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Origin and global spread of an endemic chytrid fungus lineage linked to the bullfrog trade

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1 **Origin and global spread of an endemic chytrid fungus lineage linked to the bullfrog**
2 **trade**

3
4 **Abstract**

5 The amphibian-killing fungus *Batrachochytrium dendrobatidis* (Bd) exhibits relevant
6 genetic diversity, including the Global Panzootic Lineage (Bd-GPL), associated with
7 worldwide amphibian declines. However, the evolutionary history and dispersal of
8 geographically restricted lineages, such as Bd-BRAZIL, remain unclear. Here, we
9 mapped global historical Bd records, investigated the temporal and geographic origins of
10 Bd-BRAZIL, and evaluated the role of the international bullfrog (*Aquarana catesbeiana*)
11 trade in its spread. We selected bullfrogs as a focal species due to their relevance in
12 global trade, tolerance to Bd, and their status as the only amphibian species bred in
13 captivity in Brazil and most South American countries. We used non-invasive genetic
14 techniques on museum specimens and compiled global Bd occurrence data. Integrating
15 genotypic data from traded bullfrogs, phylogenetic analyses, and historical trade routes,
16 we suggest Bd-BRAZIL likely spread from Brazil to other regions via the bullfrog trade.
17 This hypothesis is supported by: i) historical trade links between Brazil and East Asia; ii)
18 detection of Bd-BRAZIL in bullfrogs exported from South America to the USA; and iii)
19 its high prevalence in wild frogs across five Brazilian states and in bullfrog farms (51 %
20 of Bd-positive individuals). Our study underscores the critical role of the global
21 amphibian trade in the dispersal of Bd lineages and highlights the need for enhanced
22 import controls and pathological assessments to mitigate the inadvertent spread of
23 pathogens. Future studies should expand data coverage, apply advanced genotyping, and
24 further explore the evolutionary origins of Bd-BRAZIL to enhance understanding of its
25 emergence and impacts.

26
27 **Keywords:** *Batrachochytrium dendrobatidis*; Bd-BRAZIL lineage; bullfrog trade;
28 chytridiomycosis; historical pathogen records; pathogen spread.

29 1. Introduction

30 *Batrachochytrium dendrobatidis* (Bd) is a pathogenic fungus distributed
31 worldwide, causing chytridiomycosis, an infectious disease in amphibians (Longcore et
32 al., 1999; Scheele et al., 2019). This disease has led to the decline of over 500 species,
33 with at least 90 of them already extinct across the globe (Fisher and Garner, 2020;
34 Scheele et al., 2019). Understanding the origin and spread of this pathogen is critical to
35 unraveling how chytridiomycosis rapidly escalated into a global crisis.

36 The initial out-of-Africa hypothesis proposed the African continent as the likely
37 origin of Bd, based on museum specimens of *Xenopus laevis* infected with the fungus,
38 dating back to 1938 (Weldon et al., 2004). This hypothesis suggested that the
39 international trade of this frog species facilitated the global spread of the pathogen.
40 However, more recent discoveries of older Bd records in other regions worldwide, such
41 as Japan (1902) (Goka et al., 2009), the United States of America (1888) (Talley et al.,
42 2015), and Brazil (1894) (Rodriguez et al., 2014), have challenged this view, indicating
43 that the history of Bd was more complex than previously thought.

44 Historically, two main hypotheses were proposed to explain the global emergence
45 of chytridiomycosis. The "endemic pathogen hypothesis" (EPH), or enzootic hypothesis,
46 posits that the fungus has been globally distributed since its origin, with recent amphibian
47 extinction events triggered by environmental changes, such as shifts in climatic
48 conditions, that increased the pathogen's virulence (Pounds et al., 2006; Rachowicz et al.,
49 2005). The "novel pathogen hypothesis" (NPH), or epizootic hypothesis, suggests that Bd
50 originated in a specific region (initially proposed as Africa) (Weldon et al., 2004), and
51 spread globally (Daszak et al., 1999), causing rapid and unprecedented declines in more
52 susceptible amphibian populations (Lips et al., 2008, 2006; Vredenburg et al., 2010).
53 While this dichotomy has since been replaced by more integrative evolutionary and
54 epidemiological models, these early frameworks laid the foundation for ongoing
55 investigations into the origins and global spread of Bd.

56 A major shift in understanding Bd origins came with the advent of genotyping
57 and the characterization of its complex genetic diversity (Bataille et al., 2013; Byrne et
58 al., 2019; Farrer et al., 2011; Rosenblum et al., 2013; Schloegel et al., 2012). Beyond the
59 widely distributed Bd-GPL, known as the 'global panzootic lineage', four other main Bd
60 lineages have been identified (Byrne et al., 2019; O'Hanlon et al., 2018). These include
61 Bd-CAPE, found in South Africa, West Africa, locally in Europe (Farrer et al., 2011),
62 and more recently in Central America (Byrne et al., 2019), Bd-ASIA-1 found in the
63 Korean Peninsula, while Bd-ASIA-2/Bd-BRAZIL (hereafter Bd-BRAZIL) was present in
64 Brazil and Korea (O'Hanlon et al., 2018; Rodriguez et al., 2014), and likely in Japan
65 (Goka et al., 2021). Finally, Bd-ASIA-3 was found in Southeast Asia (Byrne et al., 2019).
66 The Bd lineage diversity in East Asia, combined with recent molecular evidence and the
67 Asian origin of the sister species *B. salamandrivorans* (Bsal), has led to the proposal of
68 the 'out-of-Asia' hypothesis (O'Hanlon et al., 2018). This hypothesis, supported by data
69 from both nuclear and mitochondrial genomes, posits that Bd-GPL, the lineage linked to
70 global declines (Byrne et al., 2019; Farrer et al., 2011; O'Hanlon et al., 2018), emerged in
71 Asia in the mid-20th century (O'Hanlon et al., 2018).

72 The study employed three different dating methods to estimate the time of origin
73 of Bd, which resulted in: a nuclear molecular clock indicating an origin in 1898 (with a

74 range of HPD 95 % from 1809 to 1941), a mitochondrial clock suggesting 1962 (with a
75 range of HPD 95 % from 1859 to 1988), and a mitochondrial clock using empirical dates
76 of first Bd detection as calibration proposing 1975 (with an interval of HPD 95 % from
77 1939 to 1989). Nevertheless, the origins of endemic or geographically restricted lineages
78 remain elusive. Concurrently, numerous studies have investigated historical infection
79 patterns across diverse global regions employing museum specimens (Monzon et al.,
80 2020) and novel methods of genotyping lineages (Byrne et al., 2017; Ghosh et al., 2021).
81 Moreover, these studies have probed the relation between the global amphibian trade and
82 pathogen transmission (Altherr and Lameter, 2020; Hughes et al., 2021), providing
83 crucial historical data for reevaluating the origins of Bd lineages.

84 Despite these advances, the origin of endemic or geographically restricted
85 lineages, such as Bd-BRAZIL, remains poorly understood. The Bd-BRAZIL lineage,
86 previously thought to be endemic to Brazil, is frequently detected in North American
87 bullfrogs (*Aquarana catesbeiana*, hereafter bullfrogs) from commercial markets and
88 farms (Garner et al., 2006; Ribeiro et al., 2019; Schloegel et al., 2012). As one of the
89 most widely traded amphibian species globally, bullfrogs are known for their tolerance to
90 chytridiomycosis and their potential role as carriers of Bd (Falaschi et al., 2020; Fisher
91 and Garner, 2020; Ribeiro and Toledo, 2022). The international bullfrog trade, which
92 began in the 1920s for farming and frog leg consumption, may have facilitated the global
93 dispersal of Bd lineages, including Bd-BRAZIL, to different regions worldwide (Altherr
94 et al., 2011; Falaschi et al., 2020; FAO, 2021). Evaluating historical records of Bd-
95 BRAZIL and mapping bullfrog trade routes are therefore critical to unraveling the origin
96 and global spread of this lineage.

