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Panzootic chytrid fungus exploits diverse amphibian host environments through plastic infection strategies

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Abstract

While some pathogens are limited to single species, others can colonize many hosts, likely contributing to the emergence of novel disease outbreaks. Despite this biodiversity threat, traits associated with host niche expansions are not well understood in multihost pathogens. Here, we aimed to uncover functional machinery driving multihost invasion by focusing on *Batrachochytrium dendrobatidis* (*Bd*), a pathogen that infects the skin of hundreds of amphibians worldwide. We performed a meta-analysis of *Bd* gene expression using data from published infection experiments and newly generated profiles. We analysed *Bd* transcriptomic landscapes across the skin of 14 host species, reconstructed *Bd* isolates phylogenetic relationships, and inferred the origin and evolutionary history of differentially expressed genes under a phylogenetic framework comprising other 12 zoosporic fungi. *Bd* displayed plastic infection strategies when challenged by hosts with different disease susceptibility. Our analyses identified sets of differentially expressed genes under host environments with similar infection outcome. We stressed nutritional immunity and gene silencing as important processes required to overcome challenging skin environments in less susceptible hosts. Overall, *Bd* genes expressed during amphibian skin exploitation have arisen mainly via gene duplications with great family expansions, increasing the gene copy events previously described for this fungal species. Finally, we provide a comprehensive gene data set that can be used to further examine eco-evolutionary hypotheses for this host-pathogen system. Our study supports the idea that host environments exert contrasting selective pressures, such that gene expression plasticity could be one of the evolutionary keys leading to the success of multihost pathogens.

KEYWORDS
Batrachochytrium dendrobatidis, molecular phenotypic plasticity, multihost pathogen evolution, pathogenic fungus, transcriptomic meta-analysis

1 | INTRODUCTION

Multihost pathogens represent an exceptional case of species evolution in heterogeneous environments (Woolhouse et al., 2001). These pathogens can colonize and proliferate in different species while evolving adaptations, plausibly, through the interplay between virulence and transmission (Acevedo et al., 2019; Anderson & May, 1982). The process of colonization of a specific host environment (i.e., the host with their own environmental conditions and associated symbionts) can drive pathogen diversification if host alternation does not constrain adaptation. When host alternation is prevalent and, hence, host environments fluctuate, multihost pathogens might display the same infection strategy, becoming what has been called "jack of all trades and master of none". At the same time, multihost pathogens

are resource specialists and can be masters of some or, even, all their hosts (Hellgren et al., 2009; Remold, 2012). To exploit their specific resources in different host environments, multihost pathogens can develop either a compromised or a plastic infection strategy to optimize their fitness (Mason, 2016). Characterizing pathogen evolutionary dynamics inducing different infection strategies is essential to understand host shifting success and to predict the pathogen's ability to colonize other species (De Fine Licht, 2018). Knowledge about ecological and evolutionary mechanisms that allow pathogens to infect multiple hosts can ultimately contribute to curbing emerging infectious diseases in wild populations (Woolhouse et al., 2005).

Outbreaks of multihost pathogens in amphibians, snakes, and bats demonstrate that the ability to exploit novel host environments can occur rapidly with dramatic consequences to naïve populations (Daszak et al., 2000). One of the best examples is amphibian chytridiomycosis, an emerging infectious disease caused by the batrachochytrid fungi *Batrachochytrium dendrobatidis* (hereafter *Bd*) (Longcore et al., 1999) and *B. salamandrivorans* (*Bsal*) (Martel et al., 2013). Of particular importance is the hypervirulent *Bd* Global Panzootic Lineage (*Bd*GPL) that emerged at the end of the 20th century causing extinctions and severe declines in hundreds of amphibian species worldwide (Farrer et al., 2011; Fisher et al., 2021; Fisher & Garner, 2020; O'Hanlon et al., 2018; Scheele et al., 2019). Batrachochytrids are osmotrophic fungi that exploit vertebrate substrates targeting keratinized areas in the amphibian skin (Van Rooij et al., 2015). Batrachochytrid infection in amphibians arose as a novel trait with the evolution of particular genetic features, including several types of proteases, chitin-binding proteins, and Crinkler effectors (Abramyan & Stajich, 2012; Farrer et al., 2017; Joneson et al., 2011; Sun et al., 2016, Sun et al., 2011), which are expressed during host exploitation (Ellison et al., 2017). Expansion of different gene families has occurred in multiple species of the kingdom Fungi in relation to ecological specialization, particularly for genes encoding enzymes to degrade host tissues in symbiotic fungi (Leducq, 2014). While developing in amphibian skin, *Bd* exhibits an archetypal chytrid lifecycle: flagellated waterborne zoospores encyst, penetrate the substrate through rhizoids, maturate to thalli, and asexually reproduce into zoosporangium (Longcore et al., 1999; Van Rooij et al., 2015). This process causes systemic effects that can alter amphibian skin function and structure with lethal outcomes for susceptible hosts (Voyles et al., 2009). In less susceptible species, amphibian hosts rely on multiple strategies to avoid or control disease progression maintaining skin integrity, including skin microbiome interactions, immune system activation, and behavioural traits to reduce exposure (Brannelly et al., 2021). Not surprisingly, *Bd* infection outcome is highly variable across amphibian species and both biotic and abiotic context-dependent (Zamudio et al., 2020).

