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ScienceDirect
Molecular Phylogenetics and Evolution 44 (2007) 622–633
MOLECULAR PHYLOGENETICS AND EVOLUTION
www.elsevier.com/locate/ympev

Nuclear and mtDNA-based phylogeny of southern African sand lizards, *Pedioplanis* (Sauria: Lacertidae)

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Received 14 August 2006; revised 20 April 2007; accepted 28 April 2007
Available online 18 May 2007

Abstract

The diversity of lacertid lizards in Africa is highest in the southern African subcontinent, where over two-thirds of the species are endemic. With eleven currently recognized species, *Pedioplanis* is the most diverse among the southern African genera. In this study we use 2200 nucleotide positions derived from two mitochondrial markers (ND2 and 16S rRNA) and one nuclear gene (RAG-1) to (i) assess the phylogeny of *Pedioplanis* and (ii) estimate divergence time among lineages using the relaxed molecular clock method. Individual analyses of each gene separately supported different nodes in the phylogeny and the combined analysis yielded more well supported relationships. We present the first, well-resolved gene tree for the genus *Pedioplanis* and this is largely congruent with a phylogeny derived from morphology. Contrary to previous suggestions *Heliobolus*/*Nucras* are sister to *Pedioplanis*. The genus *Pedioplanis* is monophyletic, with *P. burchelli*/*P. laticeps* forming a clade that is sister to all the remaining congeners. Two distinct geographic lineages can be identified within the widespread *P. namaquensis*; one occurs in Namibia, while the other occurs in South Africa. The *P. undata* species complex is monophyletic, but one of its constituent species, *P. inornata*, is paraphyletic. Relationships among the subspecies of *P. lineoocellata* are much more complex than previously documented. An isolated population previously assigned to *P. l. pulchella* is paraphyletic and sister to the three named subspecies. The phylogeny identifies two biogeographical clades that probably diverged during the mid-Miocene, after the development of the Benguella Current. This probably led to habitat changes associated with climate and, in conjunction with physical barriers (Great Escarpment), contributed towards speciation within the genus *Pedioplanis*.

Keywords: Lacertidae; *Pedioplanis*; ND2; 16S rRNA; RAG-1; Phylogeny; Southern Africa; Taxonomy

1. Introduction

The family Lacertidae has long been regarded as a part of the Scleroglossa (Estes et al., 1988). Although recent molecular evidence revealed a polyphyletic Scleroglossa (Townsend et al., 2004), the Lacertiformes, (including teiids, gymnophthalmids, amphisbaenians, as well as lacertids) received strong support for monophyly. The monophyly of the Lacertidae has been uniformly accepted and is supported by both molecular studies (Harris et al., 1998a; Fu, 1998, 2000) and a number of morphological synapomorphies (Estes et al., 1988; Arnold, 1989). The group is characterized by sexually dimorphic presacral vertebrae counts, hemipenial and jaw muscle characters, and the closure of the temporal fenestra by the postfrontal bone.

Lacertid diversity is greatest in the Palearctic region, however, southern Africa is also characterized by a diverse assemblage of these lizards encompassing some eight genera and 45 species. Twenty-eight species are endemic to the subcontinent (Arnold, 1989; Branch, 1998; Spawls et al., 2002), representing a lacertid diversity hotspot within sub-Saharan Africa. To date, only members of the genus *Meroles* (Harris et al., 1998b; Lamb and Bauer, 2003) have been investigated from a phylogenetic perspective using

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1055-7903/$ - see front matter © 2007 Elsevier Inc. All rights reserved.
doi:10.1016/j.ympev.2007.04.021

molecular sequence data. As a consequence phylogenetic relationships between and within most lacertid genera remain uncertain.

The genus *Pedioplanis* represents the most species-rich lacertid genus in southern Africa (eleven species). Members of the genus occupy diverse habitats including montane grassland, coastal fynbos, succulent Karoo, Nama Karoo, arid and moist savannah, and the Namib Desert (FitzSimons, 1943; Branch, 1998). All species are endemic to southern Africa except *P. namaquensis*, *P. undata* and *P. benguellensis*, which extend their range into southern Angola. Boulenger (1921) and FitzSimons (1943) assigned most of the species now placed in *Pedioplanis* to the subgenus *Mesalina* within the large genus *Eremias*. Szczerbak (1975) regarded *Eremias* polyphyletic and divided the African sand lizards into five genera, including *Mesalina.* Balletto (1968) suggested that the subgeneric name *Pedioplanis* Fitzinger, 1843 was applicable to southern African *Mesalina* and following taxonomic convention this name has been used almost exclusively since the 1980s, whereas *Mesalina* sensu stricto is applied to North African and Asian taxa.

Pedioplanis shares a number of morphological characters, including the presence of a posterior projection and posterolateral process of the septomaxilla and fused frontal bones, with other derived lacertid genera (Arnold, 1991). Among these forms, however, Arnold (1989, 1991) found no support for the collective monophyly of the southern African genera, but rather demonstrated that *Pedioplanis* and *Meroles* shared numerous putative synapomorphies with Saharo-Eurasian genera. Allozyme (Mayer and Berger-Dell'mour, 1988) and mtDNA data (12S rRNA, 16S rRNA and Cyt-b; Harris et al., 1998b) inferred a sister-genus relationship between *Pedioplanis* and *Meroles*, but Fu (2000) using a larger mtDNA data set (12S rRNA, 16S rRNA, Cyt-b, CO1, $tRNA^{Val}$ and $tRNA^{Thr}$) placed *Meroles* as sister to a monophyletic *Pedioplanis* + *Tropidosaura*. Arnold (1991) considered the large number of features shared by both *Pedioplanis* and *Meroles* as parallelisms and identified 13 putative synapomorphies of *Pedioplanis*, one of which, the outer connectors of the hemipenis armature running close together dorsally or fused, is uniquely derived in the genus.

Within *Pedioplanis* the status of the members of two species or species complexes have remained inadequately resolved. Although now treated as separate species, *P. undata*, *P. inornata*, *P. gaerdesi* and *P. rubens* were previously collectively referred to as the *P. undata* species complex. Mertens (1954, 1955) recognized three subspecies in the complex, whereas Mayer and Berger-Dell'mour (1987) based on morphology and protein electromorphs recognized up to seven forms. Recent work (e.g., Arnold, 1989, 1991; Branch, 1998) recognized five of these as valid specific entities, whereas the suggestion by Mayer and Berger-Dell'mour (1987) that *P. undata* and *P. inornata* could each be divided into northern and southern forms has not been subsequently corroborated. In the spotted sand lizard, *P. lineoocellata*, two subspecies are widely recognized, *P. l. lineoocellata*, and *P. l. pulchella* (Branch, 1998). Bauer and Branch (2001) suggested that these two subspecies should be elevated to full species given that they are allopatric and exhibit substantial morphological and ecological differences. The nominate race has slightly overlapping and keeled scales on the back that are smaller than those on the forelimbs, whereas *P. l. pulchella* has smooth, juxtaposed scales on the dorsum that are comparable in size to those of the forelimb, which are not overlapping and smooth on the back. Specimens from Lüderitz Bay in Namibia are sometimes treated as a third subspecies, *P. l. inocellata* (Mertens, 1955), named for its dull, dark gray body (occasionally with four faint dorsal stripes) and lack of large flank spots (Branch, 1998).

