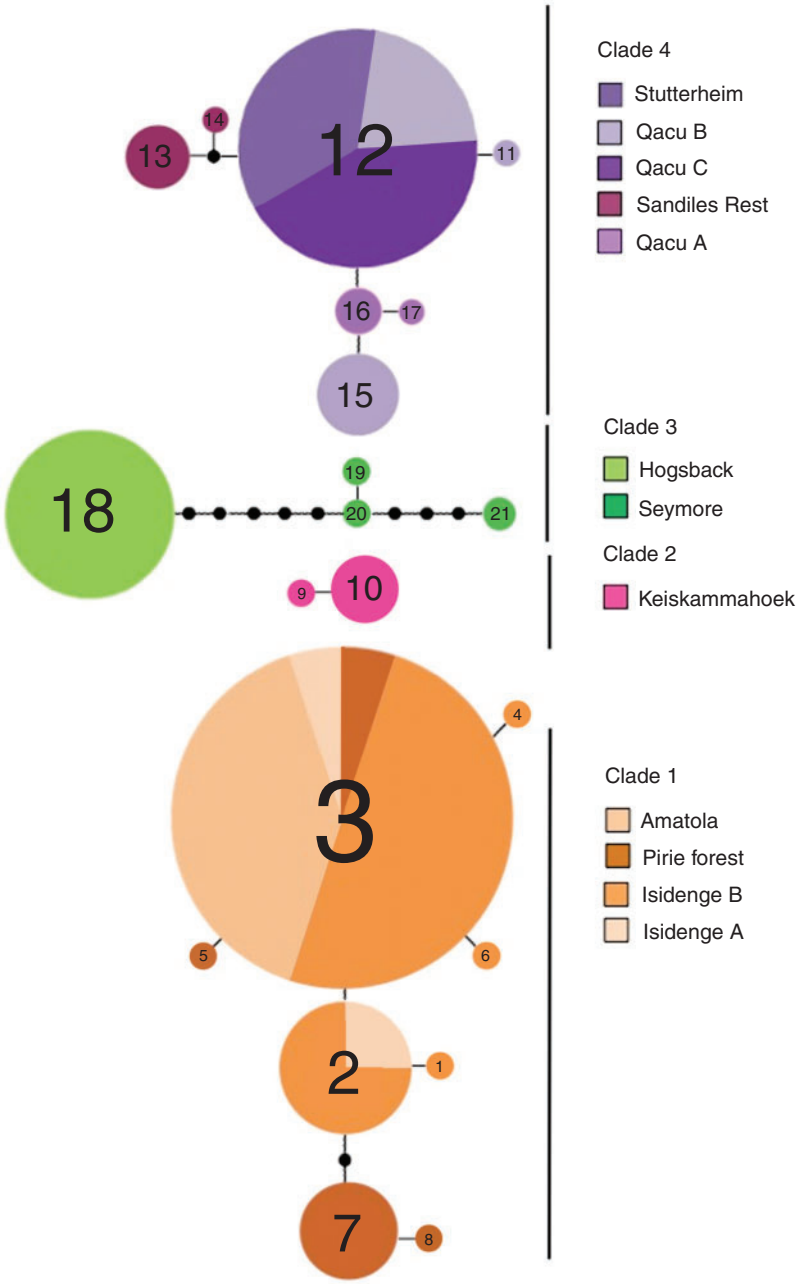

Fig. 3. Haplotype network for COI of *Peripatopsis moseleyi* consisting of four haploclusters. Solid, black circles represent unsampled haplotypes or mutational step difference.

Total evidence topology (COI + 18S rRNA)

The partial 18S rRNA locus comprised 560 bp. The two new 18S rDNA sequences were deposited in GenBank (accession numbers MF327499–MF327500). The tree topology derived exclusively from the 18S rRNA placed Fort Fordyce basal, while the remaining specimens formed a single clade (topology not shown). The Fort Fordyce specimens were 1.43% different from the rest of the *P. sedgwicki* samples. The combined COI + 18S rRNA yielded 1208 bp. The combined DNA sequence topology retrieved from the BI analyses retrieved a monophyletic *P. sedgwicki*, and the same three clades evident from the COI topology (Fig. 5).

