WILEY-BLACKWELL

Cladistics (2016) 1–32

Cladistics

10.1111/cla.12154

Unmasking alpha diversity, cladogenesis and biogeographical patterning in an ancient panarthropod lineage (Onychophora: Peripatopsidae: *Opisthopatus cinctipes*) with the description of five novel species

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Accepted 27 January 2016

Abstract

Speciation and biogeographical patterning in the velvet worm *Opisthopatus cinctipes* was examined under a null hypothesis that numerous discrete lineages are nested within the species. A total of 184 *O. cinctipes* specimens, together with a single specimen of each of the two congeneric point endemic sister species (*O. roseus* and *O. herbertorum*), were collected throughout the forest archipelago in the Eastern Cape, KwaZulu-Natal and Mpumalanga provinces of South Africa. All specimens were sequenced for two partial mitochondrial DNA loci (*COI* and *12S rRNA*), while a single specimen from each locality was sequenced for the nuclear *18S rRNA* locus. Evolutionary relationships were assessed using maximum-likelihood and Bayesian inferences, while divergence time estimations were conducted using BEAST. A Bayesian species delimitation approach was undertaken to explore the number of possible novel lineages nested within *Opisthopatus*, while population genetic structure was examined for the *COI* locus using ARLEQUIN. Phylogenetic results revealed that *O. cinctipes* is a species complex comprising seven geographically discrete and statistically well-supported clades. An independent statistical approach to species delimitations circumscribed ca. 67 species. Results from divergence time estimation and rate constancy tests revealed near constant net diversification occurring throughout the Eocene and Oligocene with subdivision of ranges during the Miocene. Gross morphological characters such as leg pair number within *O. cinctipes* were invariant, while dorsal and ventral integument colour was highly polymorphic. However, scanning electron microscopy revealed considerable differences both between and within clades. The caveats associated with both morphological and algorithmic delineation of species boundaries are discussed. The five novel *Opisthopatus* species are described.

Delineating operational taxonomic units (OTUs) is a critical component of biology that impacts virtually every sphere of study, ranging from ecology and physiology to conservation. What operational criteria should be utilized to define species is a highly contentious matter, as numerous species concepts exist that require different criteria to be satisfied before a species can be considered a valid operation taxonomic unit (Sites and Marshall, 2003; Agapow et al., 2004; Bond and Stockman, 2008). Most taxonomists would concur that multisource data derived from independent character classes offer the best evidence for the diagnoses of novel species. However, a combined integrative systematic approach may often yield conflicting evolutionary outcomes, particularly in instances where character incongruence exists, impairing the ability of taxonomists to weigh objectively the limits of defined species. In instances where cryptic speciation has been detected the problem of effective species diagnosis

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becomes more problematic due to incongruence present between traditional morphological and molecular characters. Evolutionary inferences derived from DNA sequences [particularly the mitochondrial cytochrome *c* oxidase subunit 1 (*COI*) data and to a lesser extent nuclear loci] are widely used to describe alpha diversity and identify cryptic species, and have gained recent momentum (albeit with considerable criticism) due to the advocacy of the "Barcoding of Life" initiative (Hebert et al., 2003a,b, 2004a,b; de Salle et al., 2005; Hickerson et al., 2006). Cryptic speciation has been discovered in several eumetazoan lineages (Hedin and Wood, 2002; Baker et al., 2004; Boyer et al., 2007; Daniels et al., 2009; Daniels, 2011a, 2011b; McDonald and Daniels, 2012; Engelbrecht et al., 2013; Fernández and Giribet, 2014; Medina et al., 2014). Molecular systematic studies of cryptic species have revealed substantial hidden diversity and localized endemism, negating the traditional paradigm of species with cosmopolitan distributions and forcing a rethink of units for conservation priority (Bickford et al., 2006). One of the conundrums of cryptic speciation is how to delineate the lineages because they frequently exhibit deep genetic differentiation with limited morphological differentiation. Recent advances in this regard include multilocus coalescent-based methods and Bayesian inference approaches to species delimitation, offering an algorithmic solution to delineating species boundaries in cryptic complexes (Leaché and Fujita, 2010; Yang and Rannala, 2010; Zhang et al., 2011; Medina et al., 2014), although there are notable problems with this methodological approach (Zhang et al., 2011). The analysis of data derived from independent data sets, including gross morphology, microscopic ultrastructure, and nuclear and mitochondrial genome sequencing, is anticipated to facilitate identification of concrete targets for constructing meaningful species diagnoses, even where cryptic diversity is present (Edgecome and Giribet, 2008).

Phylogenetically early branching, ancient lineages characterized by obligatory microclimatic specialization and low dispersal capabilities are ideal candidate taxa to explore cryptic speciation mechanisms as these taxa will exhibit marked spatio-temporal structure. Onychophora, commonly referred to as velvet worms, represent such a relictual Pangean panarthropod clade that is exclusively terrestrial and typically confined to indigenous forested areas, where they occur in saproxylic environments such as decaying logs and leaf litter, in which microclimate is stable (Murienne et al., 2014). Velvet worm species have historically been characterized by remarkable stasis in morphology, rendering the taxonomy of the group, particularly at the species level, largely unstable. The dubious taxonomy of velvet worm species has been compounded by a history of exclusive reliance on highly variable morphological characters (Reid, 1996). Where velvet worms have recently been subjected to molecular DNA sequencing endeavours in combination with scanning electron microscopy (SEM), pronounced alpha diversity has been unmasked (Trewick, 2000; Daniels et al., 2009, 2013; Oliveira et al., 2011, 2012a; McDonald and Daniels, 2012; Ruhberg and Daniels, 2013). These studies have demonstrated that the existing taxonomy is largely inaccurate, and has severely underestimated alpha diversity, thus highlighting the need for closer systematic scrutiny.

In South Africa two velvet worm genera (*Peripatopsis* and *Opisthopatus*) are present that historically contained eight and three described species, respectively (Hamer et al., 1997; Ruhberg and Hamer, 2005). Recent modern systematic studies resulted in a two-fold increase in species diversity within *Peripatopsis* (Daniels et al., 2009, 2013; McDonald et al., 2012; Ruhberg and Daniels, 2013), with several new species awaiting formal description (S. R. Daniels, unpubl. data). Many of the novel discovered species were nested within three geographically widespread species complexes (the *P. capensis*, *P. moseleyi* and *P. balfouri* species complexes; McDonald et al., 2012; Daniels et al., 2013; Ruhberg and Daniels, 2013). Results from these studies indicate high levels of localized endemicity, accentuating the need for the conservation of the fauna and its forest habitat. In contrast, no molecular systematic study has been undertaken on *Opisthopatus*. Of the three described *Opisthopatus* species, two (*O. herbertorum* and *O. roseus*) are point endemics, whereas the third (*O. cinctipes*) is unique among South African velvet worms in that it has an extensive and discontinuous distribution (Hamer et al., 1997; Ruhberg and Hamer, 2005). The forested areas where *O. cinctipes* has been recorded are isolated and fragmented along the eastern and southern margins of South Africa and cover < 0.5% of the total land surface in the country (White, 1981; Mucina and Rutherford, 2006). Allopatric populations of *O. cinctipes* occur from the coastal margins and adjacent interior of the eastern portions of the Eastern Cape into KwaZulu-Natal province, along the Drakensberg Mountain escarpment into Swaziland and the Mpumalanga province in north-east South Africa (Hamer et al., 1997). Within its distribution range the species is confined to isolated forest patches from sea level to high-altitude mountains and is distributed in temperate and tropical biomes that are bisected by several dry corridors, large rivers and grasslands. The species is present in two major forest types, the Afrotemperate and Indian Ocean Coastal Belt (IOCB) forests. Afrotemperate forests are discontinuous, restricted to high altitudes in the interior, and are separated from each neighbouring forest by dry lowland barriers (Mucina and Rutherford, 2006). Afrotemperate forests are intolerant of fire

regimes, and are limited in size by the frequency of fires in the surrounding fynbos (a Mediterranean heathland), grasslands and savanna biomes, hence they are generally small and located in deep gorges (Mucina and Rutherford, 2006). These forests are referred to as the Afrotemperate archipelago due to their small sizes and discontinuous distribution. IOCB forests occur along the coastal margins of the Eastern Cape and KwaZulu-Natal in South Africa and represent a young habitat type because these areas were historically inundated during episodic marine transgressions. Forest patches have undergone significant contraction and expansions in response to climatic oscillations, rainfall and fire regimes (Hamilton, 1981; White, 1981; Taylor and Hamilton, 1994; McDonald and Daniels, 2012). Climatologically, South Africa has experienced several intense mesic and xeric cycles that directly impacted terrestrial biomes, including forested areas. During the early Miocene the region was characterized by widespread temperate forested habitat, and high levels of precipitation. However, the development of the proto-Benguela current along the west coast resulted in increased aridification and the contraction of forested regions to higher elevations (White, 1978; Lawes, 1990). Consequently, during the late Miocene marked aridification was present that continued into the Pliocene ameliorations that have resulted in allopatric fragmentation of the forest habitat and have been implicated as a cladogenetic driver among velvet worms (Daniels et al., 2009; McDonald and Daniels, 2012; Myburgh and Daniels, 2015).

Given the high levels of crypsis generally present among velvet worms, the large discontinuous range of the single species, *O. cinctipes*, is anomalous. Using traditional morphological characters together with SEM and DNA sequencing of three loci, we examined phylogeographical patterns within *O. cinctipes*. Given the life history characteristics of *O. cinctipes*, in tandem with the heterogeneous and fragmented nature of its forest habitat, we investigated its evolutionary history. Specifically, we tested three hypotheses. First, we hypothesized that *O. cinctipes* is a species complex comprising several genetically isolated lineages with pronounced geographical patterning and genetic differentiation. Secondly, we hypothesized that major cladogenetic episodes in this species complex occurred during the Miocene in response to progressive climatic ameliorations and the progressive aridification that drove the contraction of the Afrotemperate forest biome in South Africa. As a corollary, we hypothesize that Afrotemperate forests in the interior of the country would harbour older lineages than populations from the IOCB. Thirdly, in contrast to the invariability of traditional gross morphological characters, we hypothesized that ultrastructural characters (i.e. pertaining to dermal papillae) would reveal diagnostic differences corresponding to genetic clades. Through this focus on Onychophora, we underline the complexities of delineating species boundaries in cases of morphologically ancient invertebrate taxa.

Materials and methods

Taxon sampling

A total of 184 *Opisthopatus cinctipes* specimens were collected from 47 localities throughout its distribution in the Eastern Cape, KwaZulu-Natal and Mpumalanga provinces of South Africa from 2006 to 2012 (Fig. 1; Table 1) (Hamer et al., 1997). In addition, a single specimen of each of the two point endemic species, *O. herbertorum* and *O. roseus*, was collected from Mount Currie and Ngele Nature Reserves, respectively. Specimens of *Opisthopatus* were hand-collected from saproxylic environments (beneath or inside decaying logs, leaf litter or under moss close to waterfalls or streams), in closed-canopy forest areas. Locality coordinates were recorded using a handheld GPS (Garmin-Trek Summit). We aimed to collect a minimum of five specimens per locality to document the genetic diversity per sample site. However, at some sample localities this was not possible as velvet worms are notoriously difficult to collect and frequently occur in low numbers, as is typical of the phylum Onychophora (S. R. Daniels, pers. observ.). Samples were placed in honey jars and killed by freezing, preserved in absolute ethanol and stored at 4 °C in a refrigerator. Specimens have been deposited in the collection of the South African Museum of Natural History (SAM-ENW-C) (Iziko Museum of Cape Town), South Africa. The two previously described subspecies (*O. c. laevis* and *O. c. natalensis*) are not recognized, in line with the two studies by Ruhberg (1985) and Hamer et al. (1997), despite Oliveira et al. (2012b) recently elevating them to species without formal examination, description or comparison with the three described *Opisthopatus* species.

Outgroup selection

Recent phylogenetic results have demonstrated that the two velvet worm families (Peripatidae and Peripatopsidae) are both monophyletic (Murienne et al., 2014). Both the South African velvet worm genera (*Peripatopsis* and *Opisthopatus*) have been recovered as monophyletic, but are not sister taxa, as they form sister groupings with the two Chilean velvet worm genera (*Metaperipatus* and *Paropisthopatus*) (Daniels et al., 2009; Allwood et al., 2010; Murienne et al., 2014). *Peripatopsis* is thought to be a sister group to *Metaperipatus* (with low or limited nodal support) while

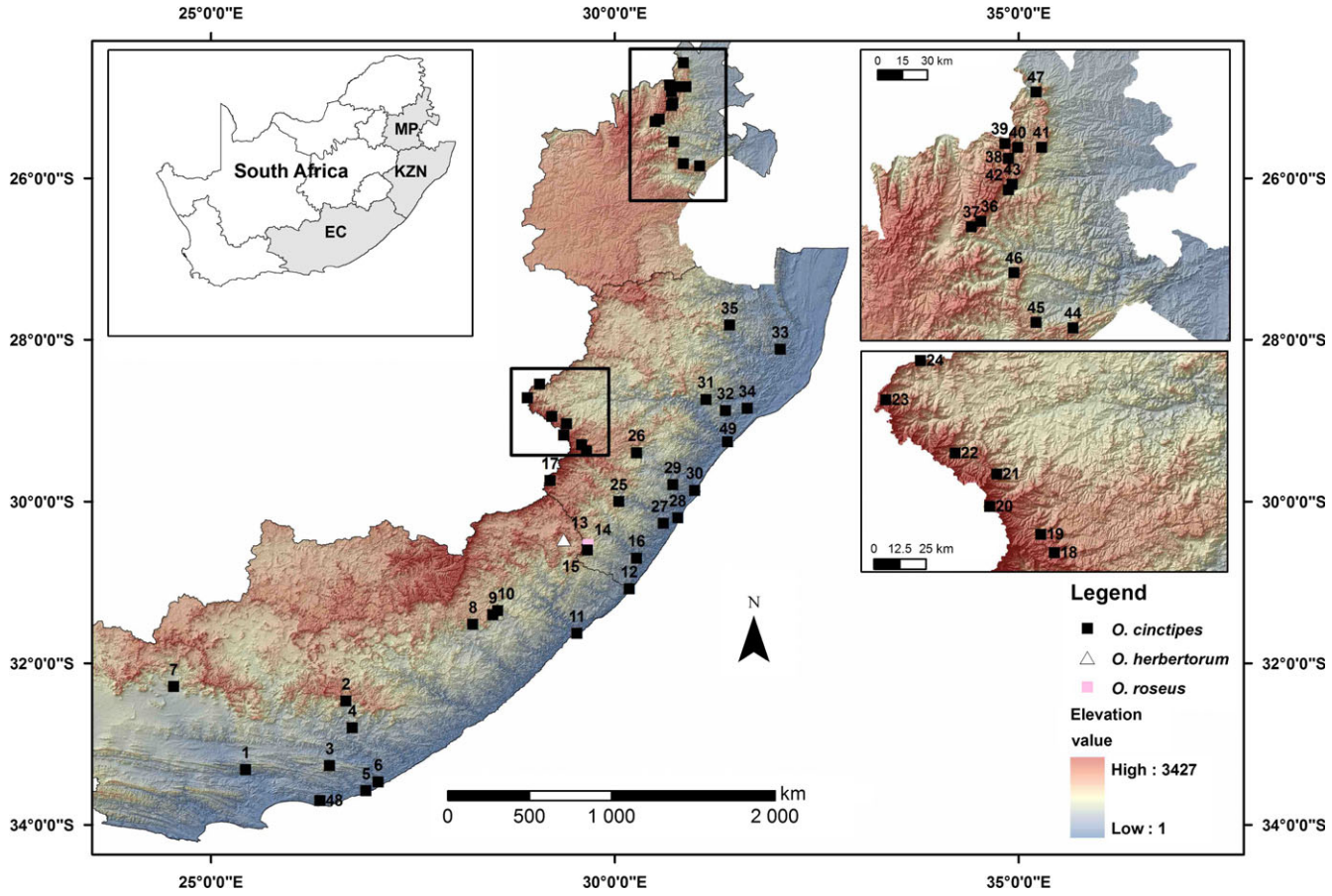

Fig. 1. List of localities where *Opisthopatus* specimens were collected throughout South Africa. Black squares represent *O. cinctipes* populations, while the white triangle and the pink square represent the two single localities for *O. herbertorum* (locality 13) and *O. roseus* (locality 14), respectively. Localities: 1, Suurberg; 2, Katberg; 3, Grahamstown; 4, Rivendell; 5, Kleinemonde River; 6, Kap River NR; 7, Graaff-Reinet; 8, Baziya; 9, Nocu; 10, Jenca Valley; 11, Port St Johns; 12, Port Edward; 13, Mount Currie NR; 14, Ngele Forest; 15, Weza Forest; 16, Oribi Gorge NR; 17, Garden Castel NR; 18, Kamberg NR; 19, Highmoor NR; 20, Injisuthi NR; 21, Monks Cowl NR; 22, Cathedral Peak NR; 23, Royal Natal NR; 24, Oliviershoek Pass; 25, Ixopo; 26, Karkloof NR; 27, Vernon Crookes NR; 28, Umkomaas; 29, Krantzkloof NR; 30, Pigeon Valley; 31, Nkandla Forest; 32, Entumeni Forest; 33, Hluhluwe-iMfolozi; 34, Ongoya Forest; 35, Ngome Forest; 36, Uitsoek Forest; 37, Buffelskloof NR,; 38, Mount Sheba NR; 39, Crystal Springs NR; 40, Graskop; 41, God's Window; 42, Lone Creek; 43, Bridal Veil; 44, Barberton; 45, Nelshoogte; 46, Kaapscehoop; 47, Mariepskop; 48, Alexandria Forest; 49, Zinkwazi. The abbreviation NR denotes Nature Reserves.

Opisthopatus is thought to be a sister group to *Paropisthopatus* (Reid, 1996). *Paropisthopatus* has not been collected in recent years and was unavailable as an outgroup in the present study. As a conservative measure, we thus used 14 outgroup species from the family Peripatopsidae, including two Chilean species (*Metaperipatus blainvillei* and *M. inae*) and 12 Australian species representing four genera. The latter species were also used by Daniels et al. (2009).

