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Short Communication

Multigene phylogeny of Malagasy day geckos of the genus *Phelsuma*S. Rocha^{a,b,c,*}, M. Vences^d, F. Glaw^e, D. Posada^c, D.J. Harris^{a,b}^a CIBIO, Centro de Investigação em Biodiversidade e Recursos Genéticos, Instituto de Ciências Agrárias de Vairão, Rua Padre Armando Quintas, 4485-661 Vairão, Portugal^b Departamento de Zoologia e Antropologia, Faculdade de Ciências, Universidade do Porto, Praça Gomes Teixeira 4099-002, Portugal^c Departamento de Bioquímica, Genética e Inmunología, Facultad de Biología, Universidad de Vigo, Vigo 36310, Spain^d Technical University of Braunschweig, Zoological Institute, Spielmannstr. 8, 38106 Braunschweig, Germany^e Zoologische Staatssammlung München, Münchhausenstr. 21, 81247 München, Germany

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1. Introduction

Geckos of the genus *Phelsuma* are amongst the most conspicuous lizards of the Malagasy region. The genus contains around 43 extant species, of which ca. 29 occur in Madagascar, and 25 of these are endemic to this island. *Rhopropella ocellata*, endemic to a small region of Namibia and South Africa is, together with *Lygodactylus* sp., the closest relative of *Phelsuma*, which is considered a monophyletic genus (Austin et al., 2004). With Madagascar as their main centre of diversity, *Phelsuma* are also present on almost all neighbouring islands and locally at the East African coast (Fig. 1). The Comoros harbour seven species (five of them endemic), the Mascarenes have seven endemic extant species (two extinct species are known: *P. gigas* and *P. edwardnewtoni*), with one subspecies endemic to Agalega island (1100 km north of Mauritius), one (non-endemic) species in two islands of the Aldabra archipelago, two endemic species in the granitic islands of the Seychelles, one endemic species in the island of Pemba off the East African coast of Tanzania, and one species in the Andaman islands, off Myanmar, in the Bay of Bengal. Within Madagascar, *Phelsuma* species are found in a variety of habitats spanning the island, mainly in primary forest regions, either dry southern seasonal forests (e.g. *P. breviceps*), scrublands (e.g. *P. mutabilis*, *P. modesta*, *P. hielscheri*), western coastal forests (e.g. *P. abbotti*), low and mid-altitude humid forests (*P. madagascariensis*, *P. guttata*, *P. lineata* ssp., *P. quadriocellata* ssp.) and even in some high-altitude regions (*P. barbouri*, *P. malamakibo*).

Despite extensive work on *Phelsuma* taxonomy, ecology, biogeography and ethology, even its alpha-taxonomy is not fully understood. Many taxa, especially subspecies, are based only on

chromatic characters and not well defined. In several cases color transitions/polymorphisms can be observed and thus some species may represent artificial taxa based on local color morphs. Previous studies, based on phenetic characters (Loveridge, 1942; Mertens, 1962; Glaw et al., 1999; Van Heygen, 2004), have led to recognition of eight species groups of Malagasy species. Close relationships between some of the groups were postulated (e.g. *P. guttata*- and *P. madagascariensis*-group and the Mascarene Islands species with the *P. modesta*-group) but some species were not assigned to any particular phenetic group (e.g. *P. vanheygeni*).

The main potential difficulty with *Phelsuma* taxonomy is thus its reliance on highly variable coloration characters, which has led to the description of a large number of species and subspecies. Taxon sampling has always been incomplete in previous molecular studies of *Phelsuma* (Radtkey, 1996; Austin et al., 2004; Sound et al., 2006; Rocha et al., 2007; Raxworthy et al., 2007; Harmon et al., 2008), making it difficult to evaluate intrageneric relationships. Additionally, the validity of many taxa is yet to be assessed using molecular markers.

Here, we present the most comprehensive molecular phylogenetic analysis of *Phelsuma* to date, based on a near-complete taxon sampling of all but two species, as well as most subspecies, and on a multilocus dataset including both fast-evolving mitochondrial genes and more moderate-to-slow-evolving nuclear DNA markers.

2. Materials and methods

2.1. Biological material, DNA isolation and sequencing

Tissue samples (tail tips) of 104 specimens of *Phelsuma* and five additional taxa (outgroup) were obtained during fieldwork in Madagascar, Seychelles, the Comoros and Tanzania in the period 2000–2006 and preserved in 70–100% ethanol. Additional samples were obtained from breeders. All recognized *Phelsuma* species, with the exception of *P. masohoala* and *P. guimbeaui*,