97 In this study, we propose two key hypotheses: i) the Bd-BRAZIL lineage
98 originated in Brazil, and ii) its global spread was driven by the international trade of
99 bullfrogs. To investigate the origin and direction of the intercontinental spread of Bd-
100 BRAZIL, we compiled historical records of Bd occurrences on a global scale, shedding
101 light on the historical distribution of this pathogen. We focused on regions where the Bd-
102 BRAZIL lineage has been detected and where bullfrog trade is a well-established
103 economic activity, including Brazil, the USA, the Korean Peninsula, and Japan. By
104 integrating genotypic and sequencing data from both Bd isolates and traded bullfrogs
105 with a comprehensive dataset on international bullfrog trade, we reconstructed the
106 potential global spread of this lineage. Our findings provide new insights into the origin
107 and global spread of Bd-BRAZIL, underscoring the critical role of the global amphibian
108 trade in pathogen introduction.

109 **2. Materials and Methods**

110 **2.1. Historical Bd records**

111 To investigate the historical distribution of Bd, we compiled and analyzed Bd
112 occurrence records from two primary sources: museum specimens sampled in this study
113 and published literature.

114 **2.1.1. Museum specimens**

115 We analyzed 2,280 museum specimens of amphibians collected from different
116 regions worldwide between 1815 and 2014, including Africa (n = 15), Asia (n = 202),
117
118

119 Europe (n = 1,963) (Butler, 2019), North America (n = 9), and South America (n = 91).
120 Most specimens were fixed in formalin, preserved in 70 % ethanol, and deposited in
121 collections worldwide (Table S1).

122 To avoid damaging museum specimens, especially older ones, we used non-
123 invasive genetic methods. Each specimen was rinsed with 70 % clean ethanol to
124 eliminate superficial contaminants from storage. After a brief drying period (Rodriguez et
125 al., 2014), we swabbed the specimens using Medical Wire swabs (MW113), following
126 standard Bd sampling procedures for preserved specimens (Cheng et al., 2011; Hyatt et
127 al., 2007). We extracted DNA from each swab using 50 µl of PrepMan Ultra (Applied
128 Biosystems), and performed qPCR to detect the presence of Bd, regardless of genotype,
129 following established protocols (Boyle et al., 2004; Lambertini et al., 2013; Retallick et
130 al., 2006). We diagnosed samples as Bd-positive with at least one zoospore genomic
131 equivalent (GE) (Hyatt et al., 2007).

132

133 2.1.2. Literature review

134 We conducted a literature review to identify additional published records of
135 historical occurrences of Bd in amphibians worldwide. Historical data were defined as
136 samples collected prior to the formal description of Bd in 1999 (Longcore et al., 1999).
137 Our review followed a systematic approach adapted from the methodology proposed by
138 Pickering et al. (2015), including predefined inclusion and exclusion criteria, keyword-
139 based database searches, and manual screening of full texts. We searched the Web of
140 Science and Google Scholar databases up to May 2025, using the following query terms
141 across titles, abstracts, and keywords:

142

143 TS= ("*Batrachochytrium dendrobatidis*" AND amphibian*) AND TS= ("museum
144 specimen*" OR "archived specimen*" OR "preserved specimen*" OR "retrospective" OR
145 "historical" OR "historic occurrence" OR "early detection" OR "first detection" OR "first
146 report")

147

148 Studies were included if they (1) were published in English, (2) were available in
149 full text (including gray literature such as theses), and (3) presented original empirical
150 data. We included records referring to amphibians (Anura, Caudata, or Gymnophiona),
151 documenting the sampling period, the oldest Bd record at each location, and, when
152 available, the identified Bd lineage. Records were derived from various sample types and
153 detection methods. We excluded studies that: (1) cited previous retrospective studies
154 without presenting new data, (2) consisted only of conference abstracts, book chapters, or
155 project reports, (3) duplicated previously published data (e.g., theses later published as
156 articles), (4) included only contemporary records (samples collected from 2000 onward);
157 or (5) did not report the date of sample collection.

158 Although we employed a comprehensive search strategy, we found that many
159 relevant studies did not include indicative terms (e.g., “museum”, “archived”,
160 “historical”) in their titles, abstracts, or keywords. Therefore, to minimize the risk of
161 overlooking eligible studies, we also performed full-text screening and manual review of
162 the reference lists from included articles. The Web of Science search returned 169
163 articles, of which 57 met the inclusion criteria. An additional 37 relevant publications

164 were identified through manual inspection. In total, we included 94 studies reporting
165 historical occurrences of Bd worldwide.

166

167 **2.2. Analysis of Bd detection probability**

168 To address potential sampling biases in historical Bd records, we analyzed
169 temporal and spatial variation in Bd detection probabilities across decades and continents.
170 Due to substantial variation in sampling effort, we use a binomial generalized linear
171 model (GLM) to appropriately account for differences in sample size and the binomial
172 nature of the detection data. Data on Bd detection and sampling effort were obtained
173 from a comprehensive global dataset, combining our historical sampling of museum
174 specimens (see Table S1) with records compiled through a literature review (see Table
175 S2). All analyses were performed in RStudio (version 1.4.1106) using R (version 4.2.2;
176 R Core Team, 2025), with a significance level of 0.05.

177 We fitted the binomial GLM using the “glm” from the “stats” package (R Core
178 Team, 2025). The response variable was specified as a two-column matrix comprising the
179 number of positive Bd detections and the number of negative detections (total samples
180 minus positives), aggregated by continent and decade. Continent and decade were
181 included as fixed-effect predictor variables to assess their influence on detection
182 probability. This modeling approach accounts for the binomial distribution of data and
183 the variation in sampling effort across continent-decade groups. To facilitate fair
184 comparisons of detection probabilities across groups with differing sample sizes,
185 predicted probabilities were generated, assuming a fixed sampling effort of 10,000
186 samples per continent-decade combination. We used the “car” package to perform an
187 ANOVA test to assess the significance of the predictor variables.

188 Observed and predicted detection probabilities were visualized using the
189 “ggplot2” package (Wickham, 2016), with observed detection rates plotted as points
190 sized by the log-transformed sample size ($\log(N + 1)$) and predicted trends represented
191 by colored lines according to continent.

192

193 **2.3. Chytrid genotyping of infected bullfrogs**

194 To determine the genotypes of Bd-infected bullfrogs, we genotyped 53 Bd-
195 positive post-metamorphic skin swab samples, previously confirmed by qPCR and
196 randomly selected from an existing dataset (Ribeiro et al., 2019). The random selection
197 was adopted to ensure an unbiased and representative subset of the available samples,
198 given the homogeneity in sampling period and geographic origin. DNA extraction and
199 qPCR were performed as described above to confirm Bd presence. From these, we
200 randomly selected 53 Bd-positive samples for genotyping. These samples were collected
201 in 2013 and 2016 from six Brazilian bullfrog farms in the state of São Paulo, Brazil.
202 Genotyping was performed using the Fluidigm Access Array microfluidic PCR platform,
203 following methods described earlier (Byrne et al., 2019, 2017). The chip-based, multiplex
204 PCR assay amplifies 192 informative marker loci specific to *Batrachochytrium* from
205 multiple samples in a single run. Among these, 191 markers are specific to Bd, and one is
206 specific to its sister species, Bsal. However, this marker was not detected in our samples.
207 Each Bd locus is approximately 150–200 base pairs long and distributed throughout the
208 Bd genome. The Fluidigm chip performs multiple PCR reactions in parallel for the 192

209 total loci across multiple samples. During this multiplexed PCR step, samples were dual-
210 barcoded for identification, and sequencing adapters were added for high-throughput
211 sequencing by Illumina MiSeq.

