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Chytrid Fungus (*Batrachochytrium dendrobatidis*) Undetected in the Two Orders of Seychelles Amphibians

Infection by the fungal pathogen *Batrachochytrium dendrobatidis* (*Bd*) is a major driver in global amphibian declines (Berger et al. 1998; Skerratt et al. 2007) and can occur

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in all three orders of Amphibia, having been most recently documented in the Gymnophiona (Doherty-Bone et al. 2013; Gower et al. 2013). *Bd* has been detected in 71 countries and 695 amphibian species (Olson and Ronnenberg 2014) but to our knowledge no screening for its presence or anecdotal evidence of chytridiomycosis has been reported from the Seychelles Archipelago, a biodiversity hotspot (Myers et al. 2000) with a very high proportion (86%) of endemic amphibian genera (Poynton 1999) (Fig. 1).

Eleven of the 12 species of Seychelles amphibians (Table 1) are globally significant. The four species of sooglossid frog are unique in being the only amphibian family endemic to an island group, and the three genera of caecilians (all endemic, comprising a radiation of six species) are the only gymnophionan genera confined to islands. The single hyperoliid frog species occurring here is endemic to the Seychelles, leaving the single ptychadenid frog as the only non-endemic amphibian (Nussbaum 1984). Being primarily fossorial, caecilians are considered difficult study organisms

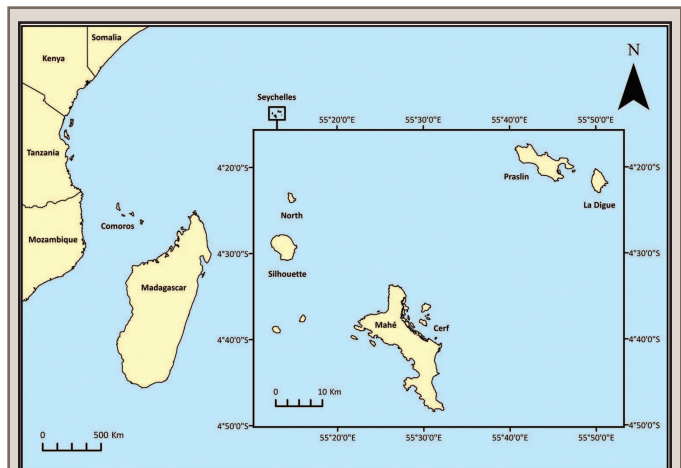


FIG. 1. Location map showing the position of the Seychelles Archipelago in relation to the African coast and Madagascar. Zoomed panel shows the main inner islands. Map generated using ArcMap 10.1 (ESRI 2012).

and collection usually requires dedicated digging (Gower and Wilkinson 2005), such that there have been few caecilian *Bd* field surveys (Gower et al. 2013). Similarly, sooglossid frogs are cryptic in their habits, undergoing direct development as part of a completely terrestrial life cycle in often inaccessible, elevated areas of moist forest (Nussbaum 1984; Nussbaum and Wu 2007). In contrast, the Seychelles Treefrog (*Tachycnemis seychellensis*: see Maddock et al. 2014), and the likely introduced Mascarene Ridged Frog (*Ptychadena mascareniensis*: see Vences et al. 2004) can be easier to encounter, forming breeding aggregations around marshes, streams, and temporary pools, especially in the evening (*T. seychellensis*) and/or after rain events (*P. mascareniensis*) (Nussbaum 1984). *Bd* is known to infect *P. mascareniensis* in mainland Africa (www.bd-maps.net/surveillance/s_species.asp; accessed 3 March 2014; Goldberg 2007), while Olson et al. (2013) identified a 50% prevalence of *Bd* in African Hyperoliidae, the family to which *T. seychellensis* belongs.

