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ORIGINAL ARTICLE

Freshwater paths across the ocean: molecular phylogeny of the frog *Ptychadena newtoni* gives insights into amphibian colonization of oceanic islands

G. John Measey[1]*, Miguel Vences[2], Robert C. Drewes[3], Ylenia Chiari[4], Martim Melo[5] and Bernard Bourles[6]

[1]*Laboratoire d'Ecologie des Sols Tropicaux, Institut de Recherche pour le Développement, 32 Avenue Henri Varagnat, 93143 Bondy Cedex, France,* [2]*Zoological Institute, Technical University of Braunschweig, Spielmannstrasse 8, 38106 Braunschweig, Germany,* [3]*Department of Herpetology, California Academy of Sciences, 875 Howard Street, San Francisco, CA 94103, USA,* [4]*Zoological Museum of Amsterdam, Mauritskade 61, 1092 AD Amsterdam, The Netherlands,* [5]*Institute of Evolutionary Biology, School of Biological Sciences, University of Edinburgh, King's Buildings, Edinburgh EH9 3JT, UK and* [6]*Institut de Recherche pour le Développement, Technopole Pointe du Diable, BP 70, 29280 Plouzane, France*

*Correspondence: G. John Measey, Laboratory of Animal Ecology, Department of Biology, University of Antwerp, B-2610 Antwerp, Belgium.
E-mail: john@measey.com

ABSTRACT

Aim Amphibians are a model group for studies of the biogeographical origins of salt-intolerant taxa on oceanic islands. We used the Gulf of Guinea islands to explore the biogeographical origins of island endemism of one species of frog, and used this to gain insights into potential colonization mechanisms.

Location São Tomé and Príncipe, two of the four major islands in the Gulf of Guinea, West Africa, are truly oceanic and have an exceptionally high biodiversity.

Methods Mitochondrial DNA is used to test the endemic status of a frog from São Tomé and compare it with congeneric taxa from tropical Africa. Existing data on surface currents, surface salinity, atmospheric circulation and bird migration in the Gulf of Guinea are summarized to address hypotheses concerning colonization mechanisms.

Results The endemic status of *Ptychadena newtoni* (Bocage) is supported here by mitochondrial DNA sequences, and analysis of this and other molecular data indicates that an East African species close to *Ptychadena mascareniensis* (Duméril and Bibron) is its nearest relative. We refute the possibility that this population was anthropogenically introduced, in favour of a natural dispersal mechanism.

Main conclusions With six endemic frogs and one caecilian, the Gulf of Guinea islands harbour a diverse amphibian fauna. Five of these species appear to have their closest relatives in East Africa. Insufficient evidence exists for transportation by storms, birds or rafts alone. However, we propose a synergy of rafting, favourable surface currents and a reduction in salinity of surface waters. Catastrophic events, or wet periods in climatic history, could allow freshwater paths to open far enough to enable continental flora and fauna to reach these and other isolated oceanic islands.

Keywords

Amphibia, Anura, Cameroon line, colonization, dispersal, Gulf of Guinea, Gymnophiona, island biogeography, phylogeny.

INTRODUCTION

Amphibians are usually described as poor dispersers (Blaustein *et al.*, 1994) with limited osmotic tolerance (Balinsky, 1981; Duellman & Trueb, 1986). In most cases their populations show strong phylogeographical structuring (Avise, 2000), which indicates low dispersal. Therefore amphibian distribution patterns are usually hypothesized to be the result of vicariance (e.g. Feller & Hedges, 1998; Biju & Bossuyt, 2003). However, recent evidence for transoceanic dispersal (Hedges *et al.*, 1992; Evans *et al.*, 2003; Vences *et al.*, 2003, 2004) and long-distance dispersal over land (Smith & Green, 2005) suggests that the long-standing biogeographical debate on vicariance vs. dispersal should be

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reconsidered, not only for amphibians (see McGlone, 2005; de Queiroz, 2005).

Intolerance of salt water explains why amphibians are normally absent from oceanic islands that have had no past connections to other land masses (Darwin, 1859). Some volcanic islands and archipelagos in the Lesser Antilles do harbour endemic frogs (Kaiser *et al.*, 1994) but, as with other Caribbean islands, they are located in an area with a complex tectonic history that is still disputed (e.g. Crother & Guyer, 1996) and that may have had past sub-aerial connections (e.g. MacPhee *et al.*, 2000). Similar controversies surround islands in the Sunda, Philippine and Pacific areas (see Vences *et al.*, 2003), many of which harbour endemic amphibians.

Vences *et al.* (2003) discovered that the two species of frog on Mayotte, an island of the Comoro archipelago in the Indian Ocean, are endemic. They provided one of the best documented examples of endemic amphibians on fully volcanic, oceanic islands, nevertheless their claim that these were the only such examples was erroneous: another indisputable example of dispersal of amphibians over a marine barrier is provided by the Gulf of Guinea islands of São Tomé and Príncipe. These volcanic islands, surrounded by deep waters of > 1800 m, harbour seven endemic amphibians: six frogs (Amphibia: Anura) and one caecilian (Amphibia: Gymnophiona). The presence of a caecilian on São Tomé is particularly interesting because they are largely subterranean amphibians that are considered highly unlikely to disperse over major marine barriers (Nussbaum, 1984; Gower *et al.*, 2002).

Although Gulf of Guinea island amphibians have been known since the mid-19th century, the significance of the islands in demonstrating amphibian overseas dispersal (Fahr, 1993) has been overlooked in several relevant accounts of amphibian biogeography (e.g. Savage, 1973; Duellman & Trueb, 1986; Hedges *et al.*, 1992; Vences *et al.*, 2003). Rafting, favoured by storms and sea currents, has been proposed as a possible mechanism of colonization of islands by amphibians (Myers, 1953; Savage, 1973; Kaiser *et al.*, 1994; Krause *et al.*, 1997; Censky *et al.*, 1998; Evans *et al.*, 2003), despite the osmotic problems of organisms with limited tolerance of salinity (Balinsky, 1981). Other aerial dispersal mechanisms depend on the occurrence of storms (especially tornadoes), which can transport smaller ontogenic stages (Simons, 1996; Elsom, 1988) or perhaps involve passive dispersal on volant vertebrates such as aquatic birds (cf. Darwin, 1859).

Here we highlight the importance of the Gulf of Guinea islands as an area for studies of overseas dispersal, especially in amphibians. We review geographical patterns in the Gulf of Guinea that could lead to dispersal events. We provide molecular confirmation of the endemicity to São Tomé of one of its amphibians [*Ptychadena newtoni* (Bocage)], rejecting the possibility that it was introduced anthropogenically, and we investigate phylogenetic relationships with other species of *Ptychadena* Boulenger. Lastly, we discuss our results in the light of three specific hypotheses for amphibian overseas dispersal: rafting, storms and bird carriage.