212 DNA extractions from swabs were purified by precipitating sodium acetate and
213 isopropanol. Before microfluidic PCR, all samples were pre-amplified in standard PCR
214 reactions with primer pools composed of the 192 primer pairs at 500 μ M for each marker.
215 Pre-amplification PCR was performed using the FastStart High Fidelity PCR System
216 (Roche), with reaction conditions as described in (Byrne et al., 2019). Pre-amplification
217 products were treated with 4 μ l ExoSAP-it (Affymetrix Inc.) and diluted 1:5 in molecular
218 grade water. The diluted products were used for microfluidic PCR and barcoding on a
219 Fluidigm Access Array IFC. Barcoded amplicons were pooled for sequencing on an
220 Illumina MiSeq using 300 bp paired-end kits.

221 The sequencing results were demultiplexed and pre-processed as described earlier
222 (Byrne et al., 2017). We used a bioinformatic R script (Byrne et al., 2019, Supporting
223 Information) to quality filter and determine variants in our results. Briefly, we filtered out
224 samples with more than 50 % missing data and loci for which more than 66 % of samples
225 were missing data. Contigs for each locus were generated by alignment using the
226 MUSCLE package in R, and alignments were visually confirmed using Geneious.
227 Concatenated alignment sequences were generated for each sample.

228 Phylogenetic analyses of concatenated gene alignments were conducted using
229 RAxML with a GTR substitution model, widely used in Bd phylogenetics for its
230 flexibility and suitability to multilocus datasets (*e.g.*, Byrne et al., 2019; Sewell et al.
231 2024). Our final phylogeny included known representative marker sequences of Bd-GPL,
232 Bd-BRAZIL, and Bd-CAPE isolates from previous genomics studies. We assessed
233 statistical support for phylogenetic relationships by rapid bootstrapping in RAxML over
234 100 replicates. Finally, we used newick utils to collapse all phylogeny branches with less
235 than 50 % bootstrap support. Swab samples within the 50 % support cut-off forming a
236 clade with a known genomic sequence were determined to be either Bd-GPL or Bd-
237 BRAZIL. Samples that did not form 50 % bootstrap supported clades with known
238 sequences were undetermined. We did not observe Bd-CAPE genotypes in our samples.
239

240 **2.4. Cytochrome-b sequencing of trade-associated bullfrog samples**

241 To explore the genetic structure of bullfrogs associated with global trade, we
242 sequenced the mitochondrial cytochrome-b (cyt-b) locus, a marker previously shown to
243 be effective in tracing bullfrog invasion history and identifying potential source
244 populations (*e.g.*, Jorgewich-Cohen et al., 2022). We analyzed DNA from swabbed frogs
245 ($n = 27$), collected from Brazilian bullfrog farms (Schloegel et al., 2010), shops in the
246 USA (Schloegel et al., 2012, 2009), and, for the first time, from bullfrog farms in
247 Pingtung County, south of Taiwan. DNA was extracted from skin swabs, and Bd
248 detection followed the protocol described by Annis et al. (2004).

249 Partial cyt-b sequences were amplified using primers and PCR conditions from
250 Ficetola et al. (2008) with GoTaq Green (Promega). These new sequences were aligned
251 with sequences from native populations previously identified (Austin et al., 2004) and
252 recent GenBank entries from native and invasive ranges using Geneious Prime. *Rana*
253 *clamitans* cyt-b was used as an outgroup sequence. We then used the unweighted pair

254 group method with arithmetic means to cluster the sequences into groups, using the HKY
255 model of sequence evolution and 100 bootstrap pseudo-replicates as a measure of
256 support.

257

258 **2.5. Understanding the scope and directionality of the global bullfrog trade**

259 We focused our trade analysis on the bullfrog, the most widely traded amphibian
260 species globally and the only amphibian legally exported from Brazil. Bullfrogs are
261 traded internationally in several forms, including live animals for farming and
262 consumption, frozen carcasses (primarily legs), and processed products. We specifically
263 concentrated our analysis on live animal trade, given its higher demonstrated potential for
264 Bd transmission. In addition to its global commercial importance, bullfrog trade is
265 supported by relatively comprehensive historical data, and previous studies have
266 consistently linked live trade pathways with Bd spread (Garner et al., 2006; Ribeiro and
267 Toledo, 2022; Schloegel et al., 2010).

268 To investigate historical trade patterns, we conducted a literature review focused
269 on the global movement of bullfrogs. Although commercial farming began in the 1930s,
270 the first officially documented exports occurred in the 1970s (Ribeiro and Toledo, 2022).
271 We prioritized data from 1970 to 2010, a period that aligns with the hypothesized
272 window for the global spread of the Bd-BRAZIL lineage, while also incorporating more
273 recent data to capture ongoing trade patterns and validate historical inferences.

274 We retrieved bullfrog trade records from the Convention on International Trade in
275 Endangered Species of Wild Fauna and Flora (CITES) database for the period 1975–2024
276 (CITES, 2025), which includes information on specimens quantities, trade years, and
277 import/export countries. Because Bd-BRAZIL was identified outside Brazil in 2009 and
278 2014, we also considered trade data covering those years. Additionally, we incorporated
279 import and export records of live amphibians in the United States from the U.S. Fish &
280 Wildlife Service for 2001–2010 (Herrel and Meijden, 2014; Schloegel et al., 2009), along
281 with updated data for 2010–2020 (Auliya et al., 2023). While these databases are
282 valuable, trade records may contain taxonomic imprecision, which can hinder accurate
283 species identification (Tlustý et al., 2023).

284 Based on this data, we built an interaction network to identify countries most
285 actively involved in bullfrog trade and characterize the structure of their exchanges.
286 Specific bullfrog trade routes were mapped in ArcGIS® to visualize potential pathways
287 for the global spread of the Bd-BRAZIL lineage. We focused on the earliest known
288 records of this lineage in each country, in relation to the established bullfrog trade flows.

289

290 **3. Results**

291 **3.1. Historical Bd records**

292 **3.1.1. Museum specimens**

293 We examined 2,280 amphibian specimens (Anura = 1,524; Caudata = 756) from
294 16 families, collected between 1815 and 2014, and representing different regions of the
295 world (Table S1). Our dataset includes very old historical specimens, reflecting an
296 expanded effort to investigate the potential presence of Bd, regardless of infection status.
297 The oldest specimen analyzed was a *Strongylopus grayii* collected in 1815 in Cape Town,
298 South Africa. Additional early records include *Pelophylax nigromaculatus* from the

299 Korean Peninsula (1884), *Alytes obstetricans* from France (1854), an *A. catesbeiana* from
300 the USA (1818), and *Leptodactylus latrans* from Brazil, also collected in 1818 (Fig. 1;
301 Table 1; Table S1).

