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Short communication

Phylogenetic affinities of Comoroan and East African day geckos (genus *Phelsuma*): Multiple natural colonisations, introductions and island radiations

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1. Introduction

The day-geckos of the genus *Phelsuma* (Gray, 1825) are amongst the most prominent reptiles from the Malagasy region. The whole genus consists of about 42 species, most of them inhabiting exclusively Madagascar. It is generally accepted that *Phelsuma* originated in Madagascar, and that from there they dispersed to several other Indian Ocean islands. In fact, they can be found on essentially every Indian Ocean archipelago: the Comoros, the Mascarenes, the Seychelles, Aldabra and even the Andamans. Having colonised these archipelagos they often radiated, producing several sets of endemic species. One of the species, *P. dubia*, mostly distributed on the western Malagasy coast, can also be found on mainland Africa, in coastal Kenya and Tanzania and in the islands of Zanzibar and Pemba (Loveridge, 1942). The island of Pemba also contains an additional endemic species, *P. parkeri* (Loveridge, 1941).

The Comoro islands, a set of four major volcanic islands located north-west of Madagascar, are inhabited by four endemic species (Fig. 1): *P. v-nigra* (with subspecies *v-nigra*, *anjouanensis*, *comoraegrandensis* and *pasteuri*), *P. nigristriata*, *P. robertmertensi* and *P. comorensis*. Additionally, *P. dubia* and *P. laticauda*, which are also found on Madagascar (and on the East African coast in the case of *P. dubia*), can be found on the Comoros (Glaw and Vences, 1994). This species abundance, as opposed to what happens for example in the Seychelles, inhabited by only two species of *Phelsuma*, is presumably due to the proximity of the Comoro Islands to Madagascar, and reflects once again the propensity of these geckos to long distance over-water dispersal.

In the absence of a thorough phylogenetic analysis, Glaw et al. (1999), in the only, so far, comprehensive study of this genus, defined nine Malagasy species groups, based on phenetic characters: *P. guttata*-group, *P. madagascariensis*-group, *P. lineata*-group, *P. mutabilis*-group, *P. laticauda*-group, *P. klemmeri*-group, *P. dubia*-group, *P. modesta*-group and *P. barbouri*-group.

Previous works by Radtkey (1996) and Austin et al. (2004) showed that both the Seychelles and the Mascarenes sets of species constitute endemic archipelago radiations, single monophyletic units that diversified as a result of both historical and ecological factors. Austin et al. (2004) also provided molecular support for at least one of the phenetic groups previously proposed, supporting close phylogenetic relationships between *P. astriatta*, *P. abbotti* and *P. madagascariensis* (*P. madagascariensis*-group). The other resolved clade in their analysis comprises *P. lineata*, *P. laticauda*, *P. quadriocellata* and *P. serraticauda*, species previously proposed to be included in two groups, *P. lineata*- and *P. laticauda*-group. However, none of these works included Comoroan species of *Phelsuma*. Thus, with the exception of the relationships within the Mascarene *Phelsuma*, the phylogenetic relationships between the extant members of the genus remain, so far, mostly unknown. *P. parkeri*, the endemic species from Pemba Island (off Tanzania) has never been included in any of the previous analyses.

Here, we used mitochondrial DNA sequence data (12S rRNA and cytochrome *b* gene fragments) from Comoroan and African (*P. parkeri*) species, together with previously published sequences from Austin et al. (2004), to assess

