



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

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6 2 **Genomic epidemiology of the emerging pathogen *Batrachochytrium dendrobatidis***  
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8 3 **from native and invasive amphibian species in Chile**  
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11 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*.

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## 40 **1. INTRODUCTION**

41 Anthropogenic activity has contributed to the current rise of fungal diseases, representing  
42 a threat to food security, animal and human health worldwide (Fisher, Gow, & Gurr, 2016).  
43 Perhaps the most striking example of this problem is chytridiomycosis, an emerging  
44 disease of amphibians caused by the fungus *Batrachochytrium dendrobatidis* (hereafter  
45 *Bd*; Longcore, Pessier, & Nichols, 1999). *Bd* occurs in all continents with amphibians, and  
46 its impact has been described as ‘the most spectacular loss of vertebrate biodiversity due  
47 to disease in recorded history’ (Skerratt et al., 2007). Initially, this pathogen was  
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3 44 considered to be a rapidly expanding clonal lineage with a single geographical origin  
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5 45 (Morehouse et al., 2003). However, further molecular analyses comprising more-  
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7 46 comprehensive methods and a wider geographical representation of isolates, revealed a  
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9 47 much more complex evolutionary history, diversity and population dynamics (Farrer et al.,  
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11 48 2011; Rosenblum et al., 2013). A hypervirulent lineage of *Bd*, termed the global panzootic  
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13 49 lineage (*Bd*GPL), is widely distributed across the globe and has been associated with  
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15 50 catastrophic and well-documented amphibian population declines and extinctions (Farrer  
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17 51 et al., 2011; Rosenblum et al., 2013). In contrast, increasing numbers of endemic  
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19 52 genotypes have been isolated from amphibians inhabiting Asia, Switzerland, South Africa  
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21 53 and Brazil (Farrer et al., 2011; Schloegel et al., 2012; Bataille et al., 2013). To date, none  
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23 54 of these have been convincingly linked to host population declines.  
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28 55 In Latin America, *Bd* is a recognized threat to many amphibian species (Lips et al.,  
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30 56 2006; Soto-Azat et al., 2013). Despite this, there is a knowledge gap on the molecular  
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32 57 diversity of *Bd* genotypes occurring in most areas within this region. For instance in Chile,  
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34 58 *Bd* is widespread, infecting native and invasive anurans, but no attempt has been  
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36 59 conducted so far to isolate the fungus or to conduct comparative molecular analyses  
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38 60 (Soto-Azat et al., 2013, 2016). This limits our capacity to adequately understand the  
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40 61 emergence and epidemiology of chytridiomycosis in the region. The aims of this study are  
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42 62 to: 1) describe the isolation and whole-genome sequencing of three *Bd* isolates collected  
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44 63 from the skin of wild amphibians inhabiting distinct geographic areas of Chile, and 2)  
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46 64 compare these genotypes with a global panel of *Bd*.  
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## 54 66 2. MATERIAL AND METHODS