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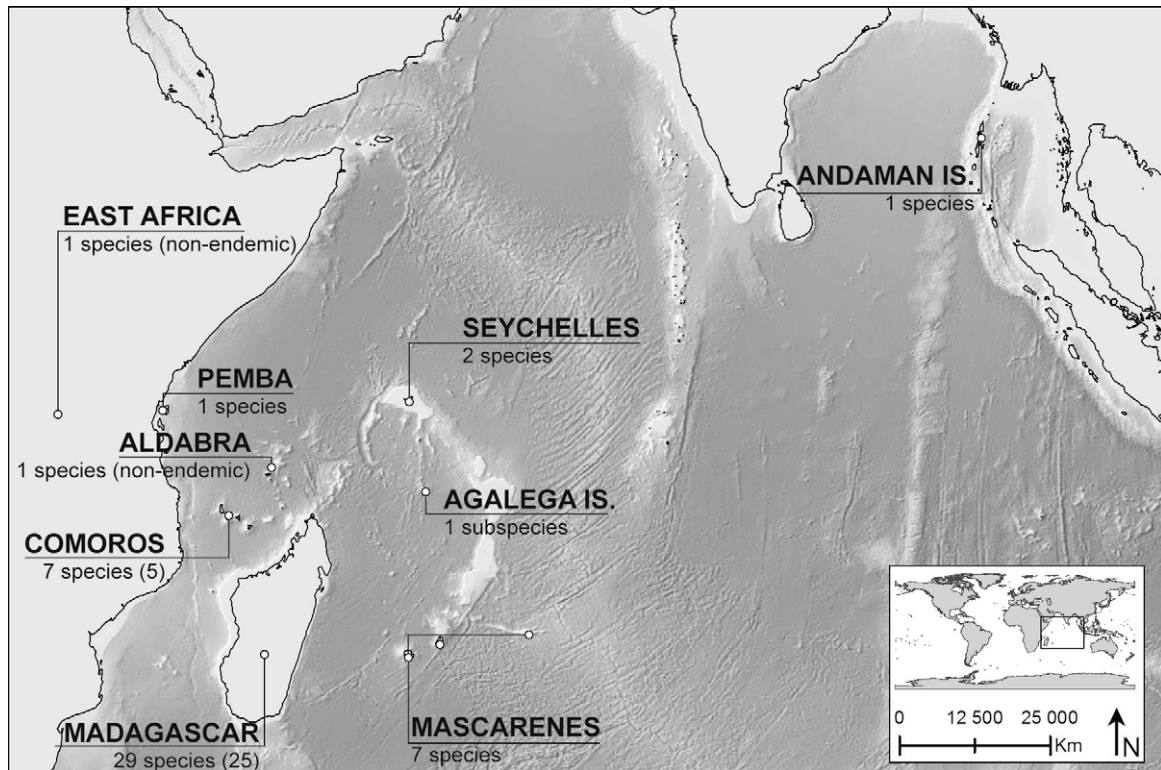


Fig. 1. Distribution of *Phelsuma* in the Indian Ocean Region and number of species per island/archipelago. The number of endemic species is given in parenthesis, when different from the total. Lighter (non-delimited) areas correspond to areas of lower sea-level.

and most recognized subspecies were represented (Table 1). We refrained from including partial sequences of *P. guimbeaui* available from Genbank (cytochrome *b*, 16S rRNA and *C-mos*) to avoid large amounts of missing data in our data set, and because this species clearly belongs into the well-studied Mascarene clade (Austin et al., 2004). For species with ample distributions, an effort was made to include samples from widespread locations to obtain preliminary information on intraspecific variation. DNA extraction followed standard salt or phenol-chloroform protocols (Kocher et al., 1989). Fragments of two mitochondrial (16s rRNA and cytochrome *b*) and three nuclear (*C-mos*, Rag-1 and Rag-2) genes were amplified via PCR. Primers used were 16sA-L and 16sB-H for 16srRNA (Palumbi et al., 1991); forward CBL14753 (Austin et al., 2004) and CBL14841 (Austin et al., 2004, modified from Kocher et al., 1989) and reverse CB3H (Palumbi et al., 1991) and CBH15579 (Rocha et al., 2007) for cytochrome *b*; G73 and G74 (Saint et al., 1998) for *C-mos*; L2408 and H2920 for Rag-1 (Vidal and Hedges, 2004) and Lung35F, Lung320R, Lung460R and 31FNVenk (Hoegg et al., 2004) for Rag-2. Amplified fragments were of 545, 714, 344, 473 and 796 bp, respectively, for 16srRNA, *Cyt-b*, *C-mos*, Rag-1 and Rag-2 fragments. All PCR amplifications were conducted in 25 μ l reactions using standard conditions. Annealing temperatures varied between 50 °C and 52 °C for 16srRNA, 42 and 53 °C for *Cyt-b*, 46 and 56 °C for *C-mos*, 52 and 57 °C for Rag-1 and 53 and 57 °C for Rag-2 and magnesium concentration varied between 1 and 4 mM. Amplification products were directly purified using a standard enzyme procedure or were sometimes cut from the gel and then purified using a gel band purification kit (Amersham Biosciences). Amplified fragments were sequenced on an ABI 3730xl automated capillary DNA sequencer. Sequences obtained for this study have been deposited in GenBank under the Accession Nos. FJ829886–FJ830328.

2.2. Sequence alignment and phylogenetic analyses

Cytochrome *b*, Rag-1 and Rag-2 gene sequences were aligned manually using BioEdit (Hall, 1999). Within *C-mos*, indels were present and thus nucleotide sequences were translated to amino acid sequences to aid the alignment. The 16srRNA nucleotide dataset contained highly variable regions and many indels, and therefore ProAlign version 0.5a3 (Löytynoja and Milinkovitch, 2003) was used. Hidden Markov Model parameters *delta* and *epsilon* were estimated from the data, while remaining parameters were set to their default values. All the columns with posterior probabilities lower than 90 were removed, for a final alignment of 384 bp. All protein-coding genes were translated to amino acids to check for stop codons that could indicate the presence of pseudogenes.

The concatenated data were filtered to remove redundant sequences, resulting in a data set with 84 sequences. The appropriate model of nucleotide substitution for each gene was determined using PAUP* 4.0b10 (Swofford, 2002) and Modeltest 3.06 (Posada and Crandall, 1998) under the AIC criterion (Akaike, 1974). Models were determined also for each codon position of the protein coding genes. χ^2 tests of sequence composition and likelihood mapping analysis (Strimmer and von Haesler, 1997), which allow an *a priori* examination of conflict versus support signal in molecular sequence data, were performed using Tree-Puzzle 5.0 (Schmidt et al., 2002).