To understand the evolution of *Bd* pathogenicity, several studies have explored its population dynamics (Morgan et al., 2007; Voyles et al., 2018), genetic diversity (Abramyan & Stajich, 2012; Farrer et al., 2017; Joneson et al., 2011; O'Hanlon et al., 2018; Rosenblum et al., 2013; Sun et al., 2011, 2016), and gene expression of both *Bd* and amphibian hosts (Ellison et al., 2014, 2017, 2020; Eskew et al., 2018; Farrer et al., 2017; Grogan et al., 2018; McDonald, Ellison, et al., 2020; McDonald, Longo, et al., 2020; Rosenblum et al., 2012; Savage et al., 2020; Silva et al., 2019), among others. The scope of these studies has been limited to genomic comparisons with few species of the phylum Chytridiomycota (Farrer et al., 2017; Joneson et al., 2011), whereas *Bd* functional machinery has been examined to understand host exploitation only in two amphibian species with similar infection outcomes (Ellison et al., 2017) or in growth media with pulverized frog skin cells (Rosenblum et al., 2012). Therefore, it remains unclear whether *Bd* can exhibit variation in gene expression (i.e., diverse molecular phenotypes) across amphibian skins, and hence transcriptional plasticity to overcome different host environments. Host species represent different environments, each with a specific skin microbiome and defence strategy (Brannelly et al., 2021), which can exert contrasting selective pressures to *Bd*. In other words, each amphibian host could be a unique ecosystem for *Bd* with its own environmental conditions due to the amphibian life history and its specific symbionts interacting with this pathogen. Accordingly, we expected that *Bd* has encountered new ecological opportunities through the exploration, colonization, and invasion of different amphibian skins after reaching naïve localities due to intercontinental trade (Farrer et al., 2011; Fisher & Garner, 2020). Under these ecological and evolutionary scenarios (i.e., the skin of naïve amphibian species), *Bd* could display a conserved infection strategy showing resilience during host alternation in inadequate/suboptimal host environments (less susceptible hosts where *Bd* is not able to degrade the amphibian skin), or exhibit trait variation to potentially enhance its fitness across different hosts (i.e., molecular phenotypic plasticity). Due to the extreme success of *Bd* as it can infect hundreds of amphibian species, we hypothesized that *Bd* can mount different/plastic infection strategies depending on the host environment through variation of its functional machinery (i.e., gene expression; see Figure 1a for a graphical representation of our hypothesis).

Here, we leverage publicly available transcriptomic datasets (Ellison et al., 2014, 2017, 2020; Eskew et al., 2018; Farrer et al., 2017; Grogan et al., 2018; McDonald, Ellison, et al., 2020; McDonald, Longo, et al., 2020; Savage et al., 2020; Silva et al., 2019) and new data from experimental infection challenges (Friday et al., 2020; Longo & Zamudio, 2017) to investigate gene expression of *Bd* across 14 different hosts species in comparison to its expression in culture samples (Ellison et al., 2017; Farrer et al., 2017; McDonald, Ellison, et al., 2020; Silva et al., 2019). We explored whether *Bd* expresses different genetic machineries to infect hosts with different infection outcomes, in which susceptible hosts could provide optimal environments whereas less susceptible hosts (i.e., partially susceptible and non-susceptible hosts) plausibly lend suboptimal environments for *Bd* proliferation. Our analyses identified sets of differentially expressed genes under different host environments with similar infection outcome (see Figure 1b for decision tree behind our classification). To trace if these differentially expressed genes represent evolutionary novelties, we inferred their origin and evolutionary history within a comparative phylogenetic framework comprising other 12 zoosporic fungi.

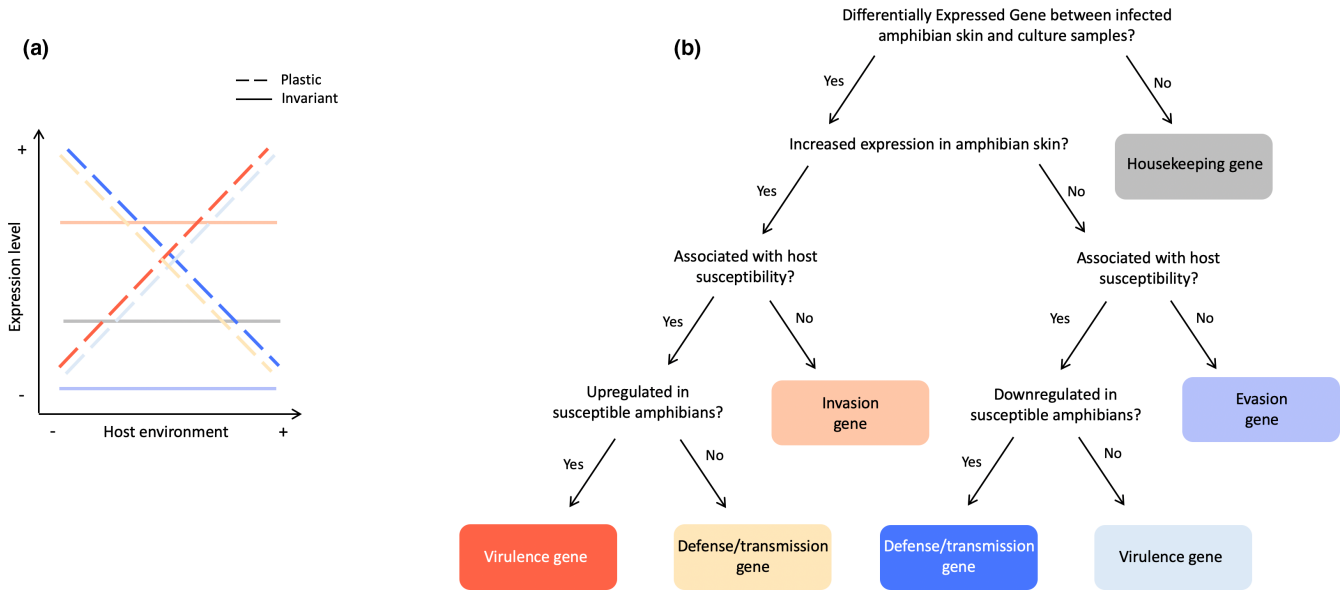

FIGURE 1 Theoretical gene expression changes driven by host environments. (a) Schematic illustration of hypothetical gene groups and their expression patterns across different hosts species (form optimal to suboptimal hosts). (b) Decision tree used to identify genes expressed during amphibian host exploitation.

