Genomic epidemiology of the emerging pathogen *Batrachochytrium dendrobatidis* from native and invasive amphibian species in Chile

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1   Rapid Communication

2   **Genomic epidemiology of the emerging pathogen *Batrachochytrium dendrobatidis***

3   **from native and invasive amphibian species in Chile**

4   Running title: Genomic epidemiology of *Batrachochytrium dendrobatidis* in Chile

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21   **Summary**

22   Emerging fungal diseases represent a threat to food security, animal and human health

23   worldwide. Amphibian chytridiomycosis, caused by the fungus *Batrachochytrium*

24   *dendrobatidis* (*Bd*), has been associated with catastrophic and well-documented

25   amphibian population declines and extinctions. For the first time, *Bd* was cultured from

26   native and non-native wild amphibians in Chile. Phylogenomic analyses revealed that

27   Chilean isolates AVS2, AVS4 and AVS7 group within the global panzootic lineage of *Bd*

28   (*Bd*GPL) in a single highly supported clade that includes a genotype previously isolated

29   from the United Kingdom. Our results extend the known distribution of *Bd*GPL in South

30   America and suggest a single and relatively recent introduction of *Bd*GPL into the country,

31   providing additional support to the role of anthropogenic activity in the global spread of this

32   panzootic lineage.

33   **Keywords:** *Batrachyla antartandica*; *Calyptocephalella gayi*; chytridiomycosis; emerging

34   infectious disease; fungal disease; *Xenopus laevis.*

35

36   **1. INTRODUCTION**

37   Anthropogenic activity has contributed to the current rise of fungal diseases, representing

38   a threat to food security, animal and human health worldwide (Fisher, Gow, & Gurr, 2016).

39   Perhaps the most striking example of this problem is chytridiomycosis, an emerging

40   disease of amphibians caused by the fungus *Batrachochytrium dendrobatidis* (hereafter

41   *Bd*; Longcore, Pessier, & Nichols, 1999). *Bd* occurs in all continents with amphibians, and

42   its impact has been described as 'the most spectacular loss of vertebrate biodiversity due

43   to disease in recorded history' (Skerratt et al., 2007). Initially, this pathogen was

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44   considered to be a rapidly expanding clonal lineage with a single geographical origin

45   (Morehouse et al., 2003). However, further molecular analyses comprising more-

46   comprehensive methods and a wider geographical representation of isolates, revealed a

47   much more complex evolutionary history, diversity and population dynamics (Farrer et al.,

48   2011; Rosenblum et al., 2013). A hypervirulent lineage of *Bd*, termed the global panzootic

49   lineage (*Bd*GPL), is widely distributed across the globe and has been associated with

50   catastrophic and well-documented amphibian population declines and extinctions (Farrer

51   et al., 2011; Rosenblum et al., 2013). In contrast, increasing numbers of endemic

52   genotypes have been isolated from amphibians inhabiting Asia, Switzerland, South Africa

53   and Brazil (Farrer et al., 2011; Schloegel et al., 2012; Bataille et al., 2013). To date, none

54   of these have been convincingly linked to host population declines.

55   In Latin America, *Bd* is a recognized threat to many amphibian species (Lips et al.,

56   2006; Soto-Azat et al., 2013). Despite this, there is a knowledge gap on the molecular

57   diversity of *Bd* genotypes occurring in most areas within this region. For instance in Chile,

58   *Bd* is widespread, infecting native and invasive anurans, but no attempt has been

59   conducted so far to isolate the fungus or to conduct comparative molecular analyses

60   (Soto-Azat et al., 2013, 2016). This limits our capacity to adequately understand the

61   emergence and epidemiology of chytridiomycosis in the region. The aims of this study are

62   to: 1) describe the isolation and whole-genome sequencing of three *Bd* isolates collected

63   from the skin of wild amphibians inhabiting distinct geographic areas of Chile, and 2)

64   compare these genotypes with a global panel of *Bd*.

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66   **2. MATERIAL AND METHODS**

67 This study was carried out in accordance with the Bioethics Committee of the Universidad

68 Andres Bello (N°13/2015), the Zoological Society of London's Ethics Committee

69 (WLE709), and the Chilean law (Agriculture and Livestock Service permit N° 351/2015).

70 In February 2015, we captured 20 tadpoles of the native marbled wood frog

71 (*Batrachyla antartandica*) in Melimoyu, southern Chile (44°6'31"S, 73°07'06"W; Figure 1a).

