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Rhinosporidiosis in African reed frogs *Hyperolius* spp. caused by a new species of *Rhinosporidium*

Patrick Scheid[1,3], Carsten Balczun[2], J. Maximilian Dehling[3], Alexander Ammon[4], Ulrich Sinsch[3,*]

[1]Laboratory of Medical Parasitology, Central Institute of the Bundeswehr Medical Service, Koblenz, Andernacherstrasse 100, 56070 Koblenz, Germany

[2]Zoology/Parasitology Group, Ruhr-Universität, Universitätsstrasse 150, 44780 Bochum, Germany

[3]Institute of Integrated Sciences, Department of Biology, Zoology Group, University of Koblenz-Landau, Universitätsstrasse 1, 56070 Koblenz, Germany

[4]Abteilung Pathologie, Bundeswehrzentralkrankenhaus, Rübenacher Strasse 170, 56072 Koblenz, Germany

ABSTRACT: We report the identification of a new *Rhinosporidium* species (Dermocystida, Mesomycetozoea) infecting amphibian hosts, while showing a species specificity for African reed frogs of the genus *Hyperolius*. Large dermal cysts (sporangia) of *R. rwandae* sp. nov. were observed in 18% of *H. lateralis* and similar cysts in 0.7% of *H. viridiflavus* surveyed. Fully developed *R. rwandae* cysts are about 500 to 600 µm in diameter and sealed from the frog tissue by a thick chitinous wall. Some cysts were filled with numerous round-oval basophilic microspores of 8 to 12 µm diameter. With the exception of legs, nodules were visible over the complete torso surface including the vocal sac of males, but the most affected skin region was the area around the cloaca. Behavior, condition, and lifespan of infected frogs do not seem to be distinct from that of healthy individuals. The mode of infection remains unknown, but we hypothesize that the infectious life stage reaches the dermis via the intraepidermal ducts of the skin glands. Molecular evidence places the new frog pathogen as a sister species of the human pathogen *R. seeberi*.

KEY WORDS: *Hyperolius lateralis* · *H. viridiflavus* · *Rhinosporidium rwandae* sp. nov. · 18S rRNA · Phylogeny · Anura · Hyperoliidae · Dermocystida

INTRODUCTION

The global decline of amphibians and the accelerated rate of species extinction are thought to be partly due to pandemic fungal pathogens causing chytridiomycosis (e.g. Berger et al. 1998, De Castro & Bolker 2005, Duffus & Cunningham 2010). The causative agent *Batrachochytrium dendrobatidis* (*Bd*) is an intracellular fungus (Phylum Chytridiomycota) that infects the keratinized tissues (skin of post-metamorphic amphibians, mouthparts of anuran larvae) and leads to the death of the host (Daszak et al. 1999). *Bd* infections have been reported from several hundred anuran and urodele species, indicating the absence of host specificity (Fisher et al. 2009, Duffus & Cunningham 2010). Recently, a second *Batrachochytrium* species (*B. salamandrivorans*) of probable Asian origin has been identified causing catastrophic die offs in salamanders in the Netherlands (Martel et al. 2013, 2014, Spitzen-van der Sluijs et al. 2013). Unlike *Bd*, the new pathogen seems to be restricted to urodele hosts (Martel et al. 2014). However, *Batrachochytrium* species are not the only pathogens which infect amphibians. Besides ranaviruses and true fungi, pathogens from 3 phyla of fungus-like microorganisms have been found to infect amphibians: Mesomycetozoea, Oomycetes and Perkinsozoa (Gleason et al. 2014).

Species in the class Mesomycetozoea are known to cause skin and muscle lesions in anurans and urodeles (Mendoza et al. 2002, Rowley et al. 2013). Nomencla-

*Corresponding author: sinsch@uni-koblenz.de

ture is subject to change and reorganization is an ongoing process (Feldman et al. 2005, Glockling et al. 2013). Mesomycetozoeans are members of an unusual clade of eukaryotic protists phylogenetically located near the animal–fungal divergence. Mesomycetozoea (synonym Ichthyosporea) split into 2 phylogenetically well-supported orders, Dermocystida and Ichthyophonida (Mendoza et al. 2002). Mesomycetozoean parasites are known to infect 18 species of amphibians. Two dermocystid genera, *Amphibiocystidium* and *Amphibiothecum* (formerly *Dermosporidium*), comprise skin pathogens, whereas *Ichthyophonus* sp. causes swelling of axial musculature and ulceration (Glenney et al. 2010, Rowley et al. 2013). *Amphibiocystidium ranae* infects some European frog and newt species producing nodular-type, externally visible skin lesions (Broz & Privora 1952, Pascolini et al. 2003, Pereira et al. 2005, González-Hernández et al. 2010, Courtois et al. 2013). Recently, another *Amphibiocystidium* species (*A. viridescens*) has been found infecting North American newts (Raffel et al. 2008). Dermocystid infections are thought not to be fatal, but self-limiting (Densmore & Green 2007). Based on their host specificity and on molecular evidence, *Amphibiocystidium* is currently considered as a sister taxon to both *Dermocystidium* and *Rhinosporidium* (Pascolini et al. 2003, Pereira et al. 2005). Finally, the mesomycetozoan *Amphibiothecum penneri* causes dermal cysts in the North American toad *Anaxyrus americanus* (Jay & Pohley 1981). Molecular evidence suggests that this species is phylogenetically more distant from *Amphibiocystidium*, *Dermocystidium*, and *Rhinosporidium* (Feldman et al. 2005).