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3 67 This study was carried out in accordance with the Bioethics Committee of the Universidad  
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5 68 Andres Bello (N°13/2015), the Zoological Society of London's Ethics Committee  
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7 69 (WLE709), and the Chilean law (Agriculture and Livestock Service permit N° 351/2015).  
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10  
11 70 In February 2015, we captured 20 tadpoles of the native marbled wood frog  
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13 71 (*Batrachyla antartandica*) in Melimoyu, southern Chile (44°6'31"S, 73°07'06"W; Figure 1a).  
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15 72 In May 2015, we captured 10 wild adults of the invasive African clawed frog (*Xenopus*  
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17 73 *laevis*) in Hualañé, central Chile (34°58'33"S, 71°51'09"W; Figure 1a). Amphibians were  
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19 74 kept for no longer than one month, with individuals of each species maintained in each of  
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21 75 two separated groups. In order to increase the efficiency of the *Bd* isolation process,  
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23 76 animals were first confirmed to be infected using a specific real-time PCR assay (Soto-  
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25 77 Azat et al., 2013). Infected animals were then euthanized under licence by immersion in a  
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28 78 diluted solution of tricaine methanesulfonate (10 g/L) for one hour. Once death was  
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30 79 confirmed, keratinized tissues of *Bd*-positive animals (i.e. the whole mouthparts of  
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32 80 tadpoles and skin sections of ventral hindlimbs and interdigital membranes of adults) were  
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34 81 removed and cultured in a fungal growth medium following the protocol developed by  
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36 82 Joyce E. Longcore ([https://umaine.edu/chytrids/batrachochytrium-dendrobatidis/directions-](https://umaine.edu/chytrids/batrachochytrium-dendrobatidis/directions-for-isolation)  
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38 83 [for-isolation](https://umaine.edu/chytrids/batrachochytrium-dendrobatidis/directions-for-isolation)). Additionally, in February 2016, during an extensive survey of *Bd* infection in  
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40 84 wild amphibians across Chile, we captured one lethargic larvae of the native Chilean frog  
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42 85 (*Calyptocephalella gayi*) in Valdivia (39°48'36"S, 73°15'21"W; Figure 1a). This individual  
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44 86 died in the field during the sampling procedure and exhibited partial depigmentation of the  
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46 87 mouthpart, a finding consistent with *Bd* infection. Therefore, we immediately transported  
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48 88 the carcass (in refrigerated conditions) to the laboratory; culture of *Bd* was performed as  
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50 89 described above. Cultures were grown at 21°C in a temperature controlled chamber and  
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52 90 were checked every day for zoosporangia development using an inverted light  
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54 91 microscope. We confirmed successful isolation (i.e. presence of free-swimming zoospores  
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3 92 and spherical bodies morphologically typical of *Bd* zoosporangia) within 2 weeks. Isolates  
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5 93 were passaged no more than three times in order to lessen the chance of genomic change  
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7 94 due to prolonged laboratory culture (Voyles et al., 2014) and were subsequently cryo-  
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9 95 archived at -80°C following Boyle et al. (2003).  
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13 96 We performed DNA extraction using the MasterPure™ Yeast DNA Purification Kit  
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15 97 (Epicentre, Wisconsin, USA). DNA extractions were quantified using a TapeStation 2200  
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17 98 (Agilent Technologies, California, USA) and Qubit 2.0 fluorimeter (Thermo Fisher  
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19 99 Scientific, Massachusetts, USA). We prepared DNA samples for sequencing on an  
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21 100 Illumina HiSeq 2000 (Illumina, California, USA). TruSeq Nano 350 gel-free sequencing  
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23 101 libraries were prepared for 125+125bp paired-end sequencing using the Illumina HiSeq  
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25 102 high output V4 chemistry.  
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29 103 Sequencing reads were first cleaned of adapter sequences and quality trimmed using  
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31 104 cutadapt v1.10 (Martin, 2011). We mapped the reads to the JEL423 reference genome  
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33 105 (GenBank assembly accession: GCA\_000149865.1) using Burrows-Wheeler Aligner  
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35 106 (BWA) v0.7.8 (Li & Durbin, 2009). We processed the resulting sequence alignment/map  
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37 107 (SAM) files using SAMtools v1.3.1 with the 'fixmate' and 'sort' programs to ready the files  
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39 108 for variant discovery. We performed the variant discovery in a two-step process using  
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41 109 freebayes version dbb6160 (Garrison & Marth, 2012). In the first step, sorted BAM files for  
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43 110 each of the isolates in the phylogeny were independently called to find variant positions.  
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45 111 The set of all variant position identified across all samples were merged into a single  
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47 112 variant call format (VCF) file. In the second step, each of the samples were re-called using  
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49 113 the positions in the merged VCF to produce a squared-off call set (a genotype call is made  
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51 114 at every locus for each isolate, including for missing data). All squared-off sample VCF  
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53 115 files were processed by vcfliib (Garrison, 2016) to break complex variants into allelic  
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55 116 primitives and vt (Tan, Abecasis, & Kang, 2015) to normalize short insertion and deletion  
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3 117 sequences (indels). We quality filtered the VCFs with bcftools version 1.3.1 (Li et al., 2009)  
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5 118 to accept only variants with sufficient supporting evidence: sites covered by 4 reads or less  
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7 119 were set to missing; potentially polymorphic sites were additionally filtered using the  
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9 120 settings recommended in the bcbio.variation.recall squaring-off pipeline (Chapman, 2016),  
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11 121 with sites not passing these filters set to homozygous reference, i.e. though there were  
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13 122 sequencing reads covering that site, there was not enough evidence to call a variant at  
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15 123 that position. We merged the processed VCF files into a single multi-sample VCF and  
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17 124 extracted a fasta file of the SNP variant calls. We conducted the phylogenetic analyses  
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19 125 using RAxML v8.2.9 with the GTRCAT model with 500 bootstrap runs. The raw read  
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21 126 sequences of AVS2, AVS4 and AVS7 are available at the NCBI Sequence Read Archive:  
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23 127 <https://www.ncbi.nlm.nih.gov/sra> under accession numbers XXX, XXX and XXX,  
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25 128 respectively.  
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### 33 130 3. RESULTS AND DISCUSSION