LOCATION AND METHODS

Description of study area – the Gulf of Guinea islands

Four islands, Bioko (aka Fernando Pó), Príncipe, São Tomé and Annobón (aka Pagalu), are the offshore part of the Cameroon line in the Gulf of Guinea (Burke, 2001). The swells on the Cameroon line are the result of past volcanic activity (Fitton, 1987; Burke, 2001), but currently only Mount Cameroon is active, having last erupted in June 2000. Bioko is separated from the West African mainland by only 32 km and sea depths of < 60 m, which suggests linkage to the mainland during the last glaciation (Lee *et al.*, 1994). This is consistent with the continental character of its biota (Mertens, 1964, 1965; Jones, 1994); hence it will not be considered further here. The other three islands have been isolated since their origins (Jones, 1994). The islands furthest away from Mt Cameroon are also most distant from the mainland, with minimum distances of at least 220 km and sea depths of > 1800 m (Table 1; Fig. 1). Dates for the origins of the three oceanic islands have been given as 31, 13 and 4.8 Ma (Príncipe, São Tomé and Annobón, respectively; Lee *et al.*, 1994), but the oldest lava flow-eruption ages give information only on the minimum age when each island was sub-aerial. Furthermore, volcanic activity persisted in all islands until as recently as the last 0.1 Ma in São Tomé (Fitton, 1987) or < 0.4 Ma (Caldeira & Munhá, 2002), although the impact of volcanic activity on the biota of these islands is unknown (but cf. Jesus *et al.*, 2005c). Therefore when and how the land surface area (and hence colonizable habitats) changed through time is only hinted at by the ages available.

Patterns of biodiversity

Isolation led to the evolution of a highly original flora, characterized by the co-occurrence of neoendemics and of Afromontane palaeoendemics (Figueiredo, 1994). A recent biogeographical study on the begonias suggested that São Tomé functioned as an important pre-Pleistocene refuge for these and possibly other plants (Plana *et al.*, 2004). The forests of São Tomé and Annobón have the highest fern diversity and density in Africa (Exell, 1944). The islands also form an important centre of endemism for many faunal groups, which gives them a unique and exceptional biota of global conservation significance (Jones, 1994; Juste & Fa, 1994).

The Gulf of Guinea islands centre of endemism is particularly striking in birds, reptiles and amphibians (Table 1). The three oceanic islands, with an area of *c.* 1000 km^2, hold 29 endemic bird species, with up to four endemic genera on São Tomé: *Amaurocichla* Sharpe, *Dreptes* Reichenow, *Thomasophantes* Hartlaub and *Neospiza* Salvadori; and another on Príncipe, *Horizorhinus* Oberholser. In comparison, the 13 main islands of the Galápagos archipelago, with an area totalling *c.* 8000 km^2, have 22 endemic species. The number of endemic birds makes up one-third of the endemics of the large Guinean Forests hotspot (Bakkar *et al.*, 1999).

Table 1 Physical geography and endemicity of plants and vertebrates* of the historically isolated islands in the Gulf of Guinea

	Príncipe	São Tomé	Annobón
Surface area (km^2)	139	857	17
Highest peak (m)	948	2024	654
Distance from closest island (km)	146	146	180
Distance from mainland Africa (km)	220	255	340
Plants	314 [26] (11)	601 [81] (14)†	208 [14] (6)
Mammals	5 [1] (0)	10 [3] (0)	2 [0] (0)
Birds	33 [6] (5)†	50 [15] (5)†††	11 [2] (1)
Fish*	23	14	N/A
Reptiles	13 [3] (6)	14 [1] (6)	1 [0] (1)
Amphibians*	3 [2] (2)	5 [4] (2)	0 [0] (0)
Amphibian species	*Hyperolius molleri*, *Leptopelis palmatus*, *Phrynobatrachus dispar*	*Schistometopum thomense*, *Hyperolius thomensis*, *Hyperolius molleri*, *Phrynobatrachus* sp. *nov.*, *Ptychadena newtoni*	None

*Only resident birds were considered. Fish refer only to freshwater species; the difference in numbers of fish species from São Tomé and Príncipe may result solely from the proportion of time spent sampling on each island. Reptiles do not include marine turtles. The previously recognized endemic frog genus *Nesionixalus* has recently been synonymized with *Hyperolius* (Drewes & Wilkinson, 2004). Numbers of species are given, those in square brackets refer to single-island endemics; in round brackets to endemics from two or three of the islands.

†Indicates presence of an endemic genus. Data from the Gulf of Guinea Islands' Biodiversity Network (http://www.ggcg.st), Feiler (1988), Fahr (1993), Feiler *et al.* (1993), Haft (1993), Nill (1993), Figueiredo (1994), Jesus *et al.* (2003), Jones & Tye (2006), T. Iwamoto (personal communication).

Each of the three oceanic islands is classified as an Endemic Bird Area (Stattersfield *et al.*, 1998), with São Tomé and Príncipe being the only small oceanic islands in the world's top 25% of Endemic Bird Areas.

As expected for oceanic islands, most endemic mammals are bats (Juste & Ibañez, 1994), but São Tomé has an endemic shrew [*Crocidura thomensis* (Bocage)] and Príncipe has an endemic subspecies of *Crocidura poensis* (Fraser). The shrews have been said to represent a 'zoogeographical mystery' (Dutton & Haft, 1996) because their metabolism and surface-to-volume ratio necessitates that they eat constantly, making them unlikely dispersers (Heim de Balsac & Hutterer, 1982). Most fish are primarily marine (with two introduced) occurring in estuarine habitats, and all known freshwater fish are secondarily adapted (T. Iwamoto, personal communication).

The reptiles and amphibians of the Gulf of Guinea islands pose some of the most difficult questions with respect to colonization. Many reptiles and nearly all amphibians are considered to have low tolerance of sea water (Balinsky, 1981). Jesus *et al.* (2005a,b,c) studied the molecular phylogenetics of the Gulf of Guinea islands' geckos and skinks, finding that although one species [*Hemidactylus mabouia* (Moreau de Jonnès)] appears to have been introduced, neither geckos nor skinks are monophyletic, suggesting multiple independent colonizations of the islands for both groups.

Fahr (1993) assumed accidental transport of eggs by waterbirds as the most likely origin for the endemic frogs of São Tomé, but the Gulf of Guinea islands are not part of any bird migration route (Jones & Tye, 2006), and the only regular visitors are Palaearctic coastal waders and seabirds [common sandpiper, *Actitis hypoleucos* (L.), whimbrel *Numenius phaeopus* (L.), and several tern species, *Sterna* spp.]. Palaearctic landbird vagrants recorded on the islands are mostly small passerines, and Afrotropical migrants do not use the islands at all (Jones & Tye, 2006). Birds that are associated with wetland habitats, and thus are the best candidates for introducing eggs of some amphibians, include the mainland ancestor of the endemic forest ibises (*Bostrychia* sp.) and aquatic species such as herons and gallinules. The ibises have their closest relatives in West Africa (Cameroon and Gabon) (Chapin, 1923; Brown *et al.*, 1982). The common moorhen [*Gallinulla chloropus* (L.)], a near-cosmopolitan species, and the green-backed heron [*Butorides striatus* (L.)], a pantropical species, have established populations on São Tomé and Príncipe in marshes and the lower reaches of rivers (Jones & Tye, 2006). As populations of these species never differentiated from mainland populations, the probability of occasional movements between the islands and the nearby mainland (e.g. Cameroon or Gabon) is high.