Mayer and Berger-Dell'mour (1988) made the first attempt to elucidate the phylogenetic relationship among the sand lizards on the basis of electrophoretic data. Their results were preliminary because of incomplete taxonomic sampling and a lack of support values for the inferred relationships. Subsequently, Arnold (1991) conducted a phylogenetic analysis of all *Pedioplanis* taxa based on morphological data. Relationships were weakly supported and most information was derived from genital characters (Arnold, 1986). The morphology, nevertheless, suggested that *P. lineoocellata* is the sister species to all other *Pedioplanis*. Arnold (1991) also found that the geographically proximal *P. burchelli* and *P. laticeps* are sister species, closely related to *P. breviceps* and that these three taxa are the sister group to (*P. inornata* (*P. husabensis* (*P. namaquensis* (*P. benguellensis* (*P. rubens* (*P. undata*, *P. gaerdesi*)))))). Arnold's (1991) phylogeny thus excluded *P. inornata* from the *P. undata* species complex.

We employed mitochondrial and nuclear DNA markers to investigate the phylogenetic relationships and evolution of *Pedioplanis*. This study specifically aims to: (i) address the phylogenetic relationship of *Pedioplanis* relative to other lacertid groups in southern Africa, (ii) determine the phylogenetic relationships among sand lizard species, (iii) establish the status of named subspecies and unnamed forms in the *P. lineoocellata* and *P. undata* species groups, respectively; and (iv) estimate times of divergence within *Pedioplanis* in an attempt to elucidate historical and ecological factors driving speciation in this group.

2. Materials and methods

2.1. Sampling

Ten of the eleven recognized species in the genus were sampled and replicate sampling resulted in a total of 100 individuals studied. Where possible sampling was done to include geographic variation within each species and also to address the validity of some of the recognized subspecies (Table 1 and supplementary materials, Figure A). The specific identities of individuals were confirmed by morphological examination of voucher specimens associated with the

Table 1
Species locality information and GenBank accession numbers of the specimens (identical sequences in each taxa were excluded and the final was based on 58 specimens) used in this study

Collection code	Museum numbers	Taxon name	Locality	GenBank Accession Nos.		
				ND2	16S rRNA	RAG-1
KTH499	—	*Australolacerta australis*	Naudesberg-Langeberg, W. Cape, S. Africa	DQ871092	DQ871150	—
KTH569	—	*A. australis*	Goedemoed-Langeberg, W. Cape, S. Africa	DQ871093	DQ871151	—
MH0531	—	*A. australis*	Zuurberg Private Nature Reserve, W. Cape, S. Africa	DQ871094	DQ871152	DQ871208
AMB6001	—	*Ichnotropis capensis*	Road to Tsumkwe, Namibia	DQ871090	DQ871148	DQ871206
AMB6067	CAS 209602	*I. capensis*	Kosi Bay, KwaZulu-Natal, S. Africa	DQ871091	DQ871149	DQ871207
AMB5589	CAS 206735	*Meroles suborbitalis*	Groenriviermond, N. Cape, S. Africa	DQ871089	DQ871147	DQ871205
AMB5629	CAS 206782	*M. knoxii*	Port Nolloth, N. Cape, S. Africa	DQ871088	DQ871146	DQ871204
AMB5921	—	*M. reticulatus*	11.3 Km S. Cape Cross, Namibia	DQ871086	DQ871144	DQ871202
MCZFS38343	—	*M. suborbitalis*	Near Grünau, Namibia	DQ871087	DQ871145	DQ871203
AMB5582	CAS 206723	*Nucras tessellata*	Groenriviermond, N. Cape, S. Africa	DQ871085	DQ871143	DQ871201
MCZFS37894	—	*Heliobolus lugubris*	Kamanjab, Namibia	DQ871084	DQ871142	DQ871200
MCZFS37870	MCZ R184277	*H. lugubris*	Kamanjab, Namibia	DQ871083	DQ871141	DQ871199
MCZFS38393	MCZ R 184524	*Pedioplanis lineoocellata pulchella*	Kgama, Limpopo, S. Africa	DQ871050	DQ871108	DQ871166
ABA21	NHMW 35385:2	*P. l. inocellata*	Lüderitz, Namibia	DQ871045	DQ871103	DQ871161
ABA20	NHMW 35360:1	*P. l. lineoocellata*	Aranos, Namibia	DQ871048	DQ871106	DQ871164
AMB6862	CAS 223974	*P. l. lineoocellata*	45 Km N. Helmeringhausen, Namibia	DQ871046	DQ871104	DQ871162
MCZFS37656	MCZ R183775	*P. l. lineoocellata*	76.2 Km E. Ugab Crossing, Namibia	DQ871047	DQ871105	DQ871163
DDT09	—	*P. l. pulchella*	Matjiesrivier Nature Reserve, W. Cape, S. Africa	DQ871051	DQ871109	DQ871167
MH0336	—	*P. l. pulchella*	Die Trap, Cederberg, W. Cape, S. Africa	DQ871049	DQ871107	DQ871165
KTH222	—	*P. laticeps*	Tankwa Karoo, Western Cape, S. Africa	DQ871069	DQ871127	DQ871185
JSM021	—	*P. laticeps*	Anysberg Nature Reserve, W. Cape, S. Africa	DQ871068	DQ871126	DQ871184
JSM018	PEMR17212	*P. laticeps*	Anysberg Nature Reserve, W. Cape, S. Africa	DQ871067	DQ871125	DQ871183
JSM015	PEMR17214	*P. laticeps*	Anysberg Nature Reserve, W. Cape, S. Africa	DQ871066	DQ871124	DQ871182
KTH346	—	*P. burchelli*	Qwa Qwa, Free State, S. Africa	DQ871065	DQ871123	DQ871181
KTH137	—	*P. burchelli*	Wamboomberg nr. Ceres, W. Cape, S.Africa	DQ871064	DQ871122	DQ871180
CF169	—	*P. burchelli*	Sneeukop, Kouebokkeveld, W. Cape, S.Africa	DQ871063	DQ871121	DQ871179
MH0334	—	*P. burchelli*	Tafelberg, Cederberg, W. Cape, S. Africa	DQ871062	DQ871120	DQ871178
MCZFS37819	—	*P. breviceps*	Gai-As, Namibia	DQ871060	DQ871118	DQ871176
MCZFS37818	—	*P. breviceps*	Gai-As, Namibia	DQ871059	DQ871117	DQ871175
AMB8473	—	*P. breviceps*	Near Gai-As, Namibia	DQ871061	DQ871119	DQ871177
ABF16	NHMW 35356:1	*P. breviceps*	Hoanib, Namibia	DQ871058	DQ871116	DQ871174
MCZFS37127	R 184164	*P. husabensis*	Northern Bank of Swakop River, Namibia	DQ871081	DQ871139	DQ871197
ABE473	—	*P. husabensis*	Ukub-W., Namibia	DQ871080	DQ871138	DQ871196
ABE451	—	*P. undata*	Palmwag, Namibia	DQ871053	DQ871114	DQ871172
ABE385	NHMW 35339:13	*P. undata*	Kunene, Namibia	DQ871054	DQ871112	DQ871170
ABE423	NHMW 35339:25	*P. undata*	Nauchas, Namibia	DQ871057	DQ871115	DQ871173
ABE415	NHMW 35339:5	*P. undata*	Uis, Namibia	DQ871053	DQ871111	DQ871169
AMB6406	CAS 214643	*P. undata*	59 Km W. Kamanjab, Namibia	DQ871055	DQ871113	DQ871171
KTH595	—	*P. inornata*	Farm Kuthula, 35 Km E. Upington, N. Cape, S. Africa	DQ871081	DQ871140	DQ871198
AMB4736	—	*P. inornata*	Richtersveld, N. Cape, S. Africa	DQ871078	DQ871136	DQ871194
ABE393	NHMW 35340:9	*P. inornata*	Fish River Canyon, Namibia	DQ871079	DQ871137	DQ871195
ABE472	—	*P. inornata*	Tsaobis Leopard Park in Swakop, Namibia	DQ871073	DQ871131	DQ871189
ABE428	NHMW 35340:5	*P. inornata*	Rössing, Namibia	DQ871072	DQ871130	DQ871188
ABE458	—	*P. inornata*	Usakos, Namibia	DQ871071	DQ871129	DQ871187
AMB6552	CAS 214789	*P. inornata*	S. Karibib, Namibia	DQ871070	DQ871128	DQ871186
ABE407	NHMW 35371:12	*P. gaerdesi*	Purros, Namibia	DQ871077	DQ871135	DQ871193