2 | MATERIALS AND METHODS

2.1 | Infected amphibian skin and in culture transcriptome data

We downloaded RNA-Seq data from *Bd* in culture studies (Ellison et al., 2017; Farrer et al., 2017; McDonald, Ellison, et al., 2020; Silva et al., 2019) and amphibian infection trials (Ellison et al., 2014, 2017, 2020; Eskew et al., 2018; Farrer et al., 2017; Friday et al., 2020; Grogan et al., 2018; Longo & Zamudio, 2017; McDonald, Longo, et al., 2020; Savage et al., 2020) and generated new infection transcriptome data for two amphibian species (see Table S1 for NCBI Sequence Read Archive, SRA, accession codes and further information about transcriptome files). Data selection was made based on sequencing strategy (Illumina sequencing technology), maximizing number of host species. Likewise, new transcriptomes from 11 skin samples of experimentally infected *Eleutherodactylus coqui* (Longo & Zamudio, 2017) and *Desmognathus auriculatus* (Friday et al., 2020) individuals were sequenced on an Illumina NovaSeq 6000 S4 2×150 flow cell after RNA extraction and cDNA library preparation using, respectively, Qiagen RNeasy Plus Mini Kit and NEBNext Ultra II Directional RNA Library Prep Kit following the manufacturers' protocols. We measured the quantity and quality of the RNA and cDNA libraries using Qubit and Bioanalyser (library RIN: RNA integrity number values >7). In total, we collated 137 transcriptomes encompassing samples from 14 different species. The researchers of the different studies determined amphibian host susceptibility from both previous literature and after performing infection experimental trials through the evaluation of survival and disease outcomes in three susceptibility categories: susceptible, partially susceptible, and non-susceptible hosts (Table S2). Species that had severe population declines due to *Bd* are considered susceptible; partially susceptible species vary their response to *Bd* in relation to, for example, environmental factors, life stages; and non-susceptible species are not showing evidence of the disease, even being naturally infected (Table S2). All the animals used in the different studies were experimentally infected with an isolate belonging to *Bd*GPL, and most individuals were captive-bred or *Bd*-naïve to the isolate used in the experimental trials (information about *Bd* isolates can be found in Table S1). Across all the studies, infected skin tissues were harvested after necessary time to complete *Bd* life cycle. In fact, *Bd* completes it life cycle in around 5 days (Longcore et al., 1999; Van Rooij et al., 2015), and in most of the experiments, skin tissues were harvested after 2 weeks of exposure.

2.2 | *Bd* gene expression pattern analyses

We applied the dual RNA-Seq approach (Westermann et al., 2012) to recover *Bd* expression profiles by in silico separating sequencing reads that mapped against the *Bd* JAM81 representative genome (GCF_000203795.1). From the newly generated transcriptomes, we filtered sequencing adapters and trimmed the 10 left-bases of the reads using Trim Galore 0.6.5 (https://github.com/FelixKrueger/TrimGalore) and Prinseq 0.20.4 (Cantu et al., 2019), respectively. The read quality for all samples was checked using FastQC 0.11.5 (https://github.com/s-andrews/FastQC) after converting publicly available transcriptomes to fastq files using SRA Toolkit fastq-dump (https://github.com/ncbi/sra-tools). We used gffread 0.11.8 (https://github.com/gpertea/gffread) to obtain the *Bd* genome gtf file and Star 2.7.3a (Dobin et al., 2013) to count number reads per gene while aligning each file against the *Bd* reference

genome (File S1 for raw count data). We considered expressed genes as the coding sequences (CDS) of *Bd* genome with reads aligned to those genetic regions. We handled zero values summing one to all genes across samples in the matrix count. To normalize expression across samples and control technical variation, we applied the median of ratios method of the R package DESeq2 (Love et al., 2014) while estimating size factors using the reference genes: glyceraldehyde-3-phosphate dehydrogenase (GAPDH), beta tubulin (TUB), and anthranilate phosphoribosyl-transferase (AnPRT) (Verbrugghe et al., 2019). We explored sample similarity using a principal component analysis (PCA) and visualized biological and technical sources of variation among samples by plotting the first five PCs. We tested the repeatability of the general *Bd* gene expression pattern across species using sample PC1 loadings with mixed effect models implemented in the R package rptR (Stoffel et al., 2017). To analyse if *Bd* expression pattern was influenced by amphibian evolutionary history, we retrieved 10,000 phylogenetic trees of the 14 host species from VertLife (Jetz & Pyron, 2018), built a strict consensus tree, and quantified phylogenetic signal by computing both Blomberg's κ and Pagel's λ for the species mean PC1 loadings using the R package phytools (Revell, 2012). We performed an ANOVA with Tukey's honestly significant difference post hoc test (Tukey's HSD post hoc) to explore correlations between sample PC1 loadings and host susceptibility category.

2.3 | Differential gene expression analyses across a gradient of host susceptibility

To determine whether mean expression per gene of the different samples in each of the three host susceptibility categories was different from the mean *Bd* expression in culture samples, we fitted the count data to negative binomial models and performed Wald tests using the R package DESeq2 (Love et al., 2014). We visualized common and unique differential expressed genes (adjust *p*-value <=.05 and one or more than one positive unit of logarithmic fold expression change) for each category using Euler diagrams with the R package eulerr (Larson et al., 2018). Based on our decision tree (Figure 1b and Table S5), we identified housekeeping genes as those without expression changes between amphibian skin and culture samples; invasion and evasion genes as those up- and downregulated, respectively in all amphibian skins; virulence genes as those upregulated in susceptible host and/or downregulated in less susceptible hosts; and defence/transmission genes as those upregulated in less susceptible hosts and/or downregulated in susceptible hosts. We annotated *Bd* genes against Uniprot (The UniProt Consortium, 2017) and Pfam (Finn et al., 2014) databases using BLAST 2.9.0 (Altschul et al., 1990) and Hmmer 3.2.1 (Johnson et al., 2010), respectively, and recovered GO and protein class information using retrieve/ID mapping of the Uniprot. We also predicted signal peptides that control protein secretion for *Bd* genes using SignalP 6.0 (Teufel et al., 2022). We computed PANTHER overrepresentation test using Uniprot annotations from all *Bd* genes versus the differentially expressed genes subsets (Mi et al., 2009). Additionally, we described transcriptomic signatures related to *Bd* sporangia and zoospores (upregulated genes in one or the other life stage) by repeating previously published differential gene expression analyses (Silva et al., 2019). Specifically, we compared sporangia samples (SRR10389434, SRR10389435, SRR10389436) and zoospore samples (SRR10389437, SRR10389438, SRR10389439). To investigate the association between life-stage related expressed genes and host susceptibility category, we compared the number of differentially expressed genes per susceptibility category that were annotated as sporangia genes and zoospore genes using Pearson's chi-square test and calculated Cramér's V using the R package rcompanion (Mangiafico, 2020).