Bayesian phylogenetic analyses were conducted using MrBayes 3.1.2 (Huelsenbeck and Ronquist, 2001). These began with a random starting tree and were run for 11,000,000 generations (20 million in the case of *Cyt-b*), and sampled every 1000 generations. Multiple runs were always performed. Convergence was checked using AWTY (Wilgenbusch et al., 2004), which provides plots of clade posterior probabilities (PPs) across post-burnin generations (cumulative function). Following Brown and Lemmon (2007), we

Table 1
Specimens used in this study, respective locations and Accession Nos.

Species	Individual	Location	GenBank Accession Nos.				
			16s	Cyt- <i>b</i>	Rag-1	Rag-2	C- <i>mos</i>
<i>P. abbotti abbotti</i>	AD1	Picard, Aldabra, 09° 24' 03.8" S, 46° 12' 21.6" E	FJ829886	FJ829974	FJ830147	FJ830238	FJ830055
<i>P. abbotti abbotti</i>	AD36	Grand Terre, Aldabra	FJ829887	FJ829975	FJ830148	FJ830239	FJ830056
<i>P. abbotti chekei</i>	FGZC 553	Ankarana, Madagascar	FJ829888	FJ829976	FJ830149	FJ830240	FJ830057
<i>P. abbotti chekei</i>	FGZC 795	Tsingy de Bemaraha, Madagascar, 18° 42' 31" S, 44° 43' 08" E	FJ829889	FJ829977	FJ830150	FJ830241	FJ830058
<i>P. abbotti chekei</i>	FGZC 947	Tsingy de Bemaraha, Madagascar, 18° 47' 03" S, 44° 46' 46" E; 177 m	FJ829890	FJ829978	FJ830151	FJ830242	FJ830059
<i>P. abbotti sumptio</i>	AP3	Assumption, 09° 44' 03.0" S, 46° 30' 01.2" E	FJ829891	FJ829979	FJ830152	FJ830243	FJ830060
<i>P. abbotti sumptio</i>	AP5	Assumption, 09° 44' 03.0" S, 46° 30' 01.2" E	FJ829892	FJ829980	FJ830153	FJ830244	FJ830061
<i>P. andamanense</i>	P-111	Andamans	FJ829893	FJ829981	FJ830154	FJ830245	FJ830062
<i>P. andamanense</i>	Pandamane	Andamans	FJ829894	FJ829982	FJ830155	FJ830246	FJ830063
<i>P. antanosy</i>	FGZC 2647	Ste. Luce, Madagascar, 24° 46.79' S, 47° 10.28' E	FJ829895	FJ829983	FJ830156	FJ830247	FJ830064
<i>P. astriata astriata</i>	AT6	Astove, 10° 04' 12.7" S, 47° 44' 20.4" E	FJ829896	FJ829984	FJ830157	FJ830248	FJ830065
<i>P. astriata astriata</i>	AT15	Astove, 10° 04' 12.7" S, 47° 44' 20.4" E	FJ829897	FJ829985	FJ830158	FJ830249	FJ830066
<i>P. astriata semicarinata</i>	APH1	Alphonse, 07° 00' 31.9" S, 52° 43' 50.4" E	FJ829898	FJ829986	FJ830159	FJ830250	FJ830067
<i>P. astriata semicarinata</i>	APH10	Alphonse, 07° 00' 31.9" S, 52° 43' 50.4" E	FJ829899	FJ829987	FJ830160	FJ830251	FJ830068
<i>P. barbouri</i>	FG/MV 2002-59	Antoetra, Madagascar	FJ829900	FJ829988	FJ830161	FJ830252	FJ830069
<i>P. berghofi</i>	ZCMV 518	Manombo Camp, Madagascar, 23° 01.699' S, 47° 43.892' E	FJ829901	FJ829989	FJ830162	FJ830253	FJ830070
<i>P. borbonica mater</i>	P-24	Basse Vallee, Reunion	DQ270574	FJ829990	FJ830163	FJ830254	FJ830071
<i>P. breviceps</i>	2000-564	Arboretum, Toliara, Madagascar	FJ829902	FJ829991	FJ830164	FJ830255	FJ830072
<i>P. cepediana</i>	PcepD	No loc. data	FJ829903	FJ829992	FJ830165	FJ830256	FJ830073
<i>P. comorensis</i>	GC22	Grand Comore, Comoros	FJ829904	DQ911694	FJ830166	FJ830257	FJ830074
<i>P. dubia</i>	Z33	Zanzibar, Tanzania	FJ829905	DQ911707	FJ830167	FJ830258	FJ830075
<i>P. dubia</i>	MH6	Moheli, Comoros	FJ829906	DQ911712	FJ830168	FJ830259	FJ830076
<i>P. dubia</i>	FG/MV 2002-2201	Ambanja, Madagascar	FJ829907	FJ829993	FJ830169	FJ830260	FJ830077
<i>P. ravenala</i>	PdubMan	Mananjary, Madagascar	FJ829908	FJ829994	FJ830170	FJ830261	FJ830078
<i>P. dubia</i>	FGZC 961	Antsalova, Madagascar	FJ829909	FJ829995	FJ830171	FJ830262	FJ830079
<i>P. dubia</i>	FGZC 962	Antsalova, Madagascar	FJ829910	FJ829996	FJ830172	FJ830263	FJ830080
<i>P. flavigularis</i>	Pflav	No loc. data	FJ829911	FJ829997	FJ830173	FJ830264	FJ830081
<i>P. flavigularis</i>	Pflav2	No loc. data	FJ829912	FJ829998	FJ830174	FJ830265	FJ830082
<i>P. grandis</i>	FG/MV 2002-2246	Antsiranana, Madagascar	FJ829913	FJ829999	FJ830175	FJ830266	FJ830083
<i>P. guentheri</i>	P-112	No loc. data	FJ829914	FJ830000	FJ830176	FJ830267	FJ830084
<i>P. guttata</i>	ZCMV 2172	Nosy Mangabe, Madagascar	FJ829915	FJ830001	FJ830177	FJ830268	FJ830085
<i>P. hielscheri</i>	FGZC 358	Isalo, Madagascar, 22° 35' 09" S, 45° 23' 57" E, 812 m	FJ829916	FJ830002	FJ830178	FJ830269	FJ830086
<i>P. inexpectata</i>	Pinexpect	No loc. data	FJ829917	FJ830003	FJ830179	FJ830270	FJ830087
<i>P. kely</i>	ZSM 607/2003	ca. 65 km south of Toamasina, Madagascar	FJ829918	FJ830004	FJ830180	FJ830271	FJ830088
<i>P. klemmeri</i>	Pklemm1	Ampasindava Peninsula, Madagascar	FJ829919	FJ830005	FJ830181	FJ830272	FJ830089
<i>P. klemmeri</i>	Pklemm2	Ampasindava Peninsula, Madagascar	FJ829920	FJ830006	FJ830182	FJ830273	FJ830090
<i>P. kochi</i>	FGZC 680	Tsingy de Bemaraha, Madagascar; 18° 42' 31" S, 44° 43' 08" E, 146 m	FJ829921	FJ830007	FJ830183	FJ830274	FJ830091
<i>P. kochi</i>	FG/MV 2002-797	Manongarivo, Madagascar	FJ829922	FJ830008	FJ830184	FJ830275	FJ830092
<i>P. kochi</i>	MV 2001-388	Ankarafantsika/Ampijoroa, Madagascar	FJ829923	FJ830009	FJ830185	FJ830276	FJ830093
<i>P. laticauda laticauda</i>	FG/MV 2002-2202	Ambanja, Madagascar	FJ829924	FJ830010	FJ830186	FJ830277	FJ830094
<i>P. laticauda laticauda</i>	FGZC 2705	Antalaha, Madagascar	FJ829925	FJ830011	FJ830187	FJ830278	FJ830095
<i>P. laticauda laticauda</i>	MY 44	Mayotte, Comoros	FJ829926	DQ911720	FJ830188	FJ830279	FJ830096
<i>P. laticauda laticauda</i>	Platic JG	Farquhars, Seychelles	FJ829927	FJ830012	FJ830189	FJ830280	FJ830097
<i>P. lineata dorsivittata</i>	FG/MV 2002-937	Montagne d Ambre, Madagascar	FJ829928	FJ830013	FJ830190	FJ830281	FJ830098