Population genetic analyses for P. sedgwicki

A total of 32 COI haplotypes in three haploclusters were retrieved for the 65 *P. sedgwicki* specimens (Fig. 6, Table 6). Haplocluster 1 corresponded to Clade 1 (Fig. 4) and was exclusive to the Fort Fordyce Nature Reserve, Haplocluster 2 corresponded to Clade 2 (Fig. 4) and included Homtini, Diepwalle and Garden of Eden, while Haplocluster 3

Table 3. Haplotype (H) frequency distribution among *Peripatopsis moseleyi* sample localities
†, newly sampled localities

Haplotype	Sample site											
	Isidenge A†	Isidenge B†	Sandiles Rest†	Qacu A	Qacu B†	Qacu C†	Seymore	Amatola	Stutterheim	Keiskammahoek	Hogsback	Pirie Forest
H01	1											
H02	6	2										
H03	2	9						8				1
H04		1										
H05								2				
H06		1										
H07												7
H08												1
H09										1		
H10										4		
H11					1							
H12					3	6			5			
H13			4									
H14			1									
H15					5							
H16				3								
H17				1								
H18											10	
H19							1					
H20							1					
H21							2					

Table 4. Summary list of the population parameters for *Peripatopsis moseleyi* in the Eastern Cape province, South Africa
N, the number of samples per locality

Sample site	*N*	No. of haplotypes (*Nh*)	No. of polymorphic sites (*Np*)	Haplotype diversity (*h*)	Nucleotide diversity (π)	Fu's F_s
Isidenge A	3	2	1	0.6667 ± 0.3143	0.0010 ± 0.0013	0.2006
Isidenge B	19	6	4	0.7427 ± 0.0719	0.0012 ± 0.0010	–3.0329
Sandiles Rest	5	2	2	0.4000 ± 0.2373	0.0012 ± 0.0012	1.0404
Qacu site A	4	2	1	0.5000 ± 0.2652	0.0008 ± 0.0010	0.1718
Qacu site B	9	3	3	0.6389 ± 0.1258	0.0021 ± 0.0016	0.9094
Qacu site C	6	1	0	0.0000 ± 0.0000	0.0000 ± 0.0000	n.a.
Seymore	4	3	5	0.8333 ± 0.2224	0.0042 ± 0.0033	0.5563
Amatola	10	2	1	0.3556 ± 0.1591	0.0006 ± 0.0007	0.4167
Stutterheim	5	1	0	0.0000 ± 0.0000	0.0000 ± 0.0000	n.a.
Keiskammahoek	5	2	1	0.4000 ± 0.2373	0.0006 ± 0.0008	0.0902
Hogsback	10	1	0	0.0000 ± 0.0000	0.0000 ± 0.0000	n.a.
Pirie forest	9	4	5	0.4167 ± 0.1907	0.0014 ± 0.0012	0.1335

corresponded to Clade 3 (Fig. 4) and contained the remaining seven sample localities. The AMOVA results revealed that 87.41% of the variation existed among populations (Va = 13.62, d.f. = 10, s.s. = 716.51, $P < 0.01$) while 12.59% of the variation occurred within populations (Vb = 1.96, d.f. = 54, s.s. = 105.93, $P < 0.01$). For Clade 1 (Fort Fordyce Nature Reserve), we could not calculate additional variation since it comprised a single locality. For Clade 2 (Homtini, Diepwalle and Garden of Eden) the among-population variation was 53.96% (Va = 1.79, d.f. = 2, s.s. = 16.11, $P < 0.01$), while the within-population variation was 46.04% (Vb = 1.53, d.f. = 8, s.s. = 12.25, $P < 0.01$). For Clade 3 (Zuurberg, Rivendell Farm, Essensbos, Ladyslipper, Port Elizabeth, Khoisan Village and Natures Valley) the among-population variation was 93.18% (Va = 5.91, d.f. = 5, s.s. = 126.87, $P < 0.01$), while the within-population variation was 6.82% (Vb = 0.43, d.f. = 20, s.s. = 8.66, $P < 0.01$). F_{ST} values between all populations were significant (Appendix 2), indicating the absence of maternal gene flow between populations, a result that was corroborated by the absence of shared haplotypes (Table 6).

Species delimitation with Bayesian GMYC

Visual inspection of post-burn-in samples from the bGMYC analysis indicated high variance in the MCMC chain. The