Knowledge about fungal and mesomycetozoean skin pathogens of African amphibians is very limited with the exception of those affecting the *Bd*-tolerant clawed frog *Xenopus laevis* (Fisher et al. 2009, Reeder et al. 2012). To the best of our knowledge, infections caused by Mesomycetozoea have not previously been detected in African amphibians (Rowley et al. 2013). However, during an extensive study of an Afromontane frog community (Sinsch et al. 2012), we encountered frog specimens which had nodular skin lesions resembling those caused by *Amphibiocystidium ranae*. Though 16 anuran species were identified dwelling in the same swamp habitat surveyed near Huye, Rwanda, only 1 reed frog species (*Hyperolius lateralis*) was heavily affected and another (*H. viridiflavus*) only rarely. In this paper, we present data on the morphological features of the *H. lateralis* pathogen causing the observed nodules and use molecular evidence for its identification as a new species within the *Rhinosporidium* genus, the first one known to infect amphibians.

MATERIAL AND METHODS

Reed frog diversity at the sampling sites

Specimens of 5 *Hyperolius* species (adults and tadpoles; Sinsch et al. 2012, Channing et al. 2013) were collected and preserved during field work in Rwanda in March and April 2011 and March 2012 (see Table 1). They were encountered in agricultural areas in the southern part of the country, near the city of Huye (2° 36.0′ S, 29° 45.4′ E, 1645 m above sea level [a.s.l.]; WGS84) and along the Rukarara River (2° 27.095′ S, 29° 27.495′ E, 2031 m a.s.l.). Liver tissue samples for molecular analyses were taken and stored separately in 98% ethanol. A total of 24 *H. lateralis* males captured during a nightly survey in an overgrown former agricultural area on March 27, 2012, were kept and observed in captivity for 4 wk. Another 271 individuals of *H. viridiflavus* were documented with digital photos *in situ*, but not collected. Species identity was corroborated by comparing the vouchers to the type specimen of *H. lateralis* in the collection of the Royal Museum for Central Africa, Tervuren, Belgium (RMCA 38902) and to one of the syntypes of *H. viridiflavus* in the collection of the Naturhistorisches Museum Wien (NHMW 22896).

Histology and microscopy

In a first step, all preserved specimens and digital photos of *Hyperolius* spp. were examined for the presence of skin efflorescences to estimate the rate of affected individuals per species. Independently of externally visible skin lesions, samples of all preserved specimens were histologically examined for pathological dermal modifications. To achieve an overview of external skin abnormalities, photos were taken at magnifications of 20 to 200× using a Keyence VHX digital microscope. Skin lesions and prominent structures of the heavily infected *H. lateralis* (ZFMK 96782) were excised with a sterile scalpel, embedded in paraffin, sectioned, stained with periodic acid-Schiff (PAS) following standard techniques, and examined with higher magnification and phase contrast with a Leica Stemi 2000C microscope equipped with a Hitachi KP-FD140F-S3 camera. The micrographs at higher magnification were taken with a Leica DM5000 B fluorescence microscope equipped with a JVC 3CCD 3-chip camera. The software used for documentation purposes was DISKUS 4.30 (Hilgers).

Nucleic acid extractions and polymerase chain reactions

Hemispherical skin lesions containing cysts with endospores were excised with a scalpel from ethanol-preserved *H. lateralis* (ZFMK 96782). DNA was extracted from the tissue following the protocol for tissue samples of the my-Budget DNA Mini Kit (Bio-Budget Technologies). The extracted DNA was used to amplify the 18S rRNA gene by polymerase chain reaction (PCR) using GoTaq® DNA Polymerase (Promega) with primers specific for Mesomycetozoea (forward: 5′-TAA GCC ATG CAT GTC AAG TAT AA-3′ and reverse: 5′-ACT AGG AAT TCC TCG TBA AGA TS-3′, Feldman et al. 2005). Using these primers, a DNA fragment of about 1400 nucleotides was expected. The PCR protocol consisted of an initial incubation at 94°C for 2 min, 40 cycles at 94°C for 1 min, 55°C for 1 min, and 72°C for 3 min, with a last extension step at 72°C for 7 min. The amplicons were separated on a 1% agarose gel, stained with ethidium bromide and visualized on a UV transilluminator.