DNA extraction, PCR and sequencing

Tissue biopsies were performed on the ventral surface of *Opisthopatus* specimens and subjected to DNA extraction using either a Qiagen DNeasy or a Macherey-Nagel kit, following the manufacturers' protocols. Extracted DNA was stored at 4 °C until required for PCR. Prior to use, a dilution of 1 μL DNA in 20 μL was made with deionized water. Two mitochondrial DNA loci, *COI* and *12S rRNA*, were selected for their relatively high mutational rates and established utility for reconstructing evolutionary relationships among a variety of panarthropod groups including Onychophora (Trewick, 2000; Daniels et al., 2009, 2013; Daniels and Ruhberg, 2010; McDonald and Daniels, 2012; Murienne et al., 2014; Myburgh and Daniels, 2015). Because mitochondrial genes constitute a single linked locus, the inclusion of nuclear markers is thus critical in avoiding bias in tree topology estimation and inferences of species boundaries. Hence, in addition to the mtDNA data, several nuclear DNA sequence markers used for the Australian velvet worms were tested. These included the primer pairs for the *FTz intron* (Rockman et al.,

Table 1
List of sample localities, corresponding to those on Fig. 1 where *Opisthopatus* specimens were sampled throughout South Africa

Locality no.	Locality	Province	Habitat	Species	*N*	Latitude	Longitude
1	Suurberg	Eastern Cape	Afrotemperate Forest	*O. cinctipes*	5	25.43	−33.32
2	Katberg	Eastern Cape	Afrotemperate Forest	*O. cinctipes*	5	26.67	−32.47
3	Grahamstown	Eastern Cape	Afrotemperate Forest	*O. cinctipes*	3	26.47	−33.27
4	Rivendall Farm	Eastern Cape	Afrotemperate Forest	*O. cinctipes*	5	26.75	−32.80
5	Kleinemonde	Eastern Cape	IOCB	*O. cinctipes*	1	26.92	−33.58
6	Kap River	Eastern Cape	IOCB	*O. cinctipes*	5	27.07	−33.47
7	Graaff-Reinet	Eastern Cape	Afrotemperate Forest	*O. cinctipes*	4	24.82	−33.43
8	Baziya	Eastern Cape	Afrotemperate Forest	*O. cinctipes*	5	28.24	−31.52
9	Nocu	Eastern Cape	Afrotemperate Forest	*O. cinctipes*	6	28.49	−31.40
10	Jenca Valley	Eastern Cape	Afrotemperate Forest	*O. cinctipes*	1	28.55	−31.35
11	Port St Johns	Eastern Cape	IOCB	*O. cinctipes*	5	29.53	−31.63
12	Port Edward	KwaZulu-Natal	IOCB	*O. cinctipes*	5	30.18	−31.08
13	Mount Currie NR	KwaZulu-Natal	Afrotemperate Forest	*O. herbertorum*	1	29.37	−30.47
14	Ngele Forest NR	KwaZulu-Natal	Afrotemperate Forest	*O. roseus*	1	29.67	−30.53
15	Weza Forest	KwaZulu-Natal	Afrotemperate Forest	*O. cinctipes*	4	29.66	−30.60
16	Oribi Gorge NR	KwaZulu-Natal	IOCB	*O. cinctipes*	5	30.27	−30.70
17	Garden Castle NR	KwaZulu-Natal	Afrotemperate Forest	*O. cinctipes*	1	29.20	−29.74
18	Kamberg NR	KwaZulu-Natal	Afrotemperate Forest	*O. cinctipes*	5	29.65	−29.38
19	Highmoor NR	KwaZulu-Natal	Afrotemperate Forest	*O. cinctipes*	3	29.59	−29.30
20	Injisuthi NR	KwaZulu-Natal	Afrotemperate Forest	*O. cinctipes*	1	29.37	−29.18
21	Monks Cowl NR	KwaZulu-Natal	Afrotemperate Forest	*O. cinctipes*	5	29.40	−29.04
22	Cathedral Peak NR	KwaZulu-Natal	Afrotemperate Forest	*O. cinctipes*	5	29.22	−28.95
23	Royal Natal NR	KwaZulu-Natal	Afrotemperate Forest	*O. cinctipes*	5	28.92	−28.72
24	Oliviershoek Pass	KwaZulu-Natal	Afrotemperate Forest	*O. cinctipes*	2	29.07	−28.55
25	Ixopo	KwaZulu-Natal	Afrotemperate Forest	*O. cinctipes*	4	30.05	−30.00
26	Karkloof NR	KwaZulu-Natal	Afrotemperate Forest	*O. cinctipes*	4	30.27	−29.40
27	Vernon Crookes	KwaZulu-Natal	IOCB	*O. cinctipes*	5	30.60	−30.27
28	Umkomaas	KwaZulu-Natal	IOCB	*O. cinctipes*	1	30.78	−30.20
29	Krantzkloof NR	KwaZulu-Natal	IOCB	*O. cinctipes*	5	30.72	−29.79
30	Pigeon Valley	KwaZulu-Natal	IOCB	*O. cinctipes*	1	30.99	−29.86
31	Nkandla Forest NR	KwaZulu-Natal	Afrotemperate Forest	*O. cinctipes*	5	31.13	−28.74
32	Entumeni Forest	KwaZulu-Natal	IOCB	*O. cinctipes*	5	31.37	−28.88
33	Hluhluwe-iMfolozi	KwaZulu-Natal	IOCB	*O. cinctipes*	5	32.05	−28.12
34	Ongoya Forest	KwaZulu-Natal	IOCB	*O. cinctipes*	5	31.64	−28.85
35	Ngome Forest	Mpumalanga	Afrotemperate Forest	*O. cinctipes*	3	31.42	−27.82
36	Uitsoek Forest	Mpumalanga	Afrotemperate Forest	*O. cinctipes*	4	30.55	−25.27
37	Buffelskloof NR	Mpumalanga	Afrotemperate Forest	*O. cinctipes*	4	30.50	−25.30
38	Mount Sheba NR	Mpumalanga	Afrotemperate Forest	*O. cinctipes*	5	30.70	−24.93
39	Crystal Springs	Mpumalanga	Afrotemperate Forest	*O. cinctipes*	5	30.68	−24.85
40	Graskop	Mpumalanga	Afrotemperate Forest	*O. cinctipes*	5	30.75	−24.87
41	God's Window	Mpumalanga	Afrotemperate Forest	*O. cinctipes*	5	30.88	−24.87
42	Lone Creek	Mpumalanga	Afrotemperate Forest	*O. cinctipes*	5	30.70	−25.10
43	Bridal Veil	Mpumalanga	Afrotemperate Forest	*O. cinctipes*	5	30.72	−25.07
44	Barberton	Mpumalanga	Afrotemperate Forest	*O. cinctipes*	5	31.05	−25.85
45	Nelshoogte	Mpumalanga	Afrotemperate Forest	*O. cinctipes*	5	30.85	−25.82
46	Kaapscehoop	Mpumalanga	Afrotemperate Forest	*O. cinctipes*	2	30.73	−25.55
47	Mariepskop Forest	Mpumalanga	Afrotemperate Forest	*O. cinctipes*	2	30.85	−24.57
48	Alexandria Forest	Eastern Cape	IOCB	*O. cinctipes*	4	26.35	−33.70
49	Zinkwazi	KwaZulu-Natal	IOCB	*O. cinctipes*	1	31.34	−29.28

N = number of samples. Nature reserves are denoted by NR. IOCB = Indian Ocean coastal Belt forest.

2001), *EPt 17* and *wingless* (Sands et al., 2009). However, these primer pairs failed to amplify in *Opisthopatus*, precluding their utility. We therefore used *18S rRNA* as a nuclear marker. A single specimen per locality was sequenced for *18S rRNA* after preliminary sequencing revealed low levels of genetic variation of this nuclear locus within one locality. This result was also corroborated by a recent *18S rRNA* study in other velvet worm taxa (Myburgh and Daniels, 2015). The latter marker has been successfully used in phylogenetic and phylogeographical studies of South African velvet worms (Daniels et al., 2009; McDonald and Daniels, 2012; Myburgh and Daniels, 2015).

For each PCR a 25-μL reaction was performed that contained 14.9 μL of molecular-grade H_2O, 3 μL of 25 mm $MgCl_2$, 2.5 μL of 10 × Mg^{2+}-free buffer, 0.5 μL of 10 mm dNTPs, 0.5 μL of each oligonucleotide primer set at 10 mm, 0.1 unit of Taq polymerase

and 2 μL of template DNA. The PCR temperature regime for all the gene fragments was 94 °C for 4 min, 94 °C for 30 s, 48 °C for 35 s and 72 °C for 30 s and a final extension step at 72 °C for 10 min. Primer pairs for the respective gene regions were as follows: LCOI-1490 and HCOI-2198 (Folmer et al., 1994) for a partial fragment of the *COI* locus; 12Sai and 12Smbi (Kocher et al., 1989) for a partial fragment of the *12S rRNA* locus; and *18S* 5F and *18S* 7R (Giribet et al., 1996) for a partial fragment of the *18S rRNA* locus. PCR products were electrophoresed on a 1% agarose gel containing ethidium bromide for 2–3 h at 90 V and products were visualized under UV light. The gel bands of DNA were excised and the DNA was extracted and purified using a QIAquick gel extraction kit. Purified PCR products were cycle sequenced using standard protocols [3 μL of the purified PCR product, 4 μL of the fluorescent-dye terminators with an ABI PRISM Dye Terminator Cycle Sequencing Reaction Kit (Perkin-Elmer) and 3 μL of a 10 μM primer solution for each primer pair]. Unincorporated dideoxynucleotides were removed by gel filtration using Sephadex G-25 (Sigma-Aldrich, St. Louis, MO, USA).

Phylogenetic analysis

Sequences were checked for base ambiguities in Sequence Navigator (Applied Biosystems, Foster City, CA, USA) and a consensus sequence was created. The protein-encoding *COI* sequences were manually aligned based on the conceptual peptide translations, which were also checked for stop codons. For *12S rRNA* and *18S rRNA*, sequences were aligned using MUSCLE v.3.8 (Edgar, 2004). Hypervariable regions that could not be aligned with confidence were excluded from the phylogenetic analyses using GBlocks v.0.91b (Castresana, 2000) under default parameters. Evolutionary relationships within *Opisthopatus* were inferred using Bayesian inference (BI) and maximum-likelihood (ML) approaches. The BI and ML analyses were conducted on both the combined mtDNA (*COI+12SrRNA*) and the total evidence. Uncorrected pairwise sequence divergence values were calculated for the *COI* locus using PAUP* v.4.10 (Swofford, 2002).

ML analysis was conducted in RAxML v.7.2.7 (Stamatakis, 2006). Heuristic searches were conducted under mixed models of sequence evolution, which allows individual model parameters of nucleotide substitution to be estimated independently for each analysis. A unique GTR+Γ model was implemented for each partition during the thorough ML tree search while CAT approximation was used during the assessment of nodal support with 1000 rapid bootstrap replicates (Felsenstein, 1985; Stamatakis, 2006). Nodal support was estimated using bootstrap resampling (1000 pseudo-replicates). Bootstrap values of $< 75\%$ were regarded as indicating poor support and those of $> 75\%$ as good support.

BI was conducted using MrBayes 3.0b4 (Ronquist and Huelsenbeck, 2003) for the large *COI* and *12S rRNA* data set as well as the reduced total evidence dataset. jModelTest v.2 (Darriba et al., 2012) was used to obtain the best-fit substitution model for each locus for the partitioned Bayesian analysis. The best-fit ML score was chosen using the Akaike information criterion (AIC) (Akaike, 1974) as this has been demonstrated to reduce the number of parameters that contribute little to describing the data by penalizing more complex models (Nylander et al., 2004). The substitution models calculated using jModelTest v.2 were used for the partitioned analysis of the combined *COI* and *12S rRNA*. For each Bayesian analysis, ten Monte Carlo Markov chains were run, with each chain starting from a random tree and 5 million generations generated, sampling from the chain every 1000th tree. This was done for combined mtDNA data (*COI+12S rRNA*) for all samples. This was repeated for the combined analyses, and the substitution models were recalculated, as we used a single specimen from each sample locality. A 50% majority rule consensus tree was generated from the trees retained (after the burn-in trees were discarded), with posterior probabilities for each node estimated by the percentage of time the node was recovered. Posterior probabilities (p*P*) of < 0.95 were regarded as not supported. Runs were repeated four times to ensure accurate topological convergence. We sought lineages within *Opisthopatus* that were recovered with high nodal support and were insensitive to algorithmic treatment as candidates for evaluating novel species boundaries. A similar approach was recently followed by Bull et al. (2013) for the widely distributed velvet worm *Euperipatoides rowelli* in the Tallaganda region of south-eastern Australia.

Population genetic structure analysis using COI

Population genetic structure analyses were performed on the *COI* data set for all the *O. cinctipes* sample localities (and excluded the single specimens of both *O. roseus* and *O. herbertorum*). The *COI* locus is the most rapidly evolving marker used in the present study and hence best capable of detecting population genetic structure as inferred by standard diversity indices, including number of haplotypes (*Nh*), haplotypic diversity (*h*), nucleotide diversity (π) and number of polymorphic sites (*Np*), while Fu's F_s (Fu, 1997) was used to determine the history of demographic stability within population. Fu's F_s with $P < 0.02$ were considered statistically meaningful. All these calculations were undertaken in ARLEQUIN v.3.01 (Schneider et al., 2000). In addition, pairwise F_{ST} among

populations were also calculated. Their significance was calculated by performing 10 000 permutations of the dataset.

DNA barcoding analyses using COI

The *COI* locus is the barcoding marker of choice. Typically, sequence divergences of known species are compared and values above a certain percentage are taken as indicative of cryptic differentiation. However, there is often an overlap between intra- and interspecific *COI* sequence divergence values—the barcoding gap. In addition, substantial geographical coverage of the species distribution is required, coupled with the inclusion of all the described species, in an attempt to differentiate conspecific groups from cryptic lineages. While the *COI* locus has been widely used in Onychophora, the ability of this marker to delineate velvet worm species and identify cryptic lineages exclusively remains largely uncorroborated. A measure of sequence divergence (uncorrected "*P*" values) derived from the *COI* data was therefore used to examine intra- and interspecific differences. We used values > 10% to recognize putative novel lineages based on our preliminary data analyses.

Divergence time estimation

No fossil velvet worms are known from the southern hemisphere Peripatopsidae. In addition, there is considerable ambiguity about the timing of separation of South Africa and Chile (Eagles, 2007; Torsvik et al., 2009). Furthermore, despite recent support for persistence of Peripatopsidae since the Jurassic (Murienne et al., 2014), the non-sister relationship of the two South African genera renders the application of an external biogeographical calibration point in our dataset problematic. Thus, we employed estimated mutation rates of these two partial mtDNA gene fragments, based on a range of mitochondrial mutation rates for the panarthropod taxa as approximations for velvet worm counterparts. For *12S rRNA*, a mutation rate of 1%/Myr was used (implemented as a normally distributed prior density with a 95% interval of 0.6–1.4%/Myr) based on brachyuran that like velvet worms constitute a panarthropod lineage whose crown group diversified in the Devonian (Schubart et al., 1998; Klaus et al., 2010). Similarly, for the *COI* locus a mean mutation rate of 2.0%/Myr was assumed with a 95% interval of 1.4–2.6%/Myr, covering a wide range of published panarthropod rates (Knowlton and Weigt, 1998; Schubart et al., 1998; Projecto-Garcia et al., 2010). This approach results in large credibility intervals, but taking the large uncertainty of molecular clock calibration into account was considered a necessary and conservative workaround to the dubious practice of biogeographically based calibration. For the nuclear *18S rRNA* gene we assumed a very broad uniform prior for the substitution rate between 0% and 2%/Myr.

Divergence time estimation was conducted using a lognormal uncorrelated relaxed clock model in BEAST v.1.7.5 (Drummond et al., 2012) for each of the two datasets. First, we used a 190-taxon mtDNA (*COI* + *12S rRNA*) dataset consisting only of *Opisthopatus* exemplars and rooted with the two *Metaperipatus*, and second, we used a 51-taxon three-locus data set (all genes) retaining only *Opisthopatus* exemplars and rooted with the two *Metaperipatus*. We applied a Yule tree prior and GTR+Γ substitution models to all partitions, with *CO1* additionally partitioned into two sets (1st and 2nd codon sites under one model; 3rd codon sites under another). For both data sets, two chains of 50 million generations were run, with sampling every 5000 chains. Stationary and effective sampling size (ESS) of parameters were investigated in Tracer v.1.6 (Rambaut et al., 2013); the initial 20% of samples was discarded as burnin, and sufficient ESS values > 200 were obtained for all parameters.

Tests of diversification rate constancy

To examine whether net diversification rates have remained constant during the evolutionary history of *Opisthopatus*, we examined log lineage through time plots for chronograms of the total evidence dataset after culling the *Metaperipatus* outgoups, using the R package LASER (Rabosky, 2006). Tests for rate constancy were implemented using the ΔAIC test statistic and six models: one-rate Yule, two-rate Yule, three-rate Yule, birth-death, density-dependent logarithmic and density-dependent exponential.

Species delimitation using Bayesian GMYC

A Bayesian implementation of the generalized 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, 200 post-burnin trees were randomly selected for analysis. To provide reliable benchmarks, all outgroups were culled except for the two *Metaperipatus* species, whose systematic validity is well established. A Markov chain was run for 100 000 generations and 50 000 generations were discarded as burnin, sampling the chain every 1000th generation. A uniform prior for the number of species was applied, with a lower bound of three (the two *Metaperipatus* species and the focal genus *Opisthopatus*) and an upper bound of 190 (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 character examination

A digital camera was used to capture images of live specimens to demonstrate their colour variation. Where possible, at least one male specimen per locality was used for gross morphological analysis and SEM. A stereomicroscope was used to observe the following gross morphological characters: number of leg pairs, dominant dorsal and ventral integument colour, distinct dorsal pattern and the presence of any unique head structures. Dorsal and ventral integument colour shows a clear ontogenetic trajectory (S. R. Daniels, pers. observ.), and hence only specimens > 1 cm in length were used in the colour analyses. Images of the dorsal and ventral integument of selected male specimens (including a colour chart as standard) were captured with a Leica DFC320 digital camera, attached to a Leica MZ 7.5 stereomicroscope, and edited using the Leica Application Suite software. These images were used to investigate differences in the integument between the different genetic clades.