2.4 | Molecular evolution analyses of chytrid isolates and differentially expressed genes

For each in vivo transcriptome, we identified genetic variants and reconstructed the phylogenetic relationships among the *Bd* isolates that were used to infect the different amphibian hosts. We performed variant detection using GATK 4.1.9.0 following the best practices for transcriptomic data (McKenna et al., 2010). We detected a total of 44,204 variants with high individual missing data percentage across samples. We excluded a total of 1462 variants that were presented in less than four samples and 3386 variants that were multinucleotide polymorphisms (MNPs). After converting the variant calling format (VCF) file to PHYLIP format using vcf2phylip (Ortiz, 2019), we inferred phylogenetic relationship of the *Bd* isolates (best tree and bootstrap support from 1000 bootstrap trees) using maximum likelihood with RAxML-NG 1.0.2 (Kozlov et al., 2019). As in the case of the amphibian host tree, we quantified phylogenetic signal of the *Bd* isolate tree by computing both Blomberg's κ and Pagel's λ for the samples PC1 loadings using the R package phytools (Revell, 2012). To infer the molecular evolution of the *Bd* genes on amphibian hosts, we analysed the CDS in a phylogenetic framework of 13 early-diverging zoosporic fungi species (see Table S3). To ensure the comparison across fungal genomes, we calculated completeness percentage using BUSCO 5.3.0 (Simão et al., 2015). After CDS translation of the genes of each genome using gffread 0.11.8 (https://github.com/gpertea/gffread), we inferred gene orthologues (derived from speciation events) and paralogues (derived from gene duplication events) using the aminoacidic sequences of the 13 studied fungi using OrthoFinder 2.3.11 (Emms & Kelly, 2019), which allowed the identification of vertically derived genes, gene duplications and gene family expansion and contraction across species (number of genes in terminal branches greater than in internal ones and vice versa, respectively), as well as putative xenologues or unassigned genes (potentially derived from horizontal transfer or lineage fusion). For each gene we also calculated the content of GC and compared codon usage tables for the unassigned genes and genes with fungal homologues using EMBOSS tools (Rice et al., 2000).

3 | RESULTS

3.1 | *Bd* expressed distinctive genetic machinery across host environments

We recovered 137 *Bd* gene expression profiles from transcriptome samples of the skin of experimentally infected amphibians (104 in vivo samples) and from in culture studies (33 in vitro samples). The maximum number of expressed genes that we detected through our analyses was 8236 (94.67% of the total genes reported in the *Bd* JAM81 representative genome, see Table S1 for expressed gene number per sample). After visualizing samples by the similarity of their gene expression profiles, we found that the first principal component (PC1) explained a high proportion of the total gene expression variance (52.07%, see Table S4 and Figure S1 for variance proportion information of the subsequent PCs) revealing several biological patterns (Figure 2) and no grouping related to technical characteristics, such as sequencing coverage (see Figure S2), *Bd* isolates (see Figure S3), or project (see Figure S4). For example, samples from the same host (*Atelopus zeteki*) shared similar gene expression profiles despite being part of two different studies and obtained by two different sequencing strategies (laser-capture microdissection and bulk RNA-Seq, respectively) (Ellison et al., 2014, 2017). Likewise, we found gene expression variation among different host species and culture samples generated under the same experimental conditions and molecular protocols during the same studies. We uncovered similar general expression patterns among *Bd* samples grown in vitro, which included two different *Bd* lineages (*Bd*GPL and *Bd*Brazil) (McDonald, Ellison, et al., 2020), as these points clustered at one end of the PC1 (Figure 2). In contrast, *Bd* gene expression in vivo showed greater variability, which was mainly explained by a quantitative gradient related to host susceptibility: expression profiles from susceptible host samples were closer to the profiles from *Bd* in vitro experiments, whereas profiles from less susceptible hosts were more distant to these in vitro samples (Figure 2). We found that PC1 loadings were repeatable across species, demonstrating that *Bd* expression profiles were at some level conserved (repeatability value $R = 0.61$, CI = [0.34, 0.77], likelihood-ratio-test $p = 8.68e\text{-}25$). We also identified a significant association between PC1 loadings and host susceptibility category (F value = 62.47, $df_{effect} = 3$ and $df_{error} = 133$, p-value <2e-16). Pairwise comparisons indicated that in susceptible hosts *Bd* displayed a different gene expression pattern from both partially susceptible (Figure 2b, Tukey's HSD post hoc: diff = 0.063, CI = [0.040, 0.085], p-value adj = 0) and non-susceptible hosts (Tukey's HSD post hoc: diff = 0.067, CI = [0.045, 0.089], p-value adj = 0) while in non-susceptible and partially susceptible hosts, *Bd* expressed similar general patterns (Tukey's HSD post hoc: diff = 0.004, CI = [−0.016, 0.024], p-value adj = .94). These differences were decoupled from the evolutionary history of the hosts (tested under Brownian Motion), as we did not find a