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36 131 We obtained three isolates across central and southern Chile (Figure 1): AVS2 from *B.*  
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38 132 *antartandica* from Melimoyu, AVS4 from *X. laevis* from Hualañé, and AVS7 from *C. gayi*  
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40 133 from Valdivia. Phylogenomic analyses showed that all Chilean isolates group within  
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42 134 *BdGPL* in a single, highly supported clade (100% bootstrap support; Figure 1b). The  
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44 135 Chilean *Bd* isolates were fixed at 99.4% of the segregating sites in *BdGPL* after filtering for  
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46 136 missing positions. There were only 2,257 variable sites exclusive to the Chilean *Bd*  
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48 137 isolates. Although we compared the genomes of AVS2, AVS4 and AVS7 with an extensive  
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50 138 global panel of *Bd*, they were shown to be highly divergent from the only known regional  
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52 139 endemic lineage (*BdBrazil*), but clustered with UKTvB, a *Bd* isolate collected from a  
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54 140 smooth newt (*Lissotriton vulgaris*) in 2009 in Kent, United Kingdom (bootstrap support  
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3 141 100%, Figure 1b). Inferring phylogenetic relationships within the *Bd*GPL lineage is difficult  
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5 142 due to potential SNP homoplasmy caused by independent loss of heterozygosity (LOH)  
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7 143 events (Rosenblum et al., 2013). We tested the topology produced using SNPs from  
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9 144 across the whole autosomal genome against a 1.66Mb region on supercontig\_1.2, which  
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11 145 displays a characteristic large-scale loss of heterozygosity (LOH) event, present in all  
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13 146 known sequenced isolates of *Bd*GPL (Rosenblum et al., 2013). The topology derived from  
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15 147 SNPs in this region (consisting of 19,913 segregating sites), recapitulated the deeply  
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17 148 diverged lineages also found in the whole genome tree, with a very flat comb-like structure  
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19 149 in the *Bd*GPL lineage due to a lack of SNPs in this region (Figure 2). All 3 Chilean isolates  
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21 150 in this study formed a clade (albeit with very little bootstrap support) with isolates from  
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23 151 Valencia (VA02), Switzerland (AoCH15), the UK (UKTvB), France (0711.1) and Canada  
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25 152 (JEL261), suggesting the clustering with the UK isolate UKTvB in the whole genome  
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27 153 analysis may not be artefact. Finally, we used the Weir and Cockerham's estimator in  
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29 154 *vcftools* to perform a sliding-window comparison of  $F_{ST}$  of the Chilean *Bd* isolates against  
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31 155 all the other *Bd*GPL isolates. In this analysis, we used a 10Kb window, with a 5kb step-  
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33 156 size along the genome to identify if any stretches of the Chilean *Bd* population were highly  
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35 157 differentiated compared to the rest of the *Bd*GPL clade (Figure 3). We identified several  
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37 158 stretches of genome where the  $F_{ST}$  estimator was more than two standard deviations  
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39 159 greater than the mean of all  $F_{ST}$  values, indicating differentiation due to positive selection  
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41 160 or reduced rates of recombination may be important at these loci in Chile. Further work is  
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43 161 required to determine if any classes of gene are functionally enriched in these high  
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45 162 divergence regions.  
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52 163 The low number of segregating sites exclusive to the Chilean *Bd* isolates, compared  
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54 164 to the total number of sites where the *Bd*GPL isolates are polymorphic, suggest a single  
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56 165 and recent instance of long-distance dispersal of *Bd* into Chile, possibly through the  
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3 166 international movement of amphibians, aquatic animals, plants or fomites (Farrer et al.,  
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5 167 2011; Schloegel et al., 2012). Molecular characterization of further isolates from Chile and  
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7 168 neighbouring countries, along with the calibration of a genome-wide molecular clock, is  
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9 169 required to confirm this hypothesis. The existence of a unique, recently introduced lineage  
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11 170 of *Bd* in Chile would be in contrast with the known history of this pathogen in the near  
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13 171 Brazil, where both *Bd*GPL and *Bd*Brazil co-exist, with evidence of multiple hybridization  