<i>P. lineata dorsivittata</i>	FGZC 488	Montagne d'Ambre, Madagascar, 12° 31' 37" S, 49° 10' 19" E, 1050 m	FJ829929	FJ830014	FJ830191	FJ830282	FJ830099
<i>P. lineata dorsivittata</i>	PlindB1	Voehemar/Iharana, Madagascar	FJ829930	FJ830015	FJ830192	FJ830283	FJ830100
<i>P. lineata dorsivittata</i>	PlindB2	Voehemar/Iharana, Madagascar	FJ829931	FJ830016	FJ830193	FJ830284	FJ830101
<i>P. lineata dorsivittata</i>	ZCMV 2029	Marojejy, Camp Simpona, Madagascar, 14° 26.199' S, 49° 44.601' E	FJ829932	FJ830017	FJ830194	FJ830285	FJ830102
<i>P. lineata lineata</i>	FG/MV 2002-299	Ranomafana, Madagascar	FJ829933	FJ830018	FJ830195	FJ830286	FJ830103
<i>P. lineata lineata</i>	FGZC 2634	Ste. Luce, Madagascar, 24° 46.79' S, 47° 10.28' E	FJ829934	FJ830019	FJ830196	FJ830287	FJ830104
<i>P. lineata lineata</i>	ZCMV 113	Ambohitsara, Madagascar; 21° 21.431' S, 47° 48.941' E	FJ829935	FJ830020	FJ830197	FJ830288	FJ830105
<i>P. lineata lineata</i>	ZCMV 813	Besariaka, Madagascar	FJ829936	FJ830021	FJ830198	FJ830289	FJ830106
<i>P. madagascariensis</i>	FG/MV 2002-500	Ambohitsara, Madagascar	FJ829937	FJ830022	FJ830199	FJ830290	FJ830107
<i>P. malamakibo</i>	FGZC 2556	Andohahela, Madagascar; 24° 32.642' S, 46° 42.847' E	FJ829938	FJ830023	FJ830200	FJ830291	FJ830108
<i>P. modesta modesta</i>	FGZC 56	Ambovombe, Madagascar	FJ829939	FJ830024	FJ830201	FJ830292	FJ830109
<i>P. modesta isakae</i>	FGZC 310	Ilasombe, Madagascar	FJ829940	FJ830025	FJ830202	FJ830293	FJ830110
<i>P. (modesta) leiogaster</i>	FG/MV 2002-1490	Toliara, Madagascar	FJ829941	FJ830026	FJ830203	FJ830294	FJ830111
<i>P. mutabilis</i>	FG/MV 2002-1494	Toliara, Madagascar	FJ829942	FJ830027	FJ830204	FJ830295	FJ830112
<i>P. mutabilis</i>	FGZC 350	Ejeda, Madagascar	FJ829943	FJ830028	FJ830205	FJ830296	FJ830113
<i>P. mutabilis</i>	FGZC 971	Antsalova, Madagascar	FJ829944	FJ830029	FJ830206	FJ830297	FJ830114
<i>P. sp. aff. mutabilis</i>	FGZC 881	Tsingy de Bemaraha, Madagascar; 18° 47' 03" S, 44° 46' 46" E, 177 m	FJ829945	FJ830030	FJ830207	FJ830298	FJ830115
<i>P. nigristriata</i>	2000/870	Mayotte, Comoros	FJ829946	FJ830031	FJ830208	FJ830299	FJ830116
<i>P. nigristriata</i>	Pnigris	Mayotte, Comoros	FJ829947	FJ830032	FJ830209	FJ830300	FJ830117
<i>P. ornata</i>	PornF1	No loc. data	FJ829948	FJ830033	FJ830210	FJ830301	FJ830118
<i>P. parkeri</i>	PB15	Pemba, Tanzania	FJ829949	DQ911750	FJ830211	FJ830302	FJ830119
<i>P. pasteurii</i>	MY65	Mayotte, Comoros	FJ829950	DQ911749	FJ830212	FJ830303	FJ830120
<i>P. pronki</i>	FGZC 2703	Moramanga region, Madagascar	FJ829951	FJ830034	FJ830213	FJ830304	FJ830121
<i>P. pusilla</i>	ZCMV 2174	Nosy Mangabe, Madagascar	FJ829952	FJ830035	FJ830214	FJ830305	FJ830122
<i>P. quadriocellata cf. bimaculata</i>	ZCMV 3211	Nosy Boraha, Madagascar	FJ829953	FJ830036	FJ830215	FJ830306	FJ830123
<i>P. quadriocellata parva</i>	ZCMV 375	Ifanadiana-Tolongoina, Madagascar, 21° 21.215' S, 47° 36.467' E	FJ829954	FJ830037	FJ830216	FJ830307	FJ830124
<i>P. quadriocellata parva</i>	FGZC 2640	Ste. Luce, Madagascar, 24° 46.50' S, 47° 09.05' E	FJ829955	FJ830038	FJ830217	FJ830308	FJ830125
<i>P. quadriocellata quadriocellata</i>	ZCMV 380	Ranomafana, Madagascar, 21° 15.699' S, 47° 27.571' E	FJ829956	FJ830039	FJ830218	FJ830309	FJ830126
<i>P. quadriocellata quadriocellata</i>	MV 2001-1060	Ambohimananarivo, Madagascar	FJ829957	FJ830040	FJ830219	FJ830310	FJ830127
<i>P. robertmertensi</i>	MY 73	Mayotte, Comoros	FJ829958	DQ911697	FJ830220	FJ830311	FJ830128
<i>P. rosagularis</i>	P-25	Gorges de la Riviere Noire, Mauritius	DQ270549	FJ830041	FJ830221	FJ830312	FJ830129
<i>P. seippi</i>	Pseippi	No loc. data	FJ829959	FJ830042	FJ830222	FJ830313	FJ830130
<i>P. serraticauda</i>	Pserratica	No loc. data	FJ829960	FJ830043	FJ830223	FJ830314	FJ830131
<i>P. standingi</i>	FGMV 2002-1562	Ifaty, Madagascar	FJ829961	FJ830044	FJ830224	FJ830315	FJ830132
<i>P. standingi</i>	FGMV 2002-2058	Ifaty, Madagascar	FJ829962	FJ830045	FJ830225	FJ830316	FJ830133
<i>P. sundbergi sundbergi</i>	PV3	Poivre, Seychelles	FJ829963	FJ830046	FJ830226	FJ830317	FJ830134
<i>P. sundbergi longinsulae</i>	MA1	Mahé, Seychelles	FJ829964	FJ830047	FJ830227	FJ830318	FJ830135
<i>P. sundbergi longinsulae</i>	CM5	Cosmoledo, 09° 42' 35.9"S, 47° 30' 28.4"E	FJ829965	FJ830048	FJ830228	FJ830319	FJ830136
<i>P. vanheygeni</i>	Pvanheig1	Madagascar	FJ829966	FJ830049	FJ830229	FJ830320	FJ830137
<i>P. v-nigra v-nigra</i>	MH 10	Moheli, Comoros	FJ829967	DQ911732	FJ830230	FJ830321	FJ830138
<i>P. v-nigra anjouanensis</i>	AJ 19	Anjouan, Comoros	FJ829968	DQ911736	FJ830231	FJ830322	FJ830139
<i>P. v-nigra comoraegrandensis</i>	GC 66	Grand Comore, Comoros	FJ829969	DQ911747	FJ830232	FJ830323	FJ830140
<i>Rhoptropella ocellata</i>	AMB5687	Namibia	FJ829970	FJ830050	FJ830233	FJ830324	FJ830141
<i>Lygodactylus luteopicturatus</i>	TZ2	Dar es Salaam, Tanzania	FJ829971	FJ830051	FJ830234	FJ830325	FJ830142
<i>Ailuronyx sp.</i>	2MA62	Mahé, Seychelles	FJ829972	n/a	FJ830235	FJ830326	FJ830143
<i>Ailuronyx seychellensis</i>	PL17	Praslin, Seychelles	n/a	FJ830052	n/a	n/a	n/a
<i>Ebenavia inunguis</i>	GC70	Grand Comore, Comoros	FJ829973	FJ830053	FJ830236	FJ830327	FJ830144
<i>Gehyra mutilata</i>	GmReu1	Reunion, Mascarenes	FJ613444	FJ830054	FJ830237	FJ830328	FJ830145