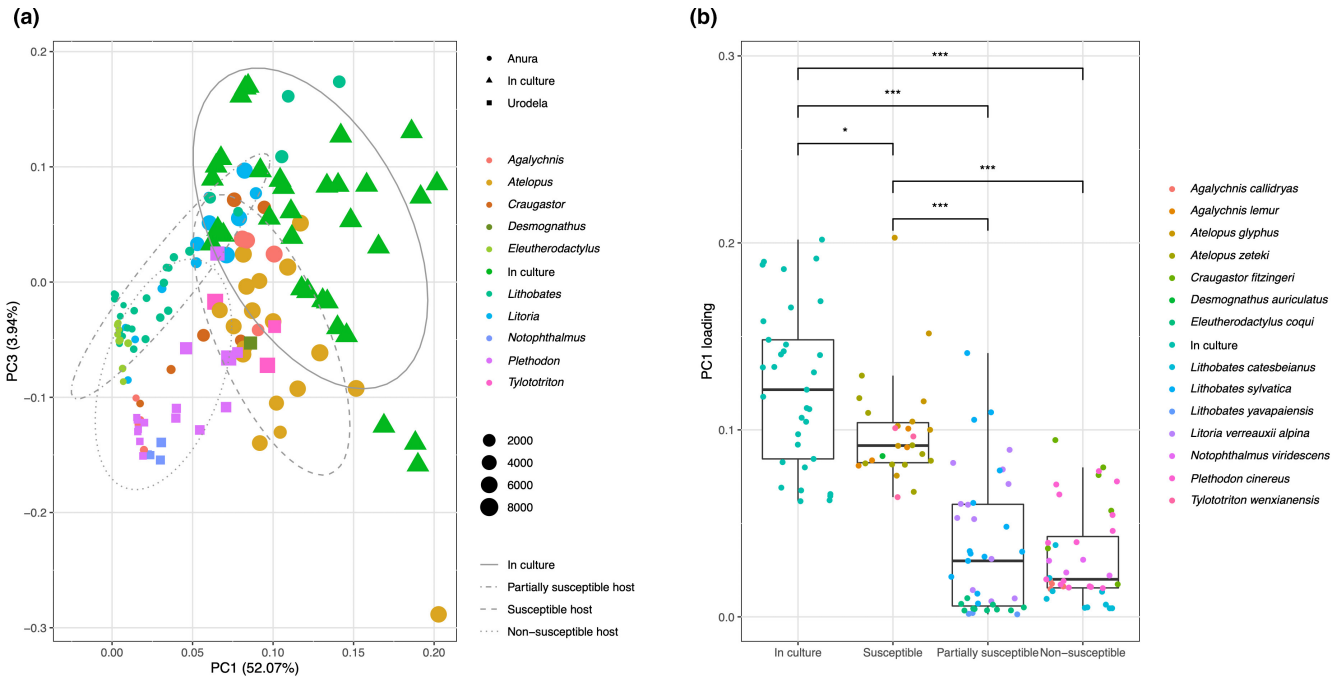

FIGURE 2 *Batrachochytrium dendrobatidis* (*Bd*) gene expression profiles across 14 amphibian hosts. (a) Principal component analysis (PCA) of *Bd* gene expression profiles for 137 samples of skin and cultures. Object shapes and colours differentiate the origin of the samples by amphibian order: Anura (●) and Urodela (■); and in culture (▲) while object size illustrates number of expressed genes. Samples are also grouped by ellipses related to host susceptibility. (b) PC1 loadings per amphibian species and host susceptibility category. The boxplot displays pairwise comparisons of *Bd* gene expression pattern per susceptibility category. Each dot represents one samples and is colour coded by environment condition (in culture and host species). Asterisks denote significant comparisons of the Tukey's honestly significant difference post hoc test (alpha < 0.05).

phylogenetic signal using the mean PC1 loading across amphibian hosts (Blomberg's $\kappa = 0.358$, p-value = .37; Pagel's $\lambda = 6.61e\text{-}05$, p-value = 1; Figure S5). Likewise, we failed to detect a strong phylogenetic signal between *Bd* isolates used in the experiments and the sample PC1 loadings of these gene expression profiles (incongruent values between Blomberg's $\kappa = 5.07e\text{-}06$, p-value = .023 and Pagel's $\lambda = 0.99$, p-value = 1.47e-69; Figure S6), however the phylogenetic tree was not well supported (branch bootstrap values <70%).

3.2 | Characterization of the *Bd* machinery for amphibian skin invasion

The colonization of amphibian skin, as opposed to the growth in culture, involved expression changes for a large number of *Bd* genes (Figure 3). In non-susceptible hosts, *Bd* expression profiles showed the greatest percent of different expressed genes (83.68%), followed by partially susceptible hosts (81.20%) and susceptible hosts (51.02%). For 12.31% of the *Bd* genes (1071), we failed to detect expression change between amphibian skin and culture samples, classifying these genes as potential housekeeping genes. We considered as evasion genes those that decreased their expression across all amphibian hosts in comparison of culture samples. We defined the genes with increased expression in the amphibian skin across all hosts as invasion genes due to their potential involvement in host exploitation (see Figure 1b and Table S5 for decision making strategy for gene designation). This genetic set was composed by 3808 genes, which were upregulated in the amphibian hosts regardless of their susceptibility category (Figure 3b). In this common genetic core, we found a battery of genes encoding peptidases, carboxypeptidases, and metalloproteases with signal peptides and extracellular locations constituting *Bd*'s secretome along with lipases, pepsins, nucleases, metal ion binding proteins, and chitin deacetylases and synthases, among others (see File S1). Additional members of the invasion gene set included genes performing their functions in the host cell nucleus, such as Crinkler effector proteins, RxLR effector proteins, DNA primases, DNA-binding transcription factors, cyclins, and cyclin-dependent kinases. These proteins might have been translocated in exocyst complexes, which components were also upregulated in the amphibian skin samples. Several genes encoding proteins with cellular defence mechanisms were highly expressed too (e.g., Dicer-like proteins, telomerases, superoxide dismutase proteins, uridylyltransferases, arsenate reductases), as well as many voltage-gated channels, transmembrane transporters, and permeases. Among the invasion gene subset, we detected numerous genes related to sporulation process. Finally, in this genetic core, we highlighted several genes annotated as viral proteins.

3.3 | Sorting *Bd* genes by host environment

From the differentially expressed *Bd* genes in amphibian hosts, we identified genes with plastic expression modulated in association with particular hosts categories (see Figure 3 and Table S5 for a representation and summary of gene class definition; and File S1 for completed information per gene). We found 62 candidate virulence genes, of which 38 were highly expressed solely in the susceptible hosts (Figure 3b). In contrast, we identified 3125 putative defence/transmission genes upregulated in partially and non-susceptible hosts or downregulated in susceptible hosts (see Table S5 for category assignation). Virulence genes were more frequently annotated as genes with a transcriptomic signature of zoospores, but with a weak association ($\chi^2 = 17.492$, p-value = 2.885e-05, Cramér's V Phi = 0.07671). In addition to the enhanced expression of extra peptidases and transmembrane transporters, to exploit susceptible hosts, *Bd* expressed genes related to cell adhesion and cell projection, predominantly through motor proteins such as dyneins and kinesins. Conversely, in the defence/transmission genetic set, we found additional genes involved in the ubiquitination and redox systems with putative defence/transmission functions as well as protein involved in response to starvation and osmotic stress, such as heat shock proteins. As consequence, *Bd* mechanisms to fight host defences could involve cell cycle arrest and even autophagy and apoptosis mechanisms. We uncovered genes related to these processes in the defence/transmission genetic subset. Our results also highlighted a large number of genes upregulated in less susceptible hosts related to gene silencing, mainly endoribonucleases and interference RNAs (RNAi), including more genes coding Dicer-like proteins and small nucleolar RNA (snoRNA), which could be involved in a bidirectional trans-kingdom communication system. We also detected proteins involved in toxin and secondary metabolites production, including members of the Velvet complex.

