To understand the evolutionary pathways that lead to emerging infections of vertebrates, here we explore the genomic innovations that allow free-living chytrid fungi to adapt to and colonize amphibian hosts. Sequencing and comparing the genomes of two pathogenic species of *Batrachochytrium* to those of close saprophytic relatives reveals that pathogenicity is associated with remarkable expansions of protease and cell wall gene families, while divergent infection strategies are linked to radiations of lineage-specific gene families. By comparing the host-pathogen response to infection for both pathogens, we illuminate the traits that underpin a strikingly different immune response within a shared host species. Our results show that, despite commonalities that promote infection, specific gene-family radiations contribute to distinct infection strategies. The breadth and evolutionary novelty of candidate virulence factors that we discover underscores the urgent need to halt the advance of pathogenic chytrids and prevent incipient loss of biodiversity.
Colonyization of the vertebrate host niche by pathogenic fungi is an ongoing process and recent expansions of host ranges are leading to an increasing global threat to animal and human health\(^1\). Amphibian chytridiomycosis is emblematic of how the emergence of fungal diseases contributes to major biodiversity loss during the current sixth mass extinction\(^2\). Within the primitive and diverse fungal phylum of Chytridiomycota, the two causative agents of chytridiomycosis Batrachochytrium dendrobatidis (\(Bd\)) and Batrachochytrium salamandrivorans (\(Bsal\)) diverged an estimated 67 million years ago to become the only taxa known to have adapted to colonize vertebrates\(^3,^4\). Yet, these pathogens demonstrate markedly different host species range, with \(Bsal\) mostly infecting a single order of hosts, the caudates (salamanders), while \(Bd\) infects over 700 species across all three orders of Amphibia\(^5,^6\). These species therefore offer a unique opportunity to elucidate the acquisition of fungal pathogenicity that allowed infection of vertebrates followed by evolution of host specificity.

Host infection by both pathogens is restricted to a similar niche in the amphibian epidermis, but results in markedly different outcomes (focal necrosis and ulceration in \(Bsal\) versus hyperplasia and hyperkeratosis in \(Bd\))\(^6\). We therefore predict that the jump of Chytridiomycota to invade amphibian hosts has been facilitated by the acquisition of common ancestral traits, whereas subsequent differentiation of infection strategies has been the result of lineage-specific adaptations. To assess these predictions, we here report the sequencing of the genomes of \(Bd\) and \(Bsal\) and comparison to those of two related saprobic chytrids; Homolaphlyctis polyrhiza (\(Hp\)) is in the same order (Rhizophydiales) as Batrachochytrium and is the closest, so far known, relative to \(Bd\), while Spizellomyces punctatus (\(Sp\)) is in a different, primarily terrestrial, order (Spizellomycetales). Differences in protease gene content for the two pathogens are then measured experimentally, revealing stage specific activity. To more broadly characterize the host–pathogen interaction during infection, we compare the in vivo transcriptomes of \(Bd\) and \(Bsal\) in a susceptible model host species (\(Tyrlootriton wenxianensis\), Tw) at late stage of infection against transcription in vitro; this reveals fundamental differences between these pathogens related to their differing infection strategies and the attendant host response.

**Results**

**Genome content of Batrachochytrium species.** We find the evolutionary adaptation to infect amphibians to be correlated with the acquisition of genes, encoding secreted proteins that are unique either to the genus Batrachochytrium, or to \(Bd\) or \(Bsal\). By sequencing both \(Bd\) and \(Bsal\) (Supplementary Table 1), we find that genome size of the host-restricted \(Bsal\) is larger (32.6 Mb) and more complex than that of the broad host range \(Bd\) (23.7 Mb) as well as those of \(Hp\) (16.7 Mb) and \(Sp\) (24.1 Mb), two-related free-living chytrid saprobes (Fig. 1). This variation in genome size is reflected in the complexity of the \(Bsal\) genome, with 10,138 protein-coding genes predicted compared to a range of 6,254–8,852 for the other chytrids (Supplementary Table 2, Supplementary Fig. 1c,d). \(Bd\) and \(Bsal\) share a core set of 5,706 gene clusters (6,403 or 6,344 genes, respectively, including paralogs), of which 542 clusters are not found in the two saprobic chytrids and include specific functions related to cell wall modification and candidate secreted effectors (discussed below) (Supplementary Fig. 1c, Supplementary Tables 3 and 4). The two Batrachochytrium species are most closely related and share large regions of synteny; both species are more closely related to \(Hp\) than to \(Sp\), and Batrachochytrium and \(Hp\) share a larger proportion of syntenic orthologs than either species shares with \(Sp\) (Fig. 1).