302 The oldest Bd-positive record in our dataset corresponds to five *Alytes*
303 *obstetricans* individuals collected in 1915 in France (from the Pyrenees), representing the
304 earliest confirmed detection of Bd in our museum sample. The second oldest positive
305 specimen was a *Megaelosia goeldii* collected in Brazil in 1964, followed by a *Rana*
306 *sauteri* from Taiwan collected in 1990 (Table S1). All other specimens analyzed,
307 including those from North America, Portugal, Italy, South Africa, and the Korean
308 Peninsula, tested negative for Bd. These findings highlight both the historical breadth of
309 our sampling effort and the documentation of new historical occurrences of Bd
310 worldwide.

311

312 **3.1.2. Literature review**

313 We identified 94 studies published between 1998 and 2025 that reported historical
314 occurrences of Bd, based on amphibian samples collected between 1835 and 2019 (Table
315 1; Table S2). Worldwide, the oldest known Bd record was from the Bolivian Andes in
316 1863 (Burrowes and De la Riva, 2017), followed by records from the USA in 1888
317 (Talley et al., 2015), Brazil and Mexico in 1894 (Basanta et al., 2021; Rodriguez et al.,
318 2014), and the Venezuelan Amazonia in 1895 (Becker et al., 2016). Notably, all these
319 oldest records originated from the Americas (Fig. 1; Table 1; Table S2).

320 A substantial proportion of historical investigations were based on specimens
321 collected in the Americas, accounting for approximately 66 % (62 out of 94) of the
322 published studies. In contrast, key regions for understanding the origin of Bd, such as
323 Asia, remain underrepresented (10.6 %; 10 out of 94). Because this geographic imbalance
324 may limit our ability to reconstruct the evolutionary history of Bd-BRAZIL, we
325 conducted a detection probability analysis (see Materials and Methods, Section 2.2;
326 Results, Section 3.2), which supported the robustness of our findings despite regional
327 disparities in sampling effort.

328 Among all reviewed studies, only ten performed genotyping of Bd lineages (Table
329 S2), and only six of these involved historical samples, *i.e.*, those collected prior to 1999
330 (Table S3). The oldest historical genotyped sample corresponded to Bd-GPL, detected in
331 Brazil in 1897, followed by Bd-BRAZIL in 1916 (Rodriguez et al., 2014) (Table S3). To
332 date, no other Bd lineages have been identified in museum specimens.

333 Bd-BRAZIL has been isolated from ten Brazilian native amphibian species and a
334 bullfrog from a frog market in the USA in 2009 and 2010, respectively (Schloegel et al.,
335 2012) (Fig. S1; Table S4; Table S5). Subsequently, Bd-BRAZIL was also detected in
336 Brazilian museum specimens collected between 1916 and 1982 (Rodriguez et al., 2014).
337 Additional isolates from various wild species were then recorded in Brazil (James et al.,
338 2015; Jenkinson et al., 2016), expanding the distribution of this Bd lineage within the
339 Brazilian Atlantic Forest. In 2014, four bullfrogs infected with Bd-BRAZIL were also

340 recorded in South Korea (O’Hanlon et al., 2018), and a few individuals in Japan,
341 including bullfrogs and native species (Goka et al., 2021) (Fig. S1; Table S5).

342

343 **3.2. Analysis of Bd detection probability**

344 We found differences in the Bd detection probabilities over time across
345 continents, except for Africa and North America, which exhibited similar probabilities
346 throughout the study period (Table S6; Figure S2). Overall, detection probability
347 increased over time on all continents, consistent with both observed and modeled
348 increases in sampling effort (Figure S2).

349 Central and South America consistently exhibited the highest detection
350 probabilities across all decades, while Asia showed the lowest, even when accounting for
351 standardized sampling effort (Figure S2). For example, in 1810, predicted detection
352 probabilities were 1.2 % in Central America and 0.5 % in South America, compared to
353 just 0.1 % in Asia. This pattern remained consistent over time. In the 1980s, detection
354 probability reached 19.6 % in Central America and 10.1 % in South America, while it
355 remained relatively low in Asia (2.5 %). These findings indicate that both historical
356 records and model outputs consistently support higher detection probabilities in the
357 Americas (Figure S2).

358

359 **3.3. Chytrid genotyping and bullfrog sequencing**

360 Out of the 53 skin swab samples from six Brazilian bullfrog farms that we
361 genotyped, we found that almost half of the samples (51.0 %) belonged to the Bd-
362 BRAZIL lineage, followed by the global pandemic lineage Bd-GPL (37.7 %), and 11.3 %
363 of the samples were categorized as undetermined, meaning that there was insufficient
364 evidence to classify them under any of the mentioned clades (Fig. S3; Table S4; Table
365 S5).

366 Our dendrogram revealed two major genetic clusters within bullfrog population,
367 consistent with previous findings (Fig. 2) (Austin et al., 2004). Samples from the invasive
368 range and those associated with the global trade generally clustered together. Two
369 genotypes from farms were grouped within the Western Lineage. Notably, bullfrog
370 genotypes from Taiwanese farms were identical to those found in multiple invasive
371 populations across Asia and in several markets in the USA. Another distinct cluster
372 within the Western Lineage included a Brazilian bullfrog farm, markets in Miami and
373 New York (USA), and invasive populations from Brazil, Cuba, China, and the Korean
374 Peninsula. A third cluster of trade and invasive bullfrog genotypes was found in the
375 Eastern Lineage. This cluster included the genotype from the bullfrog which Bd-BRAZIL
376 was first isolated (Ypsilanti, MI, USA) (Schloegel et al., 2012). This genotype was shared
377 among sequences from Brazilian bullfrog farms, a retail shop in New York, and invasive
378 bullfrogs from Brazil and Ecuador.

379

380 **3.4. Bullfrog trade**

381 We plotted a network graph illustrating global bullfrog trade and its intensity (Fig.
382 3). A total of 48 countries were involved in international bullfrog trade as exporters (n =
383 12), importers (n = 21), or both (n = 15) (Table S7). However, most of the trade is
384 concentrated among a few countries only, with the highest trade intensity observed in the

385 USA, Taiwan, the Dominican Republic, Ecuador, Brazil, China, Canada, and Mexico.
386 The USA stands out as the largest importer, while Taiwan emerges as the leading
387 exporter. The Dominican Republic, Ecuador, the USA, Brazil, and China are also
388 prominent exporters. In contrast, several countries have minimal ($n < 10$) or no
389 significant export records (Fig. 3; Table S7).

390 Importation patterns are more balanced across countries, with Canada and Japan
391 being the primary importers. Germany, France, China, and Taiwan also participate
392 significantly in the bullfrog trade. Additionally, we found records of imports with no
393 associated exporting country. The strongest commercial interactions occur between the
394 USA and its trade partners Taiwan, the Dominican Republic, Brazil, Ecuador, and China
395 (Fig. 3; Table S7).