Drewes (2002) commented on the bizarre occurrence of a high proportion of subterranean taxa amongst the herpetofaunal endemics: the caecilian *Schistometopum thomense* (Bocage) (Fig. 1, inset); three scolecophidian snakes, *Rhinotyphlops newtoni* (Bocage), *Rhinotyphlops feae* (Boulenger) and *Typhlops elegans* Peters; and a legless skink, *Feylinia polylepis* (Bocage). Like many other burrowing lower vertebrates, each of these species can be found within or beneath rotting logs on São Tomé and Príncipe (G.J.M. and R.D., personal observations), and this hints at the possible mechanism of their dispersal.

Geographical patterns around the Gulf of Guinea

Surface currents

Surface currents in the Atlantic have been determined recently as the result of a combined analysis of historical ship drifts,

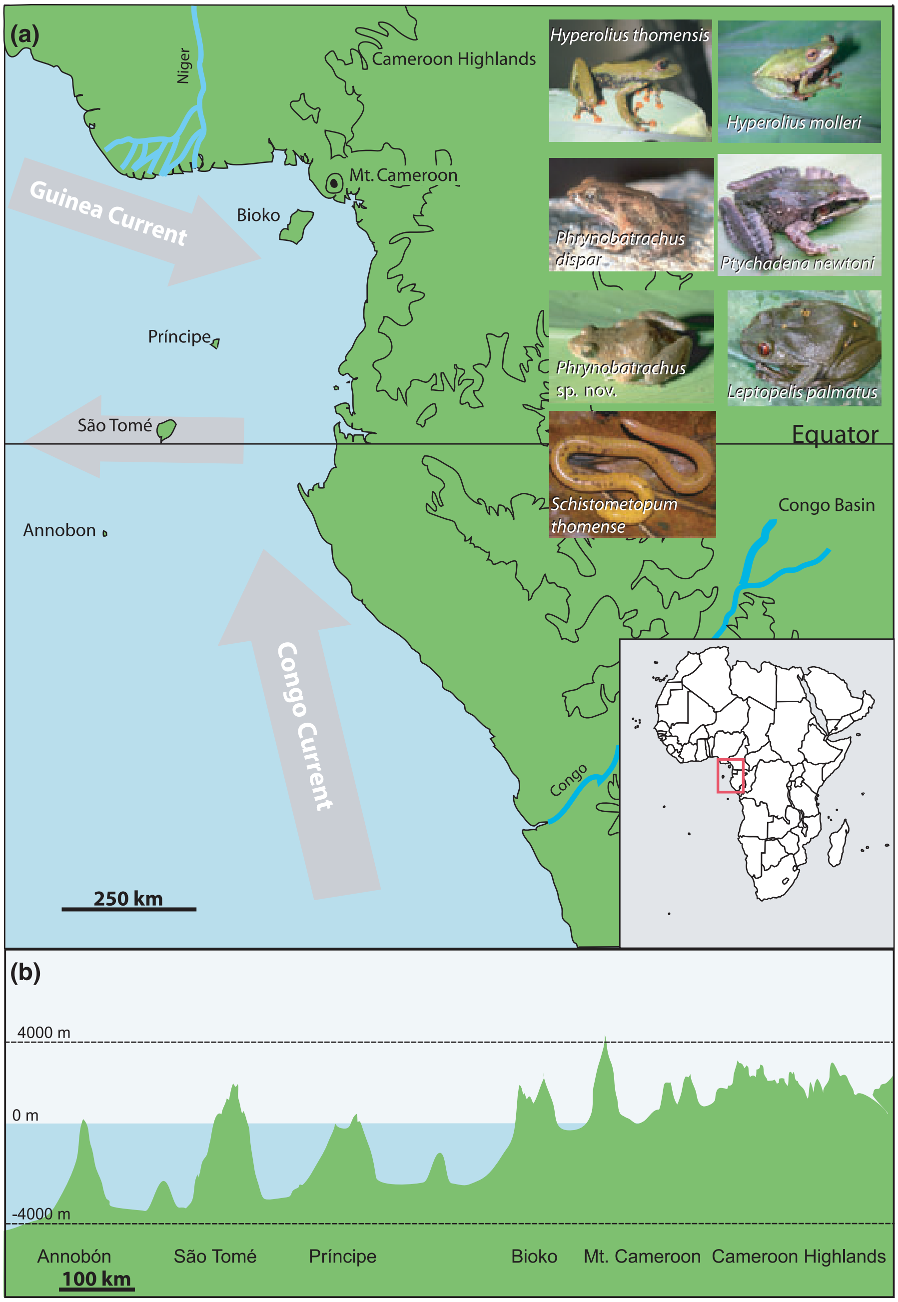

Figure 1 The Gulf of Guinea islands, lying in the Cameroon line, consist of four principal islands. (a) Arrows indicate the major sea-surface currents in the area, demonstrating the potential for objects exiting the Niger or Congo (the only rivers shown, with 500 m contours) to be taken directly to São Tomé and Príncipe. All amphibians from these islands (inset) are considered endemic, including *Ptychadena newtoni*, the closest known relatives of which appear to be East African. (b) Bioko (aka Fernando Pó) is part of the continental shelf, while Príncipe, São Tomé and Annobón (aka Pagalu) are truly oceanic islands.

hydrographical data, and surface-drifting buoy trajectories (Richardson & Walsh, 1986; Arnault, 1987; Stramma & Schott, 1999; Lumpkin & Garzoli, 2005). In summary, two perpendicular currents feature (northwards and eastwards), following the shoreline into the Gulf of Guinea and towards the Bay of Biafra (Fig. 1), and these converge to form a third westward current that carries water out along the line of the equator (see also Dupont *et al.*, 2000).

The eastward 'Guinea Current' carries surface water into the Gulf of Guinea along the southern coast of West Africa (see also Feiler, 1988; Haft, 1993), as a continuation of the North Equatorial Countercurrent (Fig. 1). This current extends to the Bay of Biafra where it becomes more diffuse, turning back westward around the equator. Moving northwards along the south-west African coast, the 'Congo Current' feeds the equatorial branch of the South Equatorial Current (Stramma

& Schott, 1999), clearly visible in ship drift data, and the mean annual Ekman surface currents. This current carries coastal waters offshore, including discharge from the Congo River (Wauthy, 1983). These opposing currents produce a swirling effect, followed by intensification westward of the South Equatorial Current.

Salinity of surface waters

Due to the difference in density, freshwater floats on underlying oceanic salted waters. Hence large rivers in spate can have a profound effect on the surface salinity of seawater in the Gulf of Guinea, and are known as tropical surface waters. These are characterized by high temperatures and low salinity, and overlie a density-discontinuous layer at the thermocline, promoting a strong and nutrient-rich upwelling effect (Binet & Marchal, 1993). The salinity of the subsurface layers reaches a maximum around the Gulf of Guinea islands, further strengthening the stratifying effect. Dessier & Donguy (1994) demonstrated large seasonal variations corresponding to the rainy seasons in the catchments of the two major river systems, the Niger and the Congo (the latter is the world's second largest river in terms of discharge volume, after the Amazon). Figure 2a shows how surface fresh waters are confined to the mouths of the Niger and Congo during the dry season. At the peak of the rainy season, the rivers' combined freshwater discharges, together with high precipitation into the Bay of Biafra, affect surface waters in the entire Gulf of Guinea (Fig. 2b). This forms a characteristic 'freshwater path' leaving the mouth of the Congo, also known as the Congo plume.