As our primary objective was to investigate the genetic variation within *Opisthopatus* we preserved our specimens in absolute ethanol. Cognizance should be taken that specimens preserved in absolute ethanol will generally yield a suboptimal image due to tissue shrinkage, as dehydration renders specimens brittle and easily damaged (Ruhberg and Daniels, 2013). A single male specimen representing each sample locality was dissected into two sections that included the anterior (head) and the posterior (with genitalia) sections. Prior to imaging, the samples were critical-point dried, mounted in carbon cement and sputter-coated with a thin layer of gold. SEM was undertaken at the Central Analytical Facility in the Department of Geology at the University of Stellenbosch using a Leo 1430VP scanning electron microscope. Beam conditions during surface analysis were 7 kV and approximately 1.5 nA, with a working distance of 13 mm and a spot size of 150. During the present study, we focused specifically on the dorsal and ventral integument structure, because these characters have been shown to be useful in delineating cryptic species boundaries in velvet worms (McDonald et al., 2012; Oliveira et al., 2012b; Daniels et al., 2013; Ruhberg and Daniels, 2013). We examined the scale ranks and shape of the dorsal integumentary primary dermal papillae. In addition, using a digital caliper we measured (in mm) two standard dimensions: total length (TL) from the anterior most point of the head to the posterior end of the body and the diameter of the body (DB) in line with oncopod 10 (Reid, 1996). These two measures have been used in the description of novel velvet worm species (Ruhberg and Daniels, 2013).

Results

Combined mtDNA topology (COI + 12S rRNA)

The *COI* and *12S rRNA* fragments comprised 610 and 281 bp, respectively. Sequences were deposited in GenBank (*COI* accession numbers KR906983–KR907172; *12S rRNA* accession numbers KR906793–KR906982). For the *COI* locus, a TVM+I+Γ ($-\ln L = 1147.16$) model was selected. For the *12S rRNA* locus, an HKY (Hasegawa et al., 1985)+I+Γ ($-\ln L = 4845.39$) model was selected. The BI and ML analyses retrieved nearly identical tree topologies, and hence only the ML tree is shown (Fig. 2). The combined mtDNA topology retrieved a monophyletic *Opisthopatus*. However, *O. cinctipes* is paraphyletic and a species complex, characterized by multiple genetically distinct clades that exhibits strong geographical structure. Deeper nodal relationships were poorly supported while the terminal nodes were statistically well supported. Specimens from the southern Afrotemperate forests in the Drakensberg Mountains in KwaZulu-Natal province formed a basal clade comprising *O. cinctipes* specimens from Garden Castle sister group to *O. roseus* sister group to *O. herbertorum* Kamberg and Highmoor. The second clade comprised *O. cinctipes* specimens from Graaff-Reinet sister group (albeit with no statistical support) to one clade of specimens from the Highveld of Mpumalanga and included Gods Window sister group to Bridal Veil, Mount Sheba and Crystal Springs, as well as specimens from Kaapscehoop, Uitsoek, Buffels and Nelshoogte. The third clade comprised *O. cinctipes* specimens exclusively from the Eastern Cape province, with Kleinemonde sister group to Suurberg and the latter clade sister group to Kap river, Alexandria forest, Rivendell, Grahamstown and Katberg. Clade four comprised specimens from Barberton on the Mpumalanga Lowveld as sister group to a clade of *O. cinctipes* specimens from the northern Drakensberg Mountain, Karkloof NR specimens as sister group to Royal Natal NR and Oliviershoek, and Ngome Forest as sister group to Injisuthi NR, Cathedral Peak NR and Monks Cowl NR. Clade five comprised specimens from the north-eastern interior of the Eastern Cape province, with Nocu specimens sister group to Baziya and Jenca Valley. The latter clade was sister group to clade six and comprised specimens from the Highveld of the Mpumalanga province, with Mariepskop sister group to Graskop, God's Window, Bridal Veil and Lone Creek. Clade seven, containing specimens from

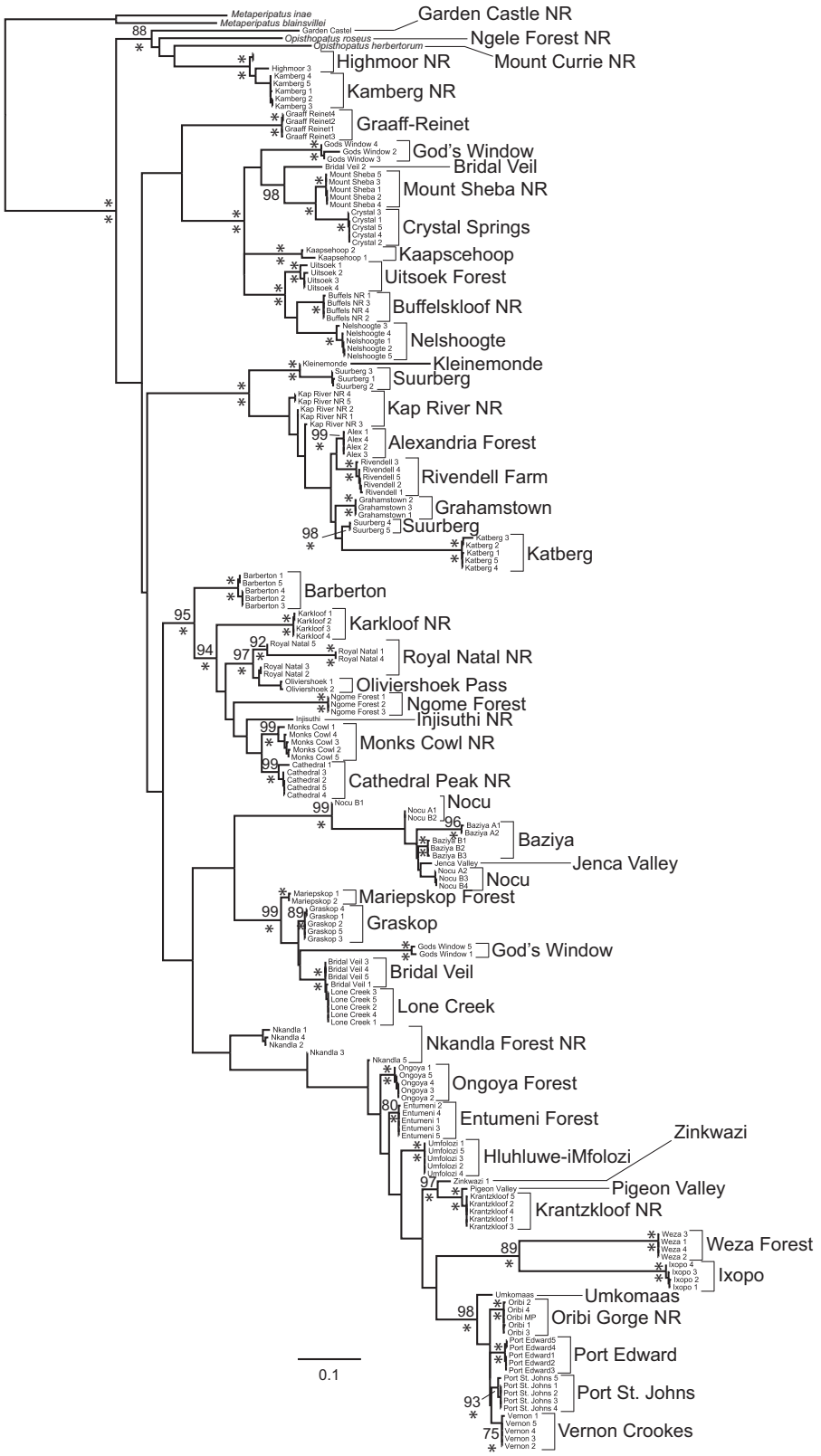

Fig. 2. ML tree topology of the *Opisthopatus* species complex based on two mtDNA (*COI +12S rRNA*) loci. Numbers above nodes indicate nodal support for bootstrapping and posterior probabilities, respectively. Nodes with 1.00 p*P* and 100% bootstrap support are marked with an asterisk. The Australian outgroups were removed from the topology. Scale bar = 0.1 changes per position.

Nkandal Forest NR, was basal and sister group to a group of specimens from the IOCB forest, with Ongoya forest sister to Entumeni, Hluhluwe-iMfolozi, Zinkwazi, Pigeon Valley, Krantzkloof, Weza, Ixopo, Umkomaas, Oribi Gorge, Port Edward, Port St Johns and Vernon Crookes.

Total evidence tree (COI + 12S rRNA + 18S rRNA)

For the *18S rRNA* locus, a 412-bp fragment was amplified per sample and combined with the mtDNA data to yield a total of 1303 bp. The *18S* sequences were deposited in GenBank under accession numbers KR907173–KR907223. The substitution model for the locus was SYM+Γ ($-\ln L = 1574.83$). Analyses of the *18S* data set alone (topology not shown) revealed a poorly resolved topology; only two clades were retrieved, namely one clade comprising *O. cinctipes* specimens from the Eastern Cape (referred to as clade 2 in Fig. 3) and a second clade comprising *O. cinctipes* specimens from the KwaZulu-Natal province IOCB forests (referred to as clade 7 in Fig. 3). For both the *COI* and the *12S rRNA* loci the substitution models for the reduced data set were recalculated (results not

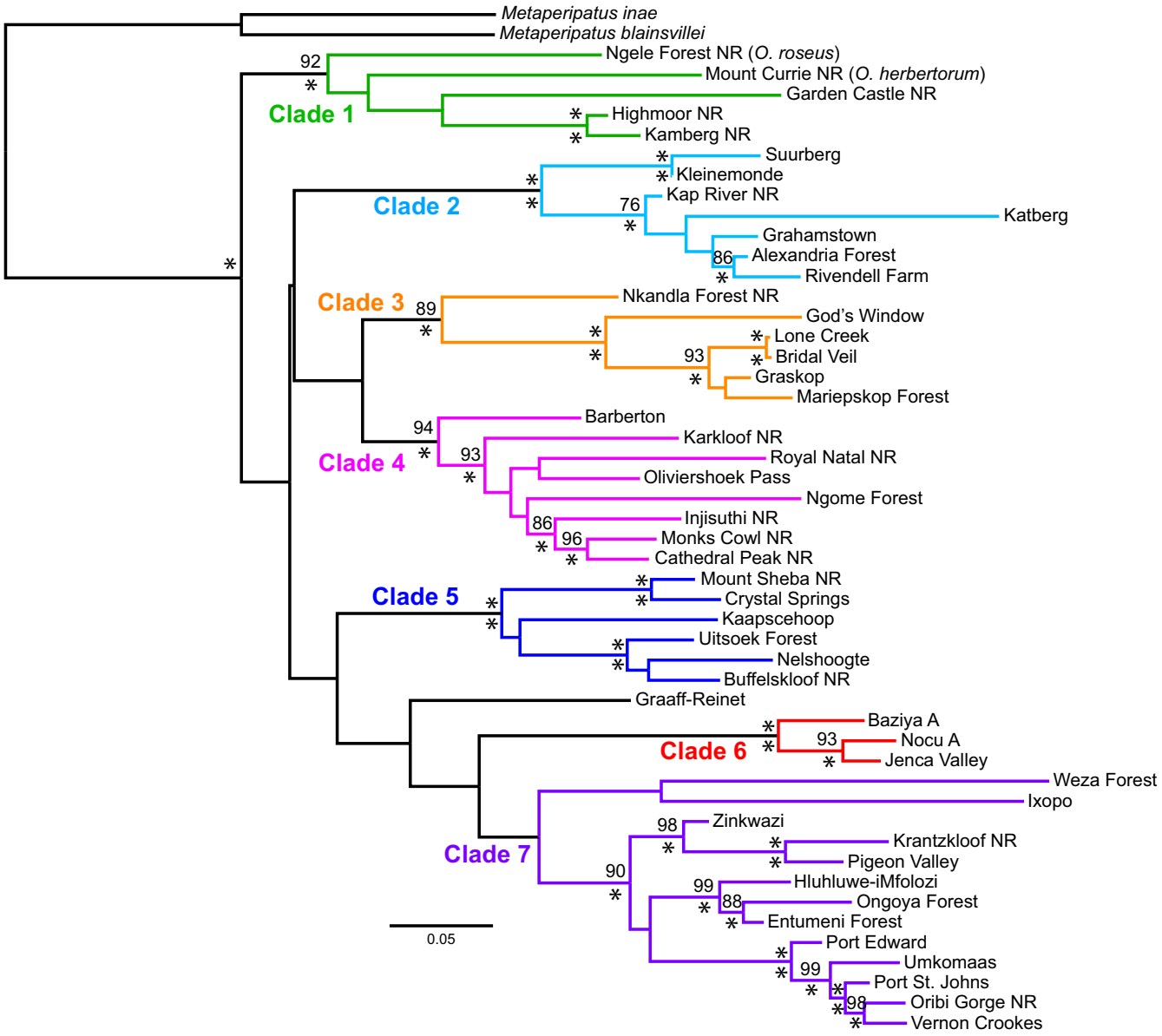

Fig. 3. ML tree topology of the *Opisthopatus* species complex based on the total evidence (*COI+12S rRNA+18S rRNA*) data set. Coloured branches correspond to distinct, geographically cohesive clades. Numbers above nodes indicate nodal support for bootstrapping and posterior probabilities, respectively. Nodes with 1.00 p*P* and 100% bootstrap support are marked with an asterisk. The Australian outgroups were removed from the topology. Scale bar = 0.05 changes per position.

shown). Both analytical methods (BI and ML) produced nearly identical topologies, and hence only the ML topology is shown (Fig. 3). The combined analyses retrieved a monophyletic *Opisthopatus* while *O. cinctipes* was again retrieved as a paraphyletic species complex. Deeper nodal relationships were poorly supported while terminal nodes had good statistical support (> 0.95 p*P*/> 75%). Seven statistically well-supported clades were retrieved (Fig. 3). Clade 1 comprised specimens from the southern Drakensberg Mountains in KwaZulu-Natal province with *O. roseus* (Ngele forest) sister group to *O. herbertorum* (Mount Currie) and *O. cinctipes* (Garden Castle NR, Highmoor NR and Kamberg NR). Clade 2 comprised *O. cinctipes* specimens from the Eastern Cape coast and adjacent interior (Kleinemonde River sister to Suurberg with the latter being sister group to Kap River NR, Katberg, Grahamstown, Rivendell and Alexandria Forest). Clade 3 comprised *O. cinctipes* specimens from Nkandla Forest NR sister group to specimens from the Mpumalanga Highveld (God's Window, Lone Creek, Bridal Veil, Mariepskop and Graskop). Clade 4 comprised *O. cinctipes* specimens from Barberton sister group to specimens from the northern Drakensberg Mountains (Karkloof sister to Royal Natal sister group to Olivierhoek Pass, Ngome forest, Injisuthi, Monks Cowl and Cathedral Peak). Clade 5 comprised the remainder of the *O. cinctipes* specimens from Mpumalanga Highveld (Mount Sheba sister to Crystal Springs with the latter forming the sister group to specimens from Kaapschehoop, Uitsoek, Buffels and Nelshoogte). Clade 6 comprised *O. cinctipes* specimens from the north-eastern interior of the Eastern Cape with Baziya being sister group to specimens from Nocu and Jenca Valley. Clade 7 comprised *O. cinctipes* specimens from Ixopo and Weza sister group to a clade comprised exclusively of specimens from the IOCB forests of KwaZulu-Natal province.

Population genetic structure analysis using COI

The TCS analyses retrieved a total of 119 *COI* haplotypes for the 184 *O. cinctipes* specimens sequenced. The number of polymorphic sites within sample localities was generally low, with the exception of three sample localities (Bridal Veil, God's Window and Nkandla) where a high number of polymorphic sites was detected. Due to the large sequence divergences between localities (see Results below) haplotypes could not be connected into a single framework at the 95% confidence level. In addition, no haplotypes were shared between localities, suggesting the absence of maternal gene flow between sample sites. The latter result is corroborated by the large and statistically significantly F_{ST} values between all sample localities indicating marked population differentiation and genetic substructure (results not shown). Among *O. cinctipes* populations, 90.21% of the variation was present between sample sites (V_a = 41.43, df = 46, SS = 7725.15, $P < 0.01$), while 9.79% of the variation was present within sample sites (V_b = 4.49, df = 139, SS = 624.73, $P < 0.01$). Haplotype diversity (*h*) was generally high while the nucleotide diversity (π) was low (Table 2). The high *h* diversity value within localities indicates moderate mutation rates or differential selection pressures, while the low π value within localities can be attributed to small isolated populations with low abundance and gene flow that has undergone severe bottlenecks (Table 2). Fu's F_s was only statistically significant for the Suurberg sample locality ($P < 0.02$). Ten of the sample sites had negative Fu's F_s values while the remainder had positive values. Negative values can be attributed to an excess of low-frequency polymorphisms consistent with population expansions or positive directional selection, while positive values indicate an excess of intermediate polymorphisms due to recent population bottlenecks or balancing selection. In our study the negative value is probably the result of population expansions, while the positive values probably reflect population bottlenecks.

DNA barcoding analyses using COI

Uncorrected intraspecific pairwise *COI* sequence divergence values within *O. cinctipes* sampled localities were generally low and < 1% for 24 sample localities representing 49% of sample sites, < 3% at five sample localities representing 6% of sample sites, < 5% at three sample localities representing 10% of sample sites, < 11% at two sample localities representing 4% of sample sites and > 15% at three sample localities representing 6% of sample sites at Nkandla, God's Window and Bridal Veil. By contrast, within clades, sequence divergence ranged from 4.91 to 19.01%, with a mean of 12.00%. Between the seven clades the uncorrected sequence divergence ranged from 11.63 to 16.06%, with a mean of 19.27%. As benchmarks, sequence divergence between the two described *Opisthopatus* species (*O. roseus* and *O. herbertorum*) was 12.95%. Divergence between the two Chilean outgroup species *M. blainvillei* and *M. inea* was 13.60%, while the uncorrected sequence divergence within the two Australian outgroup genera ranged from 5.08 to 10.49% among the five *Ooperipatus* species, and from 4.91 to 8.52% among the six *Planipapillus* species.