Similar to \(Bd\), heterozygous positions were abundant in \(Bsal\) and found throughout the genome (Supplementary Fig. 2a, Methods). This level of heterozygosity (161,831 total; 4.96 kb) is similar to those found in the non-GPL \(Bd\) isolates using the same methods (for example, \(Bd\) ACCON 141,620 = 6.05 kb, \(Bd\) CAPE TF5a1 122,352 = 5.23 kb), while BdGPL strains have less heterozygosity (for example, \(Bd\) JEL423 50,711 = 2.16 kb)\(^7\). Similar to \(Bd\), the \(Bsal\) genome appears to be diploid, with potential examples of trisomy, based on depth of coverage, allele balance and SNP phasing (Methods).

**Protease family expansion and activity.** Several of the expanded and lineage-specific protein families are highly expressed during in vivo infection of the salamander \(T. wenxianensis\) (Tw). Using RNA-Seq, we compared gene expression for parallel infections by \(Bsal\) and \(Bd\) or growth of each pathogen in culture. Of the chytrid genes that were significantly upregulated in vivo (\(n = 550\)), a large proportion was unique to \(Bsal\) (\(n = 327\); 60%), unique to \(Bd\) (\(n = 43\); 8%) or unique to the genus Batrachochytrium (\(n = 44\); 8%). Furthermore, about half of the Batrachochytrium, \(Bsal\) and \(Bd\) upregulated genes were secreted (55% and 47%, respectively). The fact that these secreted proteins are both largely not present in the saprobic chytrids based on ortholog identification, and that they show increased transcription during host colonization, suggests that the transcriptional response is focused on a unique host-interaction strategy in each species. Further, these upregulated genes likely include key virulence factors, acquired for colonization of a specific host species group for each pathogen within a vertebrate host class. Indeed, these include the M36 metalloproteases implicated in pathogenicity in chytrids\(^7,^8\) as well as as least two large families of secreted proteins (Tribes 1 and 4) with no recognizable functional domains, which are very highly expressed and may represent novel virulence factors unique to \(Bsal\) (Fig. 1, Supplementary Figs 3 and 4).

The M36 metalloprotease family known to mediate host invasion in other systems\(^9,^{10}\) is markedly expanded in \(Bsal\) (Supplementary Fig. 5), concordant with the aggressive necrotic pathology that this pathogen causes. Both \(Bsal\) (\(n = 110\)) and \(Bd\) (\(n = 35\)) have expanded M36 families compared to lower counts in the free-living saprochryid \(Sp\) and \(Hp\) (\(n = 2\) and 3, respectively). Genus and species-specific expansions in the M36 metalloproteinase family suggest both ancestral gene-family expansions underpinning adaptation to vertebrates, and species-specific expansions that are potentially contributing to the delineation of the host species group and pathogenesis. Phylogenetic analysis revealed a subclass of closely related M36 metalloproteases that are shared across both pathogens that we term the Batra Group 1 M36s (G1M36) (Fig. 2a). Species-specific gene-family expansion is illustrated by the presence of a novel secreted clade of M36 genes (\(n = 57\)) unique to \(Bsal\), which we have termed the Batra Group 2 M36s (G2M36) (Supplementary Table 5). These G2M36s are entirely encoded by non-syntenic regions of the \(Bsal\) genome (Fig. 1), supporting a recent species-specific expansion. Although most G1M36s and G2M36s are strongly upregulated in salamander skin, eight G1M36s (19%) appear more highly expressed in vitro (Fig. 2b), suggesting complex regulatory circuits underlie this subclass of protease in \(Bsal\). Regulation and activity of the expanded proteases is complex and life stage specific (Fig. 2c,d, Supplementary Table 6). The upregulation of secreted G1M36s and the associated protease activity in \(Bd\) zoospores compared to \(Bd\) sporangia points to a crucial role of these proteases during early host colonization in \(Bd\), for example, during insertion of their germ tube into the epidermal cells\(^11\). In contrast, the low
protease activity in Bs al zoospores, but high activity in the maturing Bs al sporangia suggests a role during later stages of pathogenesis, for example, in breaching the sporangial wall of developing sporangia and subsequent spread to neighbouring host cells.