Liver tissue samples of the 5 *Hyperolius* species occurring sympatrically in southern Rwanda were used to sequence a fragment of the 16S mitochondrial rRNA gene. DNA was extracted using the DNeasy Blood and Tissue Kit (Qiagen) following the manufacturer's protocol. PCR was used to amplify fragments of approximately 550 bp of the 16S mitochondrial rRNA gene using the standard primers 16SAL (5′-CGC CTG TTT ACT AAA AAC AT-3′) and 16SBH (5′-CCG GTC TGA ACT CAG ATC ACG T-3′). Amplification followed the standard PCR conditions (Palumbi 1996) with the following thermal cycle profile: 120 s at 94°C, followed by 33 cycles of 94°C for 30 s, 53°C for 30 s, and extension at 65°C for 60 s. All amplified PCR products were verified using electrophoresis on a 1% agarose gel stained with ethidium bromide. PCR products were purified using the Highpure PCR Product Purification Kit (Roche Diagnostics). A negative control using water instead of template DNA was carried out for each PCR.

Sequence and phylogenetic analysis

PCR amplicons of the endospores were bidirectionally sequenced in house on a 3130xl Genetic Analyzer platform (Applied Biosystems) using the same primers as for PCR. Sequences were assembled by DNA baser (Heracle BioSoft) and identified by a BLAST search against GenBank entries (Altschul et al. 1990). Phylogenetic trees were calculated for the 18S rRNA alignment using either the maximum likelihood algorithm in MEGA 5.2 (Tamura et al. 2011) or Bayesian inference in MrBayes (Huelsenbeck & Ronquist 2001). Branch support was calculated based on 1000 bootstraps. The calculated tree was manually refined using FigTree v1.4.2 (http://tree.bio.ed.ac.uk/software/figtree/). The partial sequence of the 18S rRNA gene of *Rhinosporidium rwandae* is deposited in GenBank (accession number: KP172222).

Sequencing reactions for the *Hyperolius* samples were performed with the DYEnamic ET Terminator Cycle Sequencing Premix kit (GE Healthcare) and then run on a MegaBACE 1000 automated sequencer (GE Healthcare). A sequence from *Afrixalus quadrivittatus* (Hyperoliidae) was used as an outgroup. The sequences were aligned using the MUSCLE algorithm (Edgar 2004) implemented in MEGA 5 (Tamura et al. 2011) and alignments were corrected by eye. The final matrix consisted of 901 bp and was used for phylogenetic analysis (maximum likelihood). Sequences are deposited in GenBank (*H. cinnamomeoventris*: JQ966568; *H. kivuensis*: JQ966567; *H. lateralis*: JQ966569; *H. rwandae*: JQ863711; *H. viridiflavus*: JQ966570).

RESULTS

Host life history and infection susceptibility

At the study sites, 5 reed frog species were recorded: *Hyperolius cinnamomeoventris*, *H. kivuensis*, *H. lateralis*, *H. rwandae*, and *H. viridiflavus*. The habitat preferences of adult *H. lateralis* and *H. cinnamomeoventris* are sedgy areas with small overgrown irrigation channels in which tadpoles of the 2 species are frequently found, sometimes in syntopy with those of *H. kivuensis* (Sinsch et al. 2012). In contrast, adult *H. viridiflavus* prefer more open agricultural areas with broad and open irrigation channels, sharing this habitat with *H. kivuensis* and *H. rwandae,* and occasionally with *H. cinnamomeoventris* (Sinsch et al. 2012). Tadpoles of the first 3 species are commonly found within the same channels.

Skin lesions as external symptoms of infection were observed in only 2 of the species, *H. lateralis* and *H. viridiflavus*, whereas none of the specimens of the other species exhibited signs of skin infection (Table 1, Fig. 1). None of the tadpoles examined showed nodular skin lesions resembling those of adults. Prevalence of skin infection differed markedly between *H. lateralis* and *H. viridiflavus*. Of the 44 adult specimens of *H. lateralis* examined, 8 had skin lesions (18.2%)

Table 1. *Hyperolius* specimens surveyed visually or histologically for skin infections. Scaling of externally visible skin lesion as a surrogate of infection intensity: A = a single small nodule; B = several small nodules, light infection of urostyle/pelvic area; C = one or a few large nodules including urostyle/pelvic area; D = more than 10 large nodules, infected urostyle skin protuberant

Species	Stage	Visual examination		Infection intensity (n)				Histological examination of skin		Sequencing
		No. total	No. infected	A	B	C	D	No. total	No. infected	of pathogen
H. cinnamomeoventris	Adult	9	none					9	none	-
	Tadpole	17	none							
H. kivuensis	Adult	13	none					13	none	-
	Tadpole	19	none							
H. lateralis	Adult	44	8	0	6	1	1	24	4	1 (ZFMK 96782)
	Tadpole	18	none							
H. rwandae	Adult	9	none					9	none	-
	Tadpole	1	none							
H. viridiflavus	Adult	293	2	1	1	0	0	22	none	-
	Tadpole	21	none							