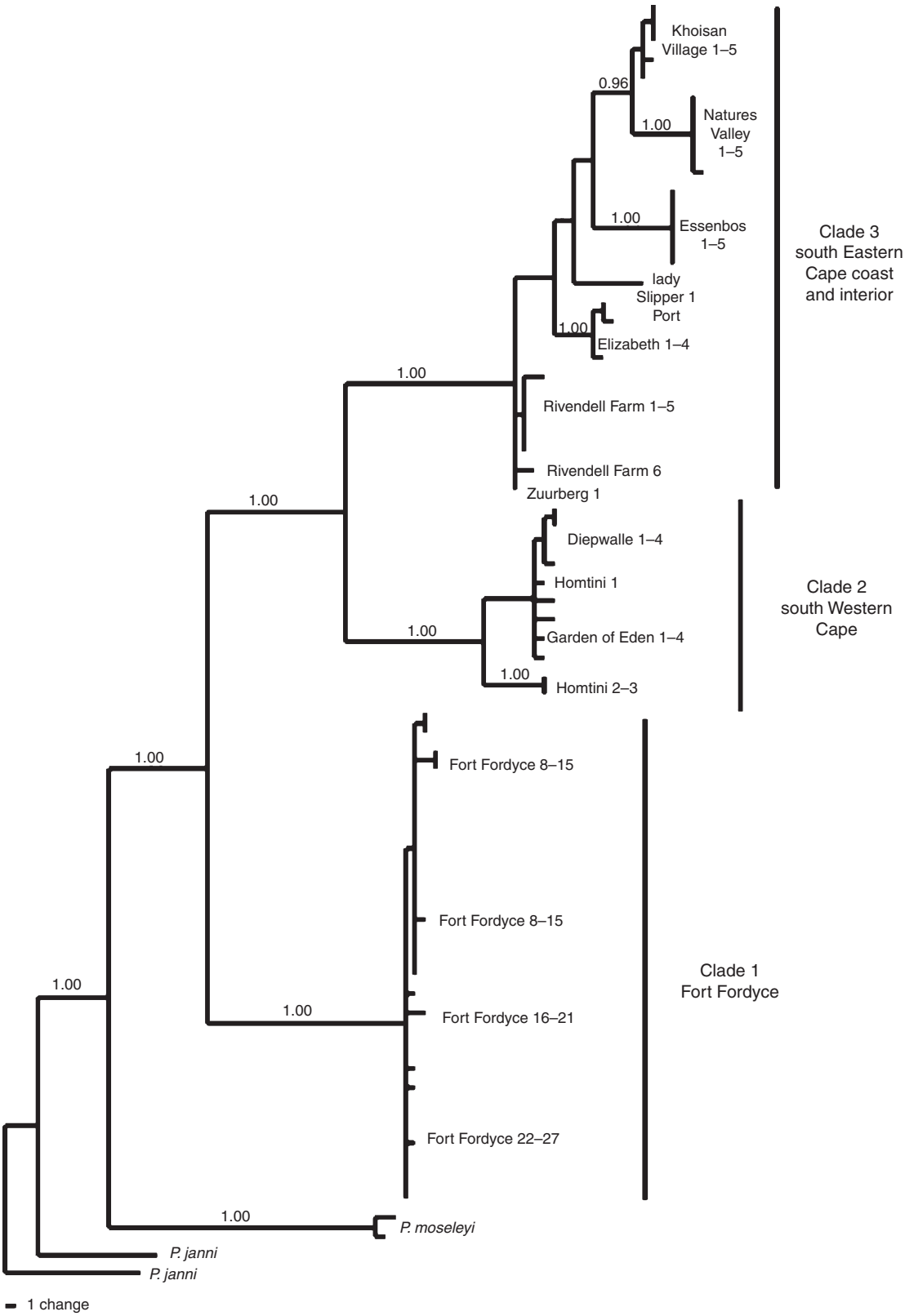

Fig. 4. A Bayesian Inference topology of *Peripatopsis sedgwicki* based on the COI mtDNA sequence data. Only PP values >0.95 are shown.

Table 5. Uncorrected 'p' sequence divergence among *Peripatopsis sedgwicki* sample sites for the COI locus
Sample sites correspond to Fig. 1*B* and Table 1

Sample site	13	14	15	16	17	18	19	20	21	22
13. Fort Fordyce	–									
14. Rivendell Farm	6.03–6.49	–								
15. Zuurberg	6.03–6.49	0.16–0.46	–							
16. Diepwalle	6.96–7.58	5.26–5.72	5.10–5.26	–						
17. Essenbos	6.65–7.11	2.32–2.63	2.17	5.86–6.02	–					
18. Homtini	7.27–7.73	5.57–6.19	5.41–5.72	0.31–2.01	6.17–6.48	–				
19. Ladyslipper	6.80–7.27	2.17–2.48	2.01	5.56–5.71	2.32	5.86–6.17	–			
20. Port Elizabeth	6.81–7.43	1.24–1.71	1.09–1.40	5.86–6.17	2.47–2.78	5.86–6.33	1.85–2.16	–		
21. Garden of Eden	7.27–7.90	5.57–6.04	5.41–5.58	0.31–0.62	6.17–6.33	0.31–2.01	5.86–6.03	5.86–6.18	–	
22. Khoisan Village	6.51–7.13	1.86–2.19	1.71–1.86	5.88–6.10	1.85–2.01	6.19–6.65	2.02–2.18	1.71–2.33	6.03–6.34	–
23. Natures Valley	6.74–7.46	2.35–2.80	2.19–2.48	6.09–6.51	1.73–2.02	6.40–6.97	2.49–2.78	2.65–3.25	6.08–6.51	1.09–1.40

threshold value across the post-burn-in samples was 22.5 GMYC clusters with a standard deviation of 16.1 (results not shown).