Variation of cell-surface proteins in Bd and Bs al. Another notable family of proteins markedly expanded in both Bd and Bs al compared to the free-living chytrids is characterized by multiple copies of the CBM18 domain (Fig. 3a). This domain is predicted to bind chitin and most copies of these proteins contain secretion signals that will target them to the cell surface or extracellular space. Species-specific differences are notable in the pronounced truncation of the lectin-like CBM18s of Bs al, suggesting a fundamental difference in capacity to bind some chitin-like molecules. In comparison, CBM18 genes in Bd are three-fold longer and harbour on average eight CBM18 domains compared with only 2.6 for Bs al. The lack of any significant changes in regulation of CBM18 upon exposure of sporangia to chitinases renders their role in protecting the fungi from host chitinase activity by fending off the fungal chitin unlikely (Fig. 3b, Supplementary Table 6). Rather, we hypothesize that the CBM18s play a role in fungal adhesion to the host skin or in dampening the chitin-recognition host response.

Expanded content of repetitive elements. Expansions of the M36 metalloprotease and CBM18 gene-family expansions coincide with an increased occurrence of repeat-rich regions in both pathogens. The fraction of the genome classified as repetitive sequence is 17% and 16% for Bd and Bs al compared to 3.7% and 4.5% for Sp and Hp, respectively (Supplementary Fig. 2). However, the dramatic differences in composition of repetitive regions between Bd and Bs al suggest independent acquisition of repeat-rich regions by both pathogens. While Bs al is rich in Alu elements (1.8 Mb; 5.6% of the genome), these are completely absent in Bd, Hp and Sp. Conversely, Bd is rich in DNA elements and long tandem repeats (LTR) (2.5 Mb; 10.9% of the genome), which are massively reduced or even absent in the other three chytrids. These differences suggest independent acquisition and diversification of repeat-rich regions contributing to genomic diversification of these species. The lack of the RNA-dependent RNA polymerase involved in RNAi defence may contribute to proliferation of repetitive elements in Bd and Bs al, though this gene is also missing from Hp.

The known association of gene-sparse, repeat-rich regions of the genome with high densities of virulence effectors in well-studied eukaryotic plant pathogens led us to examine gene-sparse regions of Bd and Bs al. Notably we found regions of low-gene density include homologues of a class of virulence effectors termed Crinkler and Necrosis (CRN) genes, previously found in the Phytophthora and Lagenidium genera of Oomycetes. CRN-like genes in Bd had long intergenic regions consistent with a gene-poor repeat-rich environment (averaging 1.4 kb; Supplementary Fig. 6, Supplementary Table 7)—a trait shared with Phytophthora infestans (ref. 16). While previously noted in Bd, we find the CRN-like family is more widely distributed among the Chytridiomycota than previously realised. We identified 162 CRN-like genes in Bd, 10 in Bs al, 11 in Hp and 6 in Sp (Fig. 4a), many of which (n = 55) belong to a single subfamily (known as DXX); genes in Bd contain one of two N-terminal motifs (Supplementary Fig. 7). As CRN-like genes did not appear highly expressed during advanced Tw infection (Fig. 4b), we next examined expression during additional life cycle stages. In both Bd and Bs al, some CRN-like genes were more highly expressed in the zoospore life stage compared to the sporangia life stage (Fig. 4c, Supplementary Table 6). However, incubation of Bd zoospores with Tw tissue for 2 h showed an increased expression of CRN genes, whereas Bs al zoospores were associated with decreased expression, indicating that CRN genes are of interest in the early infection stage of Bd, but not Bs al; the notable expansion of CRN-like genes in Bd suggests that they are of importance, however their function remains unknown. These data corroborate recent findings showing that the CRN-like family occur broadly though patchily throughout microbial eukaryotes as effectors in inter-organismal conflicts.