Divergence time estimates

The divergence time estimates suggest that the *Opisthopatus* species complex originated sometime during the Cretaceous [mean: 111.3 Mya, 95% highest posterior density (HPD): 80.6–153.0 Mya] and diversified during the Late Cretaceous (mean: 87.7 Mya,

Table 2
Summary list of the population parameters for each of the *Opisthopatus cinctipes* sample locality

Locality no.	Locality	*N*	No. of haplotypes	No. of polymorphic sites	Haplotype diversity (± SD)	Nucleotide diversity (± SD)	Fu's F_s
1	Suurberg	5	4	2	0.900 ± 0.161	0.001 ± 0.000	−2.293*
2	Katberg	5	3	6	0.800 ± 0.164	0.004 ± 0.003	1.342
3	Grahamstown	3	2	1	0.666 ± 0.314	0.001 ± 0.001	0.200
4	Rivendall Farm	5	3	6	0.800 ± 0.164	0.004 ± 0.003	1.090
5	Kleinemonde River	1	1	0	1.000 ± 0.000	0.000 ± 0.000	NA
6	Kap River NR	5	2	7	0.400 ± 0.237	0.004 ± 0.003	3.366
7	Graaff-Reinet	4	3	2	0.833 ± 0.222	0.001 ± 0.001	−0.657
8	Baziya	5	5	56	1.000 ± 0.126	0.054 ± 0.003	1.088
9	Nocu	6	4	28	0.866 ± 0.129	0.026 ± 0.016	3.714
10	Jenca Valley	1	1	0	1.000 ± 0.000	0.000 ± 0.000	NA
11	Port St Johns	5	2	5	0.400 ± 0.237	0.003 ± 0.002	2.639
12	Port Edward	5	3	3	0.800 ± 0.164	0.002 ± 0.002	0.276
15	Weza Forest	4	2	2	0.500 ± 0.265	0.001 ± 0.001	1.098
16	Oribi Gorge NR	5	3	4	0.700 ± 0.214	0.002 ± 0.002	0.276
17	Garden Castle NR	1	1	0	1.000 ± 0.000	0.000 ± 0.000	NA
18	Kamberg NR	5	2	1	0.600 ± 0.175	0.000 ± 0.001	0.626
19	Highmoor NR	3	2	24	0.666 ± 0.314	0.026 ± 0.020	5.023
20	Injisuthi NR	1	1	0	1.000 ± 0.000	0.000 ± 0.000	NA
21	Monks Cowl NR	5	5	16	1.000 ± 0.126	0.011 ± 0.007	−0.874
22	Cathedral Peak NR	5	3	18	0.700 ± 0.218	0.011 ± 0.007	3.065
23	Royal Natal NR	5	3	63	0.800 ± 0.164	0.062 ± 0.038	7.240
24	Oliviershoek Pass	2	2	4	1.000 ± 0.500	0.006 ± 0.007	1.386
25	Ixopo	4	4	8	1.000 ± 0.176	0.006 ± 0.005	−0.768
26	Karkloof NR	4	3	2	0.833 ± 0.222	0.001 ± 0.001	−0.657
27	Vernon Crookes NR	5	3	3	0.700 ± 0.218	0.001 ± 0.001	−0.185
28	Umkomaas	1	1	0	1.000 ± 0.000	0.000 ± 0.000	NA
29	Krantzkloof NR	5	2	1	0.400 ± 0.237	0.000 ± 0.000	0.090
30	Pigeon Valley NR	1	1	0	1.000 ± 0.000	0.000 ± 0.000	NA
31	Nkandla Forest NR	5	5	99	1.000 ± 0.126	0.096 ± 0.005	1.715
32	Entumeni Forest NR	5	3	4	0.700 ± 0.218	0.002 ± 0.002	0.276
33	Hluhluwe-iMfolozi NR	5	2	1	0.400 ± 0.237	0.000 ± 0.000	0.090
34	Ongoya Forest NR	5	3	3	0.700 ± 0.218	0.002 ± 0.002	0.276
35	Ngome Forest NR	3	1	0	0.000 ± 0.000	0.000 ± 0.000	NA
36	Uitsoek Forest NR	4	3	12	0.833 ± 0.222	0.010 ± 0.007	1.835
37	Buffelskloof NR	4	3	2	0.833 ± 0.222	0.001 ± 0.001	−0.887
38	Mount Sheba NR	5	3	2	0.700 ± 0.218	0.001 ± 0.001	−0.475
39	Crystal Springs NR	5	2	1	0.400 ± 0.237	0.000 ± 0.000	0.090
40	Graskop	5	1	0	0.000 ± 0.000	0.000 ± 0.000	NA
41	God's Window	5	5	113	1.000 ± 0.126	0.109 ± 0.066	1.842
42	Lone Creek	5	1	0	0.000 ± 0.000	0.000 ± 0.000	NA
43	Bridal Veil	5	3	83	0.700 ± 0.218	0.054 ± 0.033	6.893
44	Barberton	5	2	2	0.600 ± 0.175	0.001 ± 0.001	1.687
45	Nelshoogte	5	4	7	0.900 ± 0.161	0.005 ± 0.003	−0.226
46	Kaapschehoop	2	1	0	0.000 ± 0.000	0.000 ± 0.000	NA
47	Mariepskop Forest NR	2	2	2	1.000 ± 0.500	0.003 ± 0.004	0.693
48	Alexandria Forest NR	4	3	2	0.833 ± 0.222	0.001 ± 0.001	−0.887
49	Zinkwazi	1	1	0	1.000 ± 0.000	0.000 ± 0.000	NA

*$P < 0.02$.
N is the number of samples per locality.

95% HPD: 62–118 Mya) (Fig. 4). While the precision of these age estimates is limited, we note that our relaxed molecular clock approach resulted in age ranges that overlap significantly with the HPD intervals obtained by Murienne et al. (2014). Diversification of the seven major clades within the *O. cinctipes* species complex occurred throughout the Palaeocene and Eocene (range 27.0–84.2 Mya). Initial diversification in Clade 1 occurred in the Eocene or early Oligocene (mean: 37.4 Mya, 95% HPD: 26.4–50.9 Mya), a range approximately similar to that for Clade 2. Eocene to Oligocene diversification was estimated in Clade 3 (mean: 37.1 Mya, 95% HPD: 27.05–51.6 Mya). Diversification in the Palaeocene or Eocene was estimated for Clade 4 (mean: 48.4 Mya, 95% HPD: 32.6–68.2 Mya) and Clade 7 (mean: 32.5 Mya, 95% HPD: 23.8–45.2 Mya). Clade 5 was estimated to be between Late Cretaceous and Eocene in age (mean:

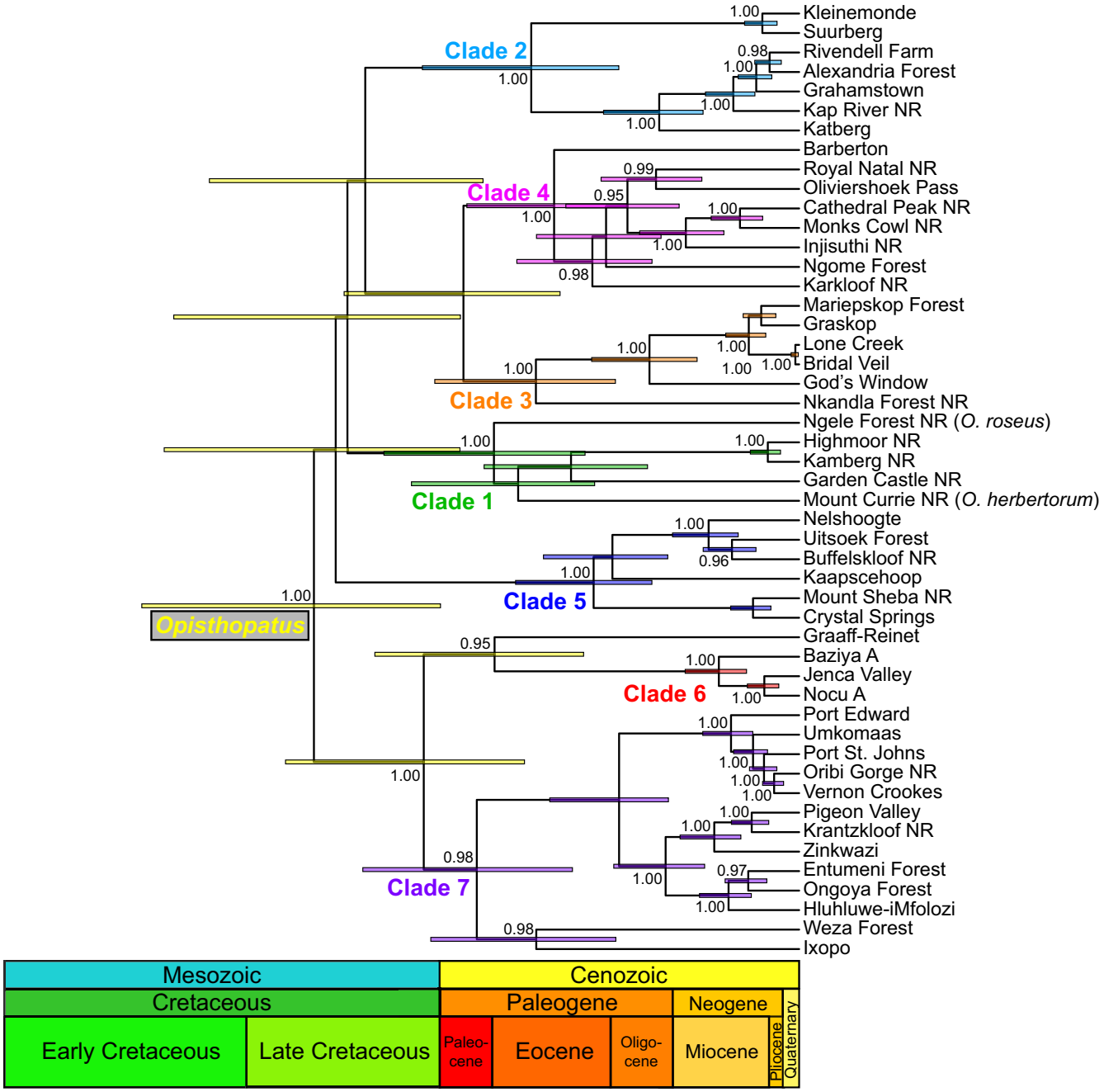

Fig. 4. Chronogram of the *Opisthopatus* species complex based on all molecular sequence data (three genes). Bars on nodes indicate 95% HPD intervals for node ages. Coloured branches correspond to distinct, geographically cohesive clades. Outgroups have been removed for clarity.

55.2 Mya, 95% HPD: 38.6–74.9 Mya). Clade 6 was not supported in the BEAST analyses. Highly similar ages and HPD intervals were recovered with both the mitochondrial and the three-locus datasets.

Rate of net diversification through time

The log lineage through time (LTT) plot based on the three-locus matrix of *Opisthopatus* revealed a fairly linear trajectory (Fig. 5). The best constant rate model selected by LASER was the one-rate Yule model (AIC = 141.8), whereas the best rate variable model (and best model overall) was the two-rate Yule model (AIC = 138.6). However, the shift point computed under the two-rate Yule model occurred 5.34 Mya, after which a five-fold rate decrease was estimated (between 0 and 5.34 Mya), largely an effect of under sampling lineages in the total evidence matrix. For comparison, the

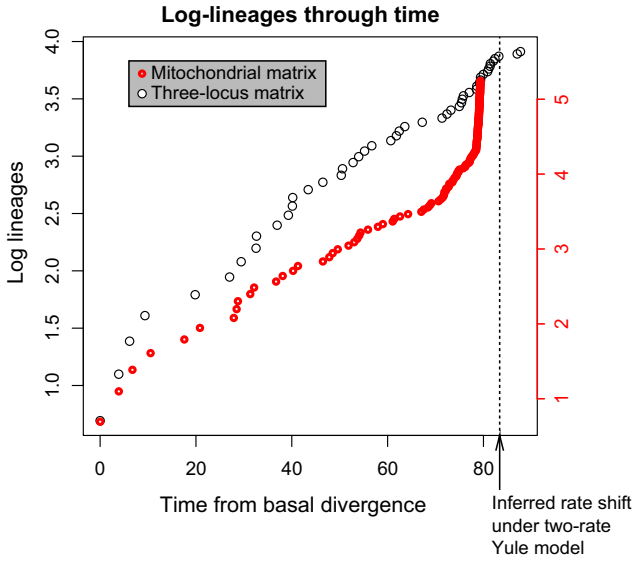

Fig. 5. Log lineage through time (LTT) plot based on the *O. cinctipes* species complex based on the three genes (black circles) or mtDNA only (red circles). The dotted line indicates the shift point in diversification rate inferred by LASER under the Yule two-rate model. Note the near-constant diversification of *Opisthopatus* after basal divergence inferred by either data set.

LTT plot of the 190-taxon chronogram showed the characteristic upturn of log lineages through time exhibited by sampling multiple exemplars of each species. In either case, a rate of net diversification was estimated for the first ca. 70 Myr of *Opisthopatus* diversification based on either data set.

Species delimitation

Visual inspection of post-burnin samples from the bGMYC analysis confirmed stationarity of the MCMC sampler. The threshold value across the post-burnin samples was 67 GMYC clusters with a standard deviation of 5 (Fig. 6a). To compare the fit of the GMYC model to the data, we mapped the log ratio of coalescence branching rate to the Yule model branching rate, and found the median of this parameter to be 1.5 and greater than zero for 99% of samples (Fig. 6b). The mean value of this parameter indicates good fit of the GMYC model to the empirical dataset.

As with most BI approaches, the results of bGMYC analysis are highly contingent upon the priors set for the threshold value. As a hypothetical example, we ran a separate analysis where the prior distribution for the number of species was set to a uniform distribution ranging from 3 to 10 GMYC clusters. The threshold value across the post-burnin samples was a strongly skewed distribution with a median of ten GMYC clusters, with most clusters corresponding to the seven major clades defined above. We also obtained a single cluster that included both *Metaperipatus* species, whose systematic validity is not in question. While the results of about ten species may seem more plausible, under this extreme prior distribution the fit of the GMYC model to the data was demonstrably poor, with a mostly negative distribution of log ratio of coalescence branching rate to the Yule model branching rate.

Morphological character examination

Both dorsal and ventral integument colour was highly polymorphic among *O. cinctipes* specimens as well as within each of the seven genetically defined clades (Fig. 7; Table 3). The two morphologically defined species, *O. herbertorum* and *O. roseus*, were always pearl white and rose pink, respectively (Fig. 7a). In *O. cinctipes* specimens the dorsal integument colour varied remarkably, ranging from rose pink to blue black, with an arrangement of brown and black being the most common morphotype (Fig. 7a). Similarly, ventral integument colour among *O. cinctipes* specimens exhibited considerable variation that ranged from pearl white to mottled brown with the ventral organs clearly evident (Fig. 7b, Table 3). All specimens possessed a distinct thin mid-dorsal line that ranged in colour from black to white. Somatic pigments often leached from the specimens following preservation in absolute ethanol, limiting the diagnostic value of colour, particularly in preserved specimens. In certain specimens, a band of orange or white was present immediately posterior to the head, while some specimens were characterized by a lateral orange-brown band along each flank of the body. Colour showed a distinct ontogenetic pattern in *O. cinctipes*. For example, at Mount Sheba juvenile specimens were light creamy pink, while adults were slate black. Leg pair number was a constant 16 for all *O. cinctipes* specimens, while in *O. roseus* and *O. herbertorum* leg pair numbers were 17 and 18, respectively. Leg pair number was invariant in six of the seven clades and thus cannot be regarded as diagnostic (Table 3). Furthermore, no head copulatory organs were observed in any of the specimens. Light microscopy examination of the arrangement of the accessory and primary dorsal papillae showed dramatic differences between the specimens in the seven clades.