used Bayes Factors (BF) to identify the appropriate partitioning strategy for the coding genes. Because in all cases BFs clearly (>150, “very strong”; Kass and Raftery, 1995) favoured partitioning by codon position with rate variation, partitions by gene and codon position were used in the analysis of the concatenated dataset (hereinafter called *combined* dataset), that ran for 50,000,000 generations. Cumulative plots of PPs and their comparison across runs were also performed to assess convergence. Separate analyses of (1) the concatenated mtDNA fragments (*mitochondrial* dataset) and (2) the concatenated nuclear fragments (*nuclear* dataset) were also performed, and run for 30 and 25 million generations, respectively. Substitution-model parameters were always unlinked across partitions. Topology and branch lengths were linked across all partitions, but each partition was allowed to have its own rate (*prset* = variable).

Maximum likelihood (ML) analyses were performed on individual genes and on the *combined* dataset using GARLI (Zwickl, 2006) under the models specified by the AIC in Modeltest with 5000 to 1,000,000 generations after better scoring topology (*genthreshfortopterm*) as a termination condition (five different analyses for each dataset). As no significant differences in the topology were observed with increasing numbers of generations, bootstrap support was calculated using 5000 *genthreshfortopterm* for each replicate. Several independent GARLI runs, each starting with a different random tree topology, were performed. Because distant outgroup taxa can influence the relationships within the ingroup taxa, and correctly rooting rapid radiations may be difficult (Shavit et al., 2007), we also conducted ML searches without the outgroup taxa to ensure that incorrect rooting was not breaking otherwise supported relationships within the ingroup. We used SH-tests (Shimodaira and Hasegawa, 1999) as implemented in PAUP*, to test the fit of individual gene trees to the different partitions. SH-tests were implemented with the RELL approximation with 1000 bootstrap replicates.

3. Results and discussion

3.1. Alignment, gene composition, gene variability and phylogenetic analysis

After removing ambiguous positions in the 16srRNA gene dataset, 384 positions were used for phylogenetic inference. In the cytochrome *b*, *C-mos*, *Rag-1* and *Rag-2* datasets no positions were excluded. The analysed fragments had very different levels of variation. After excluding the outgroup taxa, 16s had 133 variable (34.6%) and 102 parsimony informative sites (PI: 26.6%), while *Cyt-b* had 431 variable (60.4%) and 425 PI sites (57.9%). *Rag-1* was the slowest evolving gene, with 60 variable and 33 PI sites (12.7% and 6.9%, respectively). *C-mos* and *Rag-2* showed intermediate values of variation: there were 53 (15.4%) variable and 34

(9.9%) PI sites at *C-mos* and 129 (16.2%) variable and 78 (9.9%) PI positions at *Rag-2*. Despite the overall low variation in the nuclear genes, they still exhibited considerable phylogenetic signal – 67.2%, 66.3% and 65.4% resolved quartet topologies respectively for “*C-mos*”, “*Rag-1*” and “*Rag-2*” – as revealed by the four-cluster likelihood mapping analysis. Moreover, combining all the genes increases the phylogenetic signal up to 95.7% (sum of quartets falling in “signal” regions in the triangular plot of probability vectors, in the *combined* dataset). A number of heterozygous positions were observed at the nuclear genes (36 at *C-mos*, 33 at *Rag-1* and 42 at *Rag-2*), and coded as ambiguities.

Analysis with and without the outgroup (the latter not shown) recovered the same ingroup relationships. Judging from the similar results compared across runs, GARLI was unlikely to have been trapped in local optima. For all individual and combined dataset estimates, ML and Bayesian topologies and support values were largely concordant (Supplementary Figs. 1–3). While the SH tests (Table 2) rejected the null hypothesis of congruence among most of the individual gene fragments used, no individual dataset rejected the *combined* topology. Fig. 2 represents the tree obtained from the *combined* dataset. We consider this tree as our best estimate of the phylogeny of *Phelsuma* (*mitochondrial* and *nuclear* phylogenies are also presented in Supplementary Fig. 2).

Highly supported clades (PP ≥ 99) in the combined analysis are depicted in different colors, and the same individuals are also identically color-coded in the mtDNA, nuclear and individual trees. The *mitochondrial* and *combined* datasets highly supported the same groups. Most of these were also recovered using the combined nuclear dataset, the few incongruent clades from the nuclear analysis being poorly supported (Suppl. Fig. 2).