3.4 | Origin and evolution of differentially expressed *Bd* genes

We identified a total of 5830 candidate gene families (orthogroups) with one or more *Bd* genes across our early-diverging fungi phylogeny. A total of 133 of these orthogroups were classified as *Bd* species-specific families. This number represents 36.2% of the total identified gene families across all the studied fungi (Figure S7) and included 8130 of the 8700 *Bd* genes. Our results showed a total of 1665 terminal gene duplications in *Bd* terminal branch. We found that almost all of those duplications (95.56%) were grouped in orthogroups with invasion gene members (genes that were upregulated in the explored amphibian skins, Figure 4). We defined orthogroups with terminal duplications in *Bd* branch as gene families under expansion and considered these events as evolutionary innovations (see File S1). Likewise, we identified 570 unassigned or orphan genes acquired during the *Bd* evolutionary history, which, for the majority, increased their expression during the exploitation of amphibian skin (395 invasion and 118 defence/transmission genes). These candidate genetic novelties might have risen as de novo genes, horizontal gene transfers (HGT), and/or duplications of existing genes followed by neofunctionalization. Alternatively, *Bd* species-specific gene families

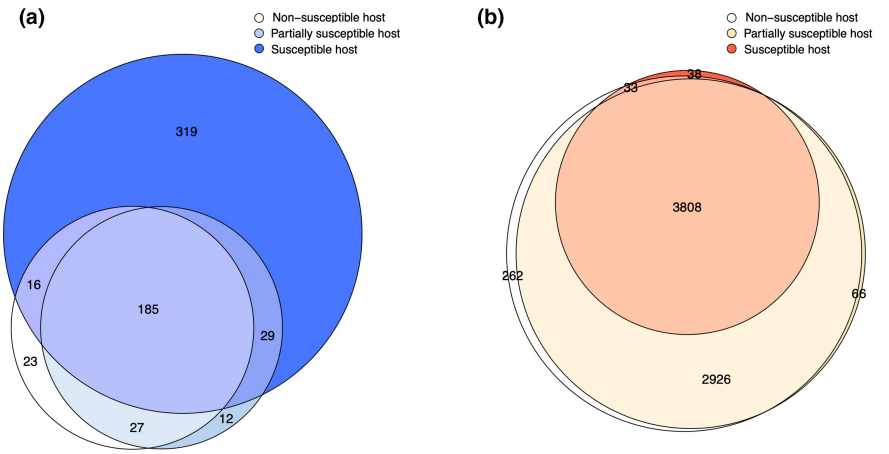

FIGURE 3 Differentially expressed *Batrachochytrium dendrobatidis* (*Bd*) genes. Euler diagram showing the numbers of upregulated (a) and downregulated (b) genes in amphibian skin across host categories.

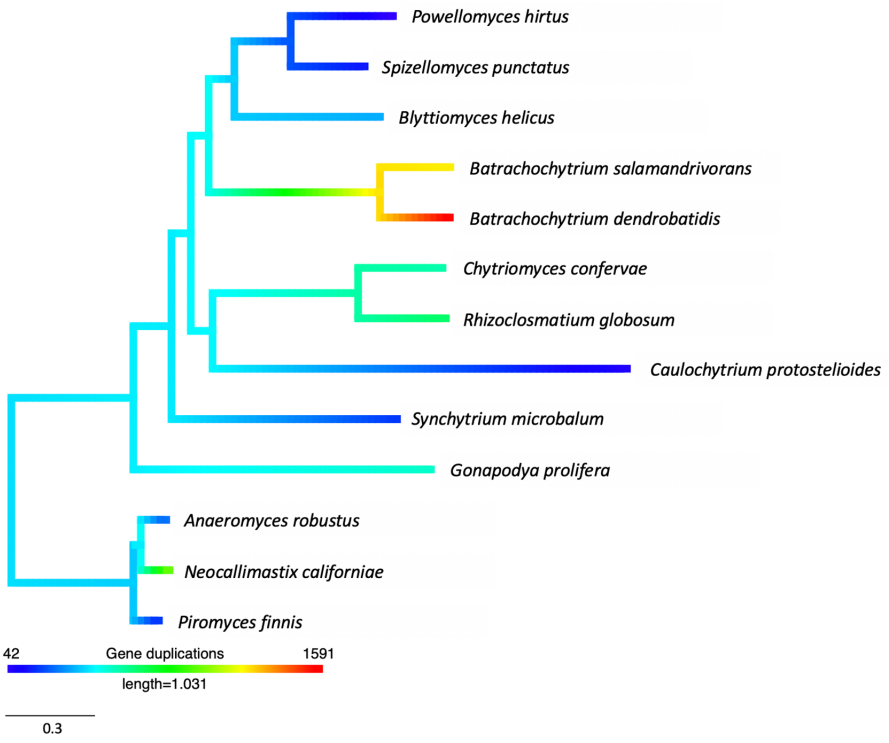

FIGURE 4 Molecular evolution of *Batrachochytrium dendrobatidis* (*Bd*) genes. Phylogenetic tree of 13 zoosporic fungi showing duplications for the gene families containing the homologues of the *Bd* upregulated genes in amphibian skin (invasion genes).

and orphan genes might be the result of gene losses across this phylogeny and orthologues might exist in unsampled species (or in these same species with incomplete genomic records, see Table S3 for genome completeness percentage). Among these candidate *Bd* specific gene families, we found several families encoding, for instance, effector proteins, serine/threonine-protein kinases, beta-secretases, arginine exporters, beta-lactamases, which *Bd* might have acquired to respond to interactions with diverse host environments more efficiently. Additionally, we detected differences in codon usage between *Bd* genes with fungal homologues and *Bd*-specific ones (sum difference = 1.558, see Table S6 for detail in codon usage).