72 In May 2015, we captured 10 wild adults of the invasive African clawed frog (*Xenopus*

73 *laevis*) in Hualañé, central Chile (34°58'33"S, 71°51'09"W; Figure 1a). Amphibians were

74 kept for no longer than one month, with individuals of each species maintained in each of

75 two separated groups. In order to increase the efficiency of the *Bd* isolation process,

76 animals were first confirmed to be infected using a specific real-time PCR assay (Soto-

77 Azat et al., 2013). Infected animals were then euthanized under licence by immersion in a

78 diluted solution of tricaine methanesulfonate (10 g/L) for one hour. Once death was

79 confirmed, keratinized tissues of *Bd*-positive animals (i.e. the whole mouthparts of

80 tadpoles and skin sections of ventral hindlimbs and interdigital membranes of adults) were

81 removed and cultured in a fungal growth medium following the protocol developed by

82 Joyce E. Longcore (https://umaine.edu/chytrids/batrachochytrium-dendrobatidis/directions-

83 for-isolation). Additionally, in February 2016, during an extensive survey of *Bd* infection in

84 wild amphibians across Chile, we captured one lethargic larvae of the native Chilean frog

85 (*Calyptocephalella gayi*) in Valdivia (39°48'36"S, 73°15'21"W; Figure 1a). This individual

86 died in the field during the sampling procedure and exhibited partial depigmentation of the

87 mouthpart, a finding consistent with *Bd* infection. Therefore, we immediately transported

88 the carcass (in refrigerated conditions) to the laboratory; culture of *Bd* was performed as

89 described above. Cultures were grown at 21°C in a temperature controlled chamber and

90 were checked every day for zoosporangia development using an inverted light

91 microscope. We confirmed successful isolation (i.e. presence of free-swimming zoospores

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92   and spherical bodies morphologically typical of *Bd* zoosporangia) within 2 weeks. Isolates

93   were passaged no more than three times in order to lessen the chance of genomic change

94   due to prolonged laboratory culture (Voyles et al., 2014) and were subsequently cryo-

95   archived at -80°C following Boyle et al. (2003).

96   We performed DNA extraction using the MasterPure™ Yeast DNA Purification Kit

97   (Epicentre, Wisconsin, USA). DNA extractions were quantified using a Tapestation 2200

98   (Agilent Technologies, California, USA) and Qubit 2.0 fluorimeter (Thermo Fisher

99   Scientific, Massachusetts, USA). We prepared DNA samples for sequencing on an

100   Illumina HiSeq 2000 (Illumina, California, USA). TruSeq Nano 350 gel-free sequencing

101   libraries were prepared for 125+125bp paired-end sequencing using the Illumina HiSeq

102   high output V4 chemistry.

103   Sequencing reads were first cleaned of adapter sequences and quality trimmed using

104   cutadapt v1.10 (Martin, 2011). We mapped the reads to the JEL423 reference genome

105   (GenBank assembly accession: GCA_000149865.1) using Burrows-Wheeler Aligner

106   (BWA) v0.7.8 (Li & Durbin, 2009). We processed the resulting sequence alignment/map

107   (SAM) files using SAMtools v1.3.1 with the 'fixmate' and 'sort' programs to ready the files

108   for variant discovery. We performed the variant discovery in a two-step process using

109   freebayes version dbb6160 (Garrison & Marth, 2012). In the first step, sorted BAM files for

110   each of the isolates in the phylogeny were independently called to find variant positions.

111   The set of all variant position identified across all samples were merged into a single

112   variant call format (VCF) file. In the second step, each of the samples were re-called using

113   the positions in the merged VCF to produce a squared-off call set (a genotype call is made

114   at every locus for each isolate, including for missing data). All squared-off sample VCF

115   files were processed by vcflib (Garrison, 2016) to break complex variants into allelic

116   primitives and vt (Tan, Abecasis, & Kang, 2015) to normalize short insertion and deletion

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117   sequences (indels). We quality filtered the VCFs with bcftools version 1.3.1 (Li et al., 2009)

118   to accept only variants with sufficient supporting evidence: sites covered by 4 reads or less

119   were set to missing; potentially polymorphic sites were additionally filtered using the

120   settings recommended in the bcbio.variation.recall squaring-off pipeline (Chapman, 2016),