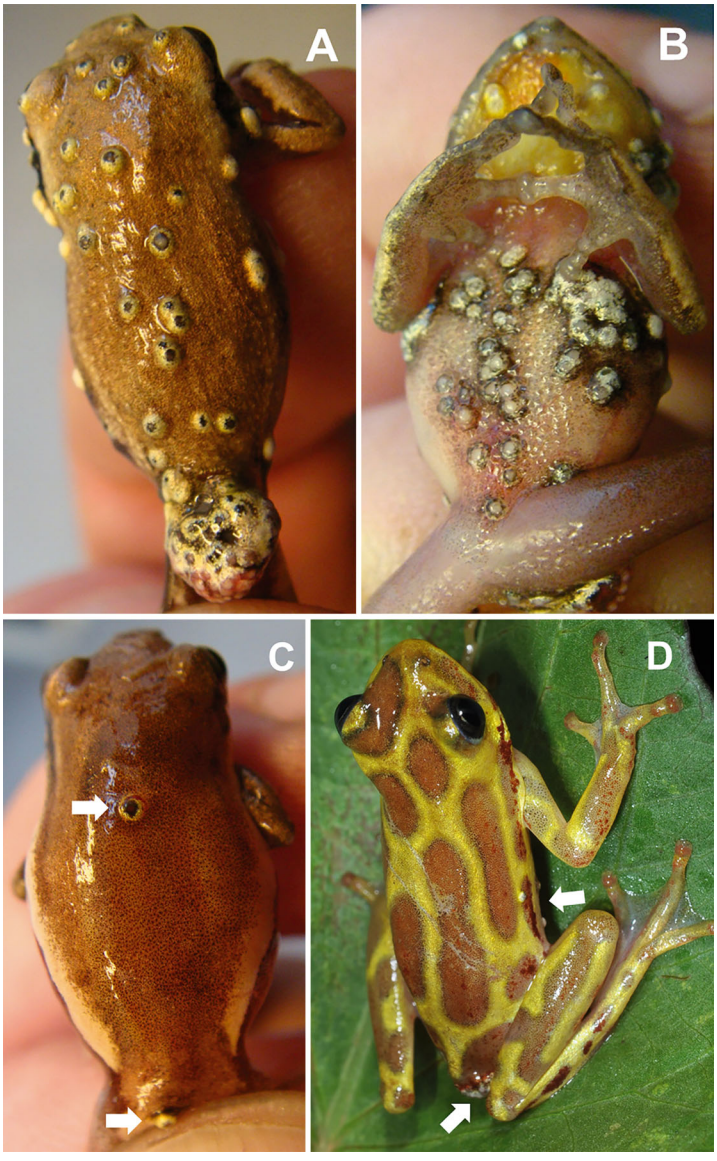

Fig. 1. (A,B) *Hyperolius lateralis* male, 22 mm snout-vent length (SVL), heavily infected with *Rhinosporidium rwandae* and collected on March 27, 2012, in Huye, Rwanda (ZFMK 96782). (C) *H. lateralis* male, 21.5 mm SVL (ZFMK 96783); early stage of infection with 2 skin efflorescences (marked by arrows). (D) *H. viridiflavus* male, 29 mm SVL; intermediate stage of infection, collected on April 20, 2011, in Huye, Rwanda

varying from a single efflorescence (Fig. 1C) to body-wide damaged skin (Fig. 1A,B), but only 2 of the almost 300 adult specimens of *H. viridiflavus* observed had lesions (ca. 0.7%; Table 1). Infected *H. lateralis* did not differ significantly from those that were apparently healthy with respect to age (0.5 vs. 0.4 yr; *t*-test, p = 0.305; details of age estimation will be published elsewhere) or snout-vent length (SVL, both 20.7 mm; *t*-test, p = 0.879). There was no specific skin region of first appearance of nodules: in 3 *H. lateralis* it was the urostyle/pelvic area, in another specimen the dorsal skin and in yet another the lateral skin.

Collection of specimens (predominately males) occurred during the night, when males produced advertisement calls to attract females to the reproduction site. We did not notice any difference in calling site selection or calling intensity *in situ* between infected and healthy males. Agility and feeding behavior of even the heavily affected male (Fig. 1A,B) in captivity did not apparently vary from that of other *H. lateralis* males. Infected (n = 3) and healthy males (n = 20) survived the 4 wk period in captivity. None of the initially healthy males developed skin nodules. The skin lesions of infected individuals did not increase in size or number. The heavily affected male was sacrificed 1 wk after capture for further histologi-

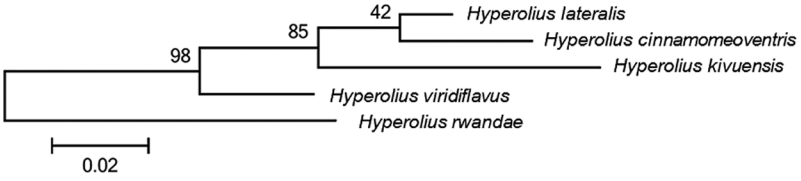

Fig. 2. Maximum likelihood phylogram of *Hyperolius* species from southern Rwanda with *Afrixalus quadrivittatus* as outgroup (not shown) based on 487 bp of the mitochondrial 16S rRNA gene. Numbers above nodes are bootstrap support values

cal and molecular examination. Consequently, it remains unknown whether the individual would have survived long-term.