Morphological variation in P. sedgwicki

Specimens from Garden of Eden, Diepwalle, Zuurberg, Rivendell Farm, Essenbos and Khoisan Village all had 19 leg pairs, while specimens from Homtini, Natures Valley and Ladyslipper had specimens with 20 leg pairs. Port Elizabeth and Fort Fordyce Nature Reserve were the only two populations that exhibited variation in leg pair number among specimens. In the Port Elizabeth population the number of leg pairs varied between 19 and 20 whereas in the Fort Fordyce Nature Reserve locality the number of leg pairs varied between 22 and 23. Clade 1 (Fort Fordyce Nature Reserve) was morphologically distinct on the basis of leg pair numbers; however, Clades 2 and 3 could not be differentiated on the basis of leg pair numbers. All specimens had a claw present on the genital leg pair. Dorsal colour was highly variable within clades, with orange and brown being the dominant colours (Table 7).

Discussion

Phylogeography of P. moseleyi

Peripatopsis moseleyi comprised four clades and haploclusers (Figs 2, 3), indicating that populations occupying forest fragments in the south Eastern Cape are genetically structured, with limited maternal dispersal between sample localities. The latter result was corroborated by the marked sequence divergence values observed between the four clades as well as high and statistically significant F_{ST} values. Clade 1 (Isidenge, Amatola and Pirie forests), Clade 2 (Keiskammahoek) and Clade 4 (Qacu, Sandiles Rest and Stutterheim) all occur is close geographic proximity; however, they are genetically discrete, suggesting that the historic contractions and expansions of forests in this region left a significant and complex impact on the phylogeography of *P. moseleyi*. Furthermore, these results demonstrate the low level of dispersal of velvet worms between geographically adjacent areas. For example, Sandiles Rest (Clade 4) is geographically close to specimens from Clade 1, such as Isidenge (Clade 1), yet is genetically highly divergent, and sister to specimens that occur roughly 40 km to the north-east. The close evolutionary relationship between *P. moseleyi* specimens from Sandiles Rest, Stutterheim and Qacu potentially indicates a historical forest connection between these three areas, to the exclusion of the closer areas. McDonald and Daniels (2012) described a similar scenario for a specimen of *Peripatopsis capensis* from Rondevlei Nature Reserve that is geographically close to a clade of specimens from the Cape Peninsula, but phylogenetically more closely related to specimens from the Theewaterskloof–Overstrand clade. McDonald and Daniels (2012) suggests that a historical corridor was most likely present for the geographically distant forests along the Jonkershoek/Hottentots Holland Mountains instead of a historical link from the geographically closer clade on the Cape Penisula. Within Clade 4, the three Qacu sample sites (A, B and C) demonstrate the importance of velvet worms in detecting the importance of habitat structure and quality. Qacu A and C were sampled on the periphery of the main forest (Qacu B), with A being a true forest fragment, while Site C was an isolated degraded forest patch on a private farm. Both the peripheral populations at Qacu had low genetic diversity for all the examined parameters. Velvet worm specimens from Keiskammahoek (Clade 2) was close to samples from Isidenge (Clade 1) (Figs 2, 3) but they were genetically very discrete. The Keiskammahoek population is situated outside of the extensive Afromontane forest network that includes Isidenge clade populations and the Sandiles Rest population. The Keiskammahoek population is situated higher than those in Clade 1, while the populations in Clade 1 are situated in a valley. In contrast, samples from Hogsback and Seymore (Clade 3) are geographically distant and considerably higher in elevation. Divergence time estimations would provide valuable insight into what environmental factors contributed to the observed phylogeographic patterning in *P. moseleyi*.