121   with sites not passing these filters set to homozygous reference, i.e. though there were

122   sequencing reads covering that site, there was not enough evidence to call a variant at

123   that position. We merged the processed VCF files into a single multi-sample VCF and

124   extracted a fasta file of the SNP variant calls. We conducted the phylogenetic analyses

125   using RAxML v8.2.9 with the GTRCAT model with 500 bootstrap runs. The raw read

126   sequences of AVS2, AVS4 and AVS7 are available at the NCBI Sequence Read Archive:

127   https://www.ncbi.nlm.nih.gov/sra under accession numbers <mark>XXX, XXX and XXX</mark>,

128   respectively.

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3. RESULTS AND DISCUSSION

131   We obtained three isolates across central and southern Chile (Figure 1): AVS2 from *B.*

132   *antartandica* from Melimoyu, AVS4 from *X. laevis* from Hualañé, and AVS7 from *C. gayi*

133   from Valdivia. Phylogenomic analyses showed that all Chilean isolates group within

134   *Bd*GPL in a single, highly supported clade (100% bootstrap support; Figure 1b). The

135   Chilean *Bd* isolates were fixed at 99.4% of the segregating sites in *Bd*GPL after filtering for

136   missing positions. There were only 2,257 variable sites exclusive to the Chilean *Bd*

137   isolates. Although we compared the genomes of AVS2, AVS4 and AVS7 with an extensive

138   global panel of *Bd*, they were shown to be highly divergent from the only known regional

139   endemic lineage (*Bd*Brazil), but clustered with UKTvB, a *Bd* isolate collected from a

140   smooth newt (*Lissotriton vulgaris*) in 2009 in Kent, United Kingdom (bootstrap support

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141   100%, Figure 1b). Inferring phylogenetic relationships within the *Bd*GPL lineage is difficult

142   due to potential SNP homoplasy caused by independent loss of heterozygosity (LOH)

143   events (Rosenblum et al., 2013). We tested the topology produced using SNPs from

144   across the whole autosomal genome against a 1.66Mb region on supercontig_1.2, which

145   displays a characteristic large-scale loss of heterozygosity (LOH) event, present in all

146   known sequenced isolates of *Bd*GPL (Rosenblum et al., 2013). The topology derived from

147   SNPs in this region (consisting of 19,913 segregating sites), recapitulated the deeply

148   diverged lineages also found in the whole genome tree, with a very flat comb-like structure

149   in the *Bd*GPL lineage due to a lack of SNPs in this region (Figure 2). All 3 Chilean isolates

150   in this study formed a clade (albeit with very little bootstrap support) with isolates from

151   Valencia (VA02), Switzerland (AoCH15), the UK (UKTvB), France (0711.1) and Canada

152   (JEL261), suggesting the clustering with the UK isolate UKTvB in the whole genome

153   analysis may not be artefact. Finally, we used the Weir and Cockerham's estimator in

154   vcftools to perform a sliding-window comparison of F_{ST} of the Chilean *Bd* isolates against

155   all the other *Bd*GPL isolates. In this analysis, we used a 10Kb window, with a 5kb step-

156   size along the genome to identify if any stretches of the Chilean *Bd* population were highly

157   differentiated compared to the rest of the *Bd*GPL clade (Figure 3). We identified several

158   stretches of genome where the F_{ST} estimator was more than two standard deviations

159   greater than the mean of all F_{ST} values, indicating differentiation due to positive selection

160   or reduced rates of recombination may be important at these loci in Chile. Further work is

161   required to determine if any classes of gene are functionally enriched in these high

162   divergence regions.

163   The low number of segregating sites exclusive to the Chilean *Bd* isolates, compared

164   to the total number of sites where the *Bd*GPL isolates are polymorphic, suggest a single

165   and recent instance of long-distance dispersal of *Bd* into Chile, possibly through the

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166 international movement of amphibians, aquatic animals, plants or fomites (Farrer et al.,

167 2011; Schloegel et al., 2012). Molecular characterization of further isolates from Chile and

168 neighbouring countries, along with the calibration of a genome-wide molecular clock, is

169 required to confirm this hypothesis. The existence of a unique, recently introduced lineage

170 of *Bd* in Chile would be in contrast with the known history of this pathogen in the near

171 Brazil, where both *Bd*GPL and *Bd*Brazil co-exist, with evidence of multiple hybridization

172 events between them (Jenkinson et al., 2016). This highlights the importance of

173 biosecurity measures to prevent the introduction and establishment of further *Bd* lineages

174 into Chile, as this pathogen has the capability to increase its genomic diversity through the

175 exchange of haplotypes among lineages.