Differences in host immune response. Despite Bs al and Bd being their own closest known relatives and causing a similar lethal skin disease, both vertebrate chytrids deploy strikingly different strategies during pathogenesis, reflecting their individual infection strategies in salamander hosts. The relatively mild skin pathology caused by Bd nevertheless induces a massive host response resulting in epidermal hyperplasia and hyperkeratosis coinciding with marked expression changes in genes involved in epidermal cornification, electrolyte and fluid homeostasis and immunity (Fig. 5). To examine host gene expression, we generated a de novo

Figure 1 | Relationship and genomic organization of four chytrid species. A phylogenetic tree inferred using RAxML indicates the relationships of the four chytrids (branch lengths indicate the mean number of nucleotide substitutions per site). To the right is a synteny plot, visualizing regions that span two or more orthologs between any two species as a connected grey line. Scaffold numbers are shown above each genome axis if longer than 100 kb, and the location of Ba tra. Group 1 M36s (G1M36), Bs al Group 2 M36s (G2M36), Crinklers (CRN) and the secreted upregulated Tribes 1 & 4 are depicted with coloured bars.
Figure 2 | M36 gene-family relationship and expression at differing life stages. (a) A phylogenetic tree inferred using RAxML from protein alignments of all identified M36 proteins in the four chytrids (branch lengths indicate the mean number of nucleotide substitutions per site). Bd genes are shown in green, Bsal genes in red, Sp genes in blue and Hp genes in black. (b) M36 expression was calculated (TMM normalized fragments per kilobase mapped; FPKM) across three in vitro replicates and three in vivo replicates (shown as 1, 2 and 3). Only M36 transcripts that are significantly differentially expressed in Tw are shown. A greater number are significantly differentially expressed in Bsal compared with Bd, which also include eight Bsal G1M36s that are downregulated. Trees indicate hierarchical clustering between data sets (above) and genes (left of heatmap). (c) Protease activity in Bd and Bsal. The protease concentration was determined in mature cultures (above) and spores (below) that were incubated with unsupplemented distilled water (control) or distilled water supplemented with different protease inhibitors. The results are presented as means ± s.e.m. using a non-parametric Mann–Whitney U-analysis. Significant changes compared to the control group are signed with an asterisk (of $P \leq 0.05$). (d) G1M36 and G2M36 mean fold changes in mRNA expression profiles in Bd (above) and Bsal (below). The data shows the normalized target gene quantities in spores that were incubated with skin tissue of Tw for 2 h, 3-day-old sporangia grown in TGhL and skin tissue from chytrid-infected Tw animals relative to freshly collected spores. The results are presented as means ± s.d. Significant differences in expression between each experimental group are shown in Supplementary Table 6.
assembly of Tw transcripts based on RNA-Seq data (Supplementary Table 8). Bd infection induces marked upregulation of Tw host genes involved in innate (that is, inflammatory, antimicrobial peptides and adaptive (that is, immunoglobulin, MHC) immune responses, a feature of infection that has previously been noted in some species for Bd[20]. Simultaneously, mucus is downregulated, and an absence of infiltration by immune cells in the infected skin was found. These responses suggest the host immune defences are dysregulated, which combined with loss of homeostasis, explains the lethality of the infection. In contrast, salamanders remain basically unresponsive towards the Bsal-induced necrosis and massive tissue destruction[4] that results in erosion of the skin barrier leading to subsequent, overwhelming septicemia and death. Unlike the significant upregulation of immune genes in response to Bd infection, we detected very little increased transcription linked to an immune response to Bsal (Fig. 5c). The lack of a substantial host immune response to Bsal suggests that it has immune-dampening properties in caudates, which could be attributed to one of its unique gene families.

Discussion
Our study shows that the differential expansion of putative virulence factors by at least two chytrid fungi is associated with an expanded host range of chytridiomycete fungi to include vertebrate hosts. Despite being closely related and both causing a lethal skin disease, the pathogenesis of these vertebrate-adapted fungal pathogens is strikingly different with the more host-restricted pathogen deploying a notably expanded arsenal of putative virulence factors and yet not triggering a strong host immune response. The evolutionary plasticity that has resulted in this remarkable, and thus-far uncharacterised, arsenal of fungal pathogenicity factors in these pathogens is strikingly different with the more host-restricted pathogen deploying a notably expanded arsenal of putative virulence factors and yet not triggering a strong host immune response. The evolutionary plasticity that has resulted in this remarkable, and thus-far uncharacterised, arsenal of fungal pathogenicity factors in these pathogens is strikingly different with the more host-restricted pathogen deploying a notably expanded arsenal of putative virulence factors and yet not triggering a strong host immune response. The evolutionary plasticity that has resulted in this remarkable, and thus-far uncharacterised, arsenal of fungal pathogenicity factors in these pathogens is strikingly different with the more host-restricted pathogen deploying a notably expanded arsenal of putative virulence factors and yet not triggering a strong host immune response. 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faced in generating a contiguous Bsal assembly could be improved through longer sequencing reads, or techniques that were used to ensure the high quality Bd genome. Indeed, an improved Bsal assembly may further resolve repeat families, syntenic divergence from Bd, and aneuploidy that may be present, which in turn may affect expression, life-stage and infection dynamics.

