TITLE: **Development and worldwide use of non-lethal and minimal population-level impact protocols for the isolation of chytrids from amphibians**

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ABSTRACT:

Parasitic chytrid fungi have emerged as a significant threat to amphibian species worldwide, necessitating the development of techniques to isolate these pathogens into sterile culture for research purposes. However, early methods of isolating chytrids from their hosts relied on killing amphibians. We modified a pre-existing protocol for isolating chytrids from infected animals to use toe clips and biopsies from toe webbing rather than euthanizing hosts, and distributed the protocol to interested researchers worldwide as part of the BiodivERsA project *RACE*; here called the *RML* protocol. In tandem, we developed a lethal procedure for isolating chytrids from tadpole mouthparts. Reviewing a database of use a decade after their inception, we find that these methods have been widely applied across at least 5 continents, 23 countries and in 62 amphibian species, and have been successfully used to isolate chytrids in remote field locations. Isolation of chytrids by the non-lethal *RML* protocol occured in 18% of attempts with 207 fungal isolates and three species of chytrid being recovered. Isolation of chytrids from tadpoles occured in 43% of attempts with 334 fungal isolates of one species (*Batrachochytrium dendrobatidis*) being recovered. Together, these methods have resulted in a significant reduction and refinement of our use of threatened amphibian

species and have improved our ability to work with this important group of emerging fungal pathogens.

INTRODUCTION

A major consequence of globalisation has been the increase of invasive species owing to trade in live animals and plants. A further outcome of this process is the concomitant rise of novel emerging fungal pathogens (EFPs; (Farrer *et al.* 2017)) as these infections are moved within trade networks and establish in uninfected regions – an example of fungal 'pathogen pollution' (Fisher *et al.* 2012). Whilst EFPs can affect humans, they have also been broadly detrimental to natural populations of plants and animals, leading to worldwide losses of biodiversity. This dynamic has been most apparent across amphibians, where EFPs leading to population extirpation and species extinctions have contributed to amphibians now being the most endangered class of vertebrate (Stuart *et al.* 2004; Mendelson *et al.* 2006). In particular, emergence of parasitic fungi in the genus *Batrachochytrium* (phylum Chytridiomycota, order Rhizophydiales) have played a major role in driving amphibian population and species declines worldwide (Berger *et al.* 1998; Fisher *et al.* 2009).

While a single species, *Batrachochytrium dendrobatidis* (*Bd*), was originally thought to have caused the ongoing panzootic (James *et al.* 2009), we now know that amphibian chytridiomycosis is caused by a much broader swathe of phylogenetic diversity than was previously thought (Farrer *et al.* 2011; Schloegel *et al.* 2012). Next-generation sequencing and phylogenomic analyses have shown that *Bd sensu stricto* is composed of deep genetic lineages which are emerging through international trade in amphibians (Fisher *et al.* 2007; Schloegel *et al.* 2009; Schloegel *et al.* 2010). Superimposed upon this background of trade-associated lineages of *Bd* has come the recent discovery of a new species of pathogenic chytrid, also within the Rhizophydiales, *B. salamandrivorans* (*Bsal*; Martel *et al.* 2013). This pathogen has rapidly extirpated European fire salamanders (*Salamandra salamandra*) in the Netherlands and a broad screening of urodeles has shown that *Bsal* occurs naturally in southeast Asia where it appears to asymptomatically infect salamander and newt species (Laking *et al.* 2017).

The ability to isolate and culture both *Bd* and *Bsal* has played a key role in catalysing research into their pathogenesis and virulence (Voyles *et al.* 2007; Rosenblum *et al.* 2012; Farrer *et al.* 2017), phenotypic characteristics (Piotrowski *et al.* 2004; Fisher *et al.* 2009;

130 Becker *et al.* 2017) and a wealth of experimental studies on epidemiologically relevant
131 parameters (Garner *et al.* 2009; Ribas *et al.* 2009; Rosenblum *et al.* 2012). Longcore *et al.*
132 (1999) first isolated *Bd* from infected amphibians by modifying techniques used to isolate
133 other chytrids (Barr 1987). Longcore cleaned small (< 0.5mm dia) pieces of *Bd*-infected leg
134 and foot skin by wiping them through agar and then placed skin pieces onto a clean plate of
135 nutrient agar containing penicillin G and streptomycin. This method worked well for isolating
136 from dead animals sent by courier from North and Central America. The method, however,
137 requires euthanizing potentially healthy animals if their infection status was unknown.
138 Further, it is difficult to perform this protocol in remote regions that lack suitable laboratory
139 facilities, and the lethal sampling of amphibians may be contraindicated if the species is
140 endangered, protected or located in protected areas.