AMB6507	CAS 214745	*P. gaerdesi*	29 Km W. Sesfontein, Namibia	DQ871075	DQ871133	DQ871191
ABE448	—	*P. gaerdesi*	Palmwag, Namibia	DQ871076	DQ871134	DQ871192
AMB7584	—	*P. gaerdesi*	33.2 Km E. of Ugab Crossing, Namibia	DQ871074	DQ871132	DQ871190
ABE384	NHMW 35341:8	*P. rubens*	Waterberg, Namibia	DQ871052	DQ871110	DQ871168
AMB4558	CAS 200033	*P. namaquensis*	Richtersveld, N. Cape, S. Africa	DQ871043	DQ871101	DQ871159
AMB4775	CAS 200105	*P. namaquensis*	Richtersveld, N. Cape, S. Africa	DQ871042	DQ871100	DQ871158
AMB4541	—	*P. namaquensis*	Richtersveld, N. Cape, S. Africa	DQ871041	DQ871099	DQ871157
ABD54	—	*P. namaquensis*	Otjondeka, Namibia	DQ871044	DQ871102	DQ871160
AMB7577	—	*P. namaquensis*	17 Km E. Ugab crossing, Namibia	DQ871040	DQ871098	DQ871156
AMB7121	—	*P. namaquensis*	Road to Uis, Namibia	DQ871039	DQ871097	DQ871155
ABD47	NHMW 35351:20	*P. namaquensis*	Trekkopje, Namibia	DQ871038	DQ871096	DQ871154
AMB6549	CAS 214784	*P. namaquensis*	S. Karibib, Namibia	DQ871037	DQ871095	DQ871153

The TreeBase study and matrix (three genes) Accession Nos. are S1763, M3213–M3215. Collection codes, AMB, Aaron M. Bauer tissue collection (corresponding voucher specimens pending accession in the National Museum of Namibia); ABE and ABD, Molecular Systematics Section of the Naturhistorisches Museum in Wien (voucher and/or tissue sample only); CAS, California Academy of Sciences, KTH, Krystal Tolley (tissue accessioned at the South African National Biodiversity Institute), CF & MH, Cape Fold Herp project (tissue only, no voucher specimens); JSM, Jane S. Makokha (some voucher specimens pending accession in Port Elizabeth Museum, South Africa); DDT, Dahne Du Toit (tissue only, no voucher); MCZ, Museum of Comparative Zoology, Harvard University; MCZ FS, Museum of Comparative Zoology, Harvard University field series (corresponding voucher specimens pending accession in the National Museum of Namibia); NHMW, Naturhistorisches Museum in Wien.

tissue samples. Due to the uncertain monophyly of *Pedioplanis*, several species belonging to other lacertid genera in southern Africa were incorporated as additional ingroup taxa. They include representatives of *Meroles* (*M. knoxii*, *M. suborbitalis*, and *M. reticulatus*), *Ichnotropis* (*I. capensis*), *Nucras* (*N. tessellata*), and *Heliobolus* (*H. lugubris*). Based on Arnold's (1989) morphological phylogeny of the family Lacertidae, *Australolacerta australis* was specified as the outgroup.

2.2. DNA extraction, amplification and sequencing

A piece of the tail or the entire liver of voucher specimens was preserved in 95% ethanol or saturated salt–DMSO buffer. Upon DNA extraction, tissue was homogenized in 250 µl extraction buffer containing 20 µl of a 10 mg/ml proteinase K solution. Total genomic DNA was extracted using the phenol/chloroform iso-amyl alcohol procedure as described by Palumbi et al. (1991). Two mitochondrial (ND2 and 16S rRNA) and one nuclear gene region (RAG-1) were selected for sequencing. The published vMet2 and vTrp ND2 primers (Cunningham and Cherry, 2004) and L2510 and H3080 16S rRNA primers (Palumbi, 1996) were used for mtDNA amplification and sequencing. RAG-1 lacertid specific primers were designed using *Eremias* sp. sequence from GenBank (AY662615; Townsend et al., 2004) and the program Primer3 (Rozen and Skaletsky, 2000): F211, 5′-ATTAC TTCAGTGCCACAAGA-3′ and R1392, 5′-CCTGCATC ATAGCTTCCAAC-3′. The PCR cycle profile was as follows: an initial 1 min denaturation at 94 °C, followed by 35 cycles of 35 s at 94 °C, 30 s at 50–55 °C (annealing) and 45 s at 72 °C (extension); with a final extension of 5 min at 72 °C using the Gene Amp PCR system 2700 (Applied Biosystems, Foster City, USA). The annealing temperature was set at 54, 50 and 55 °C for ND2, 16S rRNA and RAG-1 genes, respectively.

The PCR reaction mixture was separated on 0.8% agarose gels and amplified products were gel purified using Qiagen purification columns (Qiagen, Inc.). Cycle sequencing was done with the BigDye terminator v3.1 cycle sequencing kit (Applied Biosystems, Foster City, USA). Excess terminator dye was removed by gel filtration through Centri-Sep 96 multi-well filter plates (Princeton Separation). The cycle sequencing products were analyzed on either an ABI Prism 3100 or 3130 XL genetic analyzer (Applied Biosystems, Foster City, USA).