Understanding the amphibian-destroying armamentarium that chytrids harbour is crucial to making informed predictions of the risk that novel chytrid lineages pose to naïve and thus-far uninfected regions of the world. This research is a vital step towards correctly informing policy makers of the risk that specific lineages pose, enabling legislation to be enacted to stop the further advance of these pathogens into disease-free regions through biosecurity mechanisms such as restricting animal trade.

**Methods**

**Genome sequencing and assembly.** _B. salamandrivorans_ (Bsal) was sequenced using 29,503,468 paired-end reads (101 nt long) with insert sizes of 441 bp, and an additional 5,895,159 reads with insert sizes ranging from 500 bp to 2.5 kb, with a mean of 900 bp (totaling 262.9 × depth; Supplementary Table 1). The genome was assembled with Allpaths version R48539 (ref. 23) using 140 × fragment coverage. We detected high levels of heterozygosity, so included the 'haploidy' setting. The completeness, contiguity and correctness of the assembly were analysed using the GAEMR genome analysis package (http://www.broadinstitute.org/software/gaemr/). We also assessed the ability of Platanus v1.2.1 (ref. 24), which is designed for the assembly of highly heterozygous genomes. However, this tool did not surpass the Allpaths assembly. Additionally, we attempted a number of kmer-normalized assemblies, and splitting MiSeq reads to make pseudo-jump-paired reads. In general, these assemblies had fewer contigs but also less total sequence (dropping 2–5 Mb of sequence). We therefore chose to use the Allpaths assembly that was 32,636,440 nt in length, divided among 5,338 contigs (N50 10.5 kb).

_B. dendrobatidis_ (Bd) strain JEL423 was sequenced using Sanger technology and paired-end reads were generated from 4 kb plasmid, 10 kb plasmid and 40 kb Fosmid genomic clones. A total of 12.5 × sequence coverage was generated and assembled using Arachne25. A 175 kb scaffold corresponding to the mitochondrial genome was removed from this assembly for annotation and analysis. Nearly all of the sequence is in large scaffolds; 98% of the sequence is present in 17 scaffolds; 97% of the bases of Q40 quality are higher (one error every 10⁻⁴ bases).

**Heterozygosity and ploidy of Bsal in relation to other chytrids.** Genome-sequencing reads from Bsal were aligned back to the _Bsal_ genome using Burrows Wheeler Aligner (BWA) v0.7.4-r885 mem26, providing an average 169 × depth alignment. Binomial SNP Caller from Pileup format (BCSAP) v0.11 (ref. 27) with default settings was used to call variants. Bd strains harbouring trisomic and tetrasomic chromosomes had a high number of bi-allelic heterozygous positions with tri-allelic binomial probabilities (33:66 ratios) (between 1,944 and 6,654 per isolate) and very few tri-alleles (between 2 and 145 per isolate) possibly suggesting transient chromosome loss and gain as seen by in vitro passages of Bd. Even more pronounced were the 65,642 heterozygous positions in 33:66 ratios (40.56% of all heterozygous calls) across the _Bsal_ genome. However, only 38 bases had three separate alleles, 24 of which included insertion/deletion variants.

The majority of _Bsal_ heterozygous positions (136,501; 84%) were phased into two distinct haplotypes of two or more positions, all of which had 100% of the reads in accordance (using a minimal depth of 4 for each haplotype). These values are consistent with a polyploid genome arising from recent or transient duplications of one (or both) sets of the homologous chromosomes. Ploidy of the _Bsal_ genome was further analysed using depth of coverage and allele balance (tallies of bases with differing per cent of reads agreeing with the reference: 47–53% for bi- or tetra-alleles, 30–36% and 63–69% for tri-alleles, and 22–28% and 72–78% for tetra-alleles). Due to large number of contigs, they were ordered according to mean depth of coverage. Contigs with the lowest and highest mean coverage were over represented by the smallest contigs. All contigs smaller than 5 kb were therefore excluded from representation (Supplementary Fig. 2b).