141 We confronted this issue in a 2008-2014 project funded by BiodivERsA
142 (http://www.biodiversa.org) – *RACE*: Risk Assessment of Chytridiomycosis to European
143 amphibian biodiversity (Fisher *et al.* 2012). One of the objectives of this project was to adjust
144 the protocol of Longcore *et al*. (1999) to (i) reduce the need to kill adult amphibians, (ii)
145 improve rates of chytrid isolation by allowing the use of more animals, (iii) develop protocols
146 that enabled isolation in a field setting, and, (iv) integrate the data into the GPS-smartphone
147 enabled epidemiological software application *Epicollect* (Aanensen *et al.* 2009; Aanensen *et*
148 *al.* 2014). Further, 'forewarned is forearmed' and we wished to determine whether the
149 protocol was able to isolate other species of chytrid that are part of the amphibian skin
150 microbiota, and that may present a biosecurity risk. This need to more broadly characterise
151 global chytrid biodiversity was met by using resources from *RACE* to train researchers
152 worldwide in chytrid isolation techniques to provide opportunities to characterise novel
153 chytrids as they were discovered.

154 In addition to the non-lethal isolation protocol, a lethal method was developed in parallel to
155 isolate chytrids from the mouthparts of larval amphibians. We describe this method as a
156 refinement to the main isolation protocol.

157 METHODS

158 *Non-lethal field isolation of chytrids*

159 Animals were captured and held in separate plastic bags or suitable containers until ready for
160 processing (Supp. Info. *RML* Protocol 1 and Supp. Info. Swabbing Protocol 2). Using clean
161 gloves and sterilized dissection scissors or scalpel blades, the terminal 1-2mm of the
162 phalanges of the 4th hind toe (counting from the proximal toe) was clipped and laid on the
163 surface of an mTGhL + antibiotic (200 mg/L penicillin-G and 400 mg/L streptomycin
164 sulphate) agar plate. Alternatively, ~1mm toe-webbing biopsy punches were taken (Sklar
165 instruments, PA, USA) then laid on a plate. This allowed multiple animals to be processed
166 rapidly in the field. Subsequently, each tissue sample was transferred to a second plate with a
167 sterile needle or forceps then cleaned (as far as possible) of surface-contaminating bacteria
168 and fungi by dragging it through the agar-medium. The needle or forceps was then used to
169 place the tissue sample in a sterile 2 ml screw-cap microtube containing liquid mTGhL
170 medium with antibiotics (200 mg/L penicillin-G and 400 mg/L streptomycin sulphate), then
171 stored in a cool, dry place. While 4 ^{o}C appears optimal, we have successfully used shaded
172 regions of streams to cool cultures when refrigeration was not immediately available and
173 have even held tubes and plates for several days at > 10 ^{o}C until suitable storage conditions
174 were available.

175 Once back in the laboratory, samples in tubes were visually screened for evidence of yeast or
176 bacterial contamination (when the media takes on a 'cloudy' appearance), or mycelial 'balls'
177 around the toe that are evidence of non-chytrid fungal contaminants. Visibly clear samples
178 were decanted into a single well of a sterile 12-well lidded culture plate then incubated at
179 18°C for up to 4 weeks, topping up with extra medium to counter evaporation as necessary.
180 Depending on the size of the initial tissue sample, toe clips and webbing were divided into
181 several smaller samples before transferring to liquid culture media.