Atmospheric circulation

Modern atmospheric circulation in the Gulf of Guinea has been recently and succinctly summarized by Dupont *et al.* (2000), therefore only a brief description is given here. Light winds generally follow and are responsible for surface currents (see above). In addition to these, and of more importance in possible colonization routes from the mainland, are the seasonal Inclined Meterological Equator (IME) and Mid-Tropospheric Easterly (MTE) winds. In January and February, when the IME is at its southernmost position, the north-east trade winds blow off the Liberian coast into the Gulf of Guinea. In the boreal summer, the African Easterly winds (including MTE) dominate weather systems in West Africa (Berry & Thorncroft, 2005). Storms generated from the MTE move east to west, providing a potential colonization route from Gabon, the nearest land to the east of the Gulf of Guinea islands (Fig. 1). The MTE winds were presumed to be responsible for carrying pollen to sites in the Gulf of Guinea (Dupont *et al.*, 2000).

Study species

Frogs of the genus *Ptychadena* Boulenger, or ridged frogs, are widely distributed in Nilotic Egypt and sub-Saharan Africa (excluding south-western South Africa) and currently consist of 47 species (Frost, 2004). They are medium-sized frogs, typically 50–60 mm snout-vent length, and are often abundant in wetland areas and irrigated agricultural landscapes (Channing, 2001). Most often they are seen as an arcing flash disappearing with a plop into standing water. *Ptychadena newtoni* (Bocage) was first described as a São Tomé endemic, although it was synonymized with *Ptychadena oxyrhynchus* (Smith) by Andersson (1937). Later, Guibé & Lamotte (1957) placed it in the *Ptychadena mascareniensis* (Duméril and Bibron) group (Frost, 2004). However, both Perret (1976) and Loumont (1992) considered it a distinct species, and hence an island endemic, based on morphological characteristics including its rough dorsal skin, reduced dorsal folds, and a reported body size of 76 mm, the largest within the genus.

Molecular analyses of *Ptychadena newtoni*

A fragment of the mitochondrial 16S rRNA gene was amplified by PCR and sequenced from 30 specimens of the genus *Ptychadena*, in order to complement the data set of Vences *et al.* (2004), following methods outlined therein. These included tissue samples from four specimens of the presumed endemic *P. newtoni*, collected during a recent expedition to the

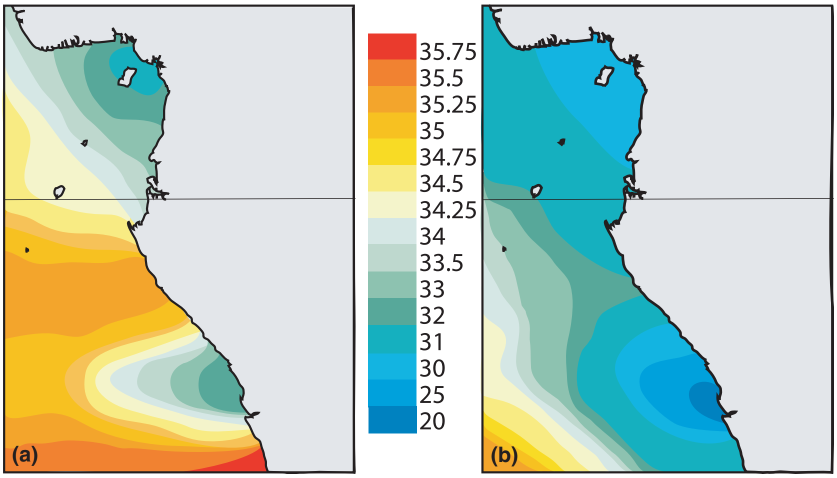

Figure 2 Sea-surface salinity (in Practical Salinity Units (psu), which is roughly equivalent to parts per thousand of salt) for the Gulf of Guinea in (a) July and (b) February, demonstrating the dramatic changes in surface salinity depending on direct rainfall and in the drainages of the Niger and Congo. Values below 30 psu characterize brackish waters and are seen extending far into the ocean, even in years of average rainfall. Data from the Mercator project (http://www.mercator-ocean.fr).

Gulf of Guinea islands (see Drewes & Wilkinson, 2004, Fig. 1 inset). For analysis we excluded haplotypes that were identical or very similar to each other (< 1% uncorrected pairwise sequence divergence); among these was the haplotype of *Ptychadena* aff. *mascareniensis* B from Cameroon, which had only slight differences from the included sequence of the same taxon from Benin.

In order to increase phylogenetic resolution within the *P. mascareniensis* complex, we sequenced fragments of the genes for cytochrome *b* (cyt *b*) and cytochrome oxidase subunit I (cox1) from representative individuals of the major lineages within the complex, and from two additional *Ptychadena* species, to obtain a data set of 1648 base pairs, using primers and PCR protocols as described by Bossuyt & Milinkovitch (2000), Chiari *et al.* (2004) and Vences *et al.* (2005). PCR conditions were 94°C for 90 s, then 10 cycles of 94°C for 30 s, 45°C (increasing 0.8°C each cycle) for 45 s, 72°C for 60 s, then 25 cycles of 94°C for 30 s, 53°C for 45 s, 72°C for 60 s, and a final extension of 10 min at 72°C for cyt *b*; and 94°C for 90 s, 35 cycles of 94°C for 30 s, 50°C for 45 s, 72°C for 60 s, final extension of 10 min at 72°C for cox1.

Fragments obtained from the amplified genes were edited and aligned manually using SEQUENCE NAVIGATOR software (Applied Biosystems Inc., Foster City, CA, USA). No gaps were present in the cox1 and cyt *b* alignments. From the 16S rDNA alignment, we excluded some areas of high variability, which needed inclusion of gaps to account for indels, and we excluded all positions with single gaps in one or more of the sequences. Maximum parsimony and maximum likelihood phylogenetic analyses were performed using PAUP* (Swofford, 2002). The Akaike information criterion, as implemented in MODELTEST (Posada & Crandall, 1998), was used to find the best-fitting substitution model for our data. To test alternative phylogenetic hypotheses, we used (a) Shimodaira–Hasegawa tests (Shimodaira & Hasegawa, 1999) which, according to Shimodaira (2002), are safe to use for this purpose but may have conservative bias; and (b) approximately unbiased tests (Shimodaira, 2002). Both statistics were calculated using the program CONSEL(Shimodaira & Hasegawa, 2001). New DNA sequences were deposited in GenBank under accession numbers DQ525918–65 (Table 2). The alignments and maximum likelihood trees were deposited in TreeBASE (http://www.treebase.org) with accession numbers M2757–8 and S1533.