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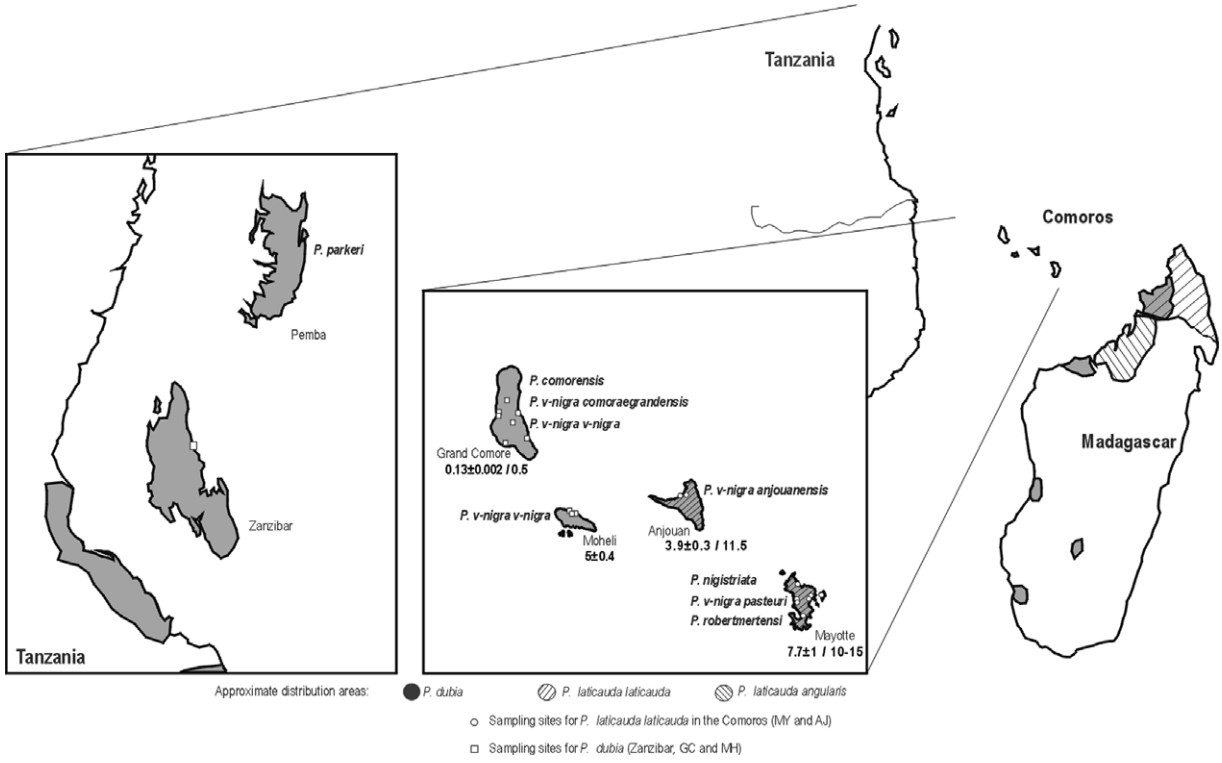

Fig. 1. Distribution of Comoroan and East African *Phelsuma* species sequenced in this study. The approximate distribution of the more widespread species (Glaw and Vences, 1994; Spawls et al., 2001) is indicated, respectively, by grey (*P. dubia*) and striped (*P. laticauda*) areas. Island ages estimates (age of the oldest exposed lavas/estimated age of the volcanic origin of the islands) follow Montaggioni and Nougier (1981); Emerick and Duncan (1982) and Nougier et al. (1986). Small white circles and squares represent, respectively, location of samples of *P. laticauda* and *P. dubia* in the Comoros and Zanzibar.

their position within the genus *Phelsuma* and to obtain broad estimates of within-species divergence of these island endemics. Furthermore, for the widespread species, *P. dubia* and *P. laticauda*, we used cytochrome *b* sequences to analyse the relationships between Malagasy, Comoroan and African haplotypes, in order to determine if these latter localities were the results of natural colonisations or anthropogenic introductions. Likelihood and Bayesian methods, and available information about island ages were also used to estimate divergence times and to test for congruency between previous divergence time estimates of main clades made by Austin et al. (2004) and others obtained using other islands ages as calibration points, and to test for congruence between obtained values using different calibration schemes.

2. Materials and methods

Geographic origins of samples used in this study are listed in Table 1. Fifty-eight *Phelsuma* tissue samples were collected from the Comoros archipelago and the East African islands of Zanzibar and Pemba. All the species from these islands are represented, with the exception of *P. nigristriata*, an endemic species from Mayotte Island. Total genomic DNA was extracted from muscle tissue (autotomized tails) preserved in pure ethanol using standard methods (Sambrook et al., 1989). Segments of two mitochondrial genes were amplified (12s rRNA, ~370 bp and cytochrome *b*, 714 bp) using universal primers 12Sa and 12Sb (Kocher et al., 1989) and CBL14753 (Austin et al., 2004) and CBH15579 (5′-TGG GAT TGA TCG TAG GAT GGC GTA-3′, Salvador Carranza, personal communication), respectively. Amplification of the 12S rRNA gene fragment followed Harris et al. (1998) and that of cytochrome *b* (cyt *b*) followed Austin et al. (2004), except that 30 s were used for annealing with temperatures varying between 44 and 53 °C depending on the species. Amplification products were directly purified using a standard enzymatic procedure, except for amplification products of cytochrome *b* from *P. comorensis*, for which unspecific bands of around 300 bp were always present and thus bands of the expected cyt *b* size were cut from the gel and then purified with a gel band purification kit (Amersham, Biosciences). Amplified fragments were sequenced on a 310 Applied Biosystem DNA sequencing apparatus (12S fragments) and on a 3730xl DNA Analyser (cyt *b*) and sequences aligned using the ClustalW (Thompson et al., 1994) application from BioEdit (Hall, 1999) with default parameters, against published sequences of other *Phelsuma* species (Austin et al., 2004). *Lygodactylus* sp., *Rhoptropella ocellata* and *Rhoptropus boultoni* (also from Austin et al., 2004) were used as outgroup.