Approximately 1/8th of the genome with the lowest coverage had the greatest proportion of tri-allelic positions. The remaining 7/8th of the genome had predominantly bi-allelic heterozygous positions, consistent with a tetraploidy. This pattern of tetraploidy with trisomy is similar to those identified in _Bd_ JEL423 (ref. 7). However, due to the low contiguous nature of the assembly, the 1/8th assembly that has evidence for triploidy could be enriched for assembly issues, which may suggest an overall diploid genome. Indeed, mapping synteny and ploidy together did not suggest whole chromosome copy number variation, and instead that tri-alleles may derive from repetitive elements (such as centromeres), or otherwise problematic regions of the assembly (Supplementary Fig. 2b). To examine this, we performed two-tailed Fisher’s exact test with q-value FDR.
of PFAM domains contained in tri-allelic compared with bi-allelic regions. We identified 13 significantly enriched terms, four of which were Glutamine amidotransferases (GATases 2, 4, 6, 7). Other likely multi-copy family members were therefore placed into an LTR/DNA category). We next took all remaining unknown families that comprised >0.3% of each genome, identified ORFs using ORFFinder, and manually checking both confirmed a number of spurious hits. OrthoMCL and manually checking both confirmed a number of unknown families that could be merged with either other unknown families or annotated families. One repeat family in \( Bsal \) had sequence similarity with \( Bd \) or annotated families. One repeat family in \( Bsal \) was relatively consistent (\( Bd = 17.1\% \), \( Bsal = 16.2\% \). However, the total repetitive sequence in \( Bsal \) amounted to an additional 1.3 Mb, and both \( Bsal \) genomes were repeat rich compared with \( Sp \) (3.7%) or \( Hp \) (4.5%). Furthermore, the composition was markedly different between the two species (Supplementary Fig. 2c,d). Specially, \( Bsal \) has about 1% (263 kb) of its genome comprised of short interspersed nuclear elements, all of which were Alu elements, which is entirely absent in \( Bd \) and \( Hp \), and comprises <0.01% of the \( Sp \) genome.

\( Bsal \) also had >10 \times the cell number of low complexity repeats (192 kb:13 kb) and nearly 20 \times the genomic occupancy of simple repeats compared with \( Bd \) (404 kb:36 kb). Conversely, \( Bd \) had 2 \times the occupancy of long interspersed nuclear elements 2 in its genome compared with that found in \( Bsal \) (195 kb:122 kb). Even more pronounced was the difference in LTR (\( Bd = 411 \text{ kb}, Bsal = 153 \text{ kb} \)) and DNA elements (\( Bd = 1.3 \text{ Mb}, Bsal = 0.5 \text{ Mb} \)). DNA elements were not identified in either of the non-amphibian-infecting chytrids. That \( Batrachochytrium \) should harbour such repeat-rich genomes compared with its closest chytrid relatives may indicate a conserved functional role, such as gene duplication, or an ancestral reduced ability to purge repetitive elements.

The largest difference in repetitive content was in the number of unclassified repeats identified in \( Bsal \) (4.2 Mb: 13%) compared with \( Bd \) (2.2 Mb: 9.24%). To characterize these repeats, we first ran an all vs all BLASTs with \( e \geq 50 \) to avoid spurious hits. OrthoMCL and manually checking both confirmed a number of unknown families that could be merged with either other unknown families or annotated families. One repeat family in \( Bd \) had sequence similarity with LTR Copia at the start, and DNA elements on the other-end (unknown families were therefore placed into an LTR/DNA category). We next took all remaining unknown families that comprised >0.3% of each genome, identified ORFs using ORFFinder, and BLASTp the largest ORF to the nr protein database. A number

![Graphs showing fold change in gene expression](image)