396 We also mapped the commercial routes focused exclusively on countries with
397 confirmed Bd-BRAZIL occurrences to reconstruct the most probable dispersal pathways
398 for this lineage. By integrating the earliest detection dates of Bd-BRAZIL in each country
399 with historical trade connections and genetic evidence from traded bullfrogs, we
400 identified several plausible transmission routes that explain its current distribution (Fig.
401 4A; Table S7). Concerning the 'out-of-Brazil' hypothesis, we identified eight routes.
402 Brazil had direct exports to the USA between 1991 and 2009, while the USA, in turn,
403 exported to the Korean Peninsula in 2004 and 2008. Although we do not have specific
404 records of Bd-BRAZIL in some countries like Ecuador, Italy, China, and Thailand, it is
405 plausible that they may have acted as intermediaries, thus contributing to the spread of
406 this Bd lineage out of Brazil. The absence of documented exports from South Korea—
407 where Bd-BRAZIL has been confirmed—to other affected regions further supports Brazil
408 as the likely origin of these dispersal events. Our analysis revealed three plausible
409 transmission routes for Bd-BRAZIL's spread through Asia (Fig. 4B; Table S7), all tracing
410 back to Japan due to the lack of recorded exports from the Korean Peninsula (Table S7).

411

412 **4. Discussion**

413 Our study provides the evidence on the origin of the Bd-BRAZIL lineage in
414 Brazil. Based on our data, we argue for the 'out-of-Brazil' hypothesis to explain the
415 current sampled diversity of Bd-BRAZIL. More specifically: i) the oldest records of Bd-
416 BRAZIL, as well as Bd-GPL, from museum frogs are from the Americas rather than East
417 Asia; ii) Bd-BRAZIL was detected in wild frogs from the Brazilian Atlantic Forest at
418 least 10 years before the presence of bullfrogs and their trade in Brazil; iii) the direction
419 and timing of bullfrog trade align with a Brazil-to-East Asia pathway, rather than the
420 reverse; iv) genetic analysis of bullfrogs in US markets support the export of South
421 American bullfrogs to the US; v) Bd-BRAZIL is more prevalent in Brazilian bullfrog
422 farms, increasing the likelihood of its exportation; and vi) while Bd-Brazil is found in
423 several native Brazilian amphibians, all other occurrences outside Brazil, except for one
424 native Japanese species, are associated with farmed bullfrogs.

425 The Bd-BRAZIL lineage, initially thought to be restricted to the Brazilian
426 Atlantic Forest, was later detected in a bullfrog from the United States frog-leg trade
427 (Schloegel et al., 2012) and, more recently, in South Korea (O'Hanlon et al., 2018) and
428 Japan (Goka et al., 2021). However, in these regions, records remain scarce and are
429 predominantly associated with a single host species, the bullfrog (Goka et al., 2021;

430 O'Hanlon et al., 2018; Schloegel et al., 2012). In contrast, Brazil has the highest number
431 of Bd-BRAZIL cases (n = 71), recorded between 1916 and 2016 in both farmed and wild
432 populations, across an extensive geographic area (~1,400 km²). Additionally, at least 10
433 native amphibian species in Brazil serve as asymptomatic hosts for this lineage
434 (Jenkinson et al., 2016; O'Hanlon et al., 2018; Ribeiro et al., 2019).

435 The high prevalence of Bd-BRAZIL in four Brazilian bullfrog farms suggests that
436 farming conditions (Ribeiro and Toledo, 2022) may favor this lineage over others, such
437 as Bd-GPL. Alternatively, its dominance may result from stochastic processes associated
438 with high host density and variable transmission dynamics, including differences in
439 infectivity, contact rates, or infection duration (VanderWaal and Ezenwa, 2016). In dense
440 populations, network structures may reduce pathogen diversity and promote single-strain
441 dominance, as observed in other multi-strain systems (Pinotti et al., 2019). Moreover,
442 recent evidence suggests that coinfection with other Bd lineages can enhance the
443 transmission of otherwise less competitive genotypes like Bd-BRAZIL, potentially
444 contributing to its persistence in high-density farming environments (Carvalho et al.,
445 2023). Further research is needed to explore these dynamics in frog farms worldwide and
446 during intercontinental live animal transport.

447 Brazil ranks among the leading global bullfrog producers, exporting large
448 quantities of live animals and their products over the past few decades (Ribeiro and
449 Toledo, 2022). The use of untreated natural pond water on farms fosters potential cross-
450 contamination between captive frogs and the environment, facilitating exchanges of Bd
451 lineages between farms and the wild (Ribeiro et al., 2019; Ribeiro and Toledo, 2022).
452 Although bullfrog trade is poorly regulated globally (Ribeiro and Toledo, 2025), we have
453 mapped a network of countries engaged in this practice. While few countries, namely the
454 USA, Taiwan, and Brazil, are major players, most exhibit low production and limited
455 global trade engagement. Notably, strong trade connections exist between countries
456 where Bd-BRAZIL has been recorded. The timeline and direction of trade suggest Brazil
457 as the origin, with subsequent spread to other regions, potentially via the USA from 1991
458 and later to the Korean Peninsula around 2004.

459 Importantly, the bullfrog is the only amphibian species legally authorized for
460 trade in Brazil (Brasil, 1998; Ribeiro and Toledo, 2025). In contrast, the illegal pet trade
461 predominantly involves other species, which are mostly exported from Brazil to Europe
462 (Máximo et al., 2021; Pistoni and Toledo, 2010). No Bd-infected individuals have been
463 identified in this illegal pet trade so far (Máximo et al., 2021). However, the recent record
464 of *Kaloula pulchra*—an Asian species native to South Asia—in Bahia, Brazil, likely
465 through the pet trade (Forti et al., 2017; Máximo et al., 2021), underscores the potential
466 for introducing exotic amphibians and possibly undetected pathogens through both legal
467 and illegal routes.

468 Although our findings support the hypothesis that Bd-BRAZIL originated in
469 Brazil and spread via bullfrog trade, important limitations must be acknowledged. Our
470 genetic analysis used the mitochondrial cytochrome-b marker, which captures only a
471 portion of genetic diversity and offers limited resolution for population structure or
472 source attribution. Nonetheless, this marker has proven useful in identifying distinct
473 bullfrog haplotypes and potential source populations in historical introduction contexts
474 (Jorgewich-Cohen et al., 2022). Our objective was not to reconstruct migration routes

475 definitively, but to assess whether sampled bullfrogs—especially those linked to Bd-
476 BRAZIL—share a genetic background compatible with known trade routes. Furthermore,
477 global Bd sampling is spatially and temporally biased, with most historical and
478 contemporary Bd detections from the Americas. However, even after accounting for
479 sampling effort, our analysis still indicated the highest Bd detection probability in the
480 Americas. Thus, our findings should be considered preliminary and interpreted in
481 conjunction with other evidence—including historical records, genotyping, and trade
482 data. Expanding the use of nuclear and genomic markers and increasing sampling in
483 underrepresented regions like Asia will be critical for understanding the evolutionary
484 origins and spread of Bd lineages.

485 Despite these limitations, our study advances understanding of Bd's historical
486 distribution. Our extended sampling of museum specimens and review of literature
487 pushed the timeline of surveillance back to several regions. For instance, we extended
488 Africa's earliest Bd sampling from 1844 (Soto-Azat et al., 2010) to 1815— though both
489 samples were negative. Nonetheless, this temporal expansion deepens our knowledge of
490 the pathogen's historical presence or absence. The oldest Bd-positive sample in our
491 museum dataset was collected in the French Pyrenees in 1915. Literature review revealed
492 earlier Bd detections than previously reported in multiple regions: Bolivia (1863)
493 (Burrowes and De la Riva, 2017), USA (1888) (Talley et al., 2015), Brazil and Mexico
494 (1894) (Basanta et al., 2021; Rodriguez et al., 2014), and Venezuela (1895) (Becker et
495 al., 2016)—all in the Americas. While previous studies proposed Bd-GPL emerged in
496 East Asia in the 20th century (O'Hanlon et al., 2018), our review identified records dating
497 back to 1897 (Basanta et al., 2021; Rodriguez et al., 2014), predating that timeline. We
498 also documented earlier Bd records for Central America (1964 *vs.* 1972), Europe (1901
499 *vs.* 1997), and the Pyrenees (1915 *vs.* 2000) than those cited by O'Hanlon et al. (2018).
500 These findings emphasize the value of revisiting historical data to refine our
501 understanding of Bd's emergence and spread.