Between May 2010 and March 2013, skin swabs were taken from wild-caught caecilians and metamorphosed anurans at multiple locations across six of the Seychelles' inner islands (Fig. 2). All sampled specimens were swabbed alive and within

24 hours of capture, using rayon-tipped MW100 fine-tip swabs (Medical Wire and Equipment, Corsham, Wiltshire, England). Swabbing protocol generally followed best-practice methods available at the time (e.g., Smith 2011). However, our sampling was undertaken by three separate research groups operating over the period, with differing primary research aims. Therefore, some deviation from standard biosecurity protocol did occur, including capture and initial handling of amphibians without gloves, and (although infrequent) housing more than one animal (but always of the same species) in the same plastic bag upon capture. The principle aim was to gather a representative sample from sites within broadly separate locations, and not to swab every individual encountered or captured. A large proportion of sooglossid frog swabbing was undertaken in the field by a single person (JL). These mostly very small anurans (except for individuals of *Sooglossus thomasseti* measuring greater than 25 mm snout-vent length) were cleaned of debris and soil by transferring them to a small plastic zip-lock bag and rinsing with fresh water sourced at the sampling locality. Although this rinsing may have removed some zoospores, it was performed to reduce the increased likelihood of PCR inhibition. The water was then drained and the animal gently restrained and swabbed within the

TABLE 1. Distribution of Seychelles amphibians (adapted from Nussbaum 1984), including the 2009 discovery of Sooglossidae on Praslin (Taylor et al. 2012) and their IUCN Red List status (IUCN 2013): LC – Least Concern; EN – Endangered; CR – Critically Endangered. Caecilian taxonomy follows Wilkinson et al. (2011).

Order	Family	Species	Islands present	Conservation status
Anura	Hyperolidae	<i>Tachycnemis seychellensis</i>	La Digue, Mahé, Praslin, Silhouette	LC
Anura	Ptychadenidae	<i>Ptychadena mascareniensis</i>	Cerf, Curieuse, Frégate, Grand Soeur, La Digue, Mahé, North ^a , Praslin, Silhouette	LC
Anura	Sooglossidae	<i>Sooglossus sechellensis</i>	Mahé, Praslin, Silhouette	EN
Anura	Sooglossidae	<i>Sooglossus thomasseti</i>	Mahé, Silhouette	CR
Anura	Sooglossidae	<i>Sechellophryne gardineri</i>	Mahé, Silhouette	EN
Anura	Sooglossidae	<i>Sechellophryne pipilodryas</i>	Silhouette	CR
Gymnophiona	Indotyphlidae	<i>Grandisonia alternans</i>	Félicité, Frégate, La Digue, Mahé, St. Anne, Silhouette	LC
Gymnophiona	Indotyphlidae	<i>Grandisonia larvata</i>	Félicité, La Digue, Mahé, Praslin, St. Anne, Silhouette	LC
Gymnophiona	Indotyphlidae	<i>Grandisonia sechellensis</i>	Mahé, Praslin, Silhouette	LC
Gymnophiona	Indotyphlidae	<i>Hypogeophis brevis</i>	Mahé	EN
Gymnophiona	Indotyphlidae	<i>Hypogeophis rostratus</i>	Cerf, Curieuse, Félicité, Frégate, Grand Soeur, La Digue, Mahé, Praslin, St. Anne, Silhouette	LC
Gymnophiona	Indotyphlidae	<i>Praslinia cooperi</i>	Mahé, Praslin	EN

^a New locality record

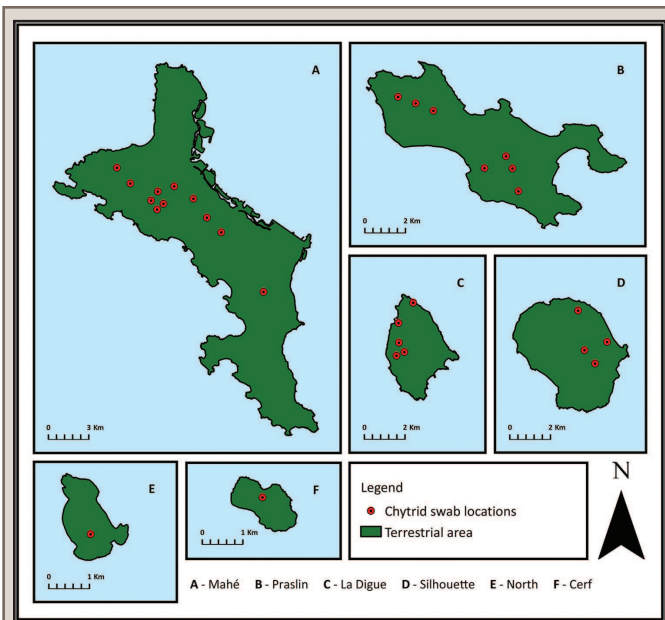


FIG. 2. Sampling localities for *Batrachochytrium dendrobatidis* across the Seychelles inner islands. Map generated using ArcMap 10.1 (ESRI 2012).