Phylogenetic analysis of the reed frog host species

Our reed frog samples from southern Rwanda resolved into 5 terminal groups in a phylogeny based on the 16S sequences (Fig. 2) which correspond to the *a priori* assignment of the specimens to 5 species based on external morphological characters. The sequences of the 5 species differ from each other by uncorrected pairwise distances of 9.3 to 15.9%. The 2 species subject to infection, *H. lateralis* and *H. viridiflavus*, were not resolved as sister clades.

Clinical and histopathological description

Supervision of the skin of the ethanol-preserved *H. lateralis* specimens (Figs. 1 & 3) revealed nodular and partially ruptured lesions. These elevated hemispherical and whitish skin efflorescences of about 1 mm diameter were observed regularly in the dorsal, lateral, and ventral skin in *H. lateralis* (Fig. 3), and additionally in the vocal sac of an uncollected *H. viridiflavus*. The urostyle and pelvic skin region was usually most affected. The efflorescences showed darkly colored internal structures of approximately 500 to 600 µm diameter.

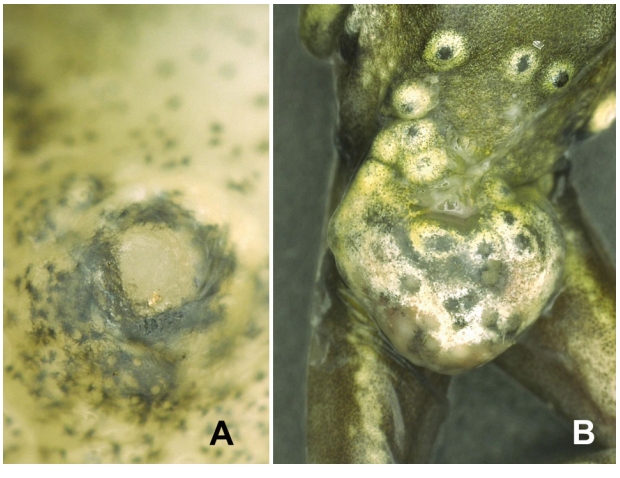

Fig. 3. Macroscopic views of skin efflorescences in the heavily infected *Hyperolius lateralis* male shown in Fig. 1A,B (ZFMK 96782). (A) Isolated efflorescence on dorsal skin and (B) cloacal region with a multitude of efflorescences

The organs in the torso of the most affected *H. lateralis* specimen (ZFMK 96782) showed no pathological changes, but early stage autolysis was detectable, particularly in liver parenchyma. Investigation of the legs did not reveal any pathological changes in the skeletal muscles and no skin nodules were present. No cysts were ever observed in internal organs or muscles.

The skin showed a multiple-row squamous epidermis containing intraepidermal ducts leading to the eccrine glands of the dermal stratum spongiosum (Fig. 4). These glands are the typical mucous and serous glands of amphibian skin (Mills & Prum 1984, Fox 1994) with about 40 and 100 µm diameter, respectively, and therefore much smaller than the cysts (Fig. 5). One efflorescence was excised with a sterile scalpel and microscopic analysis revealed spherical cysts (sporangia) subjacent to the epidermis which are bounded by thick homogenous cyst walls. These cyst walls showed an eosinophilic reaction in PAS staining and appeared to be chitin-bearing (Fig. 4). Some capsules were filled with granular eosinophilic material; others were filled with numerous round-oval basophilic microspores of 8 to 12 µm which frequently contained large inclusion bodies. The histopathological examination showed that no cellular defense action could be detected in tissue adjacent to the cysts.

Phylogenetic analysis of the 18S rRNA gene of the mesomycetozoean strain isolated from *H. lateralis*

Using the primers specific for the mesomycetozoean 18S rRNA gene in PCR, a DNA fragment of about 1400 bp was amplified. Phylogenetic analysis with sequences of the 18S rDNA of mesomycetozoeans revealed 2 phylogenetically distinct groups resembling the orders Dermocystida and Ichthyophonida (Fig. 6). The mesomycetozoean analyzed in this study forms a separate clade with *Rhinosporidium* species, namely *Rhinosporidium* sp., *R. seeberi*