RESULTS

Sequences of 16S rDNA of a total of 105 individual *Ptychadena* were available for analysis. After removal of identical or very closely related (putatively conspecific) haplotypes, and of gapped positions in the alignment, the data set consisted of 522 characters in 30 taxa. Of these, 330 were constant and 144 were parsimony-informative. MODELTEST selected a general time-reversible substitution model plus invariant sites plus gamma (GTR + I + G) with empirical base frequencies and substitution rates, a proportion of invariable sites of 0.4904 and a gamma-distribution shape parameter of 0.5131 as best fitting the data. Heuristic searches using this substitution model under the maximum likelihood optimality criterion, with 10 random addition sequences of taxa, found two trees of equal likelihood score ($-\ln L = 3739.7$). A strict consensus of these is shown in Fig. 3.

Sequences of cyt *b* and cox1 from a total of eight individuals (plus one outgroup, *Rana nigromaculata* Hallowell) were combined with 16S rDNA sequences of the same individuals for analyses. Of a total of 1648 characters in the combined data set, 1014 were constant and 442 were parsimony-informative. A general time-reversible substitution model (GTR + I + G) with a proportion of invariable sites of 0.4475 and a gamma-distribution shape parameter of 0.5793 was selected by MODELTEST as best fitting the data.

In the analysis based on 16S rDNA sequences, *P. newtoni* is found with high bootstrap support (80–93%) in a clade containing *P. mascareniensis* and several cryptic species so far considered to be *P. mascareniensis.* Interestingly, its sister species is the form named *P.* aff. *mascareniensis* A by Vences *et al.* (2004), a species occurring in eastern Africa (Tanzania, Kenya, Uganda and Egypt); however, the bootstrap support for this grouping was low (54–57%; Fig. 3). All individuals of *P. newtoni* had identical haplotypes. Their uncorrected pairwise sequence divergences to *P.* aff. *mascareniensis* A were 5.2–5.6%. The general topology of the tree recovered agrees with that published by Vences *et al.* (2004), which had a smaller taxon sampling but included more haplotypes of some taxa. Differences were found, for instance, in the basal relationships among major lineages in the *P. mascareniensis* complex, but the competing hypotheses were not supported by relevant bootstrap values in either analysis, and therefore are not discussed further.

The phylogenetic position of *P. newtoni* was strongly confirmed by the combined analysis of three genes in a reduced set of taxa. Its sister-group relationship with *P.* aff. *mascareniensis* A from eastern Africa was supported by 99% bootstrap values in both maximum parsimony and maximum likelihood analyses (Fig. 4). Separate analyses of the cyt *b* and cox1 data sets resulted in the same relationships (not shown). Both Shimodaira–Hasegawa and approximately unbiased tests significantly rejected ($P < 0.05$) nine alternative phylogenetic hypotheses in which *P. newtoni* was placed in all possible positions within and basal to the *P. mascareniensis*/*pumilio* clade, leaving the remainder of the topology unchanged.

DISCUSSION

Ptychadena newtoni endemicity

While our data do not resolve the taxonomy of the *P. mascareniensis* clade (which evidently contains many distinct as well as cryptic species), they do strongly suggest that *P. newtoni* is a distinct species endemic to São Tomé. The previously identified morphological characters, including its particularly large size, add credence to our molecular results. Moreover, this may be an example of island gigantism

Table 2 DNA sequences of the 16S rRNA gene obtained in addition to those published by Vences *et al.* (2004), and of the cox1 and cytochrome *b* genes obtained for the reduced set of taxa

Species	Origin	Locality	Voucher	Accession 16S	Accession cox1	Accession cyt *b*
Ptychadena aff. *aequiplicata*	Cameroon	Dja Reserve	CAS 199182	DQ525919	–	–
Ptychadena anchietae	Kenya	Kararacha Pond	CAS 214837	DQ525920	–	–
Ptychadena anchietae	Somalia	Karin, Bari Region	CAS 227562	DQ525921	–	–
Ptychadena anchietae	Somalia	Karin, Bari Region	CAS 227507	DQ525922	–	–
Ptychadena anchietae	South Africa	Mtunzini	No voucher	AF215404*	DQ525952	DQ525961
Ptychadena mahnerti	Kenya	Mt. Kenya	SL 171	DQ525918	–	–
Ptychadena mascareniensis	Madagascar	Nahampoana	ZSM 190/2002	AY517587*	DQ525951	DQ525960
Ptychadena aff. *mascareniensis* A	Uganda	Lake Victoria,	MVZ 234085	DQ525923	–	–
Ptychadena aff. *mascareniensis* A	Kenya	Makuru	MVZ 223624	DQ525924	–	–
Ptychadena aff. *mascareniensis* A	Kenya	Mt. Kenya	MVZ 234087	DQ525925	–	–
Ptychadena aff. *mascareniensis* A	Kenya	Mt. Kenya	MVZ 234086	DQ525926	–	–
Ptychadena aff. *mascareniensis* A	Kenya	Taita Hills	CAS 191517	DQ525927	–	–
Ptychadena aff. *mascareniensis* A	Kenya	Taita Hills	CAS 191518	DQ525928	–	–
Ptychadena aff. *mascareniensis* A	Tanzania	Kibebe farm	AC 2087	DQ525929	DQ525950	DQ525959
Ptychadena aff. *mascareniensis* C	Guinea	No precise locality	gu03.2	DQ525930	DQ525948	DQ525957
Ptychadena aff. *mascareniensis* D	Uganda	Kampala	MVZ 234084	DQ525931	–	–
Ptychadena aff. *mascareniensis* E	Central African Republic	Dzanga-Sangha Reserve	MOR DS 52	DQ525932	DQ525947	DQ525956
Ptychadena newtoni	São Tomé	São Tomé	CAS 219249	DQ525933	DQ525946	DQ525955
Ptychadena newtoni	São Tomé	São Tomé	CAS 219250	DQ525934	–	–
Ptychadena newtoni	São Tomé	São Tomé	CAS 219251	DQ525935	–	–
Ptychadena newtoni	São Tomé	São Tomé	CAS 219252	DQ525936	–	–
Ptychadena newtoni	São Tomé	São Tomé	CAS 219253	DQ525937	–	–
Ptychadena newtoni	São Tomé	São Tomé	CAS 219263	DQ525938	–	–
Ptychadena oxyrhynchus	Malawi	no precise locality	359 (6 specimens)	DQ525939	–	–
Ptychadena oxyrhynchus	South Africa	Mtunzini	No voucher	–	DQ525954	DQ525965
Ptychadena oxyrhynchus	South Africa	Kwambonambi	No voucher	AF215403*	DQ525953	DQ525962
Ptychadena aff. *porosissima* A	Tanzania	Tatanda	AC 2034	DQ525940	–	–
Ptychadena porosissima	Tanzania	Mumba	AC 2122	DQ525941	–	DQ525963
Ptychadena cf. *pumilio*	Guinea	Mont Béro	MOR Gu 212	DQ525942	DQ525949	DQ525958
Ptychadena taenioscelis	Kakamega	Kenya	NMKA 3955–1	DQ525943	–	–
Ptychadena aff. *uzungwensis*	Tanzania	Njombe	AC 1970	DQ525945	–	DQ525964
Ptychadena sp.	Tanzania	Mikumi	AC 1976	DQ525944	–	–

AC, Working collection of Alan Channing; CAS, California Academy of Sciences; MVZ, Museum of Vertebrate Zoology, Berkeley, CA, USA; MOR, Mark–Oliver Rödel collection; NMKA, National Museums of Kenya; SL, Stefan Lötters collection; ZSM, Zoologische Staatssammlung München.
*Sequences from Vences *et al.* (2004) used in combined analysis of the three genes. Letters following *Ptychadena* aff *mascareniensis* refer to those allocated in Vences *et al.* (2004).