For the cytochrome *b* gene fragment alignment was unambiguous, while the 12S rRNA gene fragment was more difficult to reliably align between species due to highly variable loop regions in the secondary structure. Thus,

Table 1
Specimens used in this study, respective locations (see Carretero et al., 2005) and accession numbers. First two letters of individual's code correspond to island of origin (AJ = Anjouan, GC = Grand Comoro, MH = Moheli, MY = Mayotte, PB = Pemba and Z = Zanzibar)

Species	Individual	Location	Accession Number 12S/Cytb
Phelsuma comorensis	GC22	Foret de La Guille	DQ911636/DQ911694
Phelsuma comorensis	GC24	Foret de La Guille	DQ911637/DQ911695
Phelsuma comorensis	GC23	Foret de La Guille	DQ911638/DQ911696
Phelsuma robertmertensi	MY73	Passamenti	DQ911639/DQ911697
Phelsuma robertmertensi	MY84	Longoni	DQ911640/DQ911698
Phelsuma robertmertensi	MY85	Longoni	DQ911641/DQ911699
Phelsuma robertmertensi	MY23	M'zouazia	DQ911642/DQ911700
Phelsuma robertmertensi	MY24	M'zouazia	DQ911643/DQ911701
Phelsuma dubia	GC9	Moroni	DQ911644/DQ911702
Phelsuma dubia	GC32	Itsoundzou	DQ911645/DQ911703
Phelsuma dubia	GC44	Foumbouni	DQ911646/DQ911704
Phelsuma dubia	MH5	Fomboni	DQ911647/DQ911705
Phelsuma dubia	MH33	Mbouerani	DQ911648/DQ911706
Phelsuma dubia	Z33	Kiwengwa, Zanzibar (Tanzania)	DQ911649/DQ911707
Phelsuma dubia	GC38	Mouadzazi	DQ911650/DQ911708
Phelsuma dubia	GC39	Mouadzazi	DQ911651/DQ911709
Phelsuma dubia	GC50	Konbani	DQ911652/DQ911710
Phelsuma dubia	GC53	Mvouni	DQ911653/DQ911711
Phelsuma dubia	MH6	Fomboni	DQ911654/DQ911712
Phelsuma dubia	MH34	Mbouerani	DQ911655/DQ911713
Phelsuma dubia	MH29	Fomboni	DQ911656/DQ911714
Phelsuma dubia	MH36	Mbatsé	DQ911657/DQ911715
Phelsuma dubia	MH38	Mbatsé	DQ911658/DQ911716
Phelsuma dubia	GC62	Belvedere (Karthala)	DQ911659/DQ911717
Phelsuma dubia	GC63	Belvedere (Karthala)	DQ911660/DQ911718
Phelsuma dubia	GC a	Moroni, Pension Laizam	DQ911661/DQ911719
Phelsuma laticauda	MY44	Chiroungoui	DQ911662/DQ911720
Phelsuma laticauda	MY6	Mamoutzu	DQ911663/DQ911721
Phelsuma laticauda	AJ35	Moutsamoudou	DQ911664/DQ911722
Phelsuma laticauda	MY7	Mamoutzu	DQ911665/DQ911723
Phelsuma laticauda	MY8	Mamoutzu	DQ911666/DQ911724
Phelsuma laticauda	MY50	Ouangani	DQ911667/DQ911725
Phelsuma laticauda	MY56	Sada road	DQ911668/DQ911726
Phelsuma laticauda	AJ10	Bazimini	DQ911669/DQ911727
Phelsuma laticauda	AJ11	Bazimini	DQ911670/DQ911728
Phelsuma laticauda	MY74	Passamenti	DQ911671/DQ911729
Phelsuma laticauda	MY86	Longoni	DQ911672/DQ911730
Phelsuma laticauda	MY87	Dzaouzi (near airport)	DQ911673/DQ911731
Phelsuma laticauda	(Platic)*	Madagascar	AY221280/AY221386
Phelsuma v-nigra v-nigra	MH10	Badjo, mountain	DQ911674/DQ911732
Phelsuma v-nigra v-nigra	MH19	Gnombéni	DQ911675/DQ911733
Phelsuma v-nigra v-nigra	MH22	Ouanani	DQ911676/DQ911734
Phelsuma v-nigra anjouanensis	AJ34	Houngouni	DQ911677/DQ911735
Phelsuma v-nigra anjouanensis	AJ19	Foret de Moya	DQ911678/DQ911736
Phelsuma v-nigra comoraegrandensis	GC10	Moroni	DQ911679/DQ911737
Phelsuma v-nigra comoraegrandensis	GC11	Moroni	DQ911680/DQ911738
Phelsuma v-nigra comoraegrandensis	GC12	Moroni	DQ911681/DQ911739
Phelsuma v-nigra comoraegrandensis	GC31	Itzanzéni	DQ911682/DQ911740
Phelsuma v-nigra comoraegrandensis	GC49	Bandanadji	DQ911683/DQ911741
Phelsuma v-nigra comoraegrandensis	GC51	Konbani	DQ911684/DQ911742
Phelsuma v-nigra comoraegrandensis	GC54	Mvouni	DQ911685/DQ911743
Phelsuma v-nigra comoraegrandensis	GC55	Mvouni	DQ911686/DQ911745
Phelsuma v-nigra comoraegrandensis	GC56	Mvouni	DQ911687/DQ911744
Phelsuma v-nigra comoraegrandensis	GC65	Belvedere (Karthala)	DQ911688/DQ911746
Phelsuma v-nigra comoraegrandensis	GC66	Belvedere (Karthala)	DQ911689/DQ911747
Phelsuma v-nigra comoraegrandensis	GC 21	Mouadja	DQ911690/DQ911748
Phesuma v-nigra pasteuri	MY65	Koualé	DQ911691/DQ911749
Phelsuma parkeri	PB15	Jondeni, Pemba (Tanzania)	DQ911692/DQ911750
Phelsuma parkeri	PB17	Jondeni, Pemba (Tanzania)	DQ911693/DQ911751
Phelsuma standingi	*	Madagascar	AY221281/AY221387
Phelsuma astriatta	*	Mahé, Seychelles	AY221273/AY221379
Phelsuma abbotti	*	Aldabra attol	AY221285/AY221391