**Figure 5 | Transcriptomes and skin histology for Tw post infection from Bsal or Bd.** (a) Skin of Tw at 10 days after exposure to Bsal (left) or Bd (right). Bsal thalli are abundantly present across the entire thickness of the epidermis (\( ) \) resulting in extensive loss of keratinocytes, whereas \( Bd \) thalli are associated with the superficial epidermal layers and hyperkeratotic foci (\( * \)). For both infections, histological evidence of an inflammatory response is lacking (Hematoxylin and eosin stain, \( \times 400 \)). (b) MA plots (showing Log2 fold change in the trimmed mean of M-values (TMM) normalized Fragments Per Kilobase of transcript per Million mapped reads (FPKM) vs average Log2 counts per million) of tw transcripts during Bsal infection (left) and Bd infection (right) compared with non-infected. Significant differential expression is highlighted in blue (Bsal) and red (Bd), where FDR \( P < 0.001 \) and \( > \)fourfold change of TMM normalized FPKM. (c) Multiple classes of immune genes (\( x \)-axis) were found differentially expressed during Bd infection, while few were found during Bsal infection. The \( y \)-axis shows the number of genes either upregulated (positive count) or downregulated during infection (negative count).
of unknown families were categorized including Sp repeat-family 6, which contained five domains belonging to LTR copia-type transposons. Similarly, Sp family 71 had four domains belonging to Gypsy transposons. This process gave categories for 92% of Sp repeats, 90% Hp repeats, 75% of Bd repeats and 62% of Bsal repeats (Supplementary Fig. 2c). Those that were not categorized included 51 Bsal repeat families, 16 Bd families, 6 Sp families and 11 Hp families.

**Gene prediction, improvement and annotation for chytrids.** Gene prediction and annotation for B. salamandrivorans (Bsal), B. dendrobatidis (Bd), S. punctatus (Sp) and H. polyrhiza (Hp) was achieved using a *de novo* Eukaryotic Annotation Pipeline (Supplementary Table 2). The genome of Hp was downloaded from Genbank (accession AFSM00000000) prior to annotation, as a gene set was not available in Genbank for this assembly, this assembly was annotated in parallel and that data is available on Figshare (https://dx.doi.org/10.1080/mnf.9.foxhage.4291310, https://dx.doi.org/10.1080/mnf.foxhage.4291313, https://dx.doi.org/10.1080/mnf.foxhage.4291274). For Sp, the available assembly and annotation was used (Genbank BioProject PRJNA37881) and RNA-Seq data was generated for Bsal and Bd and used to inform and update gene prediction, respectively. For Bsal, 78,103,411 paired-end reads (15.7 Terabases) of RNA was sequenced on three lanes encompassing 58,251 exons and 45,777 introns.

In total, 10,138 genes were identified for Bd, and only one that had been identified and not included in the two domains (also not identified as in a CRN subfamily). Including these genes provided a large increase to the bit score (1,191 – 2,671) for a new domain starting with ME(P)TIN(1,110,108)56. The HMMP from the first domain identified one new gene that had not previously been identified from Bd (BDEG_28597) and five DXX-DHA genes that had not been included in either of the domains. By now including them, we were able to obtain a higher bit score (1,576 – 1,772) for the new domain starting with R[YF]LNEVY (Supplementary Fig. 7). An updated HMM for CRN's in neither domain 1 or 2 had any further gene matches. The remaining 90 CRN's in neither domain 1 or 2 had a slightly reduced bit score (699 – 584) and only a low confidence motif: M(0.00) [LV][EQ][ST]. A tree of all identified CRN 50aa N-termini was made using RAxML v7.7.8 (ref. 52) with 1,000 bootstrap replicates with the amino acid transition model WAG (Fig. 1). The second domain (YQ[KQ]) had a bit score of 1,191 and was present in 27 genes spanning all of the DFA-DDC subfamily and most of the DFB subfamily (12,13), as well as 1/2 DBX, 5/17 DX8, 12/8 DX5 and 3/5 DXX-newDX5. The remaining 96 included a number of whole subfamilies such DN17 and newD2, and only one that had been identified and not included in the two domains (also not identified as in a CRN subfamily).

**Carbohydrate-binding module 18-containing genes.** Carbohydrate-binding proteins were identified with BLASTp searches to the Carbohydrate-Active enZymes Database (http://www.cazy.org/) using the stringent e-value cutoff of e − 50 to avoid spurious hits. Looking at only the top high scoring pair, we identified very similar numbers of Carbohydrate-Active enZymes (CBM18) for Bd, Bsal, Hp, Sp and Rp (Fig. 1 and Supplementary Table 1). To ascertain if the four chytrids secreted proteins included any large families (in addition to metalloproteases for example), we clustered all predicted secreted genes using MCL (http://micans.org/mcl/man/clmprotocols.html) with recommended settings ‘–I 1.4’. We clustered both the entire length of the protein and only the N-terminal domain, which contained the peptidase S41 domain. The largest tribe had 105 proteins, which had the highest coverage of a set of 47 domains. The second largest tribe had 61 proteins, which contained the peptidase S41 domain.