SEM revealed that the male genital openings in all examined *O. cinctipes* specimens comprised four spinous pads. The four genital pads were triangular shaped at the tip and broadened at the base. The genital pore had a cruciform shape and showed moderate levels of variation among the specimens examined. In both *O. roseus* and *O. herbertorum* the genital opening is also surrounded by four genital pads (Ruhberg and Hamer, 2005) and appears morphologically similar to those within *O. cinctipes*. SEM of the dorsal papilla,

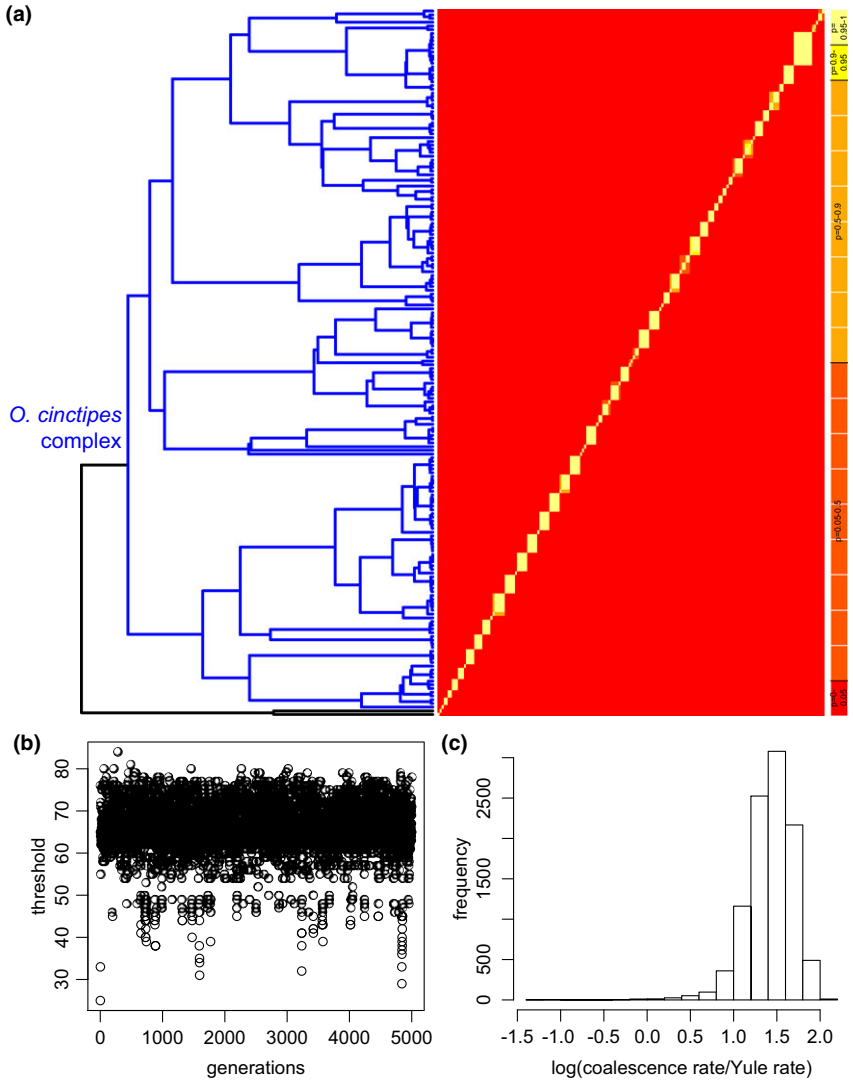

Fig. 6. (a) Species delimitation accounting for phylogenetic uncertainty for the mtDNA dataset under GMYC. The background colour represents the posterior probability that all tips nested within each node exist in a single GMYC cluster, ranging from red to white. (b) *A posteriori* distribution of species-level entities inferred by bGMYC. (c) Log ratio of coalescence rate to Yule rate for the *a posteriori* distribution. The generally positive values of this distribution indicate a good fit of the GMYC model to the data.

shape and number of scale rings revealed several diagnostic differences both within and between the seven clades (Fig. 8; Table 3).

Discussion

Integration of independent data classes is required for delimiting onychophoran species

The present study highlights the troublesome nature of defining species in instances of ancient lineages wherein several diagnostic characters yield conflicting results in the number of OTUs. Our multilocus dataset suggests that *Opisthopatus cinctipes* is a species complex characterized by several allopatric and deeply divergent clades, species paraphyly and marked genetic substructure. Although our divergence time estimation is imprecise, it does support an ancient (Eocene or older) origin of all seven *Opisthopatus* clades. Divergence of the isolated populations of *Opisthopatus* occurred later, during the Miocene, suggesting a possible role of historical climatic ameliorations as an explanatory variable for extensive range fragmentation. Traditional morphological characters such as dorsal and ventral integument colour and leg pair numbers were of limited diagnostic value for detecting the numerous phylogenetic entities within the *O. cinctipes* species complex. Specifically, strong support for the nested placement of *O. roseus* and *O. herbertorum* within the *O. cinctipes* complex indi-

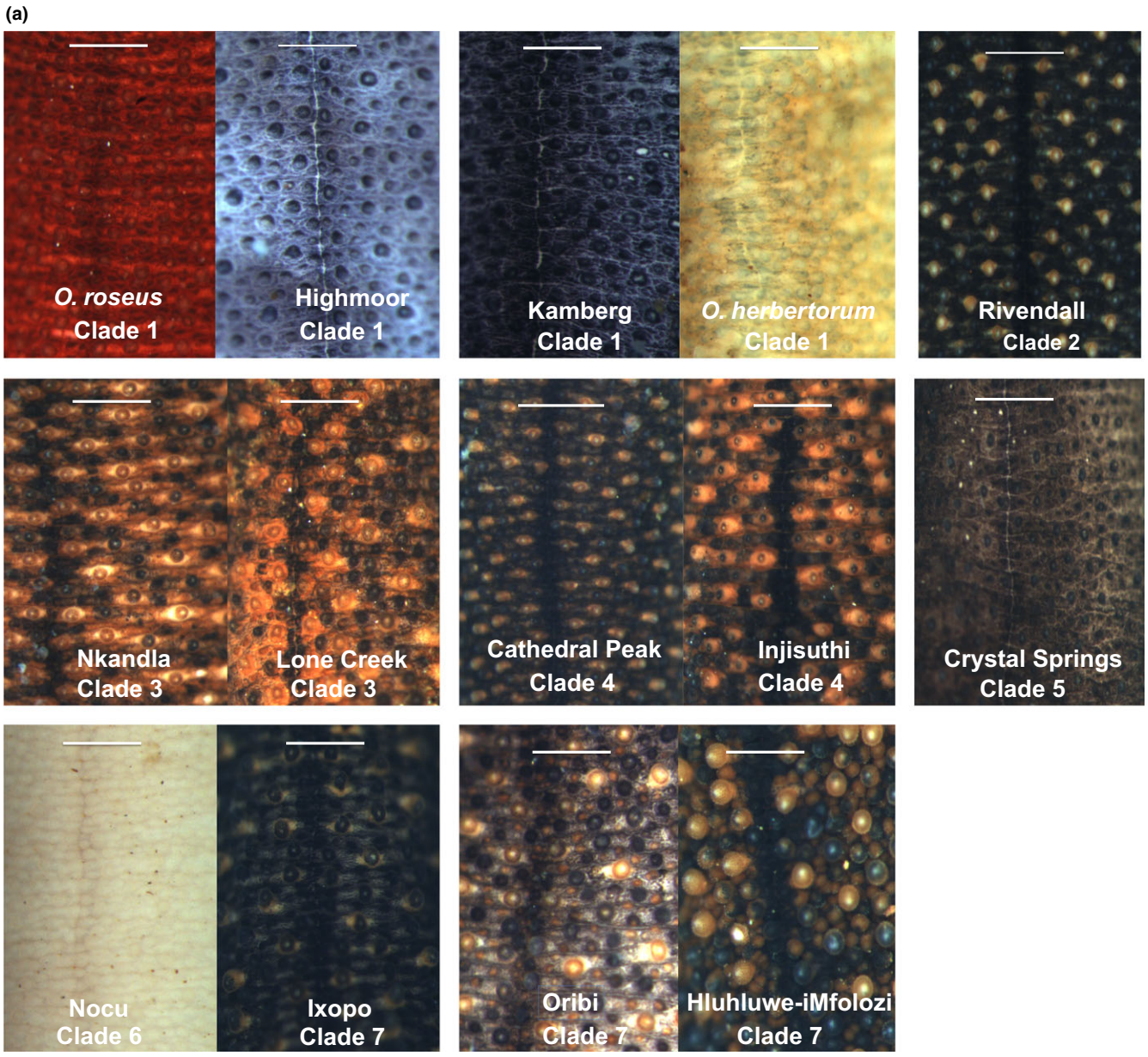

Fig. 7. (a) Light microscopic images of the dorsal integument, demonstrating the colour variation and the arrangements of the dermal papilla of selected specimens in the *O. cinctipes* species complex among the seven clades. (b) Light microscopic images of the ventral integument of selected specimens in the *O. cinctipes* species complex among the seven clades. Scale bars = 1 mm.

cates that the number of leg pairs is a poor indicator of species boundaries. This is consistent with the observation that segment number is a plastic character in other panarthropoda. For example, in the centipede *Strigamia maritima*, segment number has been shown to vary within the species with latitude and can be experimentally manipulated (Vedel et al., 2010). Similarly, genital characters did not prove useful for distinguishing *Opisthopatus* species. Unlike in many panarthropods, where the copulatory apparatus is often characterized by a lock-and-key mechanism and ensuing morphological differentiation to the level of species, fertilization via dermal insemination has been reported in the velvet worm family Peripatopsidae (Sedgwick, 1885). Within *Opisthopatus* we observed morphological invariance in the male genital anatomy using SEM, obviating inference of reproductive isolation based on genital architecture. Mating experiments could be used to assess the strength of reproductive isolation between inferred species, but these are beyond the scope of the present study.

By contrast, the arrangement of primary and secondary dermal papillae was diagnostic between and

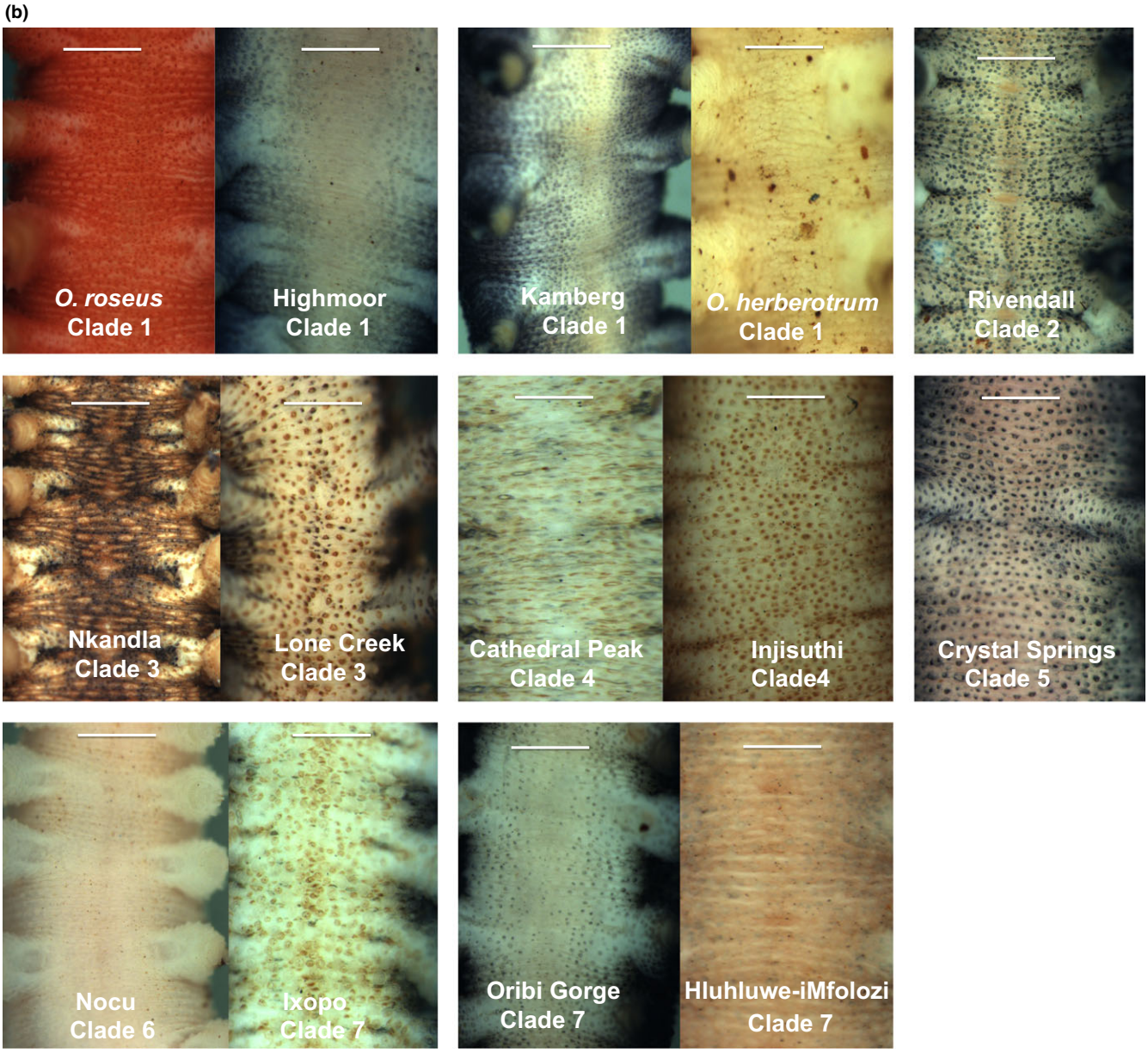

Fig. 7. continued.

within clades (Figs 3 and 8). The application of various species delimitation methods [clades established from the total (nuclear+mtDNA) evidence and mitochondrial matrices based on tree building methods, *COI* barcoding, traditional morphological characters, ultrastructural characters and Bayesian species delimitations] yielded conflicting results about the number of species that comprise the *O. cinctipes* species complex. Herein lies a conundrum, as there is considerable incongruence in the number of novel molecular OTUs between the various methods employed (ranging from 1 to > 60 species-level entities within the complex).

Consilience of delimitation methods is adjudicative of species boundaries

The question now arises as to which of these species-delineating approaches provides reasonable criteria for the recognition and diagnosis of species. Each of the methodological approaches has shortcomings, such as too few informative characters in external morphological datasets, or the tendency of bGMYC analyses based on mitochondrial data to overestimate GMYC clusters. Here we consider two alternatives. First, we postulate that only the seven geographically discrete clades are putative species. This conservative

Table 3
List of morphological features observed in each of the seven clades for the *Opisthopatus cinctipes* species complex

Locality no.	Locality	Province	Species	Clade	Leg pair number	Dorsal integument colour	Ventral integument colour	No. of scale rings
1	Suurberg	Eastern Cape	*O. cinctipes*	2	16	Olive green	Creamy white	5
2	Katberg	Eastern Cape	*O. cinctipes*	2	16	Olive green	Creamy white	12
3	Grahamstown	Eastern Cape	*O. cinctipes*	2	16	Slate black	Creamy white	NSE
4	Rivendall Farm	Eastern Cape	*O. cinctipes*	2	16	Olive green	Light brown and creamy white	6
5	Kleinemonde River	Eastern Cape	*O. cinctipes*	2	16	Slate black	Light brown and creamy white	NSE
6	Kap River NR	Eastern Cape	*O. cinctipes*	2	16	Light brown and gray	Creamy white	7
7	Graaff-Reinet NR	Eastern Cape	*O. cinctipes*	–	16	Light pink	Creamy white	5
8	Baziya	Eastern Cape	*O. cinctipes*	6	16	Light pink	Creamy white	4
9	Nocu	Eastern Cape	*O. cinctipes*	6	16	Light pink	Pearl white	7
10	Jenca Valley	Eastern Cape	*O. cinctipes*	6	16	Light pink	Pearl white	NSE
11	Port St Johns	Eastern Cape	*O. cinctipes*	7	16	Black and light brown	Cream white with light brown	NSE
12	Port Edward	KwaZulu-Natal	*O. cinctipes*	7	16	Black and light brown	Light blue and creamy white	NSE
13	Mount Currie NR	KwaZulu-Natal	*O. herbertorum*	1	18	Light blue grey and white	Pearl white	NSE
14	Ngele Forest NR	KwaZulu-Natal	*O. roseus*	1	17	Blood red	Light pink	4
15	Weza Forest	KwaZulu-Natal	*O. cinctipes*	7	16	Blue black	Creamy white	NSE
16	Oribi Gorge NR	KwaZulu-Natal	*O. cinctipes*	7	16	Slate black	Creamy white	8
17	Garden Castle NR	KwaZulu-Natal	*O. cinctipes*	1	16	Slate black	Creamy white	NSE
18	Kamberg NR	KwaZulu-Natal	*O. cinctipes*	1	16	Indigo	Light blue and white	NSE
19	Highmoor NR	KwaZulu-Natal	*O. cinctipes*	1	16	Indigo	Creamy white	4
20	Injisuthi NR	KwaZulu-Natal	*O. cinctipes*	4	16	Light brown	Creamy white	10
21	Monks Cowl NR	KwaZulu-Natal	*O. cinctipes*	4	16	Light brown	Creamy white	8
22	Cathedral Peak NR	KwaZulu-Natal	*O. cinctipes*	4	16	Blue black	Cream white with light brown	6
23	Royal Natal NR	KwaZulu-Natal	*O. cinctipes*	4	16	Light blue and brown	Light brown and white	7
24	Oliviershoek Pass	KwaZulu-Natal	*O. cinctipes*	4	16	Light brown and black	Creamy white	NSE
25	Ixopo	KwaZulu-Natal	*O. cinctipes*	7	16	Indigo	Creamy white	7
26	Karkloof	KwaZulu-Natal	*O. cinctipes*	4	16	Indigo	Cream white with light brown	NSE
27	Vernon Crookes NR	KwaZulu-Natal	*O. cinctipes*	7	16	Blue black	Creamy white	8
28	Umkomaas	KwaZulu-Natal	*O. cinctipes*	7	16	Light brown	Creamy white	NSE
29	Krantzkloof NR	KwaZulu-Natal	*O. cinctipes*	7	16	Black and brown	Creamy white	9
30	Pigeon Valley NR	KwaZulu-Natal	*O. cinctipes*	7	16	Light blue grey and white	Creamy white	NSE
31	Nkandla Forest NR	KwaZulu-Natal	*O. cinctipes*	3	16	Light brown	Creamy white	9
32	Entumeni Forest NR	KwaZulu-Natal	*O. cinctipes*	7	16	Slate black	Creamy white	NSE
33	Hluhluwe-iMfolozi NR	KwaZulu-Natal	*O. cinctipes*	7	16	Slate black	Creamy white	NSE
34	Ongoya Forest NR	KwaZulu-Natal	*O. cinctipes*	7	16	Blue black	Creamy white	8
36	Uitsoek Forest NR	Mpumalanga	*O. cinctipes*	5	16	Black and brown	Creamy white	NSE
37	Buffelskloof NR	Mpumalanga	*O. cinctipes*	5	16	Black and brown	Light brown and white	NSE
38	Mount Sheba NR	Mpumalanga	*O. cinctipes*	5	16	Slate black	Light blue and white	5
39	Crystal Springs NR	Mpumalanga	*O. cinctipes*	5	16	Blue black	Light blue and white	NSE
40	Graskop	Mpumalanga	*O. cinctipes*	3	16	Light brown and black	Light blue and white	8
41	God's Window	Mpumalanga	*O. cinctipes*	3	16	Blue black	Creamy white	NSE
42	Lone Creek	Mpumalanga	*O. cinctipes*	3	16	Light brown and black	Cream white with light brown	NSE
43	Bridal Veil	Mpumalanga	*O. cinctipes*	3	16	Blue and light brown	Creamy white	NSE
44	Barberton	Mpumalanga	*O. cinctipes*	4	16	Slate black	Creamy white	NSE

Table 3
(Continued)

Locality no.	Locality	Province	Species	Clade	Leg pair number	Dorsal integument colour	Ventral integument colour	No. of scale rings
45	Nelshoogte	Mpumalanga	*O. cinctipes*	5	16	Black and light brown	Creamy white	6
46	Kaapscehoop	Mpumalanga	*O. cinctipes*	5	16	Black and light brown	Creamy white	NSE
47	Mariepskop Forest NR	Mpumalanga	*O. cinctipes*	3	16	Black and light brown	Light brown and blue white	NSE
48	Alexandria Forest NR	Eastern Cape	*O. cinctipes*	2	16	Blue black	Creamy white	NSE
49	Zinkwazi	KwaZulu-Natal	*O. cinctipes*	7	16	Light blue	Light brown and creamy white	NSE

NSE = no scanning electron micrograph.

approach potentially results in an underestimate of the species diversity within this group. This approach also encounters such hurdles as a handful of terminal operational units that could not be placed reliably within large clades. For example, the Graaff-Reinet specimens did not belong to a specific clade, rendering the status of *O. cinctipes* specimens from this locality problematic. Secondly, we postulate that each of the sample localities represents a distinct species, based on the markedly large *COI* uncorrected pairwise distances or the number of GMYC clusters. But an approach grounded in such algorithms may be prone to overestimating species diversity, either because of the use of an arbitrary threshold for delimiting species boundaries (*COI* distances) or because of the contingency of GMYC analyses on molecular dates, which are imprecise. While our results accord with genetic isolation of all studied populations we were unable to determine through either inductive or experimental approaches which lineages within the *O. cinctipes* species complex are reproductively isolated.