FIG. 3. *Sooglossus sechellensis* from Mahé, Seychelles Archipelago.

bag, removing the need for direct handling and minimizing risk of cross-contamination. Larger anurans, and sooglossid frogs processed when field assistance was available, were sampled using the standard technique (Smith 2011). Caecilians were swabbed dorsally, laterally, and ventrally along the length of the body, and also around the vent and head. Gloves were changed between each individual except where two or more specimens had been housed in the same plastic bag. All amphibians not retained as vouchers as part of broader research aims were released at their place of capture. Swabs were kept in the dark and transferred to cool storage, mostly refrigerated within 12 h and frozen within one week of completed fieldwork.

DNA extractions and quantitative real time Taqman® polymerase chain reaction (qPCR) assays were performed at the Institute of Zoology (London, UK), using methods adapted from Boyle et al. (2004). DNA was extracted from swabs using bead beating with 0.5 mm silica beads and 60 µl PrepMan Ultra (Hyatt et al. 2007). The qPCR amplifications were performed in 25 µl



FIG. 4. *Grandisonia alternans* from Silhouette, Seychelles Archipelago.

reactions using *Bd* primers ITS-1 (forward): 5'-CCT TGA TAT AAT ACA GTG TGC CAT ATG TC-3' and 5.8S (reverse): 5'-AGC CAA GAG ATC CGT TGT CAA A-3', specific to the ITS-1/5.8S region of rDNA (Boyle et al. 2004). Standards of 100, 10, 1 and 0.1 *Bd* DNA genomic equivalents and negative controls were used in each run. Bovine serum albumin (BSA) was included in the TaqMan® master mix, to reduce inhibition of the PCR (Garland et al. 2010). Each sample was run in duplicate and no amplification in either replicate indicated a negative result.

A total of 291 skin swabs were obtained from Seychelles amphibians, representing 10 of the 12 species known to occur across the archipelago. All 213 anurans and 78 caecilians tested negative for *Bd* (comprising 66 *Sooglossus sechellensis* [Fig. 3]; 13 *S. thomasseti*, 14 *Sechellophryne gardineri*, 99 *Tachycnemis sechellensis*, 21 *Ptychadena mascareniensis*, 18 *Grandisonia alternans* [Fig. 4], 7 *G. larvata*, 10 *G. sechellensis*, 6 *Hypogeophis brevis*, 23 *H. rostratus*, 14 unidentified caecilians) (Table 2). No macroscopic presentation of chytridiomycosis-like symptoms or associated mortality was observed in any sampled individual, or in any amphibian encountered during the fieldwork. Our results suggest a widespread absence of *Bd* between 2010 and 2013 across six of the eleven amphibian-inhabited islands of the Seychelles archipelago. Some caution should be exercised in interpreting our results as indicating that the Seychelles are free of *Bd*. Many of the locations sampled were in close proximity to one another and/or linked by contiguous habitat, and so could be described as a single location for the purposes of achieving the recommended minimum sample size of 30 amphibians per site (Smith 2011), but the temporal differences between site surveys and overall limited sampling invariably resulted in this not being achieved (Table 2), leading to the possibility of type II errors. Also, the recommendation for sampling >59 individuals to detect *Bd* when infection rate is low (Skerratt et al. 2008) was achieved for only two species sampled (*T. sechellensis*, *S. sechellensis*), two islands sampled (Mahé and Praslin), and for the two pooled samples of all anurans and all caecilians. Prevalence of *Bd* infection in anurans indicates seasonal peaks in the cooler months (Berger et al. 2004; Retallick et al. 2004; Kriger and Hero 2007) even with little seasonal temperature variation (Whitfield et al. 2012). Sampling of Seychelles amphibians was not undertaken during the two (historically) coolest months of July and August. However, housing more than one animal in the same capture bag, although limited in occurrence, increased the opportunity for cross-contamination from chytrid zoospores, making detection of *Bd* more likely by fostering false-positive results for infection.