182 *Isolating chytrids from tadpoles*

183 Tadpoles often have higher burdens of infection than adults, especially long-lived tadpoles
184 (Skerratt *et al.* 2008), and have higher densities and encounter rates than adults. In some
185 situations where tadpoles were large and infections heavy, tadpoles were microscopically
186 screened with a dissecting microscope or hand lens for areas of dekeratinization of the mouth
187 parts, especially the jaw sheaths, that indicates infection (Fellers *et al.* 2001; Smith *et al.*
188 2007). Tadpoles are killed before excising their mouthparts and these preliminary
189 microscopic screens enabled us to use only a small number of animals to isolate chytrids.
190 Additionally, uninfected and naïve tadpoles that were reared in captivity were used as live

substrates to bait chytrids from adult amphibians with low levels of *Bd* infection (Bataille *et al.* 2013).

Susceptible tadpoles were reared until gills were resorbed and animals were free-swimming and feeding (developmental Gosner stage 25), because at earlier stages they are still developing the keratinized mouthparts. Each tadpole container was then immersed within a similar but larger container that held at least one chytrid-infected animal. Water exchange between the infected and bait animal containers occurred through small holes (< 0.3 mm) drilled into the bottom of the walls of the smaller internal containers. Animals were held in these conditions for between 2 and 4 weeks at species-appropriate conditions. Tadpoles were periodically examined every fourth day for the presence of the depigmented areas in the jaw sheaths that have been associated with chytrid infection.

Isolating chytrids from tadpoles first required killing by immersion in a 5 g/L solution of MS-222 (Torreilles *et al.* 2009) or other approved method. Note that anaesthetics which contain ethanol, such as phenoxyethanol (Gentz 2007), should be avoided as these will kill chytrids while MS222 is not toxic (Webb *et al.* 2005). We then dissected out keratinized jaw sheaths and cleaned the entire sheath, or sections, as above using an agar plate with antibiotics ((Longcore *et al.* 1999); Supp. Info. *RML* Protocol 1). Cleaned sections were then placed singly into sterile 12-well culture plates with 1 mL liquid media + antibiotics, or onto agar plates with 6 – 10 sections per plate, and incubated at 10 – 20 ^{o}C.

Because zoospore release may occur immediately, especially from tadpole mouthparts, cultures were examined with an inverted microscope for the presence of active zoospores every day for up to one week following the day that they were initiated. After that, checks every two days were sufficient.

Culture and diagnosis of chytrid isolates

Subsequent culture methods for *Bd* followed those of Longcore *et al.* (1999). When isolation of *Bsal* was anticipated an incubation temperature of 15 ^{o}C was required (Blooi *et al.* 2015) whereas a temperature of 18 – 22 ^{o}C is closer to the measured growth optimum of *Bd* (Longcore *et al.* 1999; Ribas *et al.* 2009). Once growth of zoospores and/or zoosporangia was observed, 100 – 500 µL volume of culture containing zoospores and zoosporangia was transferred by pipette to a new 12-well plate with liquid medium and no antibiotics, and

221 incubated at 15 – 20 °C. All successfully cultured isolates were subcultured into larger
222 volumes, then centrifuged at 1700 g for 10 min before cryopreservation. A portion of the
223 initial pellet was also be used for DNA extraction, while the remaining volume was
224 resuspended in 10% DMSO and 10% FCS in liquid media and transferred into six 2 mL
225 cryotubes for cryopreservation at -80 °C (Boyle *et al.* 2003).

226 We confirmed the identity of *Bd* and *Bsal* by quantitative PCR with an MGB Taqman probe
227 assay in either single-plex or multiplex (Boyle *et al.* 2004; Blooi *et al.* 2013). We identified
228 non-*Batrachochytrium* chytrids was achieved by sequencing appropriate regions of the
229 ribosomal RNA gene with universal fungal primers followed by comparison against OTUs
230 held in UNITE database (Unified system for DNA-based fungal species linked to
231 classification: https://unite.ut.ee) to establish a species-hypothesis for the chytrid isolate in
232 question (Schoch *et al.* 2012). If further genetic data were required, then multilocus analysis
233 or whole-genome sequencing was undertaken using chytrid-specific methods (James *et al.*
234 2009; Farrer *et al.* 2013; Farrer *et al.* 2017; Farrer *et al.* 2017).