(Carlquist, 1965), exemplified by two of the other amphibian endemics, *Leptopelis palmatus* (Peters) (see Drewes & Stoelting, 2004) and *Hyperolius thomensis* Bocage (Drewes & Wilkinson, 2004). Interestingly, *P. newtoni* shows strong genetic divergences from those taxa of which it has been considered a synonym in the past: *P. oxyrhynchus* and *P. mascareniensis*. It is remarkable that this species is deeply nested in the *P. mascareniensis* complex (Figs 3 & 4), although Loumont (1992) noted similarities to *Ptychadena anchietae* (Bocage) and *P. oxyrhynchus*. The affinity with East African samples may reflect the paucity of collections from central Africa, specifically the Congo Basin.

This confirmation of the endemicity of *P. newtoni* suggests rejection of the hypothesis that this species was introduced by human settlers. We continue by considering the implication of our results with respect to three competing hypotheses for amphibian overseas dispersal: carriage by birds, storms and rafting.

Carriage by birds

Darwin (1859) proposed carriage by birds as the most plausible explanation for the distribution of land snails on many isolated oceanic islands, and this idea is still cited as the only explanation for their distribution (Gittenberger *et al.*, 2006). Despite speculation (e.g. Fahr, 1993), we know of no record in either ornithological or herpetological literature that has reported finding amphibian eggs associated with the feet, legs or plumage of birds. However, it remains possible that eggs have been transported in this fashion.

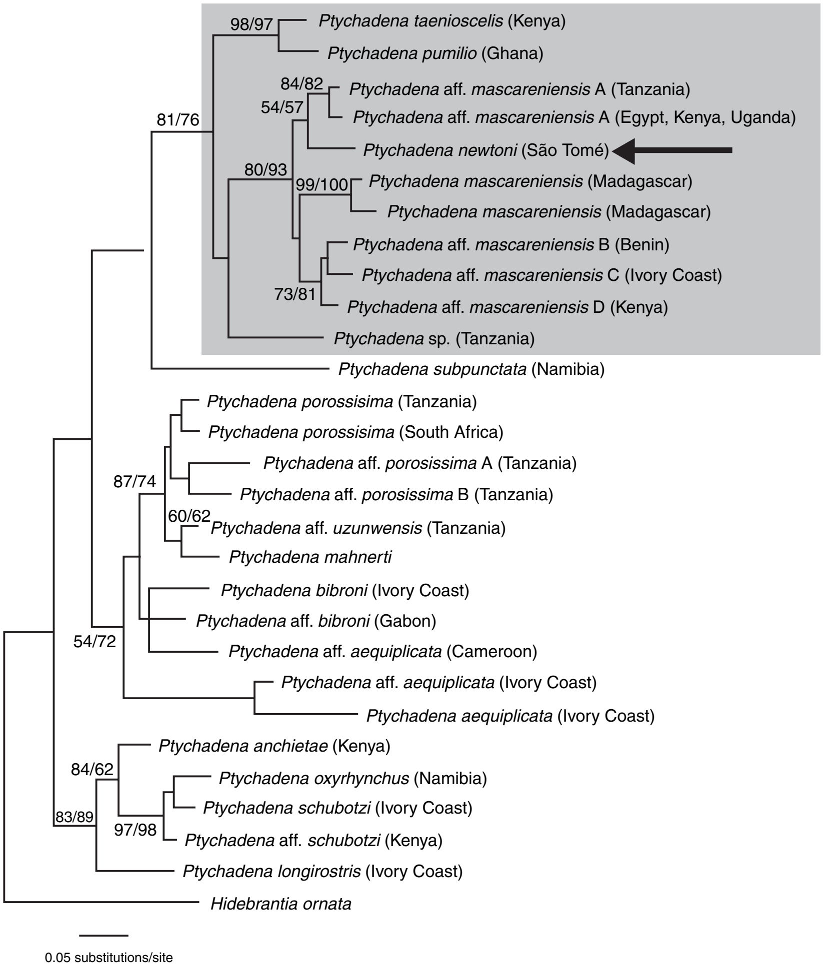

Figure 3 Maximum likelihood phylogram of species in the genus *Ptychadena* based on analysis of 522 base pairs of the mitochondrial 16S rRNA gene. Only one sequence per species or strongly divergent haplotypes were selected from the data set of Vences *et al.* (2004) and complemented with new sequences as listed (Table 2). The endemic species *Ptychadena newtoni* from São Tomé is indicated with an arrow. Numbers at nodes are support values (percentage) from maximum likelihood (100 replicates) and maximum parsimony analyses (2000 replicates), using full heuristic searches with 10 random addition sequence replicates. Values are only shown for nodes where both methods yielded values > 50%.

Although no information is available for the breeding behaviour of *P. newtoni*, other frogs in the genus deposit eggs in rafts on the surface of, and in, shallow water (see Channing, 2001), and so could become attached to the feet or plumage of birds. Frog eggs would have to avoid desiccation while being transported to São Tomé, either by direct flight or through a storm-induced medium (see below). As no east-to-west (African) migration routes are known for candidate bird species, aquatic birds would probably arrive from adjacent mainland Africa, possibly being blown to the islands in a storm. In addition, as remarked by Fahr (1993), carriage by birds does not explain the presence of the caecilian amphibian *S. thomense*, which is known to be viviparous, with juveniles measuring > 100 mm at birth (Haft & Franzen, 1996). It is also unlikely that birds would transport eggs of the tree frog *H. thomensis*, which lays eggs in tree holes on São Tomé (Drewes & Stoelting, 2004). The puddle frogs *Phrynobatrachus dispar* (Peters) and *Phrynobatrachus* sp. *nov.* lay eggs in small accumulations of water, such as those held by fallen leaves in the forest (G.J.M., personal observation), and hence are also unlikely to be dispersed by birds. Even if incidents of frogs' eggs on birds were verified, it is not parsimonious with our phylogenetic results for *P. newtoni*, and we consider it an unlikely mechanism by which any amphibians colonized the Gulf of Guinea islands.