There is very clear mitochondrial–nuclear DNA incongruence, as the rapidly evolving mitochondrial data reflect several genetically and geographically discrete clades (Fig. 2) while the slowly evolving nuclear DNA data reflected only two of the seven clades evident from the mtDNA analyses, suggesting that the marker selected is too slow to detect species boundaries within the *O. cinctipes* species complex. Fernández and Giribet (2014) made a similar observation while studying the mite harvestman *Aoraki denticulata* where a single *28S rRNA* haplotype was present for 54 specimens. In our study we found a total of 26 haplotypes (results not shown) for the 49 specimens sequenced for the *18S rRNA* locus within the *O. cinctipes* species complex. Similar problems have also been encountered while studying the mite harvestman *Metasiro* (Clouse et al., 2015). This pattern of high mtDNA structure and low nuDNA variation is clearly a problem typical of sedentary saproxylic panarthropods.

Species paraphyly has been documented in numerous panarthropod taxa and can be attributed to incorrect taxonomic designations, systematic error in phylogenetic resolution, hybridization, incomplete lineage sorting (ILS) or paralogy (Bond et al., 2001; Funk and Omland, 2003; Hendrixson and Bond, 2005). While our data does not provide evidence of hybridization or ILS due to the low evolutionary rate of the *18S rRNA*, high intraspecific diversity of the *O. cinctipes* species complex and the deeply nested position of the other *Opisthopatus* species suggest that neither hybridization nor ILS alone can account for the patterns we observed. We also did not detect stop codons in *CO1*, which could have suggested paralogy as an explanatory variable. Paralogy affecting nuclear loci that are commonly used for invertebrate popula-

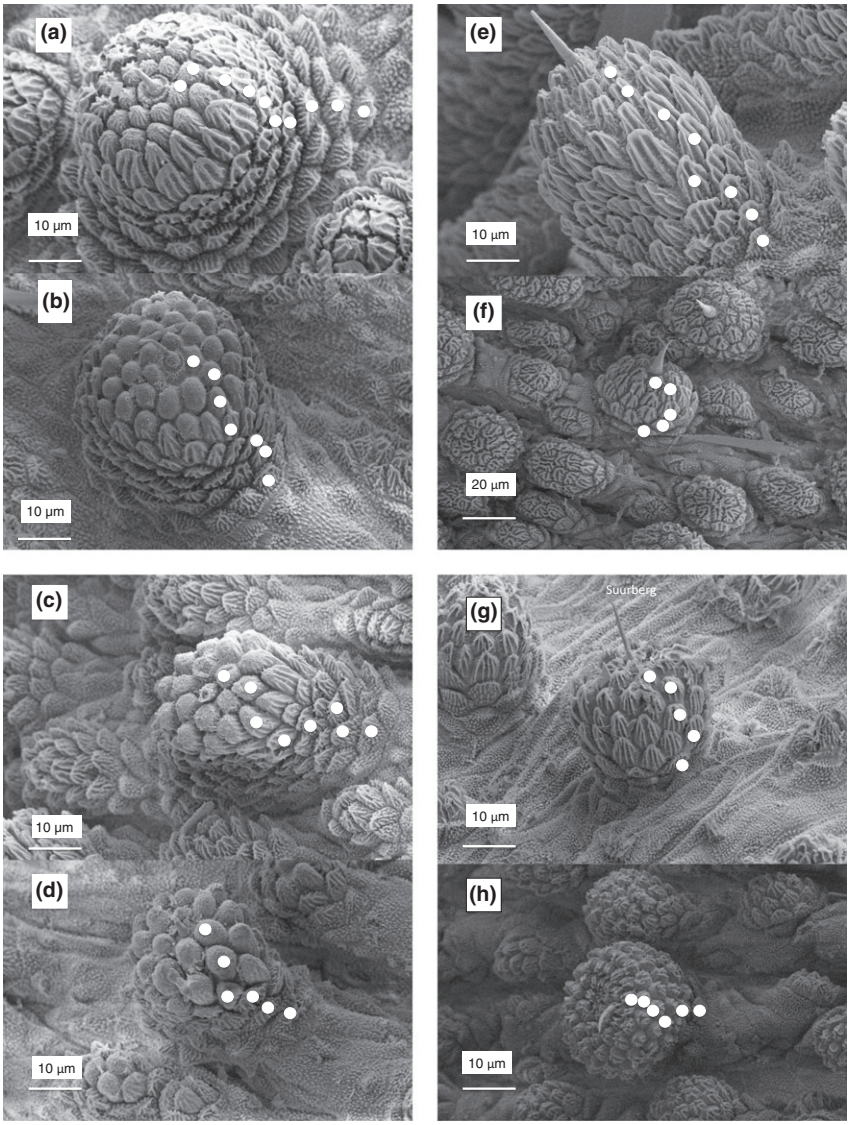

Fig. 8. Scanning electron micrographs of the arrangement of dorsal dermal papillae in the *O. cinctipes* species complex that are representatives of the seven clades. White dots indicate the number of scale rings. (a) Injisuthi, (b) Royal Natal NR, (c) Ngome, (d) Cathedral Peak NR, (e) Graskop, (f) Mount Sheba NR, (g) Suurberg, (h) Rivendell, (i) Ngele for *O. roseus*, (j) Highmoor NR, (k) Baziya, (l) Nocu, (m) Graaff-Reinet and (n) Oribi Gorge.

tion genetics have been reported in several studies (e.g. Riesgo et al., 2012; Clouse et al., 2013), but the overall congruence between the mtDNA and *18S rRNA* trees suggests this is unlikely. We thus conclude that taxonomic inaccuracy is the likely culprit behind the observed paraphyly. Were we to recognize each of the seven clades as distinct species, it would imply that one of the currently recognized species, *O. herbertorum*, should be synonymized with *O. roseus* (Clade 1). These results favour the second hypothesis and underline the discovery that higher levels of alpha diversity are potentially present within the *O. cinctipes* species complex, with five of these clades representing novel lineages. Our study demonstrates that morphology-based taxonomy, particularly the use of leg pair number and colour to delimit species, has grossly underestimated species-level diversity. Recent studies on the South African *Peripatopsis* corroborate these revelations (McDonald et al., 2012; Daniels et al., 2013; Ruhberg and Daniels, 2013). Similarly, taxonomic studies on the Neotropical Peripatidae (Oliveira et al., 2011, 2012a) have revealed marked species diversity obscured by traditional morphological characters such as leg pair numbers. However, the structure and arrangement of the secondary and primary dermal papilla were very distinct among the seven clades, indicating that these represent a useful morphological tool in differentiating cryptic lineages. Bayesian species

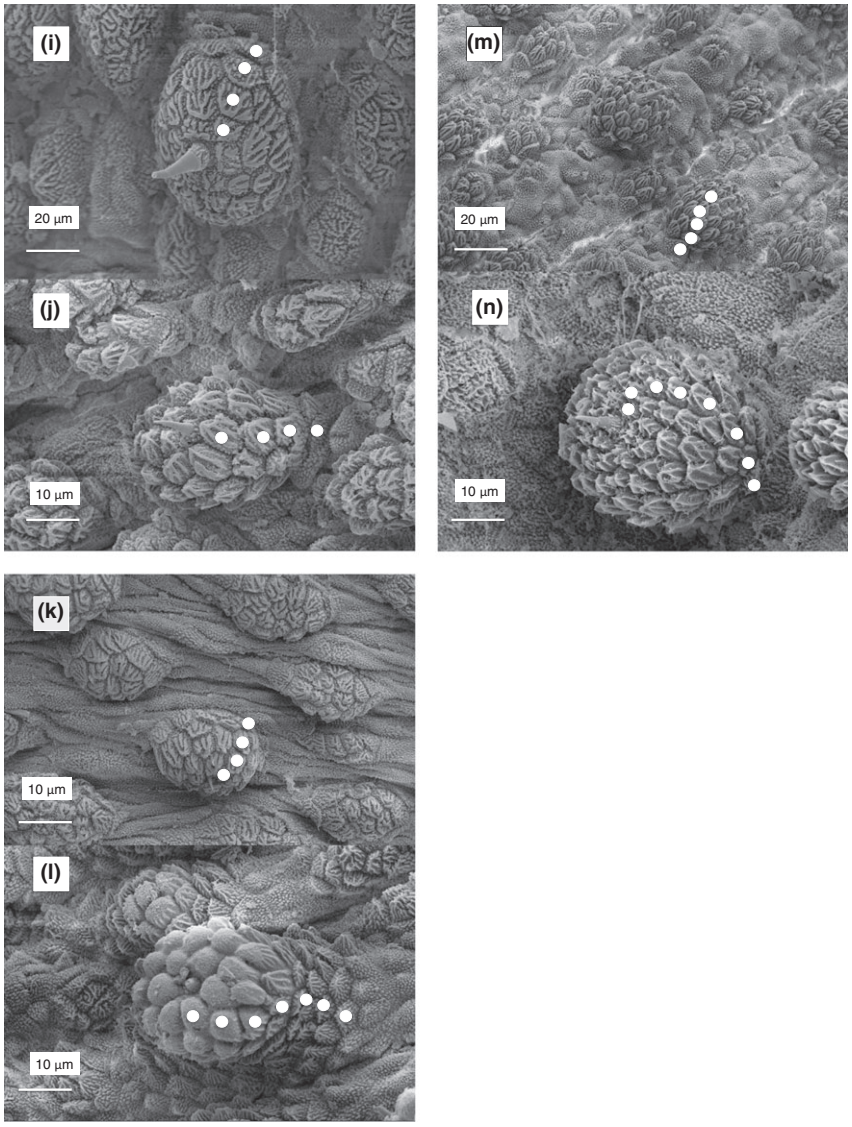

Fig. 8. continued.

delimitation methods are prone to over-splitting species in instances where significant population structure is evident, and instances where an inaccurate guide tree is used in the analyses can lead to spurious conclusions.

With few exceptions, specimens in the *O. cinctipes* species complex from the same localities were retrieved as monophyletic with good statistical support and exhibited strong genealogical and geographical exclusivity. Our results demonstrate genetic isolation among all conspecific *O. cinctipes* populations and are corroborated by the absence of shared maternal haplotypes, as evident from the marked population differentiation and statistically significant F_{ST} values across all sample localities. These results suggest maternal philopatry in *Opisthopatus* populations. Furthermore, the population genetic results corroborate the fragmented nature of the forested habitat where velvet worms typically occur and reflect their general inability to disperse across xeric boundaries as no haplotypes were shared between sample localities. Marked genetic differentiation is a pattern typical of velvet worms and other sedentary saproxylic taxa (Boyer et al., 2007; McDonald and Daniels, 2012; Daniels et al., 2013). For example, a recent study by Bull et al. (2013) on the widely distributed velvet worm *Euperipatoides rowelli* revealed the presence of six genetically distinct lineages characterized by marked genetic differentiation. During the present study we observed low haplotype and nucleotide diversity, a pattern typical of isolated populations. This can be attributed to low abundances through evolutionary time, resulting in small effective popula-

tion sizes, inbreeding and the effects of genetic drift on small populations. These populations may also reflect the impact of population bottlenecks, a result corroborated by values of Fu's F_s (Table 2).

The mean sequence divergences for the *COI* locus between the seven geographically and genetically defined *O. cinctipes* clades (> 19%) were higher than divergences between morphologically defined sister species, such as the two *Metaperipatus* species, all the *Ooperipatus* and *Planipapillus* species outgroups, and among the three *Peripatopsis* species complexes (*P. balfouri*, *P. capensis* and *P. moseleyi*) (Daniels and Ruhberg, 2010; McDonald and Daniels, 2012; Daniels et al., 2013). Most authors allude to the presence of cryptic species or the existence of a novel species when marked sequence divergence values are detected (Hebert et al., 2003a,b, 2004a,b; Boyer et al., 2007; Daniels et al., 2013). Hebert et al. (2003a) suggested that sequence divergences greater than 11% indicate separate species for a wide range of invertebrate taxa in their review of pairwise genetic (mtDNA) divergences. Implementing this cut off in this study would imply that numerous new species are nested within the *O. cinctipes* species complex. However, such a cut off is arbitrary, as it does not address recent speciation events or the presence of ancestral polymorphism. We cannot justify the untested use of this value in defining molecular taxonomic units, although we are cognizant that these results imply genetic isolation and potential reproductive divergence between the seven clades. Our results are more complex, as the mean within-clade sequence divergence is 12%, suggesting that by using this benchmark in the identification of novel lineages, we are potentially underestimating species-level diversity within *Opisthopatus*.

Colour patterns in Opisthopatus *are polymorphic and contingent upon ontogenetic stage*

We observed large-scale variation in polymorphism in dorsal and ventral body colour within the *O. cinctipes* species complex. Varying colour patterns within species complexes have also been documented for other velvet worms such as *Peripatopsis* (Daniels et al., 2009). Recent results suggest that in certain instances the colour differences in the three *Peripatopsis* species complexes (*P. balfouri*, *P. capensis* and *P. moseleyi*) may to a varying degree indicate distinct species status (McDonald and Daniels, 2012; Daniels et al., 2013; Ruhberg and Daniels, 2013). For example, within *P. moseleyi* colour was a clear diagnostic character between genetically distinct sympatric lineages that were previously considered to be conspecific, but within *P. capensis* distinct colour morphs (bright red and slate black) were genetically invariant. Colour polymorphism has also been observed in several invertebrate taxa such as butterflies (Kapan, 2001), leaf beetles, *Plateumaris sericea* (Kurachi et al., 2002) and in walking stick insects *Timema cristinae* (Nosil, 2004). Several processes are known to promote colour polymorphism. These include sensory bias, divergent and disruptive selection, environment contingent sexual selection, heterosis, disassortative mating, reproductive tradeoffs, intermittent natural selection and genetic drift.

Divergent selection and disruptive selection may be the possible drivers of the colour variation observed within the *O. cinctipes* species complex. Both forms of selection may take hold when individuals occur in isolated microhabitats and experience a different type of environment for extended periods (Gray and McKinnon, 2006). Due to their limited dispersal abilities, sedentary life history and specific microhabitat requirements, divergent and/or disruptive selection may have occurred within the *O. cinctipes* species complex leading to colour polymorphism. The polymorphism in body colour is likely to allow the velvet worm species complex to exploit a variety of habitat types, enabling camouflage and promoting crypsis. We observed clear ontogenetic changes in body colour in the *O. cinctipes* species complex, suggesting that colour per se is not a suitable diagnostic feature in differentiating putative lineages within the *O. cinctipes* species complex. The evolutionary value of these bright colour morphs is unknown.

The biogeography of O. cinctipes *suggest a role for range fragmentation and ancient dispersal among the deeply divergent clades*

We observed complex biogeographical patterns in the *O. cinctipes* species complex, with clades demonstrating a very strong correspondence to geographical regions and forest type. Within regions, multiple independent colonizations occurred in nearly every region considered in this study, resulting in several non-sister clades being monophyletic within regions, such as the Drakensberg Mountains, which are inhabited by two genetically highly divergent clades. Divergence time estimation indicates that the *O. cinctipes* species complex originated during the Cretaceous, with steady net diversifications throughout the Eocene, and rapid differentiation within populations during the Miocene. The latter result is broadly congruent with those obtained by Murienne et al. (2014) for a global study on velvet worms, while Daniels et al. (2015) also recently observed an Eocene cladogenesis for Afrotropical freshwater crabs. Similarly, a large-scale phylogeny of chameleons (a group highly dependent on forest and vegetation) also suggests an Eocene diversification at the genus level, while a species-level diversification generally occurred primarily during the Oligocene (Tolley et al., 2013). During the Paleocene and Eocene (65–38 Mya) temperatures were

higher (Deacon, 1983) and associated with wetter conditions, resulting in expansion of the forest biome and of the distribution of the ancestral species ranges. However, subsequently, climatic oscillations during the Oligocene resulted in cooler and drier climatic conditions and the contraction and fragmentation of forests throughout South Africa. During the Early Miocene, climatic conditions were warmer and wetter, and forests were presumably widespread along the coastal lowlands and mountainous interior of South Africa (Deacon, 1983). At the onset of the Middle Miocene, climatic conditions in South Africa deteriorated, resulting in a progressive decrease in precipitation (Lawes, 1990; Eeley et al., 1999) and increased aridification and shifts in the forest floral and faunal distribution patterns that persisted and intensified during the Plio/Pleistocene (Sepulchre et al., 2006). Consequently, Afrotemperate forests contracted and became confined to the high-elevation, wetter mountain slopes and deep river valleys in the eastern portions of South Africa (Lawes, 1990). These Oligocene/Miocene climatic changes resulted in vicariance and the formation of several of the observed clades.