TABLE 2. Numbers of amphibians sampled for *Batrachochytrium dendrobatidis* across six Seychelles islands. No samples were *Bd*-positive. Sampling localities shown in Fig. 2.

Species	Island	No. of sampling sites	No. of individuals sampled
<i>Ptychadena mascareniensis</i>	Mahé	1	1
	North	1	17
	Praslin	1	1
	Silhouette	1	2
<i>Sechellophryne gardineri</i>	Mahé	2	9
	Silhouette	2	5
<i>Sooglossus sechellensis</i>	Mahé	7	25
	Praslin	4	39
	Silhouette	2	2
<i>Sooglossus thomasseti</i>	Mahé	4	8
	Silhouette	2	5
<i>Tachynemid sechellensis</i>	La Digue	2	11
	Mahé	3	42
	Praslin	5	32
	Silhouette	1	14
<i>Grandisonia alternans</i>	Mahé	4	6
	Silhouette	2	12
<i>Grandisonia larvata</i>	Mahé	2	2
	Praslin	2	4
	Silhouette	1	1
<i>Grandisonia sechellensis</i>	Mahé	2	6
	Praslin	1	1
	Silhouette	1	3
<i>Hypogeophis brevis</i>	Mahé	2	6
<i>Hypogeophis rostratus</i>	Cerf	1	2
	La Digue	2	4
	Mahé	1	4
	Praslin	2	6
	Silhouette	2	7
Unidentified caecilian	Praslin	2	4
	Silhouette	1	10

Direct development—a reproductive mode adopted by the Sooglossidae and at least one Seychelles caecilian (*H. rostratus*; Nussbaum 1984)—may provide a limiting factor to the transmission of *Bd* between and among amphibians (Todd 2007; but see Longo and Burrowes 2010). The probably more vagile *T. sechellensis* and *P. mascareniensis* require water bodies for aquatic larval development, and potentially present more suitable hosts for the dispersal and spread of *Bd*. Due to previous evidence of human-mediated trans-oceanic dispersal (Vences et al. 2004), *P. mascareniensis* in particular may lend itself to continuing introduction and transportation by way of tourist and/

or domestic traffic, especially among the main islands of Mahé, Praslin, and La Digue (Fig. 1). This species' propensity for dispersal was evidenced first-hand during fieldwork following the discovery of a novel, reproducing population on North Island. Similarly, and despite no currently recorded infection or capacity as a host for *Bd* (www.bd-maps.net/surveillance/s_species.asp; accessed 21 September 2014), the recent discovery of Asian Common Toads (*Duttaphrynus melanostictus*) on the east coast of Madagascar, having likely arrived in shipping containers from Asia (Kolby 2014a), highlights a further risk to Seychelles endemic fauna as a potential disease vector. The significantly shorter shipping distances among Madagascar, the Mascarenes, and Seychelles islands undoubtedly elevates the risk of further human-mediated dispersal of this potentially invasive species.

Links between climate, temperature, and *Bd* have been documented (see Pounds et al. 2006; Bosch et al. 2007; Lips et al. 2008; Rohr and Raffel 2010; Olson et al. 2013). In tropical regions, elevated, moist, and riparian habitats are home to amphibian species considered most likely to be severely threatened by *Bd* (Wake and Vredenburg 2008). The endemic Seychelles amphibians fall into at least two, and often all three of these categories which, combined with their restricted range, highlights the requirement for targeted conservation measures (Sodhi et al. 2008). In light of the recent discovery of *Bd* in Malagasy anurans (Kolby 2014b), and given the potential susceptibility of Seychelles amphibians, continued disease monitoring warrants consideration as part of ongoing conservation work for this globally significant amphibian community. Effective implementation of Seychelles' recently approved Biosecurity Act (Animal and Plant Biosecurity Act, 2014) is consistent with maintaining such vigilance.

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