3.2. Phylogenetic relationships among species of *Phelsuma*

Our analysis recovered eight clades whose differentiation appears to have been relatively simultaneous (clades J, K, N, P, Q, B, D and E, using node labeling from Fig. 2). Most of the clades reflect previously hypothesized relationships but several novel relationships were recovered:

- (1) The recently described *P. vanheygeni* is the sister-taxon to the Seychelles radiation, and related to members of the previously designated *P. guttata* and *P. madagascariensis* species groups (which all compose clade E);
- (2) *Phelsuma antanosy*, not previously included in any published molecular phylogeny, is closely related to *P. quadriocellata*, and together with *P. lineata*, *P. kely*, *P. pusilla* and *P. comorensis* is part of a strongly supported clade (J);
- (3) *Phelsuma laticauda* and *P. serraticauda* (and *P. antanosy*) are not closely related, and the previous *P. laticauda* species group (Glaw et al., 1999) does not represent a clade. Instead,

Table 2
Shimodaira-Hasegawa test *P*-values from the tests of gene trees obtained from analysis of different data partitions. Each value reflects the fit of different individual or combined gene trees (lines) to different data partitions (columns). *P*-values below 0.05 are indicated by an asterisk.

	DATA							
	16s	<i>Cyt-b</i>	mtDNA	<i>Rag-1</i>	<i>Rag-2</i>	<i>C-mos</i>	nucDNA	Combined
<i>TREES</i>								
16s	(best)	*	*	*	*	*	*	*
<i>Cyt-b</i>	0.322	(best)	0.819	*	0.050	*	*	0.632
mtDNA	0.579	0.802	(best)	*	*	*	*	0.629
<i>Rag-1</i>	*	*	*	(best)	*	*	*	**
<i>Rag-2</i>	*	*	*	*	(best)	*	0.237	*
<i>C-mos</i>	*	*	*	*	*	(best)	*	*
nucDNA	*	*	*	0.221	0.509	0.290	(best)	*
Combined	0.449	0.644	0.773	0.064	0.107	0.072	0.209	(best)