235 *Collation of data*

236 To track and report chytrid isolation for the *RACE* project, we used a generic data collection
237 tool that allows the collection and submission of geotagged data forms from field locations,
238 *Epicollect5* (https://five.epicollect.net). This software has the advantage that it can be used on
239 mobile devices with or without internet connection, and allows the immediate sharing of data
240 across the research community. Our database at
241 https://five.epicollect.net/project/bd-global-isolation-protocol included the following data
242 fields: Date; Continent, Country, Site name; Latitude/Longitude; Wild caught or trade?;
243 Amphibian species; Life history stage; Number sampled; Chytrid isolated?; Number isolated;
244 Species of chytrid isolated; Chytrid lineage; Photograph of amphibian; Name of researchers.

245 RESULTS

246 The '*RACE* modified Longcore (*RML*) Protocol' for the non-lethal isolation of chytrids from
247 amphibians is detailed in Supp. Info. 1. Ensure that you have the relevant licences, permits
248 and permissions from ethical committees to follow the *RML* protocol 1, swabbing protocol 2
249 and isolation from larval amphibians.

250 Following the formalisation and distribution of the *RACE* protocols, our Epicollect5 project
251 summarised chytrid surveys from 2007 through to 2017 (Table 1). The Epicollect5 database
252 can be spatially visualised at
253 https://five.epicollect.net/project/bd-global-isolation-protocol/data. Figure 1 depicts the
254 isolation of amphibian-associated chytrids using the *RACE* protocols from 5 continents
255 (Africa, Asia, Australia, Europe and South America), 23 countries, 239 sampling episodes,
256 and from latitudes spanning -44.1 S (*Batrachyla antartandica*, Chile) through to 55.6 N (*Bufo*
257 *viridis,* Sweden). Chytrids have been non-lethally isolated from 1,906 animals comprising 34
258 amphibian species, of which 28 were anuran and 5 were caudatan species. Of the *Bd* isolated,
259 170 (80%) were determined to be *Bd*GPL, 5 (2%) were *Bd*CAPE, 34 (16%) were
260 *Bd*BRAZIL, 1 (>1%) was *Bd*CH and 3 (1%) were hybrids. The database also contains 5
261 records of chytrids that were non-lethally sampled from the amphibian trade.

262 *Non-lethal isolation from adult and juvenile amphibians*

263 In total, 1,152 animals were non-lethally sampled, recovering 207 chytrid isolates and
264 resulting in a recovery rate of 18% (~1 isolate per 5 animals sampled). Of these chytrids, 203
265 (98%) were *Bd*, 2 were *Rhizophydium* sp., 2 were *Kappamyces* sp. and none were *Bsal* (Table
266 1). Of the *Bd* isolated, 42 (88%) were determined to be *Bd*GPL, 5 (10%) were *Bd*CAPE, and
267 1 (2%) was *Bd*CH.

268 *Isolation of chytrids from larval amphibians*

269 In total, 784 tadpoles were sampled recovering 334 chytrid isolates and resulting in a
270 recovery rate of 43% (~1 isolate per 2 – 3 animals sampled). Isolates were recovered from 34
271 species of amphibian, all of which were anurans. These chytrid isolates were all *Bd* and, of
272 the lineages recorded, 128 (78%) were *Bd*GPL, 34 (20%) were *Bd*BRAZIL and 3 (2%) were
273 hybrids.

274 Baiting chytrid isolates from live adult animals using tadpoles was used successfully in South
275 Korean *Bombina orientalis* as previously described (Bataille *et al.* 2013). Here, six tadpoles
276 were co-housed with adult *B. orientalis*, yielding a single isolate of *Bd* for each attempt
277 equating to a rate of success of ~20%.

278 DISCUSSION

279 The *RML* protocol, based on the original suggestions of Joyce Longcore for the non-lethal
280 isolation of chytrids from amphibians, has been a success with isolates of chytrids recorded
281 from five continents. There are likely many other unrecorded uses of this method because this
282 protocol has been widely dispersed during the 5-year span (2008-2014) of the *RACE* project
283 which trained a cohort of amphibian disease researchers in these techniques.