Marked marine transgressions resulted in flooding of the coastal lowlands. The decrease in precipitation coupled with the dramatic uplift along the Eastern Cape coast and progressive climatic oscillations resulted in further contraction of Afrotemperate forests to high-altitude areas acting as micro-refugia for forest-dwelling taxa such as *O. cinctipes*, resulting in deep genetic divergences among Afrotemperate forested populations of the species. The phylogenetic tree for the *O. cinctipes* species complex supports the hypothesis that Afrotemperate forest velvet worm populations are ancestral, a result corroborated by the divergence time estimations (Fig. 3). We observed two independent dispersal and colonization events amongst both Afrotemperate and IOCB forests. For example, the IOCB velvet worm specimens are present in two highly divergent clades, 2 and 7. In Clade 2 specimens from the Eastern Cape province are sister to specimens from the Afrotemperate forests, while in Clade 7 specimens belong to the IOCB in KwaZulu-Natal province, suggesting at least two independent colonizations of this habitat. Similarly, in the Afrotemperate forest patches along the Drakensberg Mountains we observe at least two independent colonization scenarios (Fig. 3). The Drakensberg Mountains habitats have acted as a high-altitude refugium for forest-dwelling taxa. During periods of harsh climatic conditions, deeply incised gorges and cliffs on elevated plateaus may have provided a refugium for velvet worm species and other palaeoendemic forest taxa (Hughes et al., 2005; Ramdhani et al., 2008). The Drakensberg Mountains have been cited as potential refugia for several taxa (Lawes, 1990; Eeley et al., 1999; Ramdhani et al., 2008). Similarly, for the Afrotemparate Mpumalanga Highveld forests we also observed two distinct colonizations (clades 3 and 5, Fig. 3). Our results do not support a recent evolutionary origin for the IOCB clade, suggesting that the ancestral species probably survived in adjacent areas in the interior when the coastal plains were covered by marine transgressions. Evidence for the latter observation is present in the basal placement of the interior Afrotemperate forests in clades comprising IOCB specimens. For example (Fig. 6), in Clade 2, Katberg samples are placed basally. Direct comparisons of phylogeographical patterning for South African co-distributed forest-dwelling taxa is problematic as these studies did not undertake divergence time estimations (Moussalli et al., 2009; Moussalli and Herbert, 2016), did not include samples from the same geographical region as in the present study (Tolley et al., 2006) or examined phylogeographical patterning in taxa that are considerably more recent invaders of forested habitats, resulting in an absence of phylogeographic patterning (Willows-Munro and Matthee, 2011).

We consider these seven clades as evolutionary significant units (ESUs following the definition by Crandall et al., 2000) as they are distinct at the DNA level, geographically discrete and ecologically divergent (occurring in very distinct habitats). Saproxylic environments such as leaf litter and decaying logs of wood harbour a wealth of taxonomic diversity and endemism, particularly among invertebrate lineages, as well as groups that possesses deep genetic population differentiation, suggesting that they are worthy of conservation. However, the conservation of saproxylic environments in South Africa is poorly studied. Our results reveal a wealth of taxonomic diversity, a pattern that probably exists in several taxa characterized by low dispersal capability, suggesting they warrant study and conservation. Taken together, these results highlight the problem with the exclusive reliance on traditional morphological characters, and underline the need for integrative molecular systematic endeavors on eumetazoan lineages characterized by a conservative body plan such as Annelida and Platyhelminthes (King et al., 2008), which may similarly harbour marked diversity obscured by traditional taxonomic characters.

Systematics

Family Peripatopsidae Bouvier, 1905

Genus *Opisthopatus* Purcell, 1899

Type species. *Opisthopatus cinctipes* Purcell 1899

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Opisthopatus cinctipes Purcell, 1899, Annls. S.Afri. Mus. 2: 67–116.

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Opisthopatus cinctipes var. *natalensis* Bouvier, 1900, Q. J. Micr. Sci.

Peripatus (*Opisthopatus cinctipes*) Sedgwick, Cambridge Nat. Hist, 4: 24.

Opisthopatus cinctipes Bouvier, 1902, Zool. Jb. Anat. Fauna Chillensis, 2: 718–723.

Opisthopatus cinctipes Bouvier, 1904, Nouv. Arcs. Mus 6: 8.

Opisthopatus cinctipes Bouvier, 1907, Annls. Sci. nat. Zool, 5: 171–181.

Opisthopatus cinctipes var. *natalensis* Bouvier, 1907, Annls Sci. nat. Zool. 5: 181.

"Capo-Peripatus" Sedgwick, 1908, Q.J. Micr. Sci. 52: 393–397.

Opisthopatus cinctipes Clark and Smith, 1912, Misc. Coll. 65: 21.

Opisthopatus cinctipes Dubosq, 1920, Archs Zool. 59: 21–27.

Opisthopatus cinctipes Gravier and Fage, 1925, Annls. Sci. Nat. Zool. 10: 196.

Opisthopatus cinctipes Manton, 1938, Annls. Mag. Nat. Hist. 11: 478–479.

Opisthopatus cinctipes Holliday, 1942, Annls. Natal Mus. 10: 237–244.

Opisthopatus cinctipes Holliday, 1944, Annls. Natal Mus. 10: 433–436.

Opisthopatus cinctipes Manton, 1945, Nature, 156: 338.

Opisthopatus cinctipes Lawrence, 1946, Nature, 157: 304.

Opisthopatus cinctipes Choonoo, Fort Hare Papers 1: 71–119.

Opisthopatus cinctipes var *amatolensis* Choonoo, Fort Hare Papers, 1: 72.

Opisthopatus cinctipes Lawrence, 1947, Annls. Natal Mus. 11: 166–168.

Opisthopatus cinctipes var *laevis* Lawrence, 1947, Annls. Natal Mus. 11: 168.

Opisthopatus cinctipes Lawrence, 1953, in Bio. Cryptic fauna in forests. Abb. 24.

Opisthopatus cinctipes Brink, 1957, in. S. Afr. Animal. Life 4: 16–18.

Opisthopatus cinctipes var *natalensis* Brink 1957, in S. Afr. Animal Life 4: 16–17.

Opisthopatus cinctipes var *laevis* Brink 1957, in S. Afr. Animal Life 4: 16–17.

Opisthopatus cinctipes Alexander, 1957, Annls. Natal. Mus. 14: 35–43.

Opisthopatus cinctipes Storch and Ruhberg, 1977, Zoomorph, 87: 263–276.

Opisthopatus cinctipes Newlands and Ruhberg, 1978, Biogeogr. Ecol. S. Afri. 677–789.

Opisthopatus cinctipes Ruhberg and Storch, 1978, Zool. Anz. Jena. 200: 289–299.

Opisthopatus cinctipes Peters, Alberti and Ruhberg, 1979, Jb. Anat. 101: 122–135.

Opisthopatus cinctipes Storch, Alberti and Ruhberg, 1979, Zool. Anz. Jena, 203: 35–47.

Opisthopatus cinctipes Herzberg, 1979, Staatsexamensarbeit, Keil.

Opisthopatus cinctipes Ruhberg, 1985, Zoologica, 137: 1–183.

Opisthopatus cinctipes Hamer, Samways and Ruhberg, 1997. Annls. Trans. Mus. 38: 283–312.

Pregenital leg pairs: 16, the last pair of legs fully developed with four foot pads and claws. Last leg pair well developed and also used in walking.

Anal cone short.

Male genitalia composed of four pads, female genital area is longitudinal.

Dorsal integument colour highly variable and varies from slate grey to blue black to brown, while the ventral integument colour varies from creamy white to brown.

Behaviour: spirals when initially exposed.

Geographically widely distributed in discontinuous forest patches along the interior and coastal plains from the Eastern Cape into KwaZulu-Natal and the Mpumalanga provinces of South Africa.

Opisthopatus cinctipes *sensu stricto* (Clade 2, Figs 3, 7a, b, 8g, h and Table 3)

Holotype: Not designated.

Type locality: Dunbrody, near Blue Cliff Station, Uitenhage Division (formerly the Cape Colony), Eastern Cape province, South Africa.

Additional material. Two females, 3 juveniles (SAM-ENW-C007156), Katberg Forest 32°28.132′S, 26°40.653′E, Eastern Cape province, South Africa, collected S.R. Daniels and N. Solomons, 26 April 2007; 1 female, 7 juveniles (SAM-ENW-C007155), Suurberg 33°19.132′S, 25°56.226′E, Eastern Cape province, South Africa, collected S.R. Daniels and N. Solomons, 8 July 2008; 1 male, 2 damaged specimens, 3 juveniles (SAM-ENW-C007153), Rivendell farm outside Grahamstown, 32°48.674′S, 26°45.391′E, Eastern Cape province, South Africa, collected S.R. Daniels, N. Solomons, H. Ruhberg, March 2010; 4 juveniles (SAM-ENW-C007154) Grahamstown, Somerset Hill, 33°17.596′S, 26°29.548′E, Eastern Cape province, South Africa, collected V. Bills, no date; 4 females, 7 juveniles (SAM-ENW-C007151), Kap River Nature Reserve, 33°28.694′S, 27°05.458′E, Eastern Cape province, South Africa, collected S.R. Daniels and G. Baatjies on 4 January 2012; 2 male, 2 juveniles (SAM-ENW-C007150), Alexandria Forest, 33°42.087′S, 26°21.038′E Eastern Cape province, South Africa, collected S.R. Daniels, F. Gordon and G. Baatjies on 30 December 2011; 1 female, (SAM-ENW-C007152), Kap River Nature Reserve,

33°48.543′S, 27°08.474′E, Eastern Cape province, South Africa, collected A. Moussalli, D. Stuart-Fox and M. Bursey, 9 December 2005.

Diagnosis. GenBank accession numbers for Clade 2

COI: KR907003–KR907006, KR907017–KR907024, KR907046–KR907051, KR907137–KR907146.

12S rRNA: KR906793–KR906796, KR906829–KR906836, KR906862–KR906867.

18S rRNA: KR907175, KR907184, KR907191–KR907192, KR907216–KR907217.

Description.

Dorsal body primary papilla. The number of scale rings varies from five to 12 (Table 3). A similar result has been reported by Ruhberg and Hamer (2005)

Colour Pattern. Dorsal integument colour highly variable ranging from blue back to olive green. Ventral integument cream coloured with blue spots occasionally (Fig. 7, Table 3).

Leg pairs. Sixteen leg pairs.

Genital opening. Male genital pore cruciform, female genital pore a horizontal and small vertical slit with tumid lips.

Distribution. Confined to the Eastern Cape Afrotemperate and IOCB forests. Typically found under or inside decaying logs of indigenous wood, as well as in decaying *Aloe* plants along the coastal margins.

Opisthopatus roseus *sensu stricto* Lawrence 1947 Clade 1 (Figs 3, 7, 8i, j, 9a, b and Table 3)

Opisthopatus roseus Lawrence, 1947, Annls. Natal. Mus., 11: 165–168. *Opisthopatus*

Brink, 1957, in S. Afr. Animal Life, 4: 18.

Opisthopatus roseus Newlands and Ruhberg, 1978, in Werger (ed.): Biogeogr. Ecol. S. Afri. 679–682.

Opisthopatus roseus Hutchinson, 1978, New Haven & London Yale Univ. Press: 189.

Opisthopatus roseus Ruhberg, 1985, Zoologica, 137: 1–183.

Fig. 9. Photographs of the dorsal image of live velvet worms (*Opisthopatus*) to show the variation in colour among some of the described and novel species. (a) *O. roseus* Lawrence 1947, *sensu stricto* from Ngele forest, KwaZulu-Natal province; (b) *O. herbertorum* Ruhberg and Hamer (2005) from Mount Currie NR, now regarded as a junior synonym of *O. roseus*; (c) *O. drakensbergi* sp. nov., from Cathedral Peak NR, KwaZulu-Natal province; (d) *O. highveldi* sp. nov., from Graskop on the Highveld of the Mpumalanga province; (e) *O. amaxhosa* sp. nov., from Nocu from Eastern Cape province; and (f) *O. kwazululandi* sp. nov., from Pietermaritzburg, KwaZulu-Natal province. Scale bars = 5 mm.

Opisthopatus roseus Hamer, Samways and Ruhberg, 1997. Annls. Trans. Mus. 38: 283–312.

Opisthopatus herbertorum Ruhberg and Hamer, 2005, Zootaxa, 1039: 27–38.

Holotype. NM 348 and NM 349, deposited in the Natal Museum (NM), Pietermaritzburg, South Africa.

Type locality. East Griqualand, Ingeli Forest (or sometimes spelled Ngele), near Kokstad, KwaZulu-Natal province, South Africa.

Additional material. One male (SAM-ENW-C007165), Mount Currie Nature Reserve outside Kokstad, 30°28.622′S, 29°22.722′E, KwaZulu-Natal province, South Africa, collected S.R. Daniels, N. Solomons and F. van Zyl, July 2010; 1 male, 1 female, 1 juvenile damaged (SAM-ENW-C007166), Ngele forest outside Kokstad, 30°32.006′S, 29°40.907′E, KwaZulu-Natal province, South Africa, collected S.R. Daniels, N. Solomons, F van Zyl, 9 September 2007; 1 damaged specimen (SAM-ENW-C007167), Kamberg Nature Reserve, no GPS coordinates, collected M. Hamer, no date; 1 damaged specimen (SAM-ENW-C007168), Kamberg Nature Reserve, no GPS coordinates, collected M. Hamer, no date.

Diagnosis. GenBank accession numbers for Clade 1.

COI: KR907059, KR907096–KR907103, KR907169–KR907170.

12S rRNA: KR906875, KR906933–KR906940, KR906979–KR906980.

18S rRNA: KR907195, KR907210–KR907211, KR907222–KR907223.

Description.

Dorsal body primary papilla. The number of scale rings was fixed at four (Table 3).

Colour Pattern. Dorsal integument highly variable, ranging from blood red to pearl white to indigo in the Drakensberg Mountain specimens. Ventral integument in certain species blood red to pearl white (Fig. 7, Table 3).

Leg pairs. Sixteen, 17 and 18 leg pair numbers for *O. cinctipes*, *O. herbertorum* and *O. roseus*, respectively. This is the only clade where leg pair number varies. In the remaining six clades leg pair number is invariant.

Genital opening. Male genital pore cruciform, female genital pore a horizontal and small vertical slit with tumid lips.

Distribution. High-altitude indigenous Afrotemperate forest areas along the southern portions of the Drakensberg Mountains in the KwaZulu-Natal province, including Garden Castel NR, Kamberg NR and Highmoor NR in South Africa.

Remarks. Based on the genetic data presented and the paraphyletic relationship within *Opisthopatus* based on the total DNA sequence tree (Fig. 3), *O. herbertorum* Ruhberg and Hamer (2005) is nested within this clade and is here designated as a junior synonym of *O. roseus* Lawrence (1947). As *O. roseus* is the oldest published name, we include the specimen of both *O. cinctipes sensu stricto* and *O. herbertorum* in this clade under *O. roseus*. *Opsithopatus roseus sensu stricto* species appear to be confined to the southern portions of the Drakensberg Mountains in KwaZulu-Natal province, South Africa. The number of leg pairs is highly variable in this clade, and has traditionally been used to delineate the three hitherto described *Opisthopatus* species. However, several recent studies have indicated that leg pair number is a poor taxonomic tool in velvet worms (Ruhberg and Daniels, 2013; Daniels et al., 2013). Similarly, colour appears to be highly variable within *O. roseus* as in most other *Opisthopatus* species. McDonald et al. (2012) reported widespread colour variation within *Peripatopsis capensis* Grube 1866 and *P. lawrencei* McDonald et al. (2012)

***Opisthopatus highveldi* sp. nov.** Clade 3 (Figs 3, 7, 8e, 9d and Table 3)

Holotype. Two males (SAM-ENW-C007163), Graskop, 24°52.590′S, 30°53.288′E, Highveld of Mpumalanga province, South Africa, collected M. Picker, 28 May 2006.

Paratypes. Two males, 1 female, one damaged specimen, 3 juveniles (SAM-ENW-C007162), God's Window, 24°52.590′S, 30°53.288′E, Highveld of Mpumalanga province, South Africa, collected S.R. Daniels, N. Solomons, C. Kunaka and F. van Zyl, 11 June 2009.

Additional material. Seven juveniles, 2 males, 2 females (SAM-ENW-C007158), Lone Creek, Sabie Hoek, 25°06.174′S, 30°42.657′E, Highveld of Mpumalanga province, South Africa, collected S.R. Daniels, N. Solomons, C. Kunaka and F. van Zyl, 12 June 2009; 16 juveniles (SAM-ENW-C007161) Bridal Vail, Sabie, 25°04.984′S, 30°43.627′E, Highveld of Mpumalanga province, South Africa, collected S.R. Daniels, N. Solomons, C. Kunaka and F. van Zyl, 12 June 2009; 1 specimen damaged, not sexed, (SAM-ENW-C007160), Mariepskop forest, 24°34.077′S, 30°51.683′E, Highveld of Mpumalanga, South Africa, collected J.L. Horn, 27 February 2005; 1 juvenile (SAM-ENW-C007157), Graskop, 24°53.283′S, 30°52.613′E, Highveld of Mpumalanga province, South Africa, collected J.A. Neethling, 27 September 2012; 1 juvenile (SAM-ENW-C007159), God's Window, 24°52.425′S, 30°53.485′E, Highveld of Mpumalanga province, South Africa, collected J.A. Neethling, 28 September 2012; 9 males, 5 females, 8 juveniles (SAM-ENW-C007164), Nkandla Forest NR, 28°44.780′S, 31°08.079′E, KwaZulu-Natal province, collected S.R. Daniels and H. van den Worm, no date.

Dimensions. TL of the two holotypes ranged from 15.86 to 17.10 mm, while the DB ranged from 1.69 to 1.76 mm.

Dorsal body primary papilla. The number of scale rings was fixed at nine (Table 3).