2.3. Phylogenetic analysis

The sequences were visually inspected in Sequence Navigator v. 1.01 (Applied Biosystems, Foster City, USA) and alignments were performed in Clustal X (Thompson et al., 1997) using default parameters. Where necessary adjustments to the sequence alignments were made after visual inspection in MacClade v. 4.0 (Maddison and Maddison, 2002). All the sequences have been deposited in GenBank

(Table 1). Three methods of phylogenetic inference were used: parsimony (MP), maximum likelihood (ML) and Bayesian inference (BI). Congruence between the three gene partitions was tested using 100 replicates of the partition-homogeneity test (PHT) (Farris et al., 1994, 1995) in PAUP* 4.0b10 (Swofford, 2002). MP tree construction was done in PAUP* 4.0b10 with all characters unordered and equally weighted. Tree searches were conducted using heuristic tree bisection and reconnection branch-swapping (TBR) with 100 random taxon addition replicates. The support of the recovered nodes was calculated using 1000 non-parametric bootstrap replicates (Felsenstein, 1985). Using MrModeltest v. 2 (Nylander, 2004) the General Time Reversal model of nucleotide substitution with gamma shape parameters and a proportion of invariant sites (GTR + I + G) was selected for all three genes. ML was implemented in PHYML (Guindon and Gascuel, 2003) using the GTR model as input and nodal support was assessed by 1000 bootstrap replicates. BI analyses were performed with MrBayes v. 3.1.1 (Huelsenbeck and Ronquist, 2001; Ronquist and Huelsenbeck, 2003). For the combined analysis, the data from the three genes were partitioned and parameters were unlinked, allowing the assignment of different model optimizations for each DNA fragment separately. Prior models used for all three gene partitions were set to nst = 6 and rates = invgamma. Two runs of five million generations using four Markov chains were done simultaneously. Trees were sampled every hundred generations, and using the sumt command, the first 10% (5000 trees) of 50,000 trees were discarded as the burn-in. The posterior probability for each clade was determined by calculating a 50% majority rule consensus tree in PAUP* 4.0b10 (Swofford, 2002). Pairwise differences between clades or individuals was calculated in PAUP* 4.0b10 (Swofford, 2002) using a GTR correction.

2.4. Estimation of time of divergence

The hypothesis of a constant molecular clock was rejected by the likelihood ratio test (without clock-LnL 18808.24570, with clock-LnL 18863.16074; $P < 0.05$; $X^2 = 74.4683241$). The relaxed Bayesian clock implemented in Estbranches and Multidivtime was used to generate an ultrametric tree (Thorne and Kishino, 2002; Kishino et al., 2001). This method allows each gene fragment to evolve independently and the maximum likelihood estimation of transition/transversion ratios, rate heterogeneity among sites and nucleotide frequencies were determined for each gene separately using PAML v. 3.15 (Yang, 1997). As input tree a concensus topology was used. The tree was derived from the combined parsimony analysis and this topology was also similar to the results obtained from the ML and BI methods. Only 29 taxa (representing one individual of each species, all four *lineoocellata* taxa and both lineages of *namaquensis* and *inornata*) were included. Unfortunately the only fossil record for the African Lacertidae is from Morocco and has been dated to the middle Miocene, approximately 15 Mya (Estes, 1983). On this basis, we could only employ a single calibration point and the node of the ancestor of all southern African genera was constrained to lower and upper limits of 12 Mya and 18 Mya, respectively. Although a somewhat earlier scenario has been proposed by Busack and Maxson (1987), who estimated the divergence of *Heliobolus*/*Pedioplanis* from *Ichnotropis* to early Miocene (17–24 Mya) based on immunological data (serum albumin), this secondary estimate could not be used in the present study. The inferred pattern of relationships in the latter study was in conflict with ours and the age determination may have been overestimated due to an exceptionally long branch associated with *Ichnotropis* (Mayer and Benyr, 1994).

3. Results

3.1. Analysis of mitochondrial and nuclear data sets

The aligned ND2 matrix had a total of 602 characters of which 234 (38.9%) were constant, 34 (5.6%) variable but not parsimony informative and 334 (55.5%) parsimony informative. All samples of *P. lineoocellata* shared a three base pair (bp) deletion (position 437–439 in the alignment) in the ND2 region but this did not interrupt the reading frame. The corrected genetic distance comparisons among *Pedioplanis* species ranged between 7 and 34%. Exclusion of the highly variable and difficult to align sections of the 16S rRNA gene (positions 225–230, 280–292, 309–312, 332–350 in the alignment 42 characters in total) resulted in a matrix comprising 498 characters of which 220 (44.2%) were constant, 28 (5.6%) variable but not parsimony informative and 250 (50.2%) parsimony informative. The corrected genetic distance among *Pedioplanis* species ranged from 2 to 12%. The PHT did not reject the null hypothesis ($P = 0.16$) and because the two mtDNA fragments are linked and no strong conflicting nodes were present when the genes were analysed individually (data not shown) we combined the two datasets. A parsimony analysis was performed on 58 unique sequences resulting from the mitochondrial data set (the remaining 42 sequences were not included because they represented duplicate taxonomic sampling and were identical to sequences already present; the 42 identical sequences were however used to confirmed the authenticity of all sequences included). Analysis of these resulted in 56 equally parsimonious trees ($L = 2439$, CI = 0.4121 and RI = 0.740). The combined mitochondrial tree was well resolved (Fig. 1), with most of the resolution originating from the ND2 gene region (data not shown; Makokha, 2006).

Given the relatively conservative nature of the nuclear DNA fragment (Matthee et al., 2004a), only the individuals representing the fifty-eight unique mtDNA haplotypes were sequenced for the nuclear RAG-1 fragment. The aligned RAG-1 matrix comprised 1100 characters of which 679 (61.7%) were constant, 250 (22.7%) were variable but parsimony uninformative and 171 (15.6%) parsimony