(*continued on next page*)

Table 1 (*continued*)

Species	Individual	Location	Accession Number 12S/Cytb
Phelsuma madagascariensis kochi	*	Madagascar	AY221276/AY221382
Phelsuma madagascariensis grandis	*	Madagascar	AY221274/AY221380
Phelsuma madagascariensis grandis	*	Madagascar	AY221275/AY221381
Phelsuma andamanensis	*	Windoor, Andamanes	AY221277/AY221383
Phelsuma mutabilis	*	Madagascar	AY221272/AY221378
Phelsuma guentheri	*	Mascarenes	AY221311/AY221442
Phelsuma borbonica borbonica	*	Mascarenes	AY221289/AY221395
Phelsuma cepediana	*	Mascarenes	AY221294/AY221400
Phelsuma guimbeaui	*	Mascarenes	AY221330/AY221461
Phelsuma rosagularis	*	Mascarenes	AY221327/AY221458
Phelsuma ornata ornata	*	Mascarenes	AY221323/AY221454
Phelsuma serraticauda	*	Madagascar	AY221278/AY221384
Phelsuma quadriocellata	*	Madagascar	AY221282/AY221388
Phelsuma lineata	*	Madagascar	AY221279/AY221385
Rhoptropella ocellata	*	SW Africa	AY221271/AY221377
Rhoptropus boultoni	*	Twyfelfontein, Namibia	AY221269/AY221375
Lygodactylus sp.	*	Southern Africa	AY221270/AY221376

* Sequences from Austin et al. (2004).

GBlocks v0.91b (Castresana, 2000) was used to eliminate poorly aligned positions and extremely variable regions, with the following parameters: IS = 25/FS = 30/CP = 2/BL2 = 5/allowed gap position = with half; resulting in a 341 bp alignment. The Akaike Information Criterion (AIC) in Modeltest3.06 (Posada and Crandall, 1998; Posada and Buckley, 2004) was used to select an appropriate model of nucleotide substitution given the model likelihoods estimated in PAUP* (Swofford, 2000). Sequence datasets were analyzed both separately (each gene) and combined (1055 bp). For phylogeny estimation sequences from both mitochondrial genes were concatenated and collapsed into haplotypes using Collapse1.2 (a program by D. Posada, available at http://darwin.uvigo.es) with gaps treated as a fifth state. Generated datasets were used to reconstruct phylogenies using Maximum Likelihood (ML) and Bayesian (BMCMC) methods, as implemented in PHYML (Guindon and Gascuel, 2003) and MrBayes (Huelsenbeck and Ronquist, 2001) v3.1, respectively. Confidence in the ML resulting relationships was assessed using the nonparametric bootstrap technique (1000 bootstrap replicates). For the BMCMC, a combined analysis was performed in which each mitochondrial gene had independent substitution parameters and branch lengths, but the same underlying topology. Two runs with four independent chains (with default heating values) were implemented, and checked for convergence. Both in ML and BMCMC analyses model parameters values were treated as unknown and estimated during the runs. For the BMCMC, each Markov chain started with a random tree and was run for 11×10^6 generations, sampling every 1000 generations. Burnin values were always of 1001 trees, long after the log-likelihood of each run reached stability. The posterior probabilities (pP) for individual clades obtained from each analysis were checked for congruence and both analysis combined and summarized in a 50% majority-rule consensus.