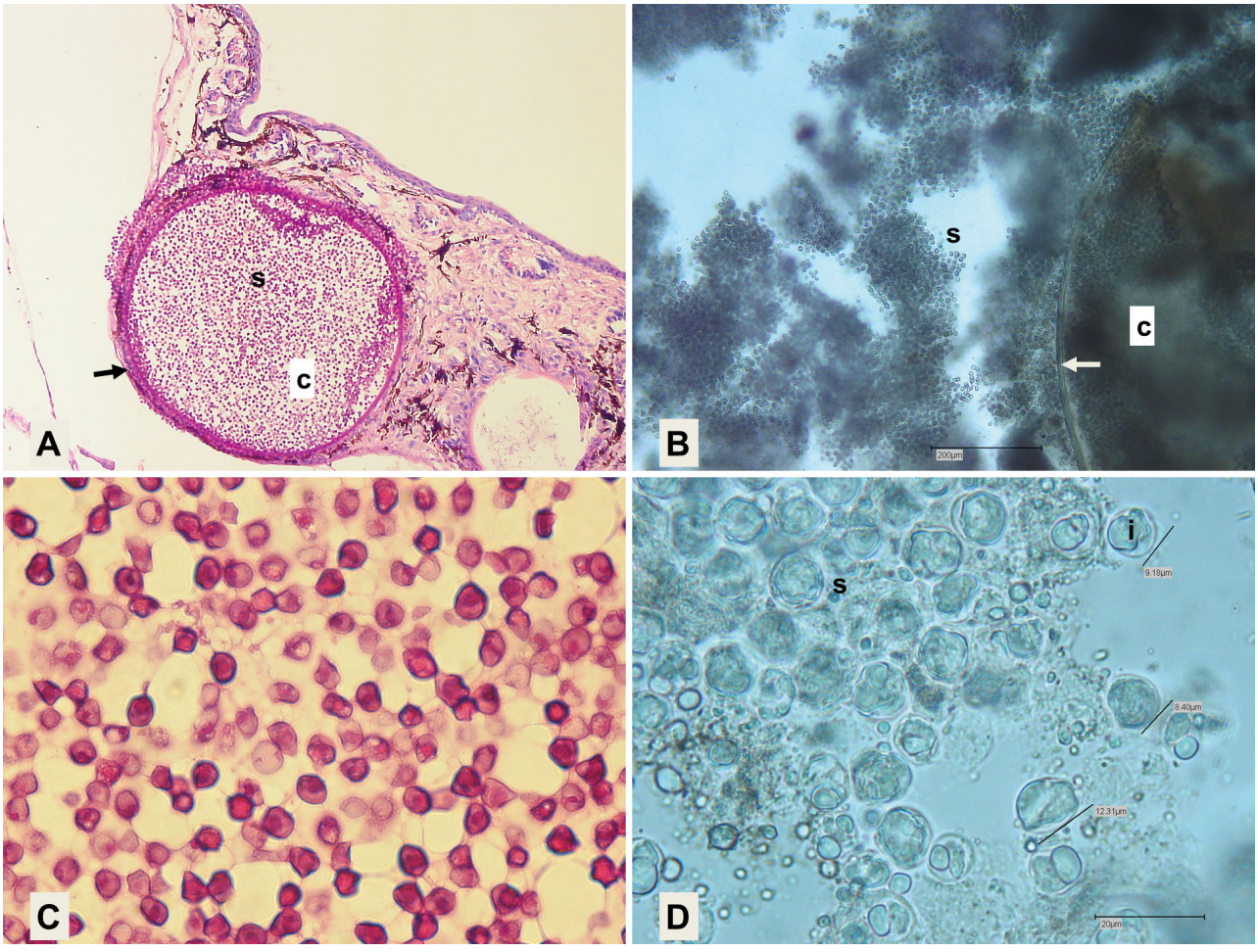

Fig. 4. Light micrographs of (A,C) periodic acid-Schiff (PAS)-stained and (B, D) unstained cysts (c) and spores (s) in the skin of the heavily infected *Hyperolius lateralis* male, including (B) a typical cross section of a subcutaneous cyst of *Rhinosporidium rwandae*. Cysts were bounded by a thick cyst wall (arrows) and spores contained inclusions (i) in the cytoplasm

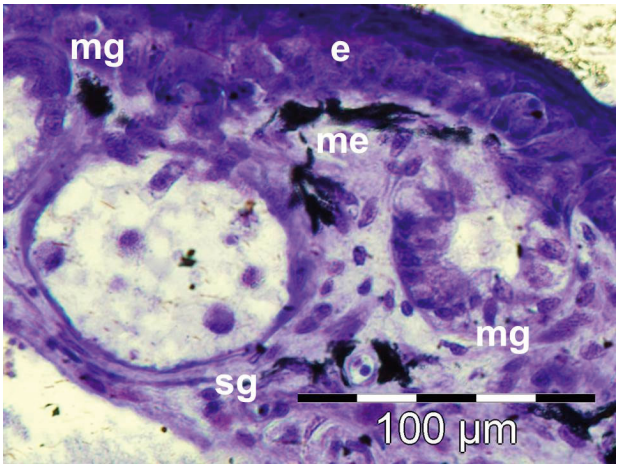

Fig. 5. Light micrograph of cresyl violet-stained skin of a *Hyperolius lateralis* male which was not infected by *Rhinosporidium*. Abbreviations: e: epidermis; me: melanophores; mg: mucous gland; sg: serous gland

and *R. cygnus*, albeit clearly separated at a significant distance. The sequence of the new *Rhinosporidium* strain differs from *R. seeberi* by an uncorrected pairwise distance of 1.1%. Due to this distance from the other members of the *Rhinosporidium* genus, we recognize the strain isolated from *H. lateralis* as a new species, *R. rwandae*.