4 | DISCUSSION

Chytridiomycosis threatens amphibian populations worldwide, however, disease risk varies greatly depending on a myriad of factors, including *Bd* lineage (O'Hanlon et al., 2018; Scheele et al., 2019; Van Rooij et al., 2015; Zamudio et al., 2020). While several studies have characterized the infection response of different amphibian species (Ellison et al., 2014, 2017, 2020; Eskew et al., 2018; Farrer et al., 2017; Grogan et al., 2018; McDonald, Longo, et al., 2020; Savage et al., 2020), our work contributes to identify *Bd*'s functional machinery involved in the process of skin invasion and persistence across a wide amphibian host range. In this study, we integrated available transcriptome data to further explore pathogen functional genomic changes arising during host-pathogen interactions. We expanded our view of this widespread multihost pathogen by characterizing its transcriptome landscapes across a diverse group of amphibians, while inferring the origin and evolutionary history of its genes. These skin expression profiles allowed us to assess the genetic machinery that *Bd* uses to infect a wide range of different host species before adaptive or coevolutionary processes since majority of animals were *Bd*-naïve. Our results not only uncovered a

common genetic machinery, but also highlighted the functional capacity of *Bd* to display molecular phenotypic plasticity in relation to host susceptibility, which is decoupled from host and *Bd* isolate phylogenetic relationships. Taken together, our findings indicate that gene expression plasticity could be one of the evolutionary keys to the emergence of this panzootic multihost pathogen predating a potential adaptation.

Consistent with previous studies (Ellison et al., 2017; Rosenblum et al., 2012), *Bd* genetic machinery in host environments significantly differed from culture. For several genes, however, we did not detect changes in expression and we proposed them as candidate housekeeping genes, expanding the number of potential references genes for expression studies (Verbrugghe et al., 2019). Culture media is often a tryptone-based broth that provides ad libitum nutrients for *Bd* to complete its lifecycle (Van Rooij et al., 2015). This favourable condition leads to the expression of a high number of genes involved in numerous different biological processes. Likewise, our results show that *Bd* can easily acquire nutrients from the skin of susceptible anurans and urodeles for growing, developing, and, accordingly, expressed a large number of genes. *Bd* expressed relatively more genes characterized with transcriptomic signatures of zoospore life stage in susceptible amphibians, plausibly indicating a more complex maturation of zoospores that could reach deeper skin layers. In these optimal environments, *Bd* could form complex vegetative structures, such as densely branched rhizoids and clusters of thalli, which, in turn, might contribute to disease progression and virulence (Fisher et al., 2021). In contrast, in less susceptible hosts, *Bd* could face what we consider challenging or hostile (suboptimal) environments. These suboptimal environments plausibly present nutritional restrictions with limitations in substrate degradation, resulting in a restrained developmental capacity. In this scenario, *Bd* expressed a battery of genes involved in cellular life cycle arrest, perhaps promoting a quick reproduction to protect itself from host defence strategies. This process might occur through a resistant sporangium that delays the release of new zoospores, which has been suggested as a mechanism that allows the persistence and transmission of this emerging pathogen (Morgan et al., 2007). Unlike the sister species, *Batrachochytrium salamandrivorans* (*Bsal*), which can form environmentally resistant nonmotile spores (Stegen et al., 2017), no resistant forms have been identified for *Bd* thus far. Variation in *Bd* life-history patterns has been demonstrated in culture under different thermal conditions (Van Rooij et al., 2015; Woodhams et al., 2008). Likewise, endobiotic (inside the cell) and epibiotic (upon the cell surface) life cycle strategies have been described in vitro observing the latest process in the explanted skin of a non-susceptible species (Van Rooij et al., 2015). Additional evidence supporting plasticity is shown by the capacity of a closely related chytrid fungus to reallocate resources by altering rhizoid morphogenesis across environments with different resource availabilities (Laundon et al., 2020).

Plasticity can be adaptive or non-adaptive with evolutionary implications in both cases. If the variation detected is adaptive, gene expression changes in response to host environments would enhance *Bd*'s ability to survive and reproduce in each host species. The repeatability of expression pattern per host environment showed by our results and previously envisioned (Ellison et al., 2017), could indicate that this molecular plasticity could promote potential adaptation (Byrne et al., 2022). In addition, we hypothesized that similar biological traits arising from shared ancestry (i.e., immune defences, behaviours, etc.) would lead to analogous environmental pressures. However, we found no phylogenetic signal in *Bd* gene expression pattern, indicating that each amphibian species represents a unique independent environment. Our analyses could point to a high adaptability of *Bd*GPL and demonstrated that differences in host susceptibility are associated with changes in the fungal gene expression. Differences in gene expression could show different infection phases, being *Bd* in less susceptible host in an early establishment phase unable to defeat the host. Among the non-susceptible species, we studied a host that has been proposed as *Bd* reservoir (Daszak et al., 2004). Accordingly, our findings could reflect gene expression reaction norms potentially through trade-offs between virulence and transmission promoted by different host environments.

This study expands previous knowledge about functional genomic elements for host exploitation and genomic innovations related to pathogenicity (Ellison et al., 2017; Farrer et al., 2017; Sun et al., 2011, 2016). Many of these genes encode extracellular proteinases part of the *Bd*'s secretome involved in biomass degradation (Lange et al., 2019), and were expressed across amphibians regardless of their susceptibility. When the nutritional substrate differed as tested in our in vitro versus in vivo comparisons, a switch in enzyme composition is expected, which was demonstrated by the increased expression of peptidases across all the studied amphibians compared to the tryptone-based broth. Like other osmotrophic fungi, after degrading the substrate, *Bd* needs to uptake nutrients, which requires membrane transports and transmembrane channels (Van Rooij et al., 2015). We identified increased expression in genes encoding transporters, which may be involved in scavenging nutrients and essential elements. Metals are one example of essential elements that provide virulence traits in many pathogenic fungi (e.g., cofactors of several peptidases) (Gerwien et al., 2018). They are also indispensable microelements for the amphibian hosts. Imbalance of metal homeostasis can be fatal for both pathogens and hosts, which compete in evolutionary arms race dynamics through pathogenesis and nutritional immunity, respectively (Hood & Skaar, 2012). On the host side, nutritional immunity can involve diametrically opposite strategies: metal sequestration or toxicity via augmentation, or via metal-catalysed generation of oxygen radicals. Pathogens must combat these strategies, for instance, by evolving efficient elimination, storage, and/or detoxification processes. The increased expression of genes encoding transporters, permeases, ion channels, and ubiquitination/deubiquitination enzymes provided evidence for the regulation of *Bd* homeostasis. In addition, competition for essential elements and changes in enzyme composition during the colonization of host environments could lead to infection progression and to the disruption of host homeostasis (Campbell et al., 2012). Finally, during its evolution, *Bd*GPL could have integrated fungal mycoviruses into its genome (Medina et al., 2019). These potential viral