Clock-like evolution of mtDNA sequences was tested by comparing likelihood scores of ML trees unconstrained and under the molecular clock. In each case, the molecular clock was rejected, and therefore divergence times were estimated using the Bayesian method of Thorne and Kishino (2002) implemented in the multidivtime package (http://statgen.ncsu.edu/thorne/multidivtime.html). This program can accommodate multiple calibration points and multiple genes, which are expected to provide improved estimates of both divergence time and rates of evolution. In this method, branch lengths are estimated without assuming a molecular clock and then times and rates are estimated by minimizing the discrepancies in branch lengths and the rate changes over branches. This method also has the added advantage of allowing the specified calibrations as upper or lower bounds on node ages. As prior ages we adopted 5 million years for the ingroup root depth (rttm, SD = 5 Myr), following Austin et al. (2004), and 0.06 substitution per site per million year for the rate at the root node (rtrate), also with a standard deviation of 0.06 (rtratesd). The rtrate and rtratesd parameters were estimated as suggested in the multidivtime manual (Rutshmann, 2005). The Brownian rate parameter was set to the default value. The MCMC analysis involved an initial burn-in of 100,000 cycles, after which the chain was sampled 10,000 times, every 100th cycle. The R8s (version 1.7) computer program (Sanderson, 1997, 2002) was also used to obtain rough estimates of divergence times, using the same calibration schemes. This program implements several methods for estimating absolute rates of molecular evolution, ranging from standard maximum likelihood methods to more experimental semiparametric and nonparametric methods that relax the stringency of the clock assumptions using smoothing methods. One of the advantages of this program is that it provides a cross-validation test that allows the user to explore the fidelity with which any of these methods explains the branch length variation, by removing each terminal branch in turn, estimating the remaining parameters of the model without the branch, predicting the expected numbers of substitution on the pruned branch and reporting the performance of this

predictions as a cross validation score. This allows the user to select the method that best explains the branch length variation (Sanderson, 2002).

Calibration times for the divergence time analysis were accommodated differently in each analysis. Island ages represent maximum ages for colonisation, i.e. upper calibration limits, the maximum age for monophyletic group inhabiting that respective island. Multidivtime allows the specification of calibration points as upper and lower limits on node ages, but it needs at least one upper and one lower limit to provide reasonable age estimates. Thus, in the absence of any appropriate lower age limit, we decided to use each island age and island age –0.5 million years as upper and lower bounds on age node priors. When using r8s to estimate node ages we introduced island ages as fixed calibration points.

For the widespread species *P. laticauda* and *P. dubia*, and because the observed level of divergence was low, haplotype networks were constructed using the program TCS v1.21 (Clement et al., 2000).

3. Results and discussion

3.1. Phylogenetic analysis

According to the AIC; the General Time Reversible model (GTR, e.g. Yang, 1994) was the best fitting model, with a gamma distribution (+G) for rate heterogeneity for 12S, and with both G and a proportion of invariable sites (+I) for Cyt *b*.

Both ML and BMCMC analyses of the combined dataset yielded highly similar estimates of the phylogenetic relationships (Fig. 2a). Although deeper relationships could not be resolved, having very low bootstrap support values (not shown), the phylogenetic affinities of the majority of the Comoroan and African species with remaining *Phelsuma*