284 In some circumstances chytrids could not be recovered from toe-clips when sampling
285 populations with persistent infection despite repeated attempts. This was particularly evident
286 when the prevalence and burden of chytrid infections in surveys was low (Swei *et al.* 2011;
287 Bataille *et al.* 2013; Laking *et al.* 2017) or when host species occupied habitats with high
288 bacterial and/or non-target fungal contaminants. In these situations we isolated chytrids from
289 tadpole mouthparts as an associated method to the *RML* protocol. The value of the *RML*
290 protocol in propelling forward research on amphibian chytridiomycosis has been very clear:
291 for instance, of the 59 scientific papers produced by *RACE*, 15 directly used isolates of *Bd*
292 that were generated by this protocol for experimental trials (Supp. Info. 3). Further,
293 subsequently many more studies using these isolates have extended our knowledge of the
294 genetic diversity of *Bd* (James *et al.* 2009; Farrer *et al.* 2011; Farrer *et al.* 2013; Jenkinson *et*
295 *al.* 2016), the development of novel diagnostics (Dillon *et al.* 2017), the genetic repertoire
296 that underpins the virulence of these pathogens (Rosenblum *et al.* 2012; Farrer *et al.* 2017)
297 and the biogeographic distributions of *Bd* diversity worldwide (Farrer *et al.* 2011; Jenkinson
298 *et al.* 2016).

299 Clearly some uncontrolled biases and unanswered questions in these studies need attention.
300 First, the majority of *Bd* isolates belong to the *Bd*GPL lineage. This could be because this
301 lineage is more widespread (and therefore more readily recovered) than other lineages (James
302 *et al.* 2015), or it could be that the intensity of *Bd*GPL infections and/or the rate of zoospore
303 production is higher than for other lineages, which would also equate to a higher rate of
304 isolation. To achieve a true and unbiased understanding of the distribution of these lineages, a
305 lineage-specific diagnostic will need to be developed and deployed. Second, if lineage-
306 specific differences in the probability of successful isolation exist, then mixed infections
307 where these lineages co-occur may not be detected. This can be controlled for by isolating
308 and genotyping many isolates from a single host and population, although this may not fully
309 account for this bias. A related bias is that not all infectious species of chytrid will respond
310 equally to culturing attempts. For instance, despite known attempts to isolate *Bsal* from

311 across its endemic southeast Asian range using the protocol, to date no successful isolations
312 of *Bsal* have been recorded. This is likely due to a combination of the low prevalence and
313 burden of infection in salamanders and newts combined with the low initial growth-rate of
314 *Bsal* (Martel *et al.* 2013; Laking *et al.* 2017). With the *RML* protocol, however, workers have
315 been able to isolate non-*Bd* species of chytrid (*e.g., Kappamyces* spp. and *Rhizophydium* sp.
316 Table 1). This diversity likely represents only a fraction of the diversity of amphibian-
317 associated chytrids that occur, and non-biased estimators of this diversity by, for instance,
318 profiling the nuclear ribosomal RNA cistron (Schoch *et al.* 2012), are sorely needed.

319 In this age of the global amphibian crisis, research on the effects of chytrid infections is
320 transitioning to attempts to mitigate their impacts (Schmeller *et al.* 2014; Garner *et al.* 2016;
321 Canessa *et al.* 2018). Both of these research streams benefit from the availability of chytrid
322 isolates, but the ethics behind these research programs can be improved. To that end, our data
323 on isolation success suggest that tadpoles are a better target for isolation than metamorphosed
324 animals. This is to some degree unfortunate, because isolation from tadpoles requires killing.
325 However we have outlined one refinement where captive reared tadpoles can be used to 'bait'
326 infections from wild-caught amphibians to isolate chytrids without killing adult amphibians.
327 Here, it is important to recognise that amphibians which have been co-housed in collections
328 should not be returned to the wild due to the danger of cross-transmission of pathogens
329 during husbandry (Walker *et al.* 2008). If it is necessary to isolate chytrids directly from wild
330 tadpoles without using bait animals, we suggest that researchers focus on more fecund
331 species with long larval periods as the focal species in aquatic amphibian communities.
332 Removal of small numbers of tadpoles when clutch sizes are in the hundreds or thousands
333 means that removals will have an insignificant ecological impact; for this reason sacrificing
334 tadpoles is preferable to killing adult animals.