Description of *Rhinosporidium rwandae*

Type host: *Hyperolius lateralis* Laurent, 1940 (Amphibia, Hyperoliidae), male ZFMK 96782 shown in Fig. 1A,B, deposited in the Forschungsmuseum Koenig, Bonn, Germany. Three infected *H. lateralis* males from the same locality were deposited as ZFMK 96783, shown in Fig. 1C, ZFMK 96784, and ZMFK 96785.

Type locality: Swamp near the city of Huye (2° 36.0′ S, 29° 45.4′ E, 1645 m a.s.l.), Rwanda.

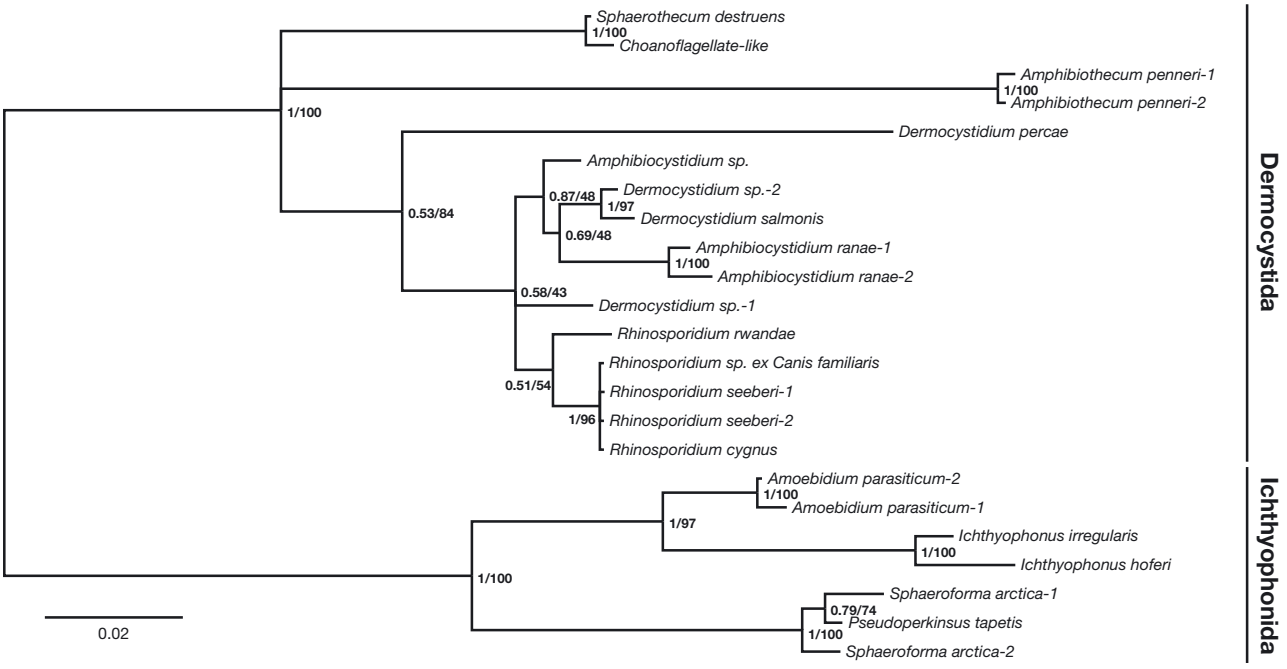

Fig. 6. Rooted Bayesian tree of 18S rRNA sequences of different mesomycetozoeans deposited in GenBank. The following sequences were used for phylogenetic reconstruction: AY372365, *Rhinosporidium* sp.; AF158369, AF118851, *Rhinosporidium seeberi*-1 and -2, respectively; AF399715, *Rhinosporidium cygnus*; EU650666, *Amphibiocystidium* sp.; DSU21336, AF533950, *Dermocystidium* sp.-1 and -2, respectively; DSU21337, *Dermocystidium salmonis*; AY692319, AY550245, *Amphibiocystidium ranae*-1 and -2, respectively; AY267345; *Sphaerothecum destruens*; L29455, Choanoflagellate-like sp.; AY772000, AY772001, *Amphibiothecum penneri*-1 and -2, respectively; AF533941, *Dermocystidium percae*; AF230303, *Ichthyophonus irregularis*; JX992717, Y16260, *Sphaeroforma arctica*-1 and -2, respectively; Y19155, AF274051, *Amoebidium parasiticum*-1 and -2, respectively; GU727527, *Pseudoperkinsus tapetis*; IHU25637, *Ichthyophonus hoferi*. Support values: posterior probability/maximum likelihood bootstrap (1000 replicates). Scale bar = substitutions per site

Site of infection: Predominantly in dorsal skin, cloacal region, less frequently in lateral and ventral skin. Mode of infection unknown.

Description: Parasites infecting the dermis of reed frogs *H. lateralis* and *H. viridiflavus*. Macroscopic, spherical cysts (sporangia) containing numerous spherical, basidophilic-staining microspores 8 to 12 µm in diameter. Cyst walls staining eosinophilic and chitin-bearing.

Hapantotype specimen: 18S rRNA gene sequence deposited in GenBank (accession number: KP-172222) and originating from type host individual ZFMK 96782.