502 Although our expanded historical review revealed earlier records of Bd presence
503 worldwide, a major limitation persists: the difficulty of genotyping historical samples.
504 Many museum specimens present challenges such as DNA degradation and potential
505 contamination among co-stored individuals (Richards-Hrdlicka, 2012), limiting lineage
506 identification. Most previous genotyping has focused on the ribosomal ITS region (Goka
507 et al., 2021; Schloegel et al., 2012), which, while useful, limited resolution. Recent
508 advances allow genotyping from swabs using multiple single-copy markers (Basanta et
509 al., 2021; Byrne et al., 2019), offering promise for museum specimens. However, this
510 technique requires relatively high pathogen loads, which are rare in degraded samples.
511 Overcoming these challenges is essential for accurately tracing Bd lineages evolution and
512 spread.

513 Our understanding of geographically restricted lineages, such as Bd-CAPE, Bd-
514 ASIA-1, Bd-ASIA-3, and Bd-BRAZIL, remains limited due to their low prevalence and
515 the uneven global sampling effort. Although our detection probability analysis helps
516 mitigate sampling bias concerns, key regions like Asia are still underrepresented in both
517 our study and the broader literature (Sreedharan and Vasudevan, 2021). This reduces the
518 chances of detecting early Bd-positive cases in these areas and limits the ability to draw
519 definitive conclusions. Thus, expanding geographic and temporal sampling, especially in

520 Asia, is essential. Despite these challenges, our results provide strong empirical and
521 genetic evidence for the Brazilian origin and international spread of the Bd-BRAZIL
522 lineage, contributing valuable insight into the complex global history of this devastating
523 fungal pathogen.

524

525 **5. Conclusion**

526 Despite significant advances in understanding the evolutionary history of Bd,
527 important gaps remain. Our research proposes the 'out-of-Brazil' hypothesis, suggesting
528 that the Bd-BRAZIL lineage originated in Brazil and subsequently spread to other
529 regions through international bullfrog trade. This hypothesis supports the notion of long-
530 term endemicity in the Brazilian Atlantic Forest, rather than in Asia.

531 Expanding historical Bd sampling efforts in Asia and characterizing lineages from
532 suspected source regions are critical next steps. Improving genetic resolution, accurately
533 identifying Bd lineages, and preserving both museum specimens and associated data are
534 essential for robust historical inference. Bd-positive records from historical museum
535 specimens serve as critical benchmarks for future investigations into the pathogen's
536 distribution and evolutionary diversification.

537 Challenges related to the global amphibian trade persist and underscore the need
538 for stronger preventive measures. These include implementing import controls,
539 conducting pathogen screenings, and establishing effective quarantine protocols to
540 safeguard native fauna (Fu and Waldman, 2022; Ribeiro and Toledo, 2025). Non-
541 invasive tools, such as environmental DNA (eDNA) protocols, are instrumental in
542 pathogen detection and in promoting “clean trade” practices (Brunner, 2020). The urgent
543 adoption of comprehensive protocols for amphibian production, trade regulation, and
544 health inspection is imperative to prevent the unintentional spread of Bd and other
545 pathogens.