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15 172 events between them (Jenkinson et al., 2016). This highlights the importance of  
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17 173 biosecurity measures to prevent the introduction and establishment of further *Bd* lineages  
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19 174 into Chile, as this pathogen has the capability to increase its genomic diversity through the  
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21 175 exchange of haplotypes among lineages.  
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26 176 *Xenopus laevis* is considered as an effective vector capable of disseminating *Bd* into  
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28 177 new regions (Soto-Azat et al., 2016) and thus represents a possible route of introduction of  
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30 178 *Bd*GPL into Chile. The earliest evidence of *Bd* in Chile is from a four-eyed frog  
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32 179 (*Pleurodema thaul*) individual collected from Concepción in 1970 (Soto-Azat et al. 2013).  
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34 180 Although, underestimation of *Bd* is a known problem when examining fixed archived  
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36 181 specimens (further discussed in Soto-Azat et al. 2009), hundreds of PCR analyses from  
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38 182 archived amphibians have failed to detect *Bd* infections prior to that date in Chile (Soto-  
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40 183 Azat et al., 2013). The exact time of introduction of *X. laevis* into Chile is unknown, but it is  
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42 184 thought to have occurred sometime in the 1970s (Jaksic, 1998). Additional means of  
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44 185 *Bd*GPL introduction, possibly assisted via tourism, research or salmon farming (Liew et al.,  
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46 186 2017), should also be considered.  
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51 187 The Chilean *Bd* isolates grouped together in our phylogenomic whole-genome  
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53 188 analysis with a genotype isolated in 2009 from the United Kingdom (UKTvB). A similar  
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55 189 phylogenetic relationship was observed when restricting the analysis to a subset of the  
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57 190 genome spanning an LOH event shared by all *Bd*GPL isolates, with isolates from other  
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3 191 European countries and a Canadian isolate also grouping with the Chilean isolates. While  
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5 192 there has been no report of mortality caused by the UKTvB, VA02, JEL261 or AoCH15\_B  
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7 193 isolates, the 0711.1 isolate from the French Pyrenees was associated with amphibian  
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9 194 mortalities. In general, *Bd*GPL has been associated with catastrophic mass mortalities and  
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11 195 population extirpations in multiple continents (Vredenburg, Knapp, Tunstall, & Briggs,  
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13 196 2010; Farrer et al., 2011; Rosenblum et al., 2013). In Chile, during the last four decades,  
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15 197 an extensive extirpation of local populations of the Darwin's frogs (*Rhinoderma darwinii*  
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17 198 and *R. rufum*) has occurred across central and southern Chile. Retrospective, cross-  
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19 199 sectional and capture-recapture data suggest that, among other factors, chytridiomycosis  
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21 200 has contributed to the occurrence of these extirpation events (Soto-Azat et al., 2013;  
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23 201 Valenzuela-Sánchez et al. 2017). The existence of the hypervirulent *Bd*GPL in Chile  
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25 202 provides additional support to this hypothesis. The susceptibility of *Rhinoderma* spp. to  
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27 203 different *Bd* genotypes, however, requires investigation (e.g. controlled infection  
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29 204 experiments), because the host-parasite interaction is a complex process that is affected  
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31 205 not only by the infectivity and virulence of the parasite but also by host-specific responses  
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33 206 and environmental factors (Woodhams et al., 2011).  
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39 207 This is the first description of Chilean genotypes of *Bd* and our study gives the first  
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41 208 insights into the origin and introduction history of this amphibian pathogen into the country.  
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43 209 Our results extend the previously known distribution of *Bd*GPL and provides additional  
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45 210 support to the role of amphibian translocations, and perhaps other international  
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47 211 movements of aquatic animals, plants or fomites, in the global spread of this panzootic  
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49 212 lineage.  
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## 222 **Competing interests**