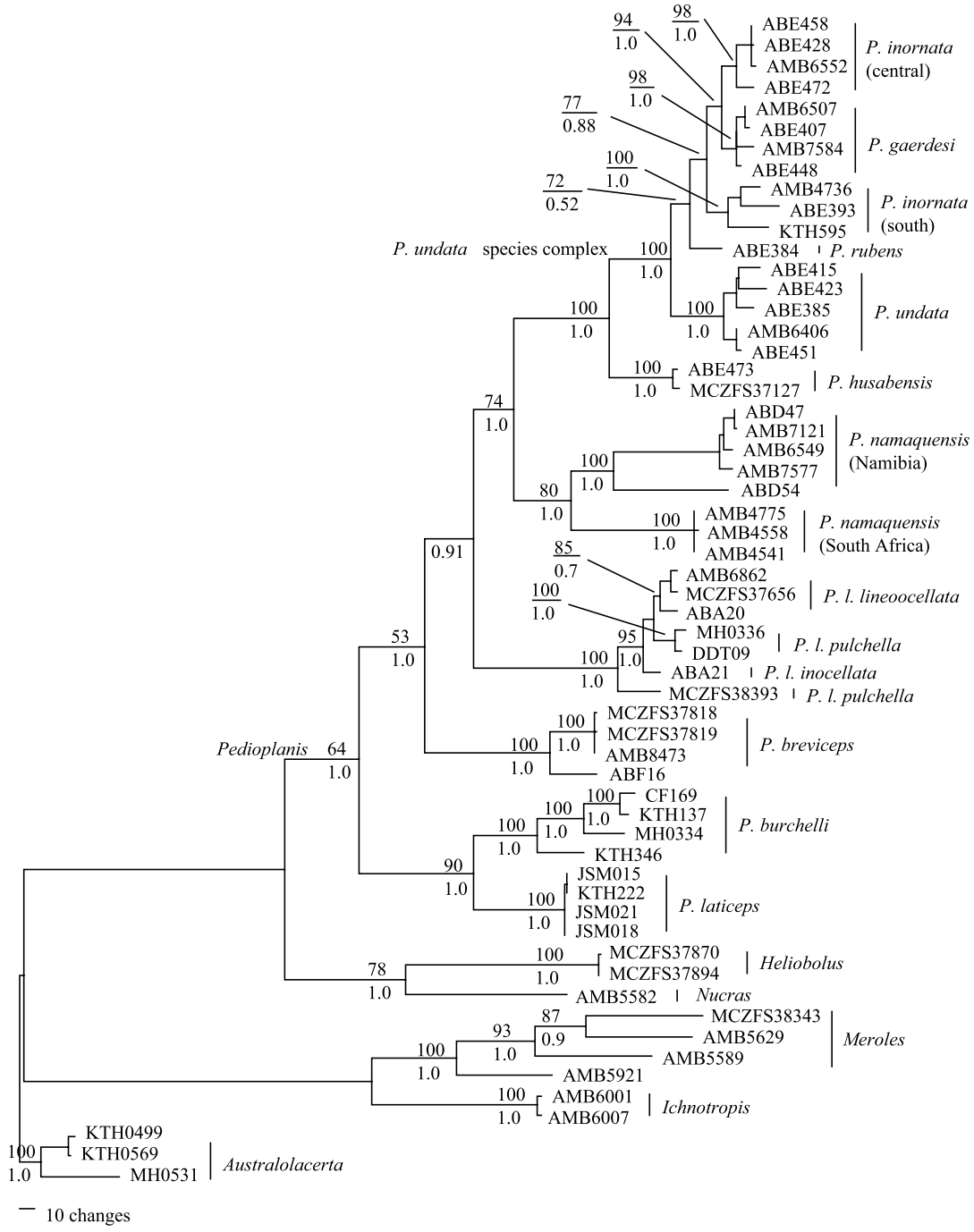

Fig. 1. A Bayesian inference analysis phylogram of the combined mtDNA data (ND2 and 16S rRNA). At nodes, bootstrap replicated (above) and Bayesian posterior probability (below) are indicated.

informative. All samples of *P. breviceps* had a 12 bp deletion (position 474–485) whereas *Meroles knoxii* and *M. suborbitalis* shared a 15 bp deletion (position 115–129). None of the deletions affected the reading frame. The corrected nuclear DNA genetic distance among *Pedioplanis* species were low and ranged between 1 and 6%. In both MP and BI, the basal nodes of the topology (those defining relationships among genera) were well resolved but most interspecific relations had little support (topology not shown).

3.2. Combined (mitochondrial and nuclear) phylogenetic analysis

The results of the PHT test between mitochondrial and nuclear genes used in this study indicated incongruence ($P = 0.02$). However, the three genes were combined because there were no strongly supported nodes that were in conflict between the trees generated by the mitochondrial and the nuclear data sets and because the combination of data frequently increases phylogenetic resolution

(Matthee et al., 2004a,b). The significant PHT results could be attributed to the conservative nature of the test (Yoder et al., 2001; Barker and Lutzoni, 2002). The concatenated dataset of 58 taxa consisted of 2200 characters of which 1305 (59.3%) were constant, 140 (6.4%) variable but parsimony uninformative and 755 (34.3%) were parsimony informative. Parsimony analysis of the combined data resulted in 72 equally parsimonious trees ($L = 2887$, $CI = 0.4465$, $RI = 0.7725$). The inclusion of the nuclear data set slightly decreased the support for recently divergent taxa (for example among the *P. undata* species complex) whereas it generally increased the support at deeper nodes, and it is likely that lineage sorting at the nuclear DNA level is not yet complete (Maddison and Knowles, 2006). Overall the MP, ML and BI trees were largely congruent (Fig. 2).