The effects of forest fragmentation within *P. moseleyi* are pronounced due to its lower dispersal capacity (Rockman *et al.* 2001; Daniels and Ruhberg 2010; McDonald and Daniels 2012; Ruhberg and Daniels 2013; Daniels *et al.* 2013). The degree of population differentiation is significant in *P. moseleyi*, due to the animals' high microclimate specificity that is compounded by the fragmented nature of the forest. This pattern is not uncommon for onychophorans and has been demonstrated in several recent studies in the Peripatopsidae (Daniels and Ruhberg 2010; McDonald and Daniels 2012; Ruhberg and Daniels 2013; Daniels *et al.* 2013, 2016). These results suggest that populations in close geographic proximity

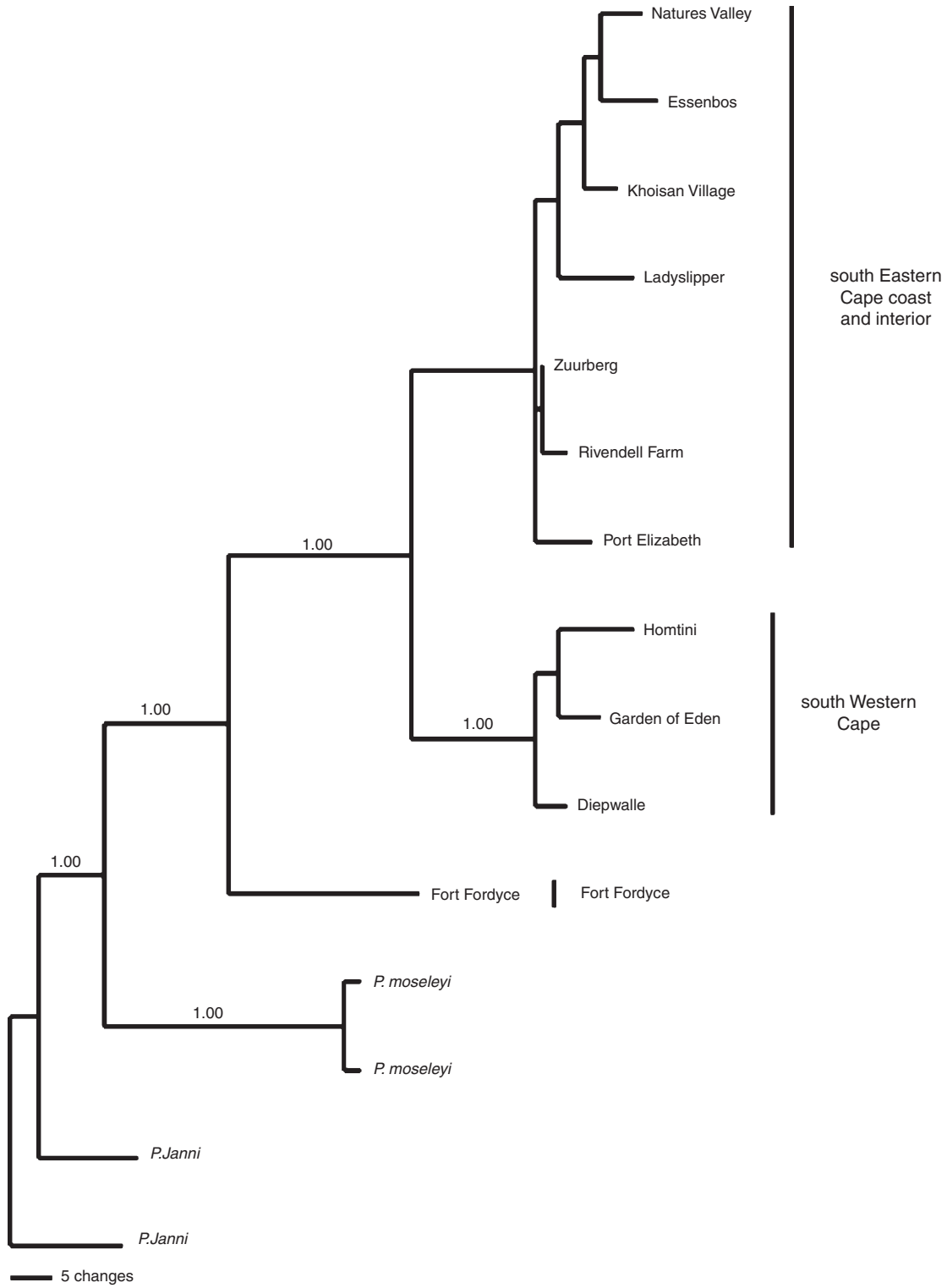

Fig. 5. A Bayesian Inference topology of *Peripatopsis sedgwicki* based on the combined DNA sequence data demonstrating the presence of three clades. Only PP values >0.95 are shown.