335 The extent to which toe-clipping effects the fitness of amphibians has been much debated
336 (*e.g.* May (2004) but see Funk *et al.* (2005)). Toe-clipping has been shown to decrease
337 amphibian survival, but this effect, when present, is linearly related to the number of toes
338 removed (McCarthy *et al.* 2004; Ulmar Grafe *et al.* 2011). For the single toe-clip that the
339 *RML* protocol requires, reduction in survival appears to be negligible (Ott *et al.* 1999; Funk *et*
340 *al.* 2005), and toe clipping is certainly preferred to killing the animal. Attention should be
341 paid to this issue, however, and, where appropriate, survival estimates should be undertaken
342 to determine the health implications of this procedure. Also, antiseptic and analgesic

343 protocols can be considered to ensure that wounds where tissue samples are excised are at
344 low risk of secondary infection (Chevalier *et al.* 2017).

345 In summary, modification of Longcore's original *Bd*-isolation protocol (Longcore *et al.*
346 1999) has enabled a broad community of scientists to engage with research on emerging
347 chytrid pathogens of amphibians. This research has had an impact worldwide, and is
348 contributing to the ongoing dialogue that is occurring between scientists, conservationists and
349 policy-makers about how we might mitigate against these infections now and into the future.

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592 **TABLE 1.** Non-lethal isolation of chytrids from adult and juvenile amphibians
593

Continent	Country	*n* Species[1]	*n* Sampled[2]	*n* Chytrid[3]	Chytrid species
Africa	Madagascar	2	145	2	*Kappamyces* sp.
	Cameroon	1	30	1	*B. dendrobatidis*
	Ethiopia	1	5	1	*B. dendrobatidis*
	South Africa	6	179	45	*B. dendrobatidis*
Asia	South Korea	2	28	10	*B. dendrobatidis*
	Taiwan	3	103	13	*B. dendrobatidis/ Kappamyces* sp.
Australia	Australia	1	2	2	*B. dendrobatidis*
Europe	Belgium	1	11	2	*B. dendrobatidis*
	France	2	261	70	*B. dendrobatidis*
	Hungary	1	15	3	*B. dendrobatidis*
	Italy	1	14	4	*B. dendrobatidis*
	Portugal	1	5	1	*Rhizophydium* sp.
	Spain	4	198	37	*B. dendrobatidis*
	Sweden	1	23	5	*B. dendrobatidis*
	Switzerland	1	30	1	*B. dendrobatidis*
	UK	4	50	8	*B. dendrobatidis*
South America	Chile	1	10	1	*B. dendrobatidis*
	French Guiana	2	66	2	*B. dendrobatidis*
Trade	n/a	4	15	5	*B. dendrobatidis*

594
595 [1]Number of amphibian species sampled, [2]total numbers of amphibians sampled, [3]number of
596 chytrids isolated
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598

599 **TABLE 2.** Isolation of *Batrachochytrium dendrobatidis* from mouthparts of larval
600 amphibians
601

Continent	Country	Host species	Larvae sampled	*Bd* isolates
Africa	Ethiopia	1	36	1
	Uganda	1	20	1
	South Africa	2	88	11
Asia	Taiwan	1	15	1
Australia	Australia	8	54	33
Europe	Belgium	2	2	2
	Netherlands	1	1	1
	France	1	138	38
	Germany	1	10	4
	Spain	3	19	7
	Switzerland	1	42	15
South America	Chile	2	28	4
	Brazil	17	353	217