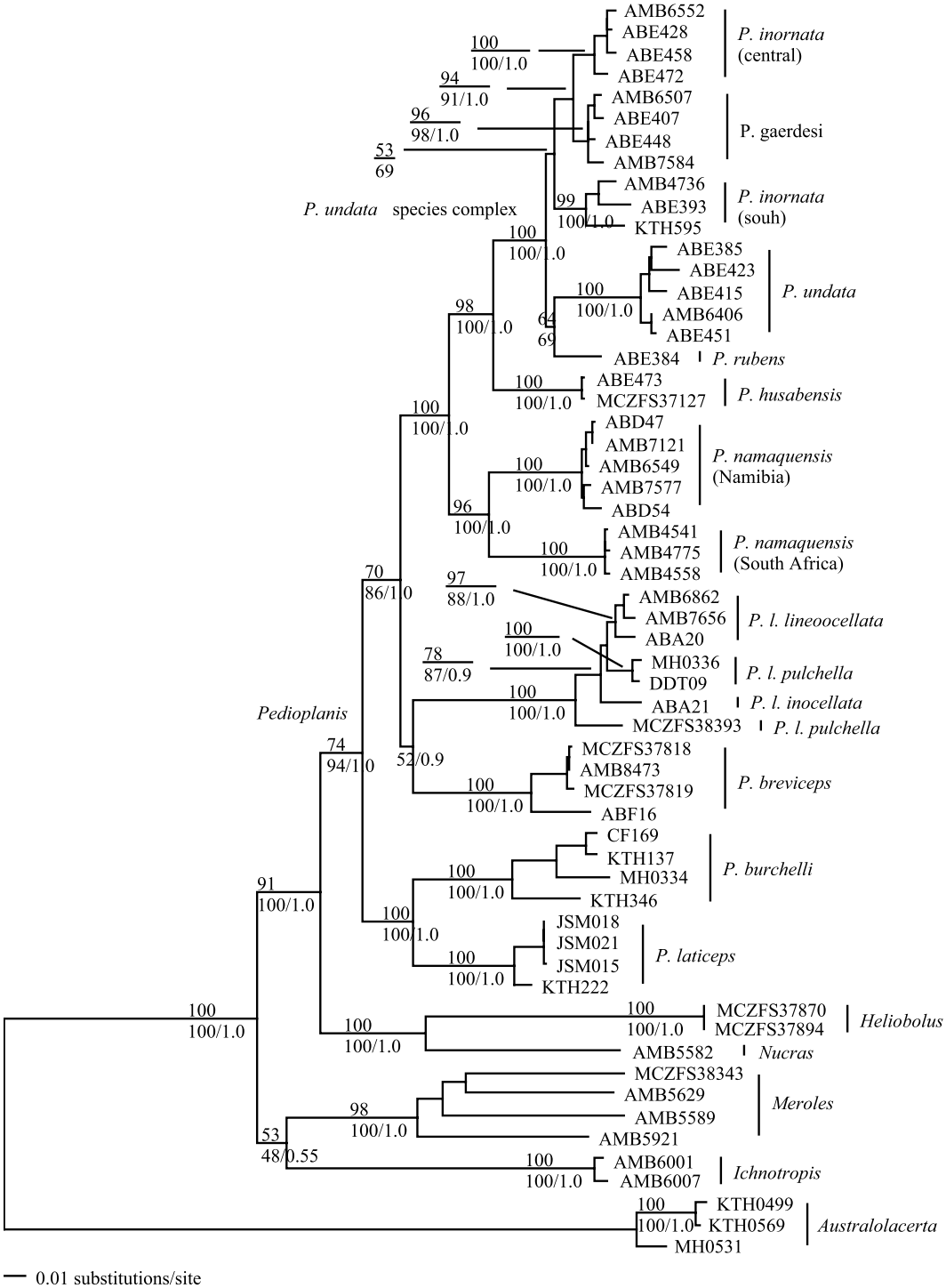

Fig. 2. Maximum likelihood phylogram based on the combined data (mitochondrial and nuclear fragments). Above the nodes are bootstrap support values derived from MP analysis below the nodes are bootstrap support from ML analysis/Bayesian posterior probabilities.

With the genus *Australolacerta* defined as the outgroup, *Meroles* forms a poorly supported clade with *Ichnotropis* (BS = 53%, ML = 48%, PP = 0.55; Fig. 2). However, the sister relationship between *Heliobolus* and *Nucras* is well supported (BS = 100%, ML = 100%, PP = 1.00). The monophyletic relationship of *Heliobolus*/*Nucras* with *Pedioplanis* is well supported (BS = 91%, ML = 100%, PP = 1.0). *Pedioplanis* is monophyletic (BS = 74%, ML = 94%, PP = 1.00) and *P. burchelli*/*P. laticeps* are sister species, and together form the sister group to the remaining species in the genus (BS = 70%, ML = 86%, PP = 1.0). Among the remaining taxa, *P. breviceps* and *P. lineoocellata* clustered as sister taxa without significant support (BS ⩽50%, ML = 52%, PP = 0.9) and together are the sister group to the remaining species of *Pedioplanis*. Collectively the subspecies of *P. lineoocellata* constitute a strongly supported clade (BS = 100%, ML = 100%, PP = 1.0). Within *P. lineoocellata*, the sample from Kgama in the Waterberg District, Limpopo Province, considered to be an isolated population of *P. l. pulchella* (Jacobsen, 1989), is sister to all other recognized subspecies (*P. l. lineoocallata*, *P. l. pulchella*, *P. l. inocellata*). Within the current concept of *P. namaquensis*, there seem to be two geographically distinct lineages, one in Namibia, the other in South Africa and together they form a monophyletic group (BS = 96%; ML = 100%; PP = 1.00). *Pedioplanis husabensis* and the *P. undata* species complex form a strongly supported clade (BS = 98%; ML = 100%; PP = 1.00). Within this species complex, the *P. undata*/*P. rubens* clade is basal, although this pattern does not receive significant support across the three methods of analyses. *Pedioplanis inornata* is paraphyletic and consists of two separate clades, a strongly supported central Namibian clade that is sister to *P. gaerdesi* and a more southern Namibian—Northern Cape clade.

3.3. Divergence time

The relaxed Bayesian clock based on the combined dataset yielded posterior molecular divergence dates with relatively narrow standard deviations (Fig. 3) as well as

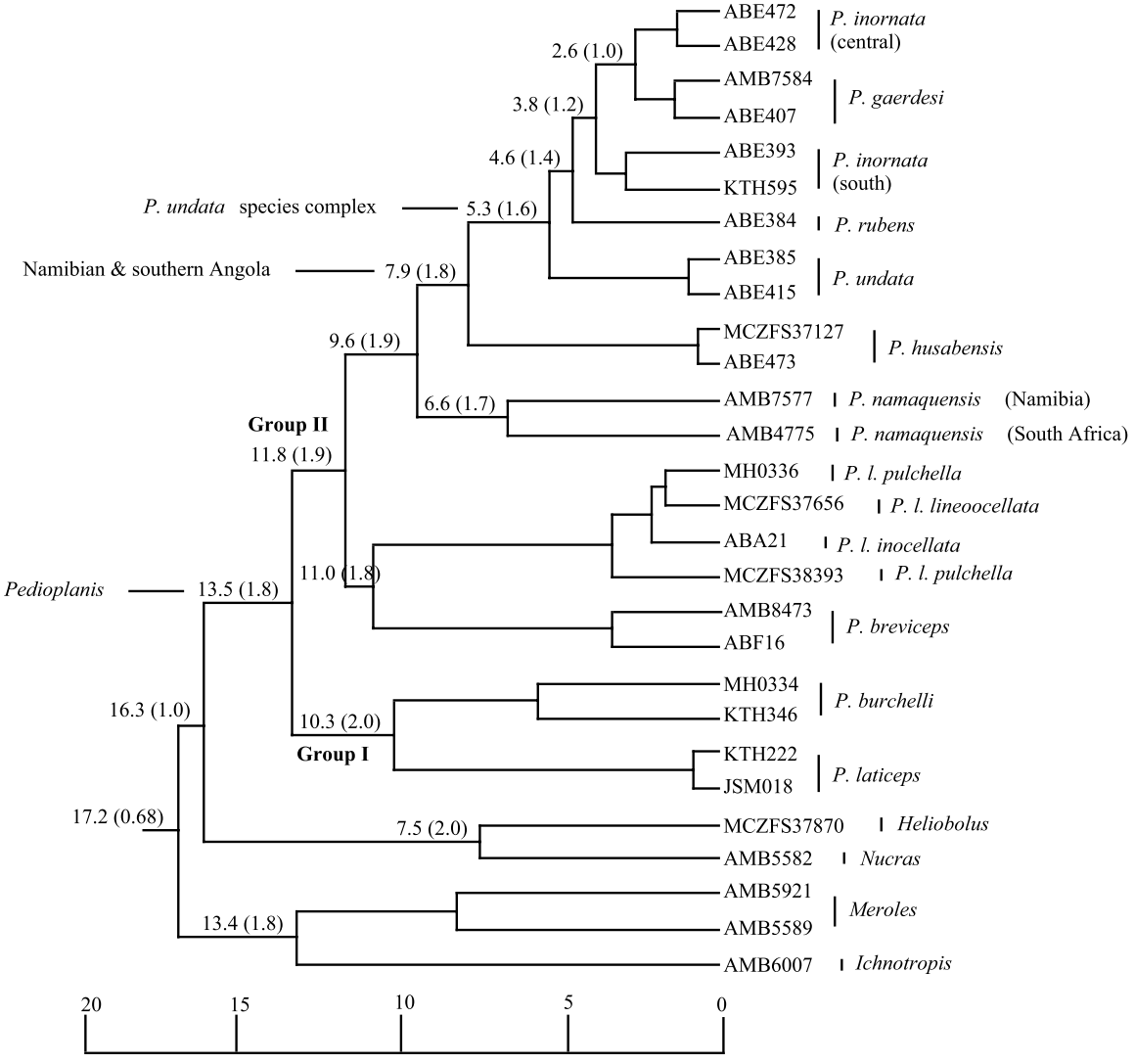

Fig. 3. Linearized tree showing times of divergence (standard error in parantheses) obtained with the relaxed molecular clock method (Miltidivtime). The roman numbers indicate the two biogeographical groupings within *Pedioplanis*.

confidence intervals (not shown). The divergence of the two biogeographical groupings (I & II, Fig. 3) within *Pedioplanis* is estimated to have occurred during the mid-Miocene (13.5 ± 1.8 Mya). Diversification within the *P. undata* species complex is approximated to have commenced in the late Miocene, with the most recent divergences dating from the Pliocene (2.6 ± 1.0 Mya). Given that only a single fossil dating point is available for this group the estimates are tentative and should be considered preliminary pending the discovery of additional, reliably dated relevant fossils. Irrespective of the accuracy of the exact date, our analyses clearly indicate that speciation of *Pedioplanis* was gradual.