cannot easily recolonise adjacent areas and demonstrate the importance that forest fragments may play in potentially aiding colonisation and dispersal events among short-range saproxylic taxa such as velvet worms and harvestmen (Trewick 2000; Boyer *et al.* 2007; de Bivort and Giribet 2010; Daniels and Ruhberg 2010; McDonald and Daniels 2012; Ruhberg and Daniels 2013;

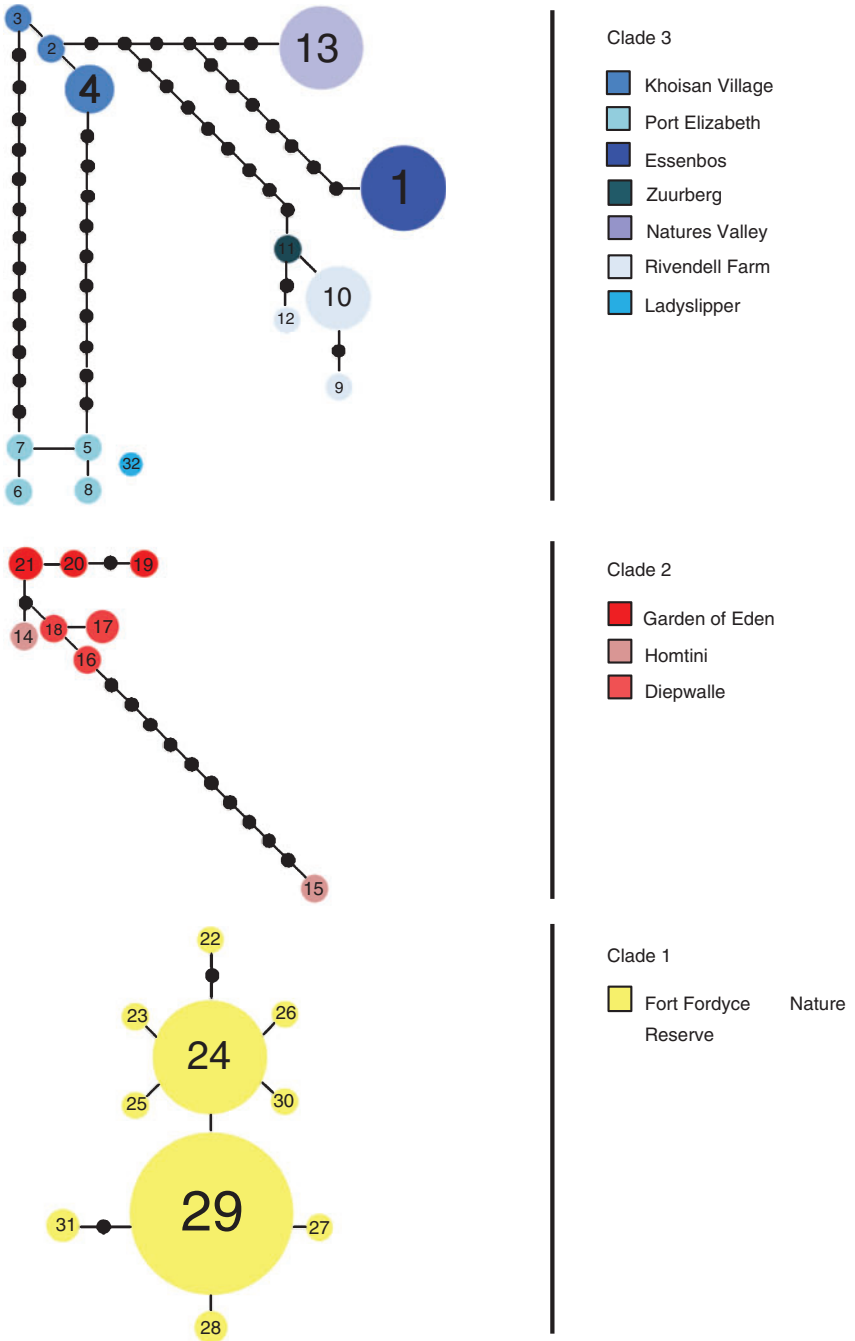

Fig. 6. Haplotype network of *Peripatopsis sedgwicki* consisting of three haploclusters. Solid, black circles represent unsampled haplotypes or mutational step difference.

Daniels *et al.* 2013). Furthermore, Daniels *et al.* (2016) also suggest that low effective populations sizes in velvet worms are likely another contributing factor to high population genetic structure.