Etymology: The species epithet derives from Rwanda. It is a noun in genitive singular.

DISCUSSION

Microscopic-morphological features and molecular evidence demonstrate that the pathogen isolated from skin nodules of *Hyperolius lateralis* is a member of the genus *Rhinosporidium* which belongs to the recently established Mesomycetozoea (Herr et al. 1999, Mendoza et al. 2002, Glockling et al. 2013). Due to the obviously smaller sizes of the mature sporangia and zoospores of *Batrachochytrium dendrobatidis* compared to *R. rwandae*, *Bd* can be ruled out as etiological agent. The microscopical and morphological examination of cysts revealed an affiliation to genera in the Mesomycetozoea. Similar details have been published for *Amphibiocystidium* spp. (Dermocystida), a known pathogen of frogs (Pereira et al. 2005, Raffel et al. 2008). However, the comparison of morphological details, especially the size of sporangia and microspores, eventually resulted in affiliation to the genus *Rhinosporidium* (Kennedy et al. 1995). The support values of the molecular phylogenetic analysis within the clade *Rhinosporidium* are comparatively low, but given the low degree of taxon sampling within this group we recommend tentative assignment of the newly identified parasite species to the genus *Rhinosporidium*. To the best of our knowledge, this is the first description of a *Rhinosporidium* species parasitizing the skin of amphibians. The currently known strains of the only other *Rhinosporidium* pathogen, *R. seeberi* (Silva et al. 2005), are the etiologic agents of rhinosporidiosis, a disease of

mucous membranes and infrequently of the skin and other tissues of endotherms (mammals: humans, cattle, cats, dogs, horses, mules; birds: ducks, parrots, swans; Kennedy et al. 1995, Leeming et al. 2007, Vilela & Mendoza 2012). Although first reported in an Argentinian patient, human rhinosporidiosis is more prevalent in India and Sri Lanka than in other geographic locations (Seeber 1900, Elston 2009). *Rhinosporidium* is a non-culturable organism and rhinosporidiosis cannot be induced in experimental animals (Arseculeratne et al. 2000). A common feature of *R. seeberi* and *R. rwandae* is the chitinous cyst wall (Bader & Grueber 1970).

It is thought that the natural habitat of *R. seeberi* is stagnant or lacustrine water, but positive evidence from the field is still lacking (Arseculeratne 2002, 2005). Analogously, we assume that infectious life stages of *R. rwandae* are probably inhabitants of the aquatic environment, and may be walled endospores with desiccation resistance, like other mesomycetozoeans (Rowley et al. 2013). If infection is based on such life stages enduring in the swamp water and does not require direct frog to frog contact, host specificity is considerably greater than in *R. seeberi* (Silva et al. 2005). In a community including 16 anuran species, the main host is clearly *H. lateralis*, while *H. viridiflavus* infections are rare and future studies will reveal if they are caused by another strain of *R. rwandae*. Distinct host specificity for multiple strains of *R. seeberi* has been shown using internal transcribed spacer 1 (ITS1), 5.8S and ITS2 sequences (Silva et al. 2005). The different strains clustered in 3 independent sister taxa, depending on their host origin, i.e. dog, human, or swan.

The mode of infection is currently unknown, but we hypothesize that infectious *Rhinosporidium* life stages may use the intraepidermal ducts of mucous and serous glands of the postmetamorphic frog skin to multiply in the stratum spongiosum of the dermis. The central dark spot of each nodule could be the inflated former opening of the duct, now filled with the cyst wall. The absence of skin glands and consequently of intraepidermal ducts in larval anurans (Bovbjerg 1963) would explain why tadpoles do not seem to be affected by rhinosporidiosis. Transmission based on vectors such as leeches, as assumed for *Ichthyophonu*s sp. (Raffel et al. 2006), is unlikely because water bodies used by *H. lateralis* adults often dry out.

R. rwandae, Amphibiocystidium spp. and *Amphibiothecum penneri* pathogens form characteristic cysts filled with numerous spores, which are typically located in the dermis of amphibians. In general, infections cause small multifocal nodules or pustules, but species assignment of pathogens based on external symptoms and spore morphology seems ambiguous without additional evidence from rDNA sequence analyses (Fredricks et al. 2000, Feldman et al. 2005, Densmore & Green 2007). Dermocystid infections are usually not fatal, but may reduce survival rate in captive newts (Raffel et al. 2008) or indirectly cause increased mortality by facilitating co-infection with *Bd* in *Rana lessonae* (Pascolini et al. 2003, Di Rosa et al. 2007). Presence of presumptive *Amphibiocystidium* cysts in the frog *Hysiboas pulchellus* in Uruguay did not affect behavior and condition of the hosts and dead or moribund frogs were never observed (Borteiro et al. 2014). The same applies to *Rhinosporidium*-infected *Hyperolius lateralis* and *H. viridiflavus*. Our future research will focus on the genetic variability of mesomycetozoeans within and among *H. lateralis* and *H. viridiflavus*, and analyze experimentally the mode of host infection by *R. rwandae*.

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