genes were expressed during host exploitation and part of the invasion genetic arsenal. The majority of described mycovirus are dsRNA and despite their high diversity in early divergent fungi, these dsRNA viruses have not being detected in *Bd* or *Bsal* yet (Myers et al., 2020; Webb et al., 2022).

Genomic elements for host exploitation described hitherto were part of the common invasion machinery used by *Bd* to persist in a gradient of host environments. Our study provides an important contribution by characterizing *Bd* responses and inferring the molecular evolution of differentially expressed genes. In advantageous host environments, *Bd* increased the expression of not only additional peptidases supporting the capacity of *Bd* to better degrade these substrates, but also more adhesion, binding, and projection proteins. These elements could confer the ability to attach, for instance, to leukocytes, which are known to be involved in the evasion of immune defences (Fites et al., 2013; Van Rooij et al., 2015). Increased pathogen virulence usually involves several mechanisms to sabotage host defences (Grogan et al., 2020). In contrast, the interactions with less susceptible hosts revealed a complex trans-kingdom communication with both host and pathogen presumably interfering on each other through gene silencing (Knip et al., 2014). In these challenging scenarios, *Bd* could protect itself through dormancy of the zoospores or resistant sporangia leading to cell cycle arrest as a result of the molecular arms race between pathogen and host. During the infection of less susceptible hosts, *Bd* upregulated also regulatory proteins members of the Velvet complex. These genes constitute conserved regulatory proteins in the kingdom fungi involved in the production of secondary metabolites, which are bioactive molecules potentially harmful to the host (Bayram & Braus, 2012). In addition, we found that *Bd* genetic armoury could have different evolutionary origin, where the majority of the invasion genes were gained de novo, potentially through gene duplication and gene family expansions or horizontal gene transfer (HGT) events. *Bd*-specific genes showed bias in codon usage. As preferred codons are more frequently encountered in highly expressed genes, *Bd*-specific genes could belong to different transcription regimes (Zhou et al., 2016). Also, the genome of different species present codon bias (Plotkin & Kudla, 2011). Hence, the difference in codon use of some *Bd* invasion genes could support the different origin of some of the *Bd*-specific genes caused by genetic assimilation from other species genomes. These results are crucial to understand species interactions during the colonization process in the skin, where, for instance, host-associated microbes can alter infection outcome (Rebollar et al., 2020). *Bd* could have acquired some of the genetic machinery from these microbes to combat resident bacteria with antifungal traits. In consequence, our findings linked how host environments present novel challenges and exert contrasting selective pressures. These results support the contribution of gene expression plasticity and the evolutionary innovations in the development of a very successful multihost pathogen.

While some of the gene expression variation described in our study could be related to features of experimental design (e.g., *Bd* isolate passage number [Langhammer et al., 2013], infection load), sequencing methodology (e.g., laser-capture microdissection or bulk RNA-Seq, sequencing coverage), stochastic changes associated with tissue harvest (e.g., cell location), and transcriptomic dynamics, our results unravelled molecular plasticity arising via the interplay with different amphibian hosts. As a result, our findings provided an explanation of the host shifting ability of this pathogen. Additionally, our study yielded a prioritized list of genes to further investigate *Bd* responses in diverse and selective host environments and their evolution, importantly for the putative xenologues. To better describe *Bd* molecular plasticity and infection strategies, the expression profile of these genes should be further explored across a much wider selection of amphibian hosts, including Asian species that have been persisting with the two chytrid fungi for a longer period of time (O'Hanlon et al., 2018). Future directions should also include experiments to study *Bd* fitness in the different host environments, enabling empirical tests of virulence and transmission trade-offs. Likewise, we recommend functional analysis in parallel using dual RNA-Seq strategies (Westermann et al., 2012) to have better estimates of how species interact with *Bd*, which can have important implications for amphibian conservation.

Our approach to analyse functional genomic plasticity can be applied to better understand the diversification of other pathogens, especially multihost fungal pathogens affecting vertebrates such as the other described batrachochytrid species and fungal pathogens infecting bats and snakes (Blehert et al., 2009; Lorch et al., 2016). Ultimately, our study aimed to uncover functional machinery driving multihost invasion and host niche expansions. Identified changes in molecular phenotypes or in the expression of some candidate genes can be analysed in a comparative framework with diverse multihost systems to explore convergence evolution. Other multihost pathogens can be under analogous pressures when colonizing their own susceptible or less susceptible host environments with homologues genes changing similarly to the differentially expressed genes described here. By characterizing this pathogen's responses under different host environments, we illustrate how plasticity in molecular phenotypes is potentially responsible for the success of another jack of all trades and master of many (Hellgren et al., 2009; Remold, 2012).

AUTHOR CONTRIBUTIONS

María Torres-Sánchez and Ana V. Longo designed the research; Jennifer Villate, Sarah McGrath-Blaser, and Ana V. Longo performed RNA extractions; María Torres-Sánchez prepared cDNA libraries, analysed the data, and wrote the original draft; all authors reviewed and approved the final manuscript.

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CONFLICT OF INTEREST

The authors declare no competing interest.

OPEN RESEARCH BADGES

This article has earned an Open Data badge for making publicly available the digitally-shareable data necessary to reproduce the reported results. The data is also available at https://github.com/TorressSanchezM/Bd_transcriptomics.

DATA AVAILABILITY STATEMENT

The newly generated transcriptomic data have been deposited in the Sequence Read Archive, (BioProject accession number PRJNA739374). Previously published data were also used for this work and SRA codes for all samples can be found in Table S1. Complete code for the analyses including parameter specifications can be found in the File S2.

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