546

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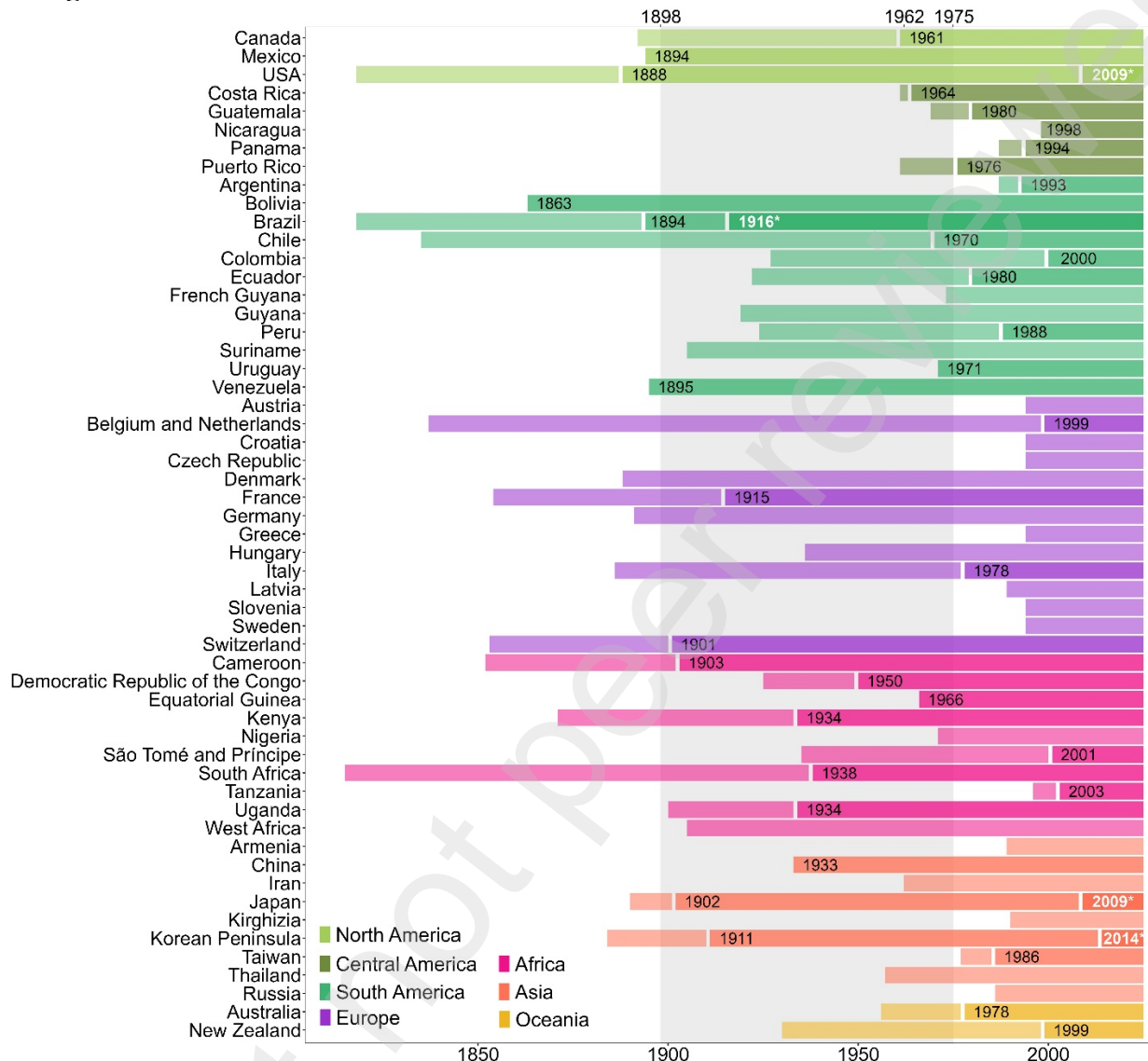
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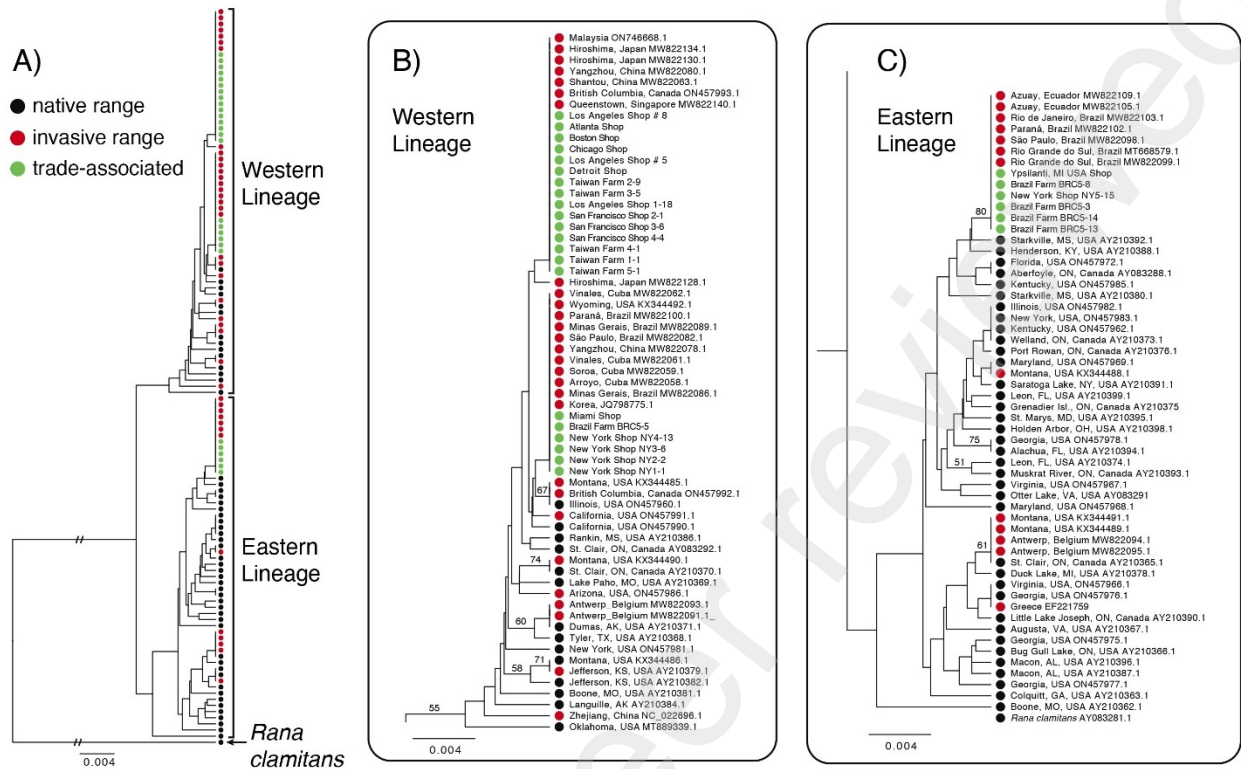
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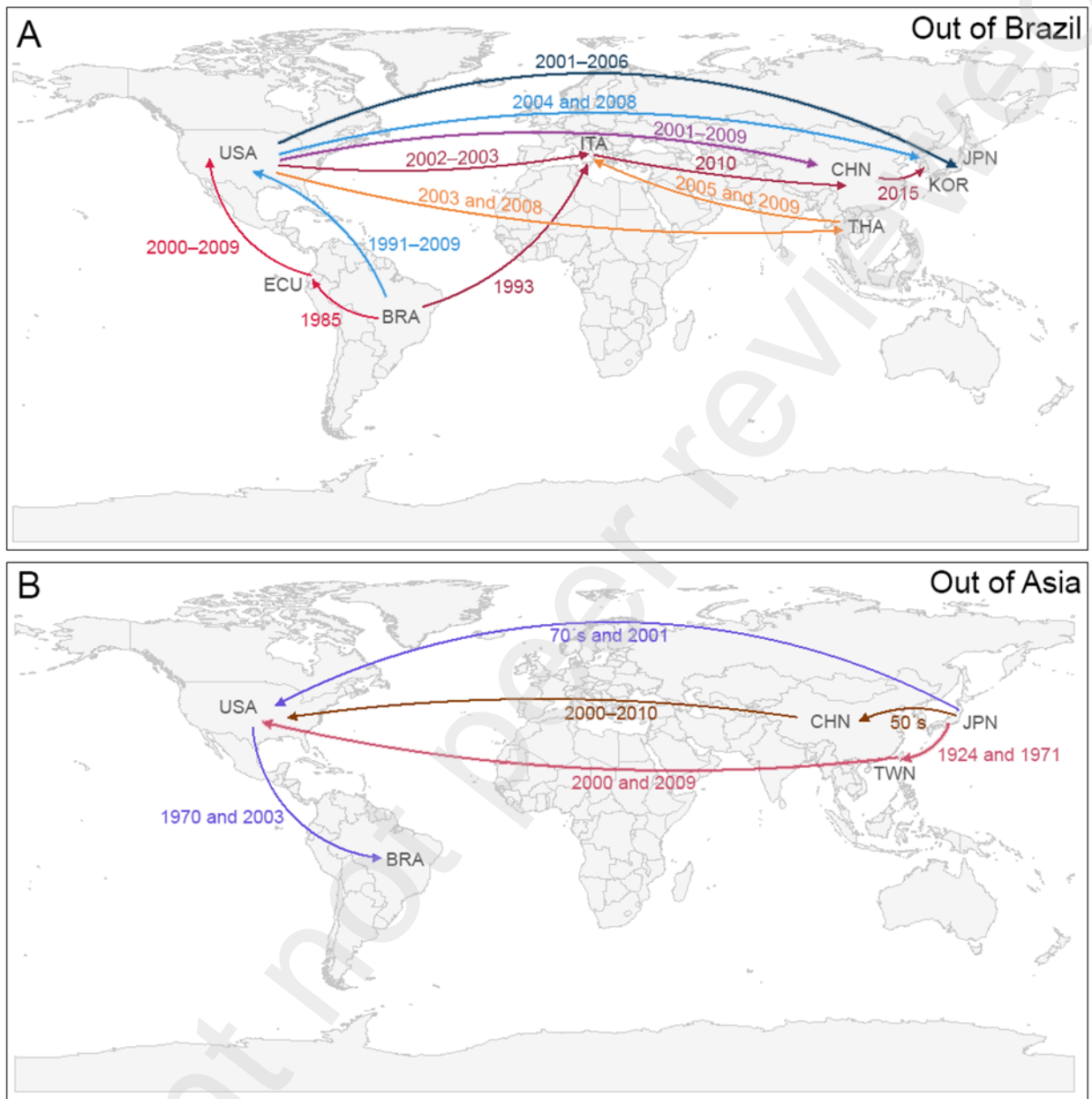


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 925 Fig. 1. Historical records of Bd detection across countries and regions. Solid bars
 926 represent sampling records (negative: light color; positive: dark color), with the
 927 corresponding year of detection indicated. Dates marked with an asterisk denote the first
 928 records of the Bd-BRAZIL lineage (see Table 1). The gray shaded area represents the
 929 estimated time frame for the origin of Bd-GPL, based on nuclear (1898) and
 930 mitochondrial DNA dating analyses—either with calibration (1975) or without
 931 calibration (1962)—as reported by O’Hanlon et al. (2018).