4. Discussion

4.1. Phylogenetic relationships of Pedioplanis

The five ingroup genera (*Nucras*, *Heliobolus*, *Ichnotropis*, *Meroles* and *Pedioplanis*) included in this study were all found to be monophyletic. When the root was placed at *Australolacerta*, there was good support for the sister taxon relationship between *Nucras* and *Heliobolus*, and this clade was retrieved as sister to a monophyletic *Pedioplanis.* The monophyly of the three genera was well supported but conflicts with previous topologies based on morphology and mtDNA (Arnold, 1989; Harris et al., 1998a; Fu, 1998, 2000). The close relationship between *Nucras* and *Heliobolus* (but not *Pedioplanis*) is consistent with Fu (2000). In this study *Meroles* is retrieved as sister genus to *Ichnotropis* although with poor support. This is in agreement with other molecularly derived patterns of relationship (Harris et al., 1998a) but is contrary to the morphological as well as combined data inferences made by the same study. Nonetheless, except for a few characters, for instance tongue color, *Meroles* and *Ichnotropis* exhibit alternative states to *Nucras*, *Heliobolus* and *Pedioplanis* (Arnold, 1989). However, relationships within the family Lacertidae are still unresolved and await improved taxonomic sampling and more data collection.

The monophyly of the genus *Pedioplanis* is well supported, corroborating previous morphological findings (Arnold, 1989, 1991) and protein electrophoresis data (Mayer and Berger-Dell'mour, 1988). Our study strongly suggest that *P. burchelli*/*P. laticeps* is the sister group to all other species in the genus *Pedioplanis* and this is in contrast to the morphologically derived phylogeny of Arnold (1991). Analyses of morphological characters suggested that *P. lineoocellata* is a sister group to all other *Pedioplanis* species, but Arnold (1991) admitted that the evidence for this was not satisfactory (the axillary mite pockets, loss of pterygoid teeth and the position of outer connectors of the hemipenes, were unreliable, of uncertain polarity or subject to ambiguous scoring). The close relationship between *P. burchelli* and *P. laticeps* based on hemipenial structure and general morphology has never been in doubt (Arnold, 1986, 1991). Indeed, these taxa have often been confused and a clear delimitation of species boundaries and ranges, especially for *P. laticeps*, is at present problematic (W.R. Branch, pers. comm.). We did not find strong support for the sister-species relationship between *P. breviceps*/*P. lineoocellata* as suggested by the BI (not shown) as well as ML tree topology. Arnold (1991) suggested that *P. lineoocellata*, *P. burchelli*, *P. laticeps*, and *P. breviceps* are all closely related since they share the derived features of exposure of the ectopterygoid as a lateral facet below the jugal bone and 25 presacral vertebrae in males. Based on the outcome of our study these "derived features" rather represent symplesiomorphic characters.

Pedioplanis namaquensis, with a wide distribution throughout Namibia and South Africa, is strongly supported as sister species to a clade consisting of *P. husabensis* and the members of the *P. undata* species complex. According to Arnold (1991) this group (*P. namaquensis* + *P. husabensis* + *P. undata* species complex) shares derived genital features. *Pedioplanis namaquensis* itself consists of two geographically distinct clades; one in the Northern Cape Province of South Africa and the other in Namibia. These lineages are separated by large genetic distance (ND2 = 18–20%, 16S rRNA = 5–6%, RAG-1 = 1–3%). Samples from southern Namibia were not available, and with our present sampling it is difficult to determine where the boundary between the two potential parapatric forms lies. Alternatively, geographically intermediate populations could reveal these two lineages to be an artifact of isolation by distance in a widespread divergent species. Interestingly, although these two lineages are molecularly distinct, they show no obvious pattern of morphological differences. Specimens described from Kalkfontein, southern Namibia by Hewitt (1926) were initially assigned subspecific status (*P. n. quadrangularis*) but FitzSimons (1943) found no morphological characters to distinguish it from the nominate race and thus did not recognize the subspecies. Bauer et al. (1993) suggested that specimens from Hoanib River in Namibia might differ from the typical form, although they did not elaborate of the specific nature of the morphological differences, and called for further investigation. Although it is difficult to delineate species based on sequence divergence only, that found between these two lineages is significantly higher than between some recognized species in this group. A population genetic revision of *P. namaquensis* is required to investigate the possible validity of *P. n. quadrangularis* and to assess the morphological variation across the range of the species in light of its significant intraspecific molecular divergence.

No tissue samples of *P. benguellensis*, a species restricted to northern Namibia and adjacent southern Angola (Branch, 1998), were included in the current study. This taxon is morphologically similar and thus regarded to be closely related to *P. namaquensis* (Arnold, 1991; W.R. Branch, pers. com.) because of a lack of clear morphological differences, Mertens (1955) considered it a synonym of *P. namaquensis*. It is possible that some "*P. namaquensis*" from northern Namibia, for instance ABD54 from Otjendeka (Table 1 and Figs. 1 and 2), may represent

P. benguellensis. Based on our analyses, this specimen is sister to the Namibian lineage of *P. namaquensis*. More specimens from this region need to be examined, both morphologically and molecularly, to assess the validity and distinctness of *P. benguellensis*.

4.2. The status of the P. undata species complex and subspecies within P. lineoocellata

Contrary to the phylogeny proposed by Arnold (1991), which placed *P. inornata* as sister species to *P. namaquensis* and *P. husabensis*, our study shows that the *P. undata* complex group is monophyletic and sister to *P. husabensis*. All the currently recognized taxa within the "*P. undata*" species complex were found to be monophyletic except *P. inornata*, which is made up of two distinct lineages, one from central Namibia and the other from southern Namibia and the Northern Cape Province of South Africa. Due to low levels of nodal support, the relationships amongst members of the *P. undata* species group remain unclear; only the sister relationship between the *P. gaerdesi* and the central Namibian lineage of *P. inornata* is well supported. The phylogenetic relationships presented here for this species complex should therefore be considered tentative. The two lineages of *P. inornata* are moderately divergent from each other (ND2 = 7–8%, 16S rRNA = 3–4%, RAG-1 = 1–2%). These levels of sequence divergence are, however, well within the ranges of between-species divergence among other recognized *Pedioplanis* species, and given the strong support for paraphyly, it is suggested that the two forms should be elevated to species level. This is consistent with Mayer and Berger-Dell'mour's (1987) suggestion that two forms of *P. inornata* occur parapatrically in Namibia, one with a limited distribution in west-central Namibia and the other widespread in southern Namibia and extending into northern South Africa. The southern form is characterized by brownish or reddish coloration and greenish spots and is correctly associated with the name *P. inornata*, which was described from the Orange River by Roux (1907). The northern form, with a distinctive grayish forebody and reddish hindbody and yellow spots, may be specifically distinct and will be the subject of further investigation by the authors. On the other hand, our data does not support the recognition of two genetically distinct forms of *P. undata* and is thus consistent with Mayer and Berger-Dell'mour (1987), who considered these "forms" as probable color morphs rather than real biological entities.