Storms

There are several reports of movement of amphibians by particular weather conditions (see Elsom, 1988; Simons, 1996) when tornadoes suck up a portion of a water body (a waterspout) containing small stages (eggs, larvae and metamorphs) of amphibians. These may be carried a short distance, normally no more than a few tens of kilometres, after which the contents are deposited in what is sometimes referred to as a 'remarkable shower' (Simons, 1996). The more violent the tornado, the greater the distance they are likely to travel, and the less likely the contents (in the case of live amphibians) are to survive, instead being torn to pieces and subjected to low temperatures. Other incidents, where more violent storms have been implicated in colonization events of anolis lizards (e.g. Calsbeek & Smith, 2003), refer to adult individuals reaching islands by being blown into sea water, and are therefore unlikely to explain amphibian colonization. Again, storms are also unlikely to account for a live-bearing and subterranean caecilian. In the unlikely event that storms were responsible for

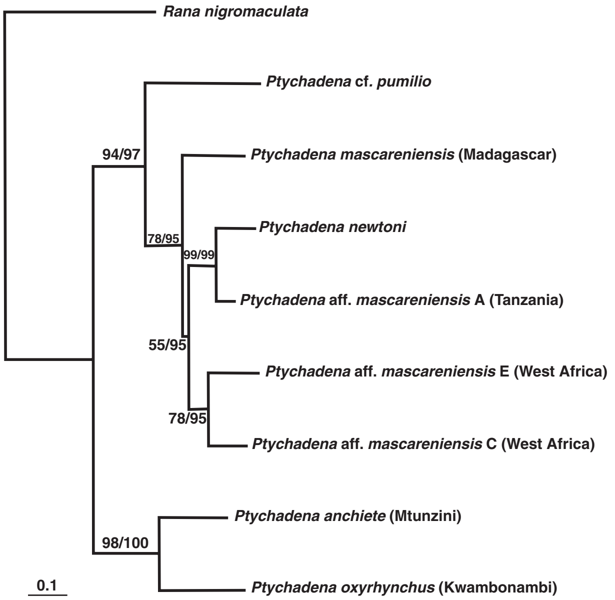

Figure 4 Maximum likelihood phylogram of selected species in the *Ptychadena mascareniensis* complex and *Ptychadena oxyrhynchus*, based on 1648 base pairs of DNA sequences of the 16S rRNA, cyt *b* and cox1 genes. Numbers at nodes are bootstrap support values (percentage) from maximum likelihood and maximum parsimony analyses (2000 replicates each).

the movement of amphibians, colonizers would probably be swept in storms from coastal areas to the north or east (see above), a scenario not parsimonious with the results presented here.

Rafting

The rafting hypothesis is congruent for data we have regarding relevant surface currents, occurrence of rafts, and the results of our molecular analysis. Colonizers may have been washed by rainwater into rivers inside the Congo catchment and thence onto rafts in the Congo River. That such conglomerations regularly leave the Congo River is well documented (Renner, 2004 and references therein). Present-day sea currents, wind movements and historical data (see above) all suggest that a raft leaving the mouth of the Congo River may arrive on the shores of the Gulf of Guinea islands. However, this would involve movement of *c.* 1000 km through the sea, during which time high salinity levels would be capable of killing any potential amphibian colonizers. The consequences of high-salinity rafting have been witnessed in the form of thousands of amphibians transported on rafts from the Rio de la Plata [predominantly the caecilian *Chthonerpeton indistinctum* (Reinhardt and Lütken)] washing up dead on the beaches of Uruguay (R. de Sa, personal communication).

A number of amphibian species appear to tolerate elevated salinity by increasing the osmotic concentration of their body fluids to maintain a favourable but relatively small gradient for the osmotic influx of water (Balinsky, 1981). This is apparently possible through their ability to accumulate high levels of urea in body fluids, associated with both urea retention and increased urea synthesis (Shoemaker *et al.*, 1992). While tolerance to 100% seawater may be restricted to very few amphibians (Balinsky, 1981; Schoener & Schoener, 1984), other data show a remarkably high survival for some species in up to 65% seawater (Romspert, 1976), with unpublished data suggesting the possibility of survival in 70% seawater for several months (A. P. Romspert, personal communication). While these data are not available for a wide range of amphibian taxa, they suggest that reduced salinity of surface waters may provide for amphibian passage. Indeed, terrestrial anurans not known for their saline tolerance (*Rana temporaria* L. and *Bufo bufo* L.) are reported to have extensive gene flow by swimming between islands separated by brackish water in the northern Baltic Sea (Seppä & Laurila, 1999). Although there are no explicit data regarding salinity tolerance of *P. newtoni* (or any *Ptychadena*), we consider it unlikely that it can tolerate 100% seawater. Thus the major obstacle to colonization through rafting is the 1000 km or so of sea from the mouth of the Congo River to São Tomé.

Existing data and the simplicity of their distribution, age and number suggest that the Gulf of Guinea islands have the potential to be a model system for studying transoceanic dispersal. We propose a novel mechanism to explain our data, which may be of use in other examples of overseas dispersal by salt-intolerant organisms.

Rafting on freshwater paths

While other workers have mentioned rafting (e.g. Hedges *et al.*, 1992) or a combination of rafting and oceanic currents (e.g. Kaiser *et al.*, 1994), none has highlighted the potential of a combined mechanism with three components: a floating raft, a favourable surface current, and a reduction in salinity of surface waters.

A transporting medium

Floating conglomerations of tree trunks (rotting and living), freshwater aquatic plants, and even soil regularly raft down large rivers in times of spate. In Brazil, floating islands or 'camalotes' have been found to contain numerous terrestrial herpetofauna, including snakes, lizards, frogs and caecilians (Iherring, 1911; Archaval *et al.*, 1979; Schiesari *et al.*, 2003). Rafts have been implicated in colonization events of many herpetofauna, including amphibians (Myers, 1953; Evans *et al.*, 2003). The phenomena of rafts and floating islands have recently been extensively reviewed, demonstrating the long-distance oceanic travel of some conglomerations carrying a diverse array of taxa (VanDuzer, 2004; Thiel & Gutow, 2005). For example, rafts are the likely dispersal mechanism used by many angiosperms that are carried into the tropical Atlantic from the Congo River, and which potentially can traverse the Atlantic in as little as 2 weeks (Renner, 2004).

The presence of rotting logs in a raft is of particular significance, because burrowing taxa such as the caecilian

S. thomense and blind snakes readily inhabit such microhabitats in the forests of São Tomé. Scolecophidian snakes have already been observed on rafting materials (Thiel & Gutow, 2005), and this provides a simple explanation for the presence of burrowing taxa on the Gulf of Guinea islands, a presence that cannot be explained credibly by storms or bird carriage.

Surface currents

There is little doubt that surface currents in the Gulf of Guinea would carry the discharge of two of the world's largest rivers (Congo and Niger), and any raft they may contain, toward Príncipe, São Tomé and Annobón, via the South Equatorial Current (Fig. 1). Although these currents show seasonal changes (Richardson & Walsh, 1986), they continue to demonstrate the same major effect. More important are the effects of large climatic changes that may intensify or reduce the currents (Binet & Marchal, 1993), resulting in long interglacial periods when the currents were favourable to the scenario we propose.

Surface salinity

The data we present (above; Fig. 2) show the strong seasonal changes in surface salinity for a 'normal year', which are directly related to rainfall in the Congo Basin. This relationship further suggests that, in historical periods when rainfall was higher in tropical Africa, freshwater path values as low as 25 psu may have reached the islands. Historical fluctuations in sea surface salinities have been inferred from foraminiferans in sediment cores (Stott *et al.*, 2004). Similar evidence suggests that major discharge pulses of freshwater exited the Congo several times over the past 13 kyr (Marret *et al.*, 2001), and that the Congo plume has extended hundreds of kilometres west from its current position for at least 150 kyr (Dupont *et al.*, 2000). We therefore consider it probable that such freshwater paths have periodically drastically reduced surface salinity as far as the Gulf of Guinea islands.