Diagnosis. GenBank accession numbers for Clade 3.

COI: KR907025–KR907034, KR907110–KR907113, KR907122–KR907126, KR907135–KR907136, KR90 6988–KR906992.

12S rRNA: KR906817–KR906821, KR906842–KR906846, KR906852–KR906861, KR906950–KR906951, KR906895–KR906899.

18S rRNA: KR907181, KR907187, KR907189–KR907190, KR907215, KR907200.

Description.

Colour pattern. Dorsal integument brown to black to indigo. Ventral integument creamy white with light brown papillae (Fig. 7, Table 3).

Leg pairs. Sixteen leg pairs.

Genital opening. Male genital pore cruciform, female genital pore a horizontal and small vertical slit with tumid lips.

Distribution. Occurs in indigenous Afrotemperate forest patches along the Highveld in Mpumalanga province, South Africa. Typically found in decaying logs of wood that are moist or under moss in close proximity to waterfalls where the animals occur in the top 1 cm of the water-soaked soil. A single allopatric population is present at Nkandla Forest on the KwaZulu-Natal coast.

Etymology. Named for the Highveld of Mpumalanga, an inland plateau with an altitude above 1500 m.a.s.l but below 2000 m.a.s.l.

Remarks. *Opisthopatus highveldi* sp. nov. is sister to Clade 4, with both being novel species.

***Opisthopatus drakensbergi* sp. nov.** Clade 4 (Figs 3, 7, 8a, b, c, d, 9c and Table 3)

Holotype. One male (SAM-ENW-C007136), Royal Natal National Park, 28°41.373′S, 28°56.246′E, KwaZulu-Natal province, South Africa, collected C. Haddad, 21 January 2011.

Paratypes. One male and 1 juvenile (SAM-ENW-C007141), Cathedral Peak NR, 28°96.184′S, 29°22.138′E, KwaZulu-Natal province, South Africa, collected G. Giribet, S. Daniels and B. de Bivort, 15 November 2011.

Additional material. Five juveniles (SAM-ENW-C007137), Royal Natal National Park, 28°43.327′S, 28°55.993′E, KwaZulu-Natal province, South Africa, collected S.R Daniels and N. Solomons, 18 January 2008; 1 juvenile (SAM-ENW-C007135), Cathedral Peak, 28°96.194′S, 29°22.134′E, KwaZulu-Natal province, South Africa, collected by C. Uys, 5 January 2005; 9 juveniles (SAM-ENW-C007143), Cathedral Peak, Rainbow Gorge, 28°57.590′S, 29°13.656′E, KwaZulu-Natal province, South Africa, collected S.R Daniels and N. Solomons, 18 January 2008; 2 juveniles (SAM-ENW-C007138), Bergville, little Switzerland, no GPS coordinates, collected S.R Daniels, C.A Matthee, 16 January 2008; 3 males, 1 female, 8 juveniles (SAM-ENW-C007144), Monks Cowl, 29°02.945′S, 29°24.399′E, KwaZulu-Natal province, South Africa, collected S.R Daniels and N. Solomons, 16 January 2008; 1 juvenile and 2 males, Karkloof, 29°24.408′S, 30°16.608′E, KwaZulu-Natal province, South Africa, donated by H. Ruhberg, kept in culture since collected 4 August 2002; 2 juveniles (SAM-ENW-C007139) Karkloof, 29°24.408′S, 30°16.608′E, KwaZulu-Natal province, South Africa, collected G. Giribet, S. Daniels and B. de Bivort, November 2011; 1 specimen, genital area damaged (SAM-ENW-C007145), Royal Natal National Park, no GPS coordinates, KwaZulu-Natal province, South Africa, no collectors information, no date; 1 juvenile (SAM-ENW-C007145) Karkloof, Glasswork, no GPS coordinates, KwaZulu-Natal province, South Africa, collected M. Hamer, 12 April 2006; 5 males, 5 females and 15 juveniles (SAM-ENW-C007146), Barberton, 25°51.597′S, 31°03.725′E, Mpumalanga province, S.R. Daniels, N. Solomons, C. Kunaka and F. van Zyl, no date provided.

Dimensions. TL of the holotype was 13.41 mm, while DB was 2.41 mm.

Diagnosis. GenBank accession numbers for Clade 4.

COI: KR907045, KR907056–KR907058, KR907074–KR907077, KR907079–KR907095, KR907040–KR 907044.

12S rRNA: KR906915, KR906872–KR906874, KR906910–KR906913, KR906915–KR906932, KR 906837–KR906841, KR906837–KR906841.

18S rRNA: KR907203, KR 907194, KR907205–KR907209, KR907186.

Description.

Dorsal body primary papilla. The number of scale rings varied from 7 to 10 (Table 3).

Colour pattern. Dorsal integument brown and slate black with a well-developed mid-dorsal line. Ventral integument is predominantly brown (Fig. 7, Table 3). Sometimes has a light brown lateral line.

Leg pairs. Sixteen leg pairs.

Genital opening. Male genital pore cruciform, female genital pore a horizontal and small vertical slit with tumid lips.

Distribution. Occurs at high altitude in allopatric indigenous Afrotemperate forest areas in the northern Drakensberg Mountains of KwaZulu-Natal province of South Africa. Found under moss on rocks after heavy summer downpours, and also in decaying logs of wood in closed-canopy forests. Two geographically isolated populations of *O. drakensbergi* sp. nov. are present at Barberton and at the Ngome forest (Ntandeka Wilderness area) based on the phylogenetic evidence (Fig. 3). The latter observation

probably represents a sampling gap as the Great Escarpment was not sampled between these Drakensberg Mountains and the two latter two eastern localities.

Etymology. Named after the Drakensberg Mountains in KwaZulu-Natal province, South Africa.

Remarks. Two species are present on the Drakensberg Mountains, *O. roseus* and *O. drakensbergi* sp. nov., restricted to the southern and northern portions of the mountains, respectively.

***Opisthopatus swatii* sp. nov.** Clade 5 (Figs 3, 7, 8f and Table 3)

Holotype. Three males (SAM-ENW-C007169), Mount Sheba Nature Reserve, 24°56.334′S, 30°42.810′E, Highveld of Mpumalanga province, South Africa, collected S.R. Daniels, N. Solomons, C. Kunaka and F. van Zyl, 10 June 2009.

Paratypes. Five males, 16 females, 7 juveniles (SAM-ENW-C007170), Mount Sheba Nature Reserve, 24°56.334′S, 30°42.810′E, Highveld of Mpumalanga province, South Africa, collected S.R. Daniels, N. Solomons, C. Kunaka and F. van Zyl, 10 June 2009.

Additional material. Eleven males, 16 females, 24 juveniles (SAM-ENW-C007171), Crystal Springs Nature Reserve, 24°51.926′S, 30°41.470′E, Highveld of Mpumalanga province, South Africa, collected S.R. Daniels, N. Solomons, C. Kunaka and F. van Zyl, 10 June 2010; 3 juveniles (SAM-ENW-C007172), Nelshoogte Plantation, 25°49.557′S, 30°51.043′E, Highveld of Mpumalanga province, South Africa, collected S.R. Daniels, N. Solomons, C. Kunaka and F. van Zyl, 10 June 2009; 1 male (SAM-ENW-C007173), Kaapschoop, Berlin Plantation, 25°33.405′S, 30°44.536′E, Highveld of Mpumalanga province, South Africa, collected S.R. Daniels, N. Solomons, C. Kunaka and F. van Zyl, 12 June 2009; 1 male, 3 juveniles (SAM-ENW-C007174), Buffelskloof Nature Reserve, 25 km south of Lydenburg, 25°19.014′S, 30°29.940′E, Mpumalanga province, South Africa, collected J.L. Horn, 15 March, 2006; 2 damaged specimens, sex not determined (SAM-ENW-C007175), Uitsoek Forest, 20 km south-east of Lydenburg, 25°16.603′S, 30°33.088′E, Mpumalanga province, South Africa, collected J.L. Horn, 1 February 2006; 1 specimen damaged not sexed (SAM-ENW-C007176), Buffelskloof Nature Reserve, 25 km south of Lydenburg, 25°18.040′S, 30°30.581′E, Mpumalanga province, South Africa, collected J.L. Horn, 14 March, 2006; 1 juvenile (SAM-ENW-C007177), Mount Shiba Nature Reserve, 5 km from Pilgrims Rest, 24°56.360′S, 30°42.545′E, Mpumalanga province, South Africa, collected J.L. Horn, 12 December 2006.

Dimensions. TL for the three male holotypes ranged from 13.34 to 19.91 mm, while DB ranged from 0.65 to 1.56 mm.

Diagnosis. GenBank accession numbers for Clade 5.

COI: KR907035–KR907039, KR907105–KR907109, KR907115–KR907121, KR907127–KR907134.

12S rRNA: KR906807–KR906816, KR906827–KR906828, KR906847–KR906851, KR906942–KR906949.

18S rRNA: KR907179–KR907180, KR907183, KR907188, KR907213–KR907214.

Description.

Dorsal body primary papilla. The number of scale rings varied from 5 to 6 (Table 3).

Colour pattern. Dorsal integument blue to slate black. Ventral integument light brown to creamy white (Fig. 7, Table 3). Juveniles light pink, fading to pearl white when preserved.

Leg pairs. Sixteen leg pairs.

Genital opening. Male genital pore cruciform, female genital pore a horizontal and small vertical slit with tumid lips.

Distribution. Occurs in indigenous Afrotemperate forest patches along the Highveld in Mpumalanga province, South Africa, often in close proximity to small streams in closed-canopy forests. Occurs in or under bark and in leaf litter.

Etymology. Named for the Swati people who inhabit this region of Mpumalanga.

Remarks. Two *Opisthopatus* species are present in Mpumalanga, *O. highveldi* sp. nov. and *O. swatii* sp. nov.

***Opisthopatus amaxhosa* sp. nov.** Clade 6 (Figs 3, 7, 8k, l, 9e and Table 3)

Holotype. One female (SAM-ENW-C007149), Jenca valley, Langeni area, 31°21.956′S, 28°33.436′E, Eastern Cape province, South Africa, collected D. Herbert and L. Davis, 18 February 2006.

Paratypes. Two females (SAM-ENW-C007148), Baziya forest, Langeni area, 31°31.250′S, 28°24.738′E, Eastern Cape province, collected D. Herbert and L. Davis, 19 February 2006.

Additional material. Two females, 2 juveniles (SAM-ENW-C007149), Nocu forest, Langeni area, 31°24.928′S, 28°29.990′E, Eastern Cape province, South Africa, collected D. Herbert and L. Davis, 18 February 2006.

Dimensions. TL of the holotype was 21.78 mm, while DB as measured in line with oncopod 10 was 2.36 mm.

Diagnosis. GenBank accession numbers for Clade 6.

COI: KR 907152–KR907163.

12S rRNA: KR906967–KR906978.

18S rRNA: KR907219–KR907221.

Description.

Dorsal body primary papilla. The number of scale rings varied from 4 to 7 (Table 3).

Colour pattern. Dorsal integument varies from light pink (fading to pearl white when preserved), blue black to indigo. Ventral integument varies from light brown with white patches (Fig. 7, Table 3).

Leg pairs. Sixteen leg pairs.

Genital opening. Male genital pore cruciform, female genital pore a horizontal and small vertical slit with tumid lips.

Distribution. Distributed in the Afrotemperate forests of north-eastern Eastern Cape province where it borders with KwaZulu-Natal province, South Africa. Found under or inside decaying indigenous logs of wood or in leaf-litter.

Etymology. Named after the amaXhosa people of the region.

Remarks. *Opisthopatus amaxhosa* sp. nov. is sister to Clade 7; specimens are rose pink when alive, but fade to pearl white when preserved.

***Opisthopatus kwazululandi* sp. nov.** Clade 7 (Figs 3, 7, 8n, 9f and Table 3)

Holotype. One male, 1 female (SAM ENW-C007178), Vernon Crookes Nature Reserve, 32°36.522′S, 27°14.417′E, KwaZulu-Natal province, South Africa, collected S.R. Daniels, F. Gordon, G. Baatjies and M. Pérez-Losada, 3 May 2011.

Paratypes. Two females and 2 males, 3 juveniles (SAM ENW-C007190), Vernon Crookes Nature Reserve, 32°36.522′S, 27°14.417′E, KwaZulu-Natal province, South Africa, collected S.R. Daniels and N. Solomons, 7 September 2007.

Additional material. Two males, 1 juveniles (SAM ENW-C007188), Oribi Gorge Nature Reserve, 30°42.376′S, 30°16.211′E, KwaZulu-Natal province, South Africa, collected S.R. Daniels and H. van den Worm (no date); 4 females, 2 males, 1 juvenile, Krantzkloof Nature Reserve, Pietermaritzburg, 29°47.303′S, 30°43.707′E, KwaZulu-Natal province, South Africa, collected S.R. Daniels and H. van den Worm (no date); 2 females, 2 juveniles (SAM ENW-C007191), Ongoya forest Nature Reserve, 28°51.569′S, 31°38.996′E, KwaZulu-Natal province, South Africa, collected S.R. Daniels and H. van den Worm (no date); 7 females 1 juvenile (SAM ENW-C007192), Entumeni forest, KwaZulu-Natal province, South Africa, collected S.R. Daniels and H. van den Worm (no date); 5 males, 1 damaged, 3 juveniles (SAM ENW-C007180), Port Edward, 31°05.348′S, 30°11.086′E, KwaZulu-Natal province, South Africa, collected S.R. Daniels, N. Solomons and F. van Zyl (no date); 2 males, 1 female, 2 juveniles (SAM ENW-C007179), Hluhluwe-iMfolozi, 28°07.441′S, 32°03.504′E, KwaZulu-Natal province, South Africa, collected S.R. Daniels, F. Gordon, G. Baatjies and M. Peréz-Losada, 1 May 2011; 1 female (SAM ENW-C007181), Port St Johns, (no GPS coordinates), Eastern Cape province, South Africa, collected by M. Bursey, 24 January 2006; 3 juveniles (SAM ENW-C007182), Zinkwazi Beach, 29°17.253′S, 31°53.587′E, KwaZulu-Natal province, South Africa, no collector information and no date; 1 specimen damaged (SAM ENW-C007183), Zinkwazi Beach, KwaZulu-Natal province, South Africa, no GPS coordinates, no date; 1 female (SAM ENW-C007185), Pigeon Valley (no GPS coordinates), Durban, KwaZulu-Natal, South Africa, collected D. Herbert (no collection date); 1 male (SAM ENW-C007186), Port St Johns, 31°35.857′S, 29°31.991′E, Eastern Cape province, South Africa, collected by C. Haddad, 10 January 2011; 2 juveniles (SAM ENW-C007187), Ixopo district, 30°00.781′S, 30°03.839′E, KwaZulu-Natal province, South Africa, collected S.R. Daniels and H. van den Worm, 10 September 2007; 4 juveniles (SAM ENW-C007184), Weza forest, 30°63.113′S, 29°71.596′E, Eastern Cape province, South Africa, collected G. Giribet, S. Daniels and B de Bivort, November 2011.

Dimensions. TL of the male and female holotypes was 15.84 and 22.10 mm, respectively, while DB was 3.50 and 3.44 mm, respectively.

Diagnosis. GenBank accession numbers for Clade 7.

COI: KR906983–KR906987, KR906993–KR907002, KR907011–KR907016, KR907064–KR907073, KR 907078, KR907104, KR907147–KR907151, KR907 164–KR907168, KR 907007–KR 90710, KR 907060–KR907063.

12S rRNA: KR906801–KR906806, KR906880–KR 906894, KR906900–KR906914, KR906941, KR906962–KR906966, KR 906797–KR906800, KR906876–KR 906879.

18S rRNA: KR907177, KR907179, KR907182, KR 907197–KR907199, KR907201–KR907204, KR907212, KR907218, KR 907176, KR907196.

Description.

Dorsal body primary papilla. The number of scale rings varied from 7 to 9 (Table 3).

Colour pattern. Dorsal integument light brown or brown with black; for some specimens the initial colour was rose pink that faded to cream upon preservation. Ventral integument light brown to creamy white (Fig. 7, Table 3).

Leg pairs. Sixteen leg pairs.

Genital opening. Male genital pore cruciform, female genital pore a horizontal and small vertical slit with tumid lips.

Distribution. Restricted to the IOCB and adjacent interior of Eastern Cape and KwaZulu-Natal province of South Africa occurring from Port St Johns to Hluhluwe-iMfolozi Nature Reserve. Typically found in closed-canopy environments, in decaying indigenous logs of wood or in the soil beneath the decaying logs, depending on the moisture availability.

Etymology. Named for its predominant distribution along the coastal margins of KwaZulu-Natal province.

Remarks. This recently derived clade is variable in dorsal and ventral integument colour but has a stable number of leg pairs and is sister to *Opisthopatus amaxhosa* sp. nov.

Acknowledgments

An SABI grant from the National Research Foundation to the senior author made this research possible, while the University of Stellenbosch is thanked for logistical support. The Fulbright Scholarship Program and the Oppenheimer Memorial Trust are thanked for financial support while the first author was on sabbatical at Harvard University, USA, where a part of the research was undertaken and written. The DNA sequencing facility and the SEM unit at Stellenbosch University are thanked for their support and help; particular thanks goes to Madelein Frazenburg. Ezemvelo-KNZ Wildlife, the Eastern Cape Wildlife and Mpumalanga Parks are thanked for permits that allowed for sampling to be conducted in nature conservation areas. Dai Herbert, C. Haddad, Vir Bills, Gonzalo Giribet, Charmain Uys, Adnan Moussalli, Michelle Hamer, Johanna Horn, Mary Bursey and Mike Picker are thanked for sample collection. Nicolas Solomons, Hein van den Worm, Gail Baatjies, Marcos Perez-Losada and Fawzia Gordon are thanked for help during fieldwork. Comments by Mark Siddall and one anonymous reviewer improved a previous draft of the manuscript. Dai Hebert, Gonzalo Giribet and Mike Picker are thanked for their willingness to share photographic images of live velvet worms. A special word of gratitude to Dawn Larsen of the South African Museum for accessioning the specimens at such short notice.

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