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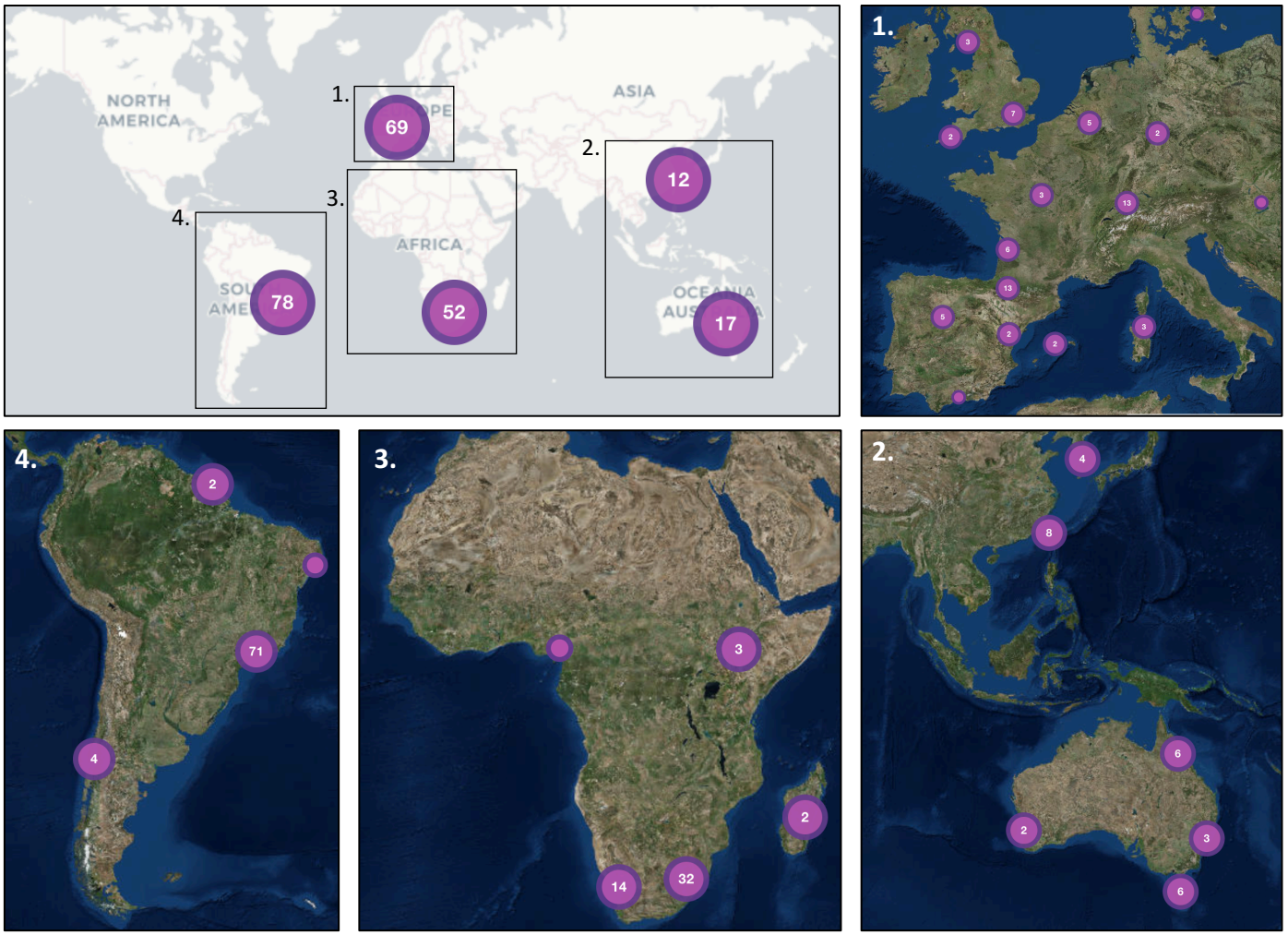

Figure 1. Worldwide distribution of sites where the *RML* Longcore protocol has been used to isolate chytrids. Numbers denote the quantity of amphibian species investigated. A browseable version of this *Epicollect 5* map can be accessed at https://five.epicollect.net/project/bd-global-isolation-protocol