In summary, our proposed mechanism of dispersal involves conglomerations of vegetation leaving the mouth of the Congo River (the largest of the rivers in the region), and being rafted by surface currents within water of reduced salinity to the Gulf of Guinea islands. The present-day catchment for the Congo River reaches as far east as the Ruwenzori Mountains, potentially bringing rafts from eastern Africa. In the mid-Miocene (15 Ma) the Congo watershed extended further east, with more rivers draining west towards the Atlantic (Goudie, 2005). This was interrupted 12–13 Ma (late Miocene) by the uprising of the present Western Rift Valley, which reduced the Congo catchment and shifted the watershed further west (Goudie, 2005). Hence the results of our molecular analyses of *P. newtoni*, which suggest an East African rather than West African ancestry, could be accounted for by an actual East African nearest relative (from the historical Miocene drainage), or a parent taxon from the poorly sampled current Congo basin. Due to the lack of fossil data for divergences in *Ptychadena* and related taxa, and of other suitable calibration points, our data set does not allow us to calculate divergence times using molecular-clock approaches. However, assuming that rates in this amphibian group concur with the generally observed rates of 0.3–1% pairwise 16S divergence per million years (Vences *et al.*, 2005), this places the divergence of *P. newtoni* from *P.* aff. *mascareniensis* A at 5.6–18.6 Ma; thus the dispersal of *P. newtoni* to the islands prior to the uplift of the Western Rift Valley is plausible.

Other Gulf of Guinea island taxa

These three components not only favour transport of amphibians, but may partly explain the presence of the observed high biodiversity of animals and plants. For example, a raft may have enough invertebrate food to explain the presence of the São Tomé and Príncipe endemic shrews (Thiel & Gutow, 2005). The phylogenetic affinities of skinks suggest that those from São Tomé are most closely related to East African species, while those from Príncipe are most closely related to specimens from Cameroon (Jesus *et al.*, 2005b). Furthermore, the phylogenetic relationships among geckos and skinks suggest multiple independent colonization events of the islands (Jesus *et al.*, 2005a,b) consistent with the arrival of several rafts, as might be expected with the proposed mechanism. Other São Tomé fauna have also been reported to have East African relatives, for example the endemic snail genus *Bocageia* Girard has its nearest relatives in the Comoros and Mt Ruwenzori (Gascoigne, 1994).

The proposed mechanism of dispersal does not rule out rafts originating from other Gulf of Guinea rivers, most notably the Niger, because prevailing oceanic currents would also favour movements towards the Gulf of Guinea islands (Fig. 1). However, the dominance of the Congo River suggests that it might be expected to be the origin for other Gulf of Guinea island amphibians. Drewes & Wilkinson (2004) affirmed the endemic status of *Hyperolius thomensis* and *H. molleri* Bedriaga, finding that they were most closely related to *Hyperolius cinnamomeoventris* Bocage collected in Uganda, although the number of taxa sampled was small. Additional support comes from a recently reported preliminary phylogeny of the genus *Phrynobatrachus* Günther (Zimkus, 2005). This showed that another of the islands' endemic frogs, *P. dispar*, is most closely related to a group of East African species: *Phrynobatrachus parvulus* (Boulenger); *Phrynobatrachus keniensis* Barbour and Loveridge; *Phrynobatrachus* cf. *minutus* (Boulenger); *Phrynobatrachus* cf *rungwensis* (Loveridge); and *Phrynobatrachus rungwensis* (Loveridge). A morphological and molecular study currently under way by J. Uyeda and co-workers (personal communication) indicates that the *Phrynobatrachus* of Príncipe and those of São Tomé are distinct species, and that phylogenetic analysis placed both in a clade with East African species.

The caecilian *S. thomense* has only one congener, *Schistometopum gregorii* (Boulenger), that has a wholly East African distribution (Nussbaum & Pfrender, 1998). Additionally, Nussbaum & Pfrender (1998) mention a single caecilian specimen housed in the Royal Museum of Central Africa, Tervuren, which is undoubtedly a member of the genus *Schistometopum* (verified by G.J.M.) and which was most probably collected in eastern Congo.

We acknowledge that there are unexplained barriers to this particular mechanism, such as how floating rafts overcame cataracts and waterfalls existing in the present day Congo River, although many possibilities exist over the last 15 Ma, including the drainage of a central African lake (Goudie, 2005). However, the proposed colonization of the Gulf of Guinea islands using rafts on freshwater paths should give pause for thought to proponents of a vicariance-dominated explanation for amphibian distributions. A marked east–west divide of the amphibian fauna of the African Intertropical Montane Region exists whereby less than half the genera are shared (Poynton, 1999). Remarkably, all five Gulf of Guinea island genera (Table 1) are shared between this east–west divide, and those investigated to date appear to have eastern origins. Rafts leaving the Congo may not only be responsible for colonizing the islands in the Gulf of Guinea. We speculate that phylogeographical examination of more taxa from the African Intertropical Montane Region may reveal other ancient east-to-west dispersal events congruent with our proposed mechanism.

We further suggest that this mechanism may have served in colonization events of other oceanic islands. Available data on surface sea currents suggest that rafts from north-western Madagascar would be taken through a vortex to the Comoro Islands, and during the monsoon rainfall in April the surface salinity drops due to output from the Betsiboka River, providing a freshwater path for rafts from Madagascar to the Comoros (data from the Naval Research Laboratory, http://www7320.nrlssc.navy.mil/global_ncom/mad.html). The two frogs from Mayotte (Vences *et al*., 2003) and many of the Comorian reptiles (Raselimanana & Vences, 2003) show affinities with the lowland fauna of north-western Madagascar.

The Gulf of Guinea islands are particularly suitable as a model region for transoceanic dispersal, and deserve more attention from researchers. More of the endemic flora and fauna of these islands need phylogenetic investigation, which may give better insights into the ancient colonization of remote oceanic islands. In summary, we urge reconsideration of the generally accepted idea that marine environments are always effective barriers to salt-intolerant taxa, because there is now evidence to suggest that they can, at least occasionally, provide freshwater paths for organisms on floating rafts to drift to and colonize remote oceanic islands.

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BIOSKETCHES

John Measey is an amphibian ecologist and has worked throughout Africa, including São Tomé. Current interests include the ecology of subterranean herpetofauna, with a focus on their population biology and dispersal abilities.

Miguel Vences is professor of evolutionary biology and has long been interested in the biogeography of amphibians and other vertebrates of Madagascar and the Comoros islands.

Robert C. Drewes is curator of herpetology at the California Academy of Sciences and has been studying African herpetofauna for over 35 years.

Ylenia Chiari is a postdoctoral researcher interested in conservation genetics of amphibians and reptiles. Over the past 3 years she has worked on conservation of Malagasy amphibians.

Martim Melo is currently assessing the status and phylogeny of the Gulf of Guinea islands' avian taxa, although his interests encompass speciation and island biogeography in general.

Bernard Bourles is a physical oceanographer at Institut de Recherche pour le Développement and has been working on the Equatorial Atlantic circulation for more than 15 years. He currently works in the Gulf of Guinea with the African Monsoon Multidisciplinary Analysis program.

Editor: Robert McDowall