176   *Xenopus laevis* is considered as an effective vector capable of disseminating *Bd* into

177 new regions (Soto-Azat et al., 2016) and thus represents a possible route of introduction of

178 *Bd*GPL into Chile. The earliest evidence of *Bd* in Chile is from a four-eyed frog

179 (*Pleurodema thaul*) individual collected from Concepción in 1970 (Soto-Azat et al. 2013).

180 Although, underestimation of *Bd* is a known problem when examining fixed archived

181 specimens (further discussed in Soto-Azat et al. 2009), hundreds of PCR analyses from

182 archived amphibians have failed to detect *Bd* infections prior to that date in Chile (Soto-

183 Azat et al., 2013). The exact time of introduction of *X. laevis* into Chile is unknown, but it is

184 thought to have occurred sometime in the 1970s (Jaksic, 1998). Additional means of

185 *Bd*GPL introduction, possibly assisted via tourism, research or salmon farming (Liew et al.,

186 2017), should also be considered.

187   The Chilean *Bd* isolates grouped together in our phylogenomic whole-genome

188 analysis with a genotype isolated in 2009 from the United Kingdom (UKTvB). A similar

189 phylogenetic relationship was observed when restricting the analysis to a subset of the

190 genome spanning an LOH event shared by all *Bd*GPL isolates, with isolates from other

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191   European countries and a Canadian isolate also grouping with the Chilean isolates. While

192   there has been no report of mortality caused by the UKTvB, VA02, JEL261 or AoCH15_B

193   isolates, the 0711.1 isolate from the French Pyrenees was associated with amphibian

194   mortalities. In general, *Bd*GPL has been associated with catastrophic mass mortalities and

195   population extirpations in multiple continents (Vredenburg, Knapp, Tunstall, & Briggs,

196   2010; Farrer et al., 2011; Rosenblum et al., 2013). In Chile, during the last four decades,

197   an extensive extirpation of local populations of the Darwin's frogs (*Rhinoderma darwinii*

198   and *R. rufum*) has occurred across central and southern Chile. Retrospective, cross-

199   sectional and capture-recapture data suggest that, among other factors, chytridiomycosis

200   has contributed to the occurrence of these extirpation events (Soto-Azat et al., 2013;

201   Valenzuela-Sánchez et al. 2017). The existence of the hypervirulent *Bd*GPL in Chile

202   provides additional support to this hypothesis. The susceptibility of *Rhinoderma* spp. to

203   different *Bd* genotypes, however, requires investigation (e.g. controlled infection

204   experiments), because the host-parasite interaction is a complex process that is affected

205   not only by the infectivity and virulence of the parasite but also by host-specific responses

206   and environmental factors (Woodhams et al., 2011).

207   This is the first description of Chilean genotypes of *Bd* and our study gives the first

208   insights into the origin and introduction history of this amphibian pathogen into the country.

209   Our results extend the previously known distribution of *Bd*GPL and provides additional

210   support to the role of amphibian translocations, and perhaps other international

211   movements of aquatic animals, plants or fomites, in the global spread of this panzootic

212   lineage.