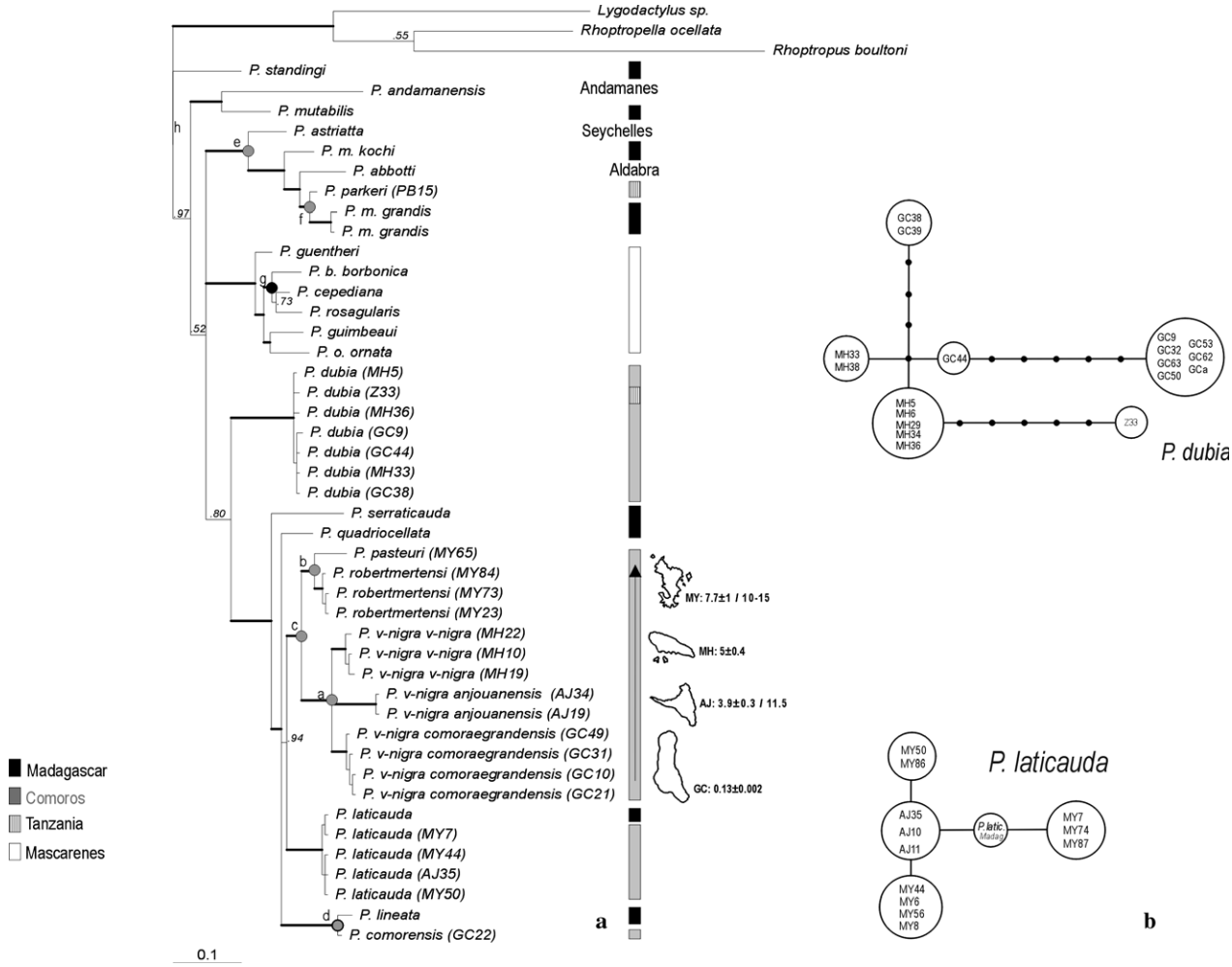

Fig. 2. (a) Phylogenetic relationships of the studied *Phelsuma* species inferred from Bayesian analysis of 1055 bp of mtDNA data. Branches in bold indicate well supported nodes with Bayesian Posterior probabilities (pP) of 1. Other important pP values are signalled in the figure (in italics). The Maximum Likelihood analysis (not shown) resulted in a similar tree topology with all the supported groups being the same. The black dot indicates the main calibration node (2.1 Myr) and grey nodes (numbered) indicate nodes for which age estimates are given, both from multidivtime and r8s software packages. Results of age estimates can be found on Table 2. (b) Haplotype networks for *Phelsuma laticauda* and *Phelsuma dubia* based on 714 bp of cytochrome *b*. Circle size is proportional to the number of individuals exhibiting the haplotype and individual code is given inside each circle (sample locations given in Table 1 and shown in Fig. 1). Small black dots represent missing/not sampled haplotypes.

included in the analysis could be estimated with high confidence. According to these analyses, *Phelsuma parkeri*, the endemic species from Pemba Island (Tanzania) clearly belongs to the *P. madagascariensis*-group (with *P. astriatta*; *P. madagascariensis* and *P. abbotti*), being consistently placed within *P. madagascariensis*, more closely related to *P. madagascariensis grandis*, from north Madagascar from which it differs by 6.77%, compared to the 12.7% sequence divergence between *P. madagascariensis grandis* and *P. madagascariensis kochi* (12S and Cyt *b*, uncorrected *p*-distances). This is the second species, together with *P. abbotti*, to be placed within *P. madagascariensis*. With four subspecies with allopatric distributions, *P. madagascariensis* is one of the most widely distributed species in Madagascar, and again here, evidence of its paraphyly further supports the treatment of at least *P. m. grandis* and *P. m. kochi* as separate species. The status of other extant subspecies remains unknown pending further molecular studies. *Phelsuma parkeri* thus represents another example within this genus of an ancient and natural transmarine dispersal and colonisation, in this case from Madagascar to Pemba Island.