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Fig. 2. Cytochrome-b dendrogram of sequences from bullfrogs. The Eastern and Western Lineages correspond to designations from (Austin et al., 2004). Only bootstrap values > 50 % for non-zero branch lengths are shown. *Rana clamitans* cyt-b was used as an outgroup sequence.



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Fig. 4. Dated bullfrog commercial routes that may show the potential spread pattern of the Bd-BRAZIL lineage from Brazil (A) and Asia (B) to other regions of the world. Same colors represent possible sequences of routes.

951 Table 1. Historical records of *Batrachochytrium dendrobatidis* (Bd) worldwide. For each
952 country, we report the sampling period, number of specimens analyzed, year of the first
953 confirmed detection of Bd, and—when available—the year of the first detection of the
954 Bd-BRAZIL lineage. NA indicates information not available in the original source. The
955 earliest sampling and detection records for each continent are highlighted in bold (see
956 Fig. 1). References correspond to published studies and museum data analyzed in this

957 study. S = reference for the first year of amphibian sampling; D = reference for the first
 958 confirmed detection of Bd; L = reference for the first detection of Bd-BRAZIL.

Country	Sampled period	First Bd detection	N specimens	Bd-BRAZIL detection	Reference
<u>North America</u>					
Canada	1892–2001	1961	963	NA	This study (S); Ouellet et al., 2005 (D)
Mexico	1894–2012	1894	1,437	NA	Basanta et al., 2021 (S, D); Ouellet et al., 2005 (S)
USA	1818 –2017	1888	18,582	2009	This study (S); Schloegel et al., 2012 (B); Talley et al., 2015 (D)
<u>Central America</u>					
Costa Rica	1961 –2011	1964	1,501	NA	de León et al., 2019 (S, D)
Guatemala	1969–2009	1980	696	NA	Cheng et al., 2011 (S); Mendelson et al., 2014 (D)
Nicaragua	1997–2011	1998	164	NA	Stark et al., 2017 (S, D)
Panama	1987–2005	1994	2,661	NA	Berger et al., 1998 (S, D)
Puerto Rico	1961 –2007	1976	2,545	NA	Burrowes et al., 2004 (S, D)
<u>South America</u>					
Argentina	1987–1993	1993	13	NA	Ouellet et al., 2005 (S); Ghirardi et al., 2011 (D)
Bolivia	1863–2016	1863	631	NA	Burrowes and De la Riva, 2017 (S, D)
Brazil	1818 –2016	1894	37,077	1916	This study (S); Rodriguez et al., 2014 (D, B)
Chile	1835–2009	1970	1,133	NA	Soto-Azat et al., 2013 (S, D)
Colombia	1927–2006	2000	980	NA	Becker et al., 2016 (S); Flechas et al., 2015 (D)
Ecuador	1922–2010	1980	82	NA	Becker et al., 2016 (S); Ron and Merino-Viteri, 2000 (D)
French Guyana	1973–1992	Not detected	3	NA	Becker et al., 2016 (S)
Guyana	1919–2011	Not detected	42	NA	Becker et al., 2016 (S)
Peru	1924–1999	1988	65	NA	Becker et al., 2016 (S); Rodriguez and Catenazzi, 2017 (D)
Suriname	1905–2016	Not detected	231	NA	Jairam, 2020 (S)
Uruguay	1971–2009	1971	33	NA	Lambertini et al., 2017 (S, D)
Venezuela	1895–2007	1895	1,390	NA	Becker et al., 2016 (S, D)

Europe

Austria	1994–2004	Not detected	24	NA	Garner et al., 2005 (S)
Belgium and Netherlands	1837–2007	1999	289	NA	Spitzen-Van Der Sluijs et al., 2014 (S, D)
Croatia	1994–2004	Not detected	8	NA	Garner et al., 2005 (S)
Czech Republic	1994–2004	Not detected	18	NA	Garner et al., 2005 (S)
Denmark	1888	Not detected	1	NA	This study (S)
France	1854–2014	1915	2,095	NA	This study (S, D)
Germany	1891–2004	Not detected	300	NA	This study (S)
Greece	1994–2004	Not detected	88	NA	Garner et al., 2005 (S)
Hungary	1936–2005	Not detected	127	NA	Vörös et al., 2018 (S)
Italy	1886–2012	1978	238	NA	This study (S); Canestrelli et al., 2013 (D)
Latvia	1989–1990	Not detected	5	NA	Ouellet et al., 2005 (S)
Portugal	1859–2004	NA	28	NA	This study (S); Garner et al., 2005 (D)
Slovenia	1994–2004	Not detected	29	NA	Garner et al., 2005 (S)
Spain	1881–2004	NA	349	NA	This study (S); Garner et al., 2005 (D)
Sweden	1994–2004	Not detected	197	NA	Garner et al., 2005 (S)
Switzerland	1853–2009	1901	1,040	NA	Peyer et al., 2010 (S, D)
United Kingdom	1994–2004	NA	466	NA	Garner et al., 2005 (S)
<u>Africa</u>					
Cameroon	1852–2019	1903	2,234	NA	Soto-Azat et al., 2010 (S); Doherty-Bone et al., 2025 (D)
Democratic Republic of the Congo	1925–1994	1950	232	NA	Seimon et al., 2015 (S, D)
Equatorial Guinea	1966–1998	1966	314	NA	Hydeman et al., 2017 (S, D)
Kenya	1871–2000	1934	188	NA	Vredenburg et al., 2013 (S, D)
Nigeria	1971	Not detected	5	NA	Ouellet et al., 2005 (S)

São Tomé and Príncipe	1935–2012	2001	468	NA	Hydeman et al., 2017 (S, D)
South Africa	1815 –2001	1938	1,049	NA	This study (S); Weldon et al., 2004 (D)
Tanzania	1996–2006	2003	187	NA	Weldon et al., 2020 (S, D)
Uganda	1900–1959	1934	325	NA	Doherty-Bone et al., 2025 (S, D)
West Africa*	1905–2011	Not detected	793	NA	Penner et al., 2013 (S)
<u>Asia</u>					
Armenia	1989	Not detected	15	NA	Ouellet et al., 2005 (S)
China	1933–2009	1933	1,258	NA	Zhu et al., 2014 (S, D)
Iran	1962–1990	Not detected	19	NA	Ouellet et al., 2005 (S)
Japan	1890–2000	1902	612	2009	Goka et al., 2009 (D); Goka et al., 2021 (B); Rios-Sotelo et al., 2018 (S)
Kirghizia	1990	Not detected	18	NA	Ouellet et al., 2005 (S)
Korean Peninsula	1884 –2004	1911	248	2014	This study (S); Fong et al., 2015 (D); O’Hanlon et al., 2018 (B)
Taiwan	1977–2009	1986	369	NA	Schmeller et al., 2022 (S, D)
Thailand	1957–2004	Not detected	123	NA	McLeod et al., 2008 (S)
Russia	1986	Not detected	26	NA	Ouellet et al., 2005 (S)
<u>Oceania</u>					
Australia	1956–2007	1978	11,423	NA	Murray et al., 2010 (S, D)
New Zealand	1930 –2010	1999	721	NA	Shaw et al., 2013 (S, D)

959 * “West Africa” includes records from Bénin, Burkina Faso, Côte d’Ivoire, Ghana, Guinea, Liberia, and
960 Sierra Leone