223 The authors declare that they have no competing interests.

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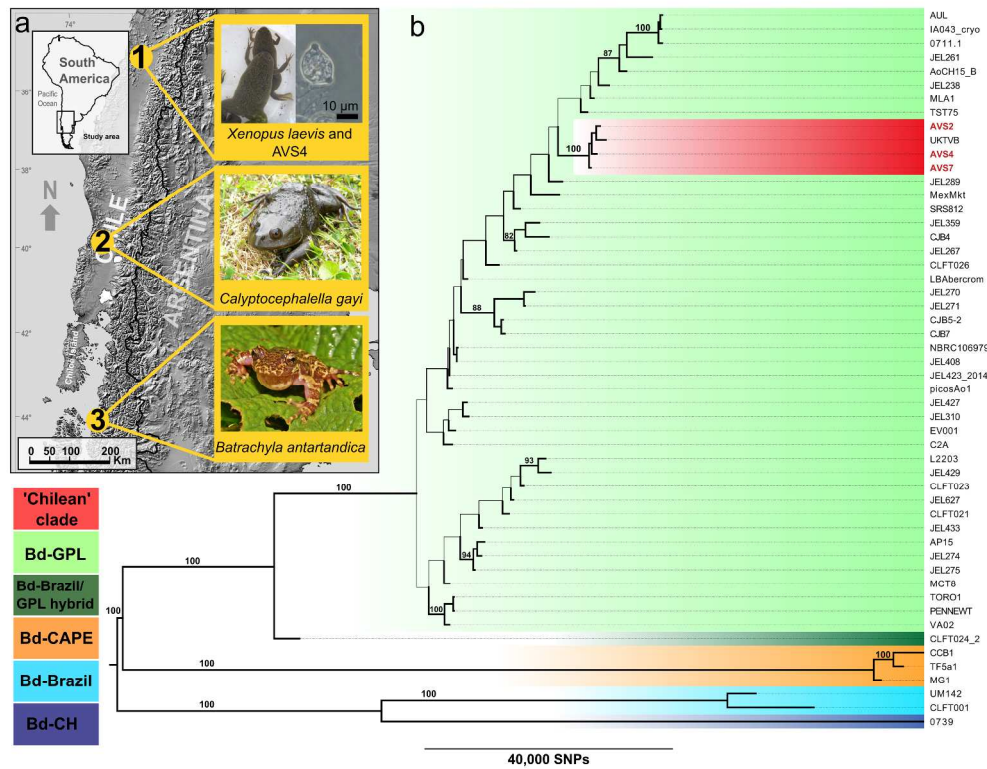


Figure 1. (a) Sites of collection, amphibian species captured and *Batrachochytrium dendrobatidis* (*Bd*) [AVS4] isolate obtained from Chile. (b) Global phylogeny of 52 isolates of *Bd* based on 363,497 segregating sites. The clade containing the Chilean *Bd* isolates is highlighted in red, and the Chilean 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.

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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.

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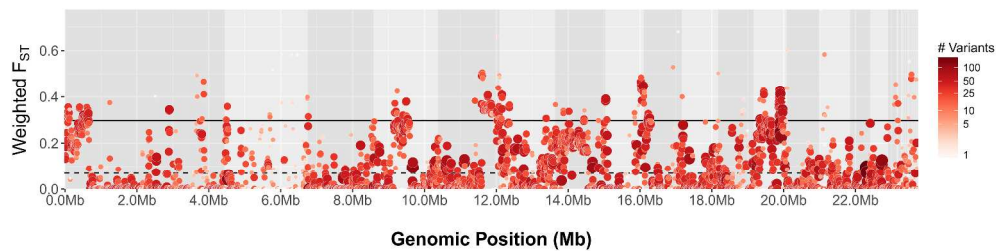


Figure 3. Sliding window analysis of population differentiation of Chilean *Bd* isolates against other *Bd*GPL isolates using Weir and Cockerham's  $F_{ST}$  estimator. Each point represents a 10Kb genomic window, with a 5kb step-size. The dashed black line represents the mean  $F_{ST}$  (0.0679). The 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.

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