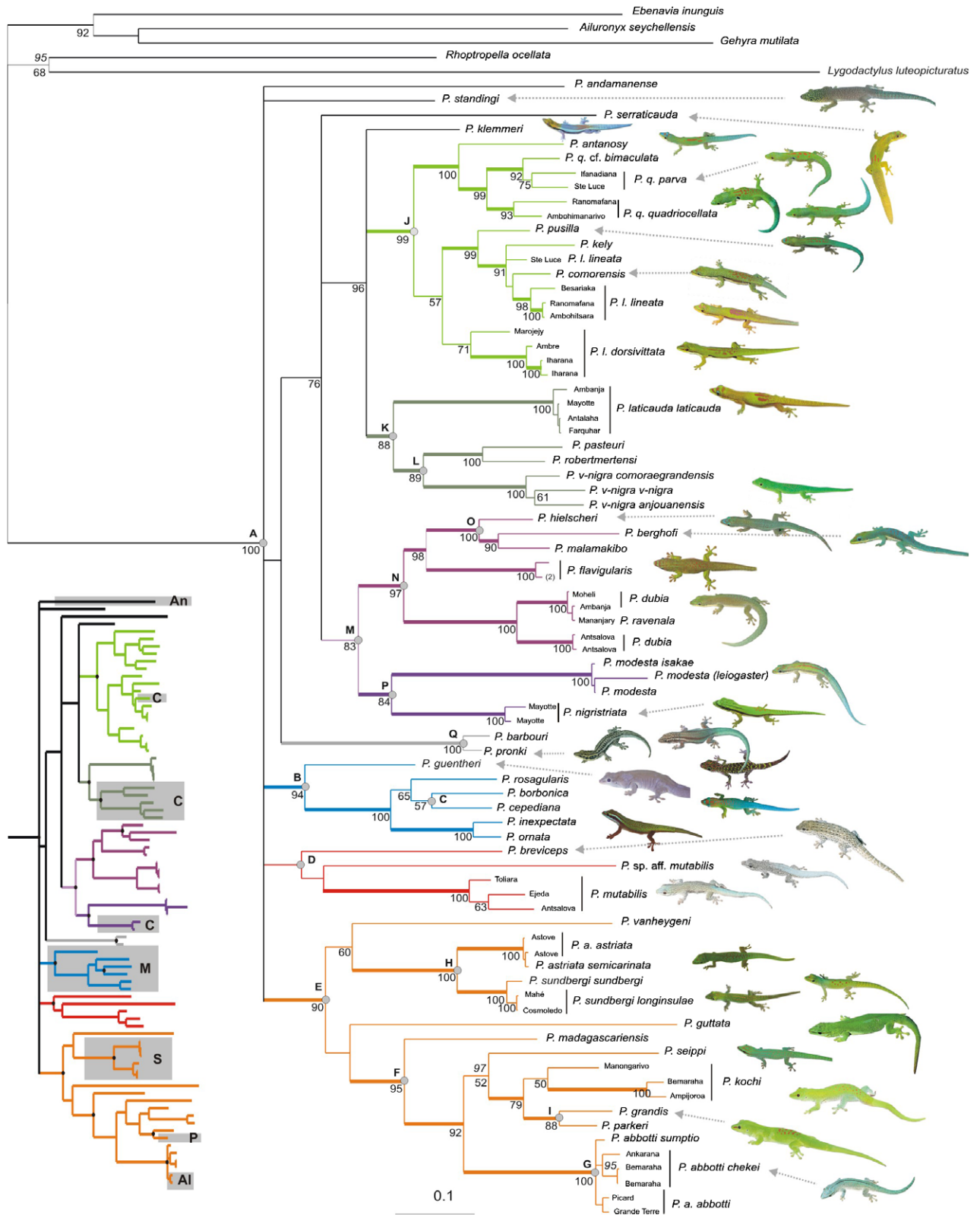


Fig. 2. Bayesian 50% majority-rule consensus phylogenetic tree of the combined data. Groups supported by PP \geq 99 are color coded (and discussed in detail in the text: blue lineage, Mascarenes group; red lineage, *P. mutabilis* group; grey lineage, *P. barbouri* and *P. pronki*; orange lineage, *P. guttata*- and *P. madagascariensis*-group; purple, *P. dubia* and *P. modesta* species group; yellow, *P. laticauda* species group and green lineage, *P. lineata* species group). Branches supported by PP \geq 99 are highlighted in bold. Bootstrap support values from ML analyses above 50 are given below corresponding branch. Relevant PP values above 95 are given above respective branches (italics). Node labels (A–M) given correspond to major clades discussed in the text. Inset figure in lower left corner reproduces schematically the same phylogenetic tree and indicates *Phelsuma* lineages not occurring on Madagascar: AI, Aldabra; An, Andamans; C, Comoros; M, Mascarenes; P, Pemba; S, Seychelles.

P. serraticauda represents an early diverging *Phelsuma* lineage and *P. laticauda* seems to be related to a group containing the endemic Comoran *P. v-nigra*. The separate position of *P. serraticauda* in our tree requires further study as it is in apparent conflict with the placement of this species in a clade with *P. laticauda*, *P. lineata* and *P. quadriocellata* in Austin et al. (2004), Raxworthy et al. (2007) and Harmon et al. (2008);

- (4) *Phelsuma klemmeri* is not closely related to *P. pronki* and *P. barbouri* (which do form a supported clade, Q), and;
- (5) *Phelsuma leiogaster* (sensu Glaw and Vences, 1994) is nested within *P. modesta*, confirming that these two taxa, which so far have not been included together in a molecular phylogeny, are probably conspecific (Nussbaum et al., 2000). They are closely related to *P. nigristriata* from the Comoros and not to the Mascarenes clade.

Beside these main novelties, our phylogeny contributes to clarifying *Phelsuma* systematics in various further aspects. Close relationships between *P. lineata*, *P. kely*, *P. quadriocellata* and *P. pusilla* (clade J) have been long hypothesized (e.g. Glaw et al. 1999) and were found for *P. lineata* and *P. quadriocellata* (the other taxa not included) in previous studies (Austin et al., 2004; Raxworthy et al., 2007; Harmon et al., 2008). The four species' close relationship is now also confirmed by our results, which also highlight that the species-level taxonomy in this clade needs to be revised. Indeed, a species status is probably warranted for several subspecies (Boumans et al., 2007).