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214   **ACKNOWLEDGMENTS**

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Competing interests

223   The authors declare that they have no competing interests.

224

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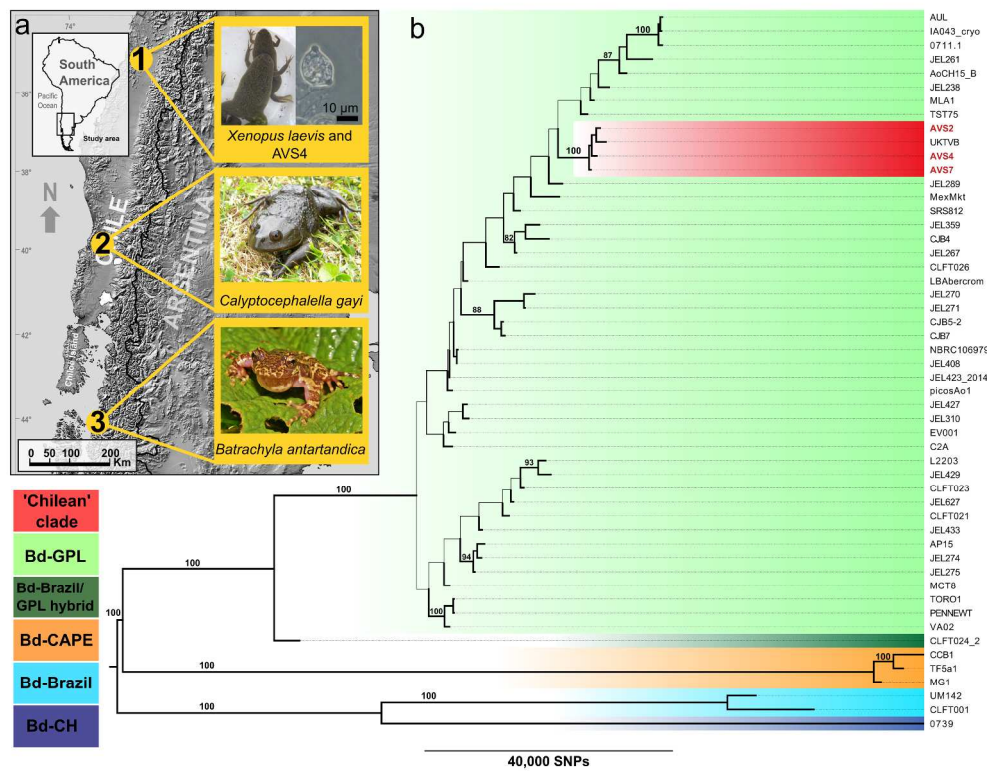
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Figure 1. (a) Sites of collection, amphibian species captured and *Batrachochytrium dendrobatidis* (*Bd*) [AVS4] isolate obtained from Chile. (b) Global phylogeny of 52 isolates of *Bd* based on 363,497 segregating sites. The clade containing the Chilean *Bd* isolates is highlighted in red, and the Chilean isolates are labelled in red: AVS4 from *Xenopus laevis* from Hualañé, AVS7 from *Calyptocephalella gayi* from Valdivia, and AVS2 from *Batrachyla antartandica* from Melimoyu. The branches of the tree are weighted (thickness) by bootstrap support (500 replicates), with branches with 80% of support and above labelled.

Figure 2. A phylogenetic tree of *Bd* isolates with proportional branch lengths, based on a 1.66Mb region of supercontig_1.2. This region includes a known loss of heterozygosity (LOH) event shared by all know *Bd*GPL isolates. The Chilean isolates (labelled in red) form a clade (circled in red) with several isolates from Europe (0711.1, AoCH15, UKTvB and VA02), and one from Canada (JEL261). The branch labels indicate bootstrap support from 500 bootstrap replicates.
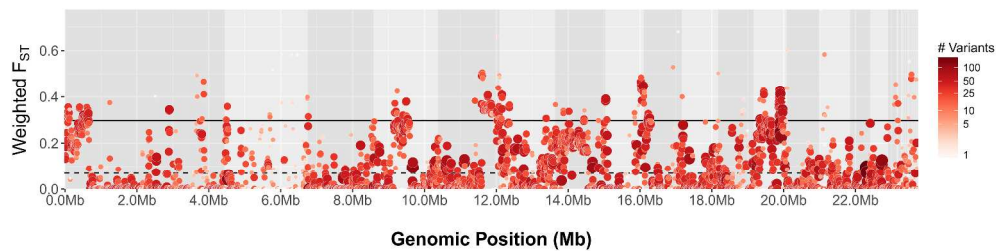
251x438mm (300 x 300 DPI)

Figure 3. Sliding window analysis of population differentiation of Chilean *Bd* isolates against other *Bd*GPL isolates using Weir and Cockerham's F_{ST} estimator. Each point represents a 10Kb genomic window, with a 5kb step-size. The dashed black line represents the mean F_{ST} (0.0679). The solid black line represents the 95% quantile threshold of the F_{ST} estimator (0.3141). Each point is sized and coloured on a log-scale by the number of variants in each window. The legend indicates the colour scale (the number of SNPs included in each window varied from 1 to 177, with a median of 24). Point size from small to large is scaled from low to high numbers of variants.

3007x751mm (72 x 72 DPI)