The Comoroan endemic species *P. v-nigra* and *P. robertmertensi,* interestingly, do form a single clade, with very high support values. This clade is further subdivided in two groups also strongly supported: *P. (v-nigra) pasteuri* with *P. robertmertensi* (with a 1.0 posterior probability and 0.95 bootstrap value (not shown)), and the remaining subspecies of *P. v-nigra*. The nucleotide sequence divergence between *P. (v-nigra) pasteuri* and the other *P. v-nigra* subspecies ranges from 12.4 to 14.3% (uncorr. *p*-dist.), while the distances between *P. v-nigra v-nigra*, *P. v-nigra anjouanensis* and *P. v-nigra comoraegrandensis* vary between 6.5 and 7.8%. The observations above indicate that *P. v-nigra* is also paraphyletic and that *P. (v-nigra) pasteuri* should be treated as a full species, as already proposed by Meirte (2004). *Phelsuma robertmertensi* is thus the most closely related species to *P. pasteuri*, with 9.7–9.9% sequence divergence. With both species being endemic to the island of Mayotte, if this phylogeny estimate continues to be supported with the addition of the remaining species of this genus, it may represent a within-island speciation phenomenon. Within the Comoros, so far, these are the only reptile taxa exhibiting this pattern, since neither *Hemidactylus* (Rocha et al., 2005) nor *Cryptoblepharus* skinks (Rocha et al., 2006) present such a structure. Mayotte has a complex topography, with several volcanoes and also a complex geological history with at least three different active volcanic phases recognized. Lava sample ages span a range of around 5 Myr, with the most recent parts of the island dating from at least 1.53 ± 0.1 Myr. *Phelsuma robertmertensi* is found in forested coastal localities while *P. pasteuri* inhabits inland forest and plantations (Carretero et al., 2005), but their distribution ranges are not well known. Without a better knowledge regarding their ecological preferences, if they occur or not in sympatry and if recent volcanic phenomena on Mayotte could have caused isolation of populations, any hypothesis about their speciation mechanism is preliminary. As to *P. v-nigra* subspecies, the branching pattern is not resolved, preventing the reconstruction of the patterns of island colonisation. Taking into account the similar levels of sequence divergence between the subspecies, the three islands might have been colonised by a common ancestor at roughly the same time. Whether these Comoroan species are a monophyletic unit or two, or the identity of their respective sister species, is still not clear, and will only be known when all *Phelsuma* species are included in a comprehensive phylogeny of the genus.

Phelsuma comorensis, the endemic species from Grand Comoro, is clearly the sister taxa of *P. lineata*, which comprises five subspecies, three of them with very restricted distributions, while the others, *P. lineata lineata* and *P. lineata elanthana*, are widespread in the eastern Malagasy coast. Nucleotide sequence divergence between *P. comorensis* and the *P. lineata* specimen analyzed is only 3.6%, a very low value, compared to the degree of divergence between other species or even subspecies, which implies that a more recent colonisation and speciation event gave rise to this Grand Comoro endemism.

Concerning *P. dubia* and *P. laticauda*, both present in Madagascar and the Comoros, and in the case of *P. dubia,* also in the East African coast, the levels of differentiation between individuals from different locations/islands is minimum, without any clear geographic structure. Such a pattern most probably reflects very recent human-aided dispersals, rather than natural colonisations (Fig. 2b). Nevertheless, within *P. dubia* the level of differentiation is higher than within *P. laticauda*, where all the haplotypes are just one mutation step away from the others (cytochrome *b* data). *P. laticauda laticauda* (the same subspecies that occurs on Comoros) has a very restricted distribution in the humid regions of northern Madagascar, while *P. dubia* occurs along the western coast of Madagascar, possibly with disjunct populations (Glaw and Vences, 1994). Therefore, it is not surprising that, despite having originated from recent introductions, the Comoroan populations of *P. dubia* exhibit more genetic variation than *P. laticauda*, simply reflecting the diversity within the "source" populations. Both species are the ones with the largest distribution areas outside Madagascar, and *P. laticauda* has been introduced even onto the Hawaii islands. It would be interesting to further examine African populations of *P. dubia* to accurately understand their origin. Certainly, the fact that both species inhabit a wide variety of habitats, including human buildings, with palms and banana trees as their preferred vegetation (Carretero et al., 2005), influences the extent to which they can be subject to human-aided dispersals, making them some of the most prone species to this kind of phenomena within the genus.

3.2. Divergence time estimates

For the relaxed Bayesian analyses performed in multidivtime, as mentioned above, we allowed some variation in island colonisation age; specifying each calibration as an

Table 2
Posterior estimates of divergence ages (±standard deviation) and 95% credibility intervals for some of the nodes inferred from the concatenated dataset using the Bayesian relaxed molecular clock implemented in multidivtime and the Langley–Fitch method implemented in r8s

Clade, node id	Calibration					
	2.1–1.6 Myr on node g *(2.1 on node g for LF)*		2.1–1.6 Myr on node g 0.5–0 Myr on node d *(2.1 on node g and 0.5 on node d for LF)*		0.5–0 Myr on node d *(0.5 on node d for LF)*	
	Age ± SD	95 % CI	Age ± SD	95 % CI	Age ± SD	95 % CI
Bayesian relaxed clock (MULTIDIVTIME) *Likelihood LF (r8s) (estimated ages only)*						
P. v-nigra, **a**	1.12 ± 0.53 *1.26*	[0.38–2.39]	1.10 ± 0.46 *1.23*	[0.35–2.1]	0.32 ± 0.28 *1.02*	[0.05–1.04]
P. robertmertensi + *P. pasteuri*, **b**	1.05 ± 0.54 *1.99*	[0.3–2.33]	0.95 ± 0.5 *1.94*	[0.28–2.08]	0.29 ± 0.26 *1.94*	[0.05–1]
P. robertmertensi + *P. pasteuri* + *P. v-nigra*, **c**	1.64 ± 0.68 *2.98*	[0.65–3.29]	1.49 ± 0.59 *2.90*	[0.61–2.96]	0.47 ± 0.39 *2.39*	[0.06–0.41]
P. lineata + *P. comorensis*, **d** *(calibration)*	0.48 ± 0.36 *0.62*	[0.03–1.4]	0.27 ± 0.13 —	[0.02–0.48]	0.13 ± 0.1 —	[0.06–0.4]
P. astriatta, **e**	3.18 ± 1.05 *4.96*	[1.62–5.62]	3.02 ± 0.95 *4.84*	[1.57–5.31]	0.97 ± 0.73 *3.99*	[0.21–2.80]
P. parkeri, **f**	1.03 ± 0.53 *1.40*	[0.28–2.36]	0.98 ± 0.50 *1.37*	[0.26–2.18]	0.31 ± 0.27 *1.13*	[0.04–0.99]
P. borbonica, **g** *(calibration)*	1.81 ± 0.14 —	[1.61–2.08]	1.8 ± 0.14 —	[1.61–2.08]	0.45 ± 0.4 *1.68*	[0.07–1.34]
Ingroup root, **h**	4.95 ± 1.43 *6.95*	[2.91–8.44]	4.68 ± 1.28 *6.7*	[2.82–7.77]	1.55 ± 1.10 *5.59*	[0.35–4.29]