Systematics of P. sedgwicki

Peripatopsis sedgwicki comprised three allopatric clades. This finding is corroborated by the phylogeny and the clustering on the haplotype network, while the difference between Clade 1 and the remaining two clades are further supported by the morphological analyses. These results suggest the presence of at least one novel lineage within *P. sedgwicki*. Using the phylogenetic species concept (Avise and Ball 1990) suggests that individuals that share similar derived traits can be viewed as a distinct species; these traits can be in the form of both genealogical and morphological traits. The combined DNA sequence analyses together with the morphology of *P. sedgwicki*

Table 6. Haplotype frequency distribution among *Peripatopsis sedgwicki* sample localities

Haplotype	Sample site										
	Fort Fordyce	Rivendell Farm	Zuurberg	Diepwalle	Essenbos	Homtini	Ladyslipper	Port Elizabeth	Garden of Eden	Khoisan Village	Natures Valley
H01					5						
H02										1	
H03										1	
H04										3	
H05								1			
H06								1			
H07								1			
H08								1			
H09		1									
H10		4									
H11			1								
H12		1									
H13											5
H14						1					
H15						2					
H16				1							
H17				2							
H18				1							
H19									1		
H20									1		
H21									2		
H22	1										
H23	1										
H24	7										
H25	1										
H26	1										
H27	1										
H28	2										
H29	10										
H30	1										
H31	2										
H32							1				

Table 7. List of morphological characters examined within the three *Peripatopsis sedgwicki* clades identified during the present study

N, number; ?, unknown character state

Population	Clade	Leg pair *N*	Presence of claw on genital leg pair	Papillar rows	Dorsal colour
Fort Fordyce NR	1	22/23	+	?	Terracotta red
Homtini	2	20	+	8	Orange, broad brown stripe
Garden of Eden	2	19	+	8	Brown-bright orange
Diepwalle	2	19	+	8	Bright orange, tan flank
Port Elizabeth	3	19/20	+	8	Bright orange, tan flank
Natures Valley	3	20	+	8	Terracotta red
Zuurberg	3	19	+	?	Orange, broad brown stripe
Rivendell Farm	3	19	+	?	Orange, broad brown stripe
Ladyslipper	3	20	+	8	Orange, broad brown stripe
Essensbos	3	19	+	?	Orange, broad brown stripe
Khoisan Village	3	19	+	?	Orange, broad brown stripe

suggest possible cladogenesis within the species according to this concept. Specimens from Clade 1 (Fort Fordyce Nature Reserve) show the great sequence divergence (7.5%) from the remaining two *P. sedgwicki* clades (Clades 2 and 3). The use of sequence divergence as an indicator for speciation is a contentious issue as there is no prescribed sequence divergence percentage that is indicative of separate species (Daniels *et al.* 2016). Studies on Peripatopsidae have demonstrated that sequence divergence

should be coupled with other indicators of speciation due to variability of sequence divergence among and within species (Daniels and Ruhberg 2010; McDonald and Daniels 2012; Daniels *et al.* 2013). This demonstrates the variability of sequence divergence of species which should be considered when designating potential putative evolutionary units; it is therefore necessary that a combination of sequence data (sequence divergence percentage and both mtDNA and total evidence tree topology) and morphology be used to infer new species (Daniels *et al.* 2016).

Further analyses should also be carried out on the south Eastern Cape coast and interior clade (Clade 3), since the holotype of *P. sedgwicki* is from Knysna (Hamer *et al.* 1997), which is close to the distribution of the south Western Cape Clade 2 (Fig. 1*B*) since the former clade is different from the latter clade, and characterised by a sequence divergence of 6% for the COI locus. The area between Clades 2 and 3 is characterised by several deep gorges and rivers that run directly into the Indian Ocean along the coast and thus limit dispersal between the two clades. The latter observation is corroborated by the haplotype network and the AMOVA results. However, the evidence for a new species within this study is based on gross morphology and further scanning electron microscopy (SEM) is required to delimit species boundaries. The use of SEM imagery in the study of Ruhberg and Daniels (2013) confirmed four novel species found within *P. moseleyi sensu lato*, which was already determined by DNA sequence data. Furthermore, for velvet worm species, the structure of the primary dermal papillae, which can be assessed only by using SEM, has been shown to be diagnostic (Ruhberg and Daniels 2013; Daniels *et al.* 2016). Ruhberg and Daniels (2013) showed how minor morphology could give insight into novel speciation. Furthermore, the high sequence divergence between the south Eastern Cape coast and interior clade and the south Western Cape clade (6%) could further support this notion of cryptic speciation of the south Eastern Cape coast and interior clade.