Clade N, containing *Phelsuma dubia*, *P. flavigularis*, *P. malamakibo*, *P. berghofi* and *P. hielscheri*, largely corroborates previous classifications (Glaw et al., 1999; Van Heygen, 2004). The placement of *P. hielscheri* close to *P. lineata* and *P. laticauda* in the analysis of Raxworthy et al. (2007) is likely due to a confusion of samples, and/or use of a very short DNA fragment for *P. hielscheri* (193 bp). Our analysis further indicates a strong differentiation of western *P. dubia* specimens (Antsalova), rendering this species paraphyletic with respect to the recently described *P. ravenala* (Raxworthy et al., 2007) and indicating that the taxonomy of these species needs revision.

Clade Q, containing *Phelsuma barbouri* and *P. pronki*, was strongly supported by the mitochondrial and nuclear data sets, contradicting their most recent classification (Van Heygen, 2004).

Clade D contains three species from south-western (*P. breviceps*) and western (*P. mutabilis*, *P. sp. aff. mutabilis*) Madagascar. The close relationships between *P. breviceps* and *P. mutabilis* had previously been hypothesized based on morphometric and scale features (Loveridge, 1942). Our results support their distinctiveness, and suggest that *P. sp. aff. mutabilis* might be a distinctive undescribed species that occurs sympatrically with *P. mutabilis* in the Tsingy de Bemaraha area in western Madagascar (Glaw and co-workers, unpublished observations).

The placement of the previous *P. madagascariensis* subspecies (clade E) supports the proposal of Raxworthy et al. (2007) to elevate these taxa to species rank. The monophyly of the Seychelles species is here confirmed, with *P. vanheygeni* as the sister taxon of this clade. Lerner (2004) excluded *P. vanheygeni* from the *P. guttata* and *P. madagascariensis* species groups (and others) due to its egg-gluing behaviour, but our molecular data imply multiple independent evolution (and/or loss) of egg-gluing behaviour: species of *P. dubia* and *P. modesta* groups (except *P. nigristriata*), *P. barbouri* and *P. vanheygeni* are egg gluers.

Our analysis confirms previously recognized island species/lineage affinities and also the monophyly of both Mascarenes and Seychelles radiation, as well as the monophyly of the Comoran radiation comprising *P. pasteuri*, *P. robertmertensi* and *P. v-nigra*. *Phelsuma nigristriata* is most closely related to *P. modesta* from

Madagascar; thus, the Comoros underwent more independent colonisations (three) than any other archipelago. *Phelsuma andamanense* seems to be an ancient lineage, lacking clear relationships to any other extant lineage.

The complex geological island setting where *Phelsuma* evolved certainly provided numerous and unique opportunities for isolation and diversification. The phylogeny proposed here should lead to a better understanding of causes, patterns and dynamics of diversification within this genus and in Madagascar. This newly generated phylogeny is also a valuable framework for further research on *Phelsuma* relationships at a phylogeographic level.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ympmv.2009.03.032.

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