interval, with island age as the upper limit and island age—0.5 Myr as the lower limit. Thus, we used the same calibration point of Austin et al. (2004), i.e., 2.1 Myr of the oldest rocks of Reunion under the assumption that the ancestor of *P. borbonica* colonised it soon after island formation, and estimates for remaining island colonisations (Comoros) were obtained. Additionally we used the estimated age of the volcanic origin of Grand Comoro (0.5 Myr) as a calibration point on node d (*P. lineata* + *P. comorensis*, Fig. 2), assuming that *P. comorensis* colonised Grand Comoro also soon after its emergence. Node ages were estimated using either each calibration point alone or both together. The cross-validation test implemented in R8s indicated that branch length variation was explained with the highest fidelity by the Langley–Fitch method (LF) (Langley and Fitch, 1973, 1974), which uses maximum likelihood to reconstruct divergence times under the assumption of a molecular clock. As a result of that, the LF method was run with the TN algorithm (which is the best and fastest for use with LF—Sanderson, 2004). Ages of some relevant nodes obtained under the different methods and calibration schemes are given on Table 2.

Our absolute age estimates do vary considerably depending on the calibration point and the method used, but this variation is higher for deeper nodes than for recent ones, where this variability is considerably reduced. However, relative ages are somewhat consistent across methods and calibrations. Number and phylogenetic distribution of calibrations are known to severely affect estimates, and using only younger calibrations may result in severe underestimation of older node ages (Porter et al., 2005). The lack of consistency in our estimates is most likely due to the lack of more calibrations points, and therefore results must be taken cautiously. Nevertheless, these estimates are roughly in agreement with previous studies that imply that diversification of main *Phelsuma* clades should have happened around 5 Myr ago (Austin et al., 2004). The time estimates for the nodes leading to the Comoroan species are all very young, in comparison to island ages (Table 2), with the single exception of the node leading to *P. comorensis*, which is dated to around 0.5 Myr (when not set as a calibration), i.e., soon after the island emergence. All the estimates of the age of the *P. v-nigra* clade were at most 1.26 Myr, and the splitting of *P. robertmertensi* and *P. pasteuri* does not seem to be older than 2 Myr. Even if we consider this set of three species as a real monophyletic group, age estimates are not older than 3 Myr, much later than islands emergence: minimum age estimates for Mayotte (*P. pasteuri* and *P. robertmertensi*) are around 7 Myr old and for Anjouan and Moheli of around 3.9 and 5 Myr old, respectively. Also, the colonisation of Pemba by the ancestor of *P. parkeri* seems to have happened around 1 Myr ago.

4. Conclusions

The Comoro islands are inhabited by at least five endemic species: *P. robertmertensi*, *P. pasteuri*, *P. v-nigra*, *P. comorensis* and *P. nigristriata*. While *Phelsuma* from the Mascarenes and the Seychelles form apparently monophyletic groups, at least two, and possibly three colonisation events from Madagascar explain the origin of the studied Comoroan endemic species, with the phylogenetic position of *P. nigristriata* still unknown. *Phelsuma dubia* and *P. laticauda* populations lack any kind of geographic structure, reflecting patterns more probably related with multiple recent and/or anthropogenic movements. *Phelsuma parkeri*,

the endemic species from Pemba Island (Tanzania) has its closest relatives within *P. madagascariensis*, a widespread and subspecies-rich group from Madagascar, representing another independent transmarine colonisation. All these colonisation events are relatively young when compared to islands age, with the possible exception of the ancestor of *P. comorensis* that appears to have colonised Grand Comoro soon after its emergence. Age estimates confirm previous results about the age of diversification of main clades within *Phelsuma*, but these estimates have to be taken carefully until a more complete phylogeny is available for this genus.

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