The relationships amongst the currently recognized subspecies of *P. lineoocellata* appear more complicated than previously thought. The specimens from the Waterberg District, Limpopo Province, South Africa previously assigned to *P. l. pulchella* (Jacobsen, 1989; Branch, 1998), are basal to all other *P. lineoocellata* specimens. In addition, the sample from Lüderitz Bay (*P. l. inocellata*) is sister to the samples assigned to *P. l. lineoocellata* and the remainder of *P. l. pulchella*. This renders the subspecies *P. l. pulchella* paraphyletic. *Pedioplanis l. lineoocellata* and *P. l. pulchella* are morphologically and ecologically distinct (FitzSimons, 1943; Branch, 1998). Based on this, Bauer and Branch (2001) proposed that the two subspecies should be raised to specific status. *Pedioplanis l. inocellata* from Lüderitz Bay is also morphologically distinct (Mertens, 1955; Haacke, 1965; Branch, 1998). The high level of divergence between the Waterberg specimen and all other specimens belonging to the species (ND2 = 9–10%, 16S rRNA and RAG-1 = 1–2%), indicates that they are an independently evolving lineage and we suggest that they too should be elevated to specific status. No previously proposed names are available for this form and a species description will be presented elsewhere. Interestingly, a population of *P. lineoocellata* from Roodeplaat (Gauteng Province), South Africa, not sampled in our study, has also been identified as morphologically distinctive (Jacobsen, 1989) and should be investigated.

4.3. Biogeography of Pedioplanis in southern Africa

There are two well defined biogeographic groupings within *Pedioplanis* (Fig. 3, Groups I and II). Group I consists of *P. burchelli* and *P. laticeps*, which are endemic to South Africa. The relaxed Bayesian clock estimate suggests that the two clades diverged during the mid-Miocene, a period charcterized by unstable climate leading to major habitat change in the region (Linder, 2003, 2005). Within group II, the rest of the species form a coherent, chiefly Namibian and southern Angolan group which consists of *P. husabensis*, *P. rubens*, *P. gaerdesi*, *P. undata* and *P. inornata*. The only exception is *P. breviceps*, which, although currently endemic to Namibia, does not group within the Namibian clade.

The divergence time estimates suggest that speciation in the Namibian/southern Angolan clade occurred from the late Miocene to the Pliocene. This is subsequent to the development of the Benguela Current along the west coast of southern Africa during the Miocene (Siesser, 1980). This event is thought to have increased aridity in the Namib Desert and might be responsible for the rapid radiation in the Namibian clade, particularly in the *P. undata* species complex (indicated by short branch lengths, Figs. 1 and 2). These events have also been associated with speciation in other lizard groups such as *Meroles* (Arnold, 1991; Lamb and Bauer, 2003), *Pachydactylus* group geckos (Bauer and Lamb, 2005), and the desert plated lizard, *Gerrhosaurus skoogi* (Lamb et al., 2003). The changes in aridity in the region could also have played an important role in habitat changes, especially the extensive sand deposition during the Miocene (Lancaster, 1990). Phylogenetic relationships indicate a steady cline from the more mesic habitat in group I, i.e., from *P. burchelli* found in Cape fynbos and montane grassland, to the extremely xeric habitats of the Namib Desert inhabited by *P. gaerdesi* in the Namibian/southern Angolan group. This is in agreement with Arnold's (1981) model of speciation in which competition,

displacement and adaptation are important processes leading to speciation as populations colonizing more extreme habitats. *Meroles*, which is a typical desert lizard group with almost all species having part of their range in the Namib Desert (Branch, 1998; Harris et al., 1998b; Lamb and Bauer, 2003), clearly fits this model. *Pedioplanis* shows similar patterns, with the more recently divergences (within group II) associated with the dry habitats of the Namib Desert or surrounding dry savanna.

In Namibia, the Western Escarpment forms a zoogeographical transition zone with savanna in the east and Namib Desert in the west. Mayer Mayer and Berger-Dell'mour (1987) classified the various forms of the *P. undata* species complex as either being Pro-Namib or Namib and/or having southern or northern transition distributions in Namibia. Although the Western Escarpment seems to be a barrier between *P. gaerdesi* and *P. undata* the two lineages of *P. inornata* are divided into northern and southern populations separated at roughly the level of the Swakop River. These west–east and north–south transition zones have also been demonstrated to have played a role in the evolution of the *Pachydactylus punctatus* group (Bauer and Branch, 1995; Bauer, 1999) and possibly in the divergence of *Trachylepis s. sulcata* from *T. s. ansorgii* (Bauer et al., 1993).

Most of the early-diverging species in the genus *Pedioplanis* have very wide distributions (for instance, *P. burchelli*, *P. lineoocellata* and *P. namaquensis*). However, isolation due to restriction to particular habitat types seems to have played a role in the divergence between closely related species in this group. The separations between *P. burchelli* and *P. laticeps*, *P. lineoocellata* and *P. breviceps*, and *P. undata* and *P. rubens* are mainly associated with habitat preference. For instance, *P. rubens* is restricted to red sandstone bedrocks in the Waterberg Plateau in Namibia and *P. husabensis* is only found in the rocky desert between the Khan and Swakop rivers in the Husab Mountains (Branch, 1998). These are the only two members of this group that are rock dwelling (Branch, 1998; Mayer and Richter, 1990). It has been suggested that they might have diverged from their relatives as a result of adaptation to a rupicolus lifestyle. Substrate specificity has been suggested to play an important role in evolution of other groups of reptiles in the region, for instance geckos (Bauer, 1999; Bauer and Lamb, 2005).

Acknowledgments

We thank Krystal Tolley, Michael Cunningham, Andrew Turner, Bill Branch, Le Fras Mouton, Dahne du toit and Marius Burger for providing some tissue samples. Benny Bytebier, Rauri Bowie and Krystal Tolley are thanked for commenting on an earlier version of this manuscript. We also thank the two anonymous reviewers whose comments and suggestions have greatly improved the manuscript. Financial contributions were received from the Department of Botany and Zoology University of Stellenbosch, National Research Foundation GUN 2053662 (RSA), National Science Foundation (USA) Grant DEB 9707568, and Villanova University (USA).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ympev.2007.04.021.

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