The use of variation in leg pair number has been shown to be unreliable in other studies delineating *Peripatopsis* species (Daniels and Ruhberg 2010; McDonald and Daniels 2012; Ruhberg and Daniels 2013; Daniels *et al.* 2013). However, within *P. sedgwicki* leg pair number has remained fairly stable among populations (Hamer *et al.* 1997): leg pair number is either 19 or 20 leg pairs. However, the specimens from Fort Fordyce Nature Reserve possessed 22 or 23 leg pairs, suggesting that leg pair number is a good species discriminator in this instance. Another consideration for the suggestion of cryptic speciation is the geographic discreteness of the Fort Fordyce population. This geographic confinement of the population to the high-altitude forest habitat (<1100 m) likely aided its allopatric divergence since the low-lying areas are dry and do not support forests. The contraction of forest periods during the Plio/Pleistocene likely resulted in the isolation and confinement of this population to higher altitude, while preventing dispersal from adjacent areas due to the development of xeric corridors, thus promoting cladogenesis. Hence we are confident that the Fort Fordyce population represents a novel species, and a taxonomic description will be undertaken in the future.

Implications for P. moseleyi *and* P. sedgwicki

Both *P. moseleyi* and *P. sedgwicki* possess high population genetic structure, suggesting limited maternal dispersal between the forest fragments in the south-eastern Cape of South Africa. These results suggests that the conservation of their forest habitat is critical in order to conserve genetic diversity within these species, particularly since they are habitat specialists. Similar patterns of marked genetic differentiation is also likely present in other invertebrate taxa that are forest habitat specialists. The detection of novel lineages within *P. sedgwicki* and its confinement to a single sample locality indicates higher levels of localised endemism in velvet worms and reiterates the need for more fine-scale sampling. A study is also planned to examine the species boundaries among the three clades within *P. clavigera* (Daniels *et al.* 2013).

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Appendix 1. FST value across the 11 sample localities

1, Essenbos; 2, Khoisan Village; 3, Port Elizabeth; 4, Rivendell Farm; 5, Zuurberg; 6, Ladyslipper; 7, Natures Valley; 8, Homtini; 9, Diepwalle; 10, Garden of Eden; 11, Fort Fordyce

	1	2	3	4	5	6	7	8	9	10	11
1	0.00000										
2	0.77904	0.00000									
3	0.98545	0.73483	0.00000								
4	0.98293	0.77544	0.32579	0.00000							
5	1.00000	0.55357	0.45455	0.20000	0.00000						
6	1.00000	0.12500	0.96078	0.96429	1.00000	0.00000					
7	1.00000	0.77448	0.86280	0.75709	1.00000	1.00000	0.00000				
8	0.99434	0.61931	0.97443	0.97446	0.98529	0.97015	0.99498	0.00000			
9	0.97613	0.64468	0.95880	0.96218	0.95104	0.90580	0.97847	0.27413	0.00000		
10	0.80716	0.33981	0.79142	0.82887	0.61569	–0.36111	0.82843	0.21252	0.30841	0.00000	
11	0.93331	0.72594	0.93119	0.93283	0.92647	0.53800	0.93878	0.89905	0.89634	0.67521	0.00000

Appendix 2. List of FST value across the 12 sample localities for *Peripatopsis moseleyi*

1, Seymore; 2, Hogsback; 3, Qacu site 1; 4, Qacu site 2; 5, Stutterheim; 6, Sandiles Rest; 7, Quaca NR; 8, Amatola; 9, Isidenge site B; 10, Pirie Forest; 11, Coert site B; 12, Keiskammahoek

	1	2	3	4	5	6	7	8	9	10	11	12
1	0.00000											
2	0.90196	0.00000										
3	0.93668	0.97268	0.00000									
4	0.95585	1.00000	0.35599	0.00000								
5	0.95585	1.00000	0.35599	0.00000	0.00000							
6	0.94207	0.98961	0.64828	0.80000	0.80000	0.00000						
7	0.94190	0.99460	0.25637	0.82558	0.82558	0.79422	0.00000					
8	0.97570	0.99530	0.98218	0.99451	0.99451	0.98880	0.99149	0.00000				
9	0.97201	0.98581	0.97880	0.98532	0.98532	0.98168	0.98345	0.16944	0.00000			
10	0.96533	0.98872	0.97537	0.98660	0.98660	0.98017	0.98276	0.79545	0.68427	0.00000		
11	0.95143	0.99674	0.97360	0.99498	0.99498	0.98265	0.98757	0.48949	–0.07565	0.63265	0.00000	
12	0.91614	0.99130	0.96257	0.99237	0.99237	0.97857	0.98386	0.99002	0.98024	0.98010	0.98661	0.00000