For Sp we identified 8,952 genes encoding 9,424 transcripts. Sp had an average of 6 exons per gene (56,727/8,952), which is greater than the average of 5 exons per gene in the genome now covered 6.9% of the genome.

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For Sp we identified 8,952 genes encoding 9,424 transcripts. Sp had an average of 6 exons per gene (56,727/8,952), which is greater than the average of 5 exons per gene in Hp, and 4.7 exons per gene in Bd and Bsal. All gene sets had high coverage of a set of 24 conserved genes, indicating that our annotation is highly complete; the updated RNA-Seq gene set was substantially improved for Bd (Supplementary Fig. 1a–c).

Genes were functionally annotated by assigning PFAM domains55, GO terms, KEGG assignment and ortholog mapping to genes of known function. HMMPR3 (ref. 46) was used to identify PFAM (release 27) and TIGRFam (release 12) domains, and BLASTx used against the KEGG v65 database (e-value < 1 × 10−10). GO terms were assigned using Blast2GO version2.35, (ref. 47), with a minimum e-value of 1 × 10−15. SignalP 4.0 (ref. 48) and TMHMM49 were used to identify secreted proteins and trans-membrane proteins respectively (Supplementary Fig. 1d). The protease composition of each chytrid was determined using top high scoring pairs from BLASTp searches (e-value < 1 × 10−10) made to the file ‘pupkin.lib’, which is a non-redundant library of 447,156 protein sequences of all the peptides and peptidase inhibitors that are included in the MEROPS database (downloaded 2nd September 2014 from http://merops.sanger.ac.uk/). Bd had 586 top hits, Sp had 158 total, Bd had 538 total and Sp had 568 total. For Hp, only 92BLASTp hits were found for Sp (e-value < 10−30) and only one that had been identified and not included in the two domains. For Sp (e-value < 10−30) and only one that had been identified and not included in the two domains. For Sp (e-value < 10−30) and only one that had been identified and not included in the two domains.
CBM18 genes for Bd. We also increased the number of CBM18 domains found in Bd from 67 to 90. For Sp, we found just 5 genes with 8 domains. For Bsal, we found 15 genes with 30 domains. For Tw, we found gene with 6 domains. None of the chytrid CBM18 genes were identified as a 1:1 ortholog. Each gene was aligned using muscle, and a tree was constructed with RAxML v7.7.8 (ref. 52) with 1,000 bootstrap replicates with the amino acid transition model WAG (Fig. 3).

Genes containing a carbohydrate esterase 4 (CE4) superfamily mainly include chitin deacetylases (EC 3.5.1.41) clustered together, and had been previously described as deacetylase-like (DE)53. Bd had 10 (previously 9 were reported)60 although one lacked a DE domain. Bsal had 6 DE CBM18s, 1 of which lacked the DE domain. Sp had 3 DE CBM18s, and Hp had none. Another group of CBM18 domains was observed from chitinase-like and was previously described as tyrosinase-like (TL). Bd had 5 (previously 3 were reported)60.

Bsal had 3, Hp had 1 and Sp had none. The third group consisted of genes with no secondary domains and was previously described as lectin-like (LL). We recovered the same 6 genes in Bd as previously reported, and similarly found 6 in Bsal, 1 in Sp and none in Hp. However, the 6 in Bsal CBM18 had all 18 modules (averaging 8 per gene), while the 6 in LL CBM18 had only 16 CBM18 modules (averaging 2.6 per gene). Bsal LL CBM18s are also considerably truncated compared with those of Bd, (mean Bsd protein length = 606, mean Bd protein length = 206). Finally, one of the CBM18 genes in Sp was drastically divergent from each of the others, and in addition to the CBM18 domain, also contained a glycosyl hydrolase family 64 (GH64), a glycosyl hydrolase family 18 (GH18), a Hormone receptor domain (HRM) and a Carbohydrate-binding (CB) domain. This gene is unlikely to be related in function to the other three groups, but could potentially be involved in the modulation of chitin recognition proteins59.

**Ortholog-based phylogenetic and functional analysis of chytrids.** To reconstruct the evolutionary relationships between the four chytrid species, we identified 1:1 orthologs with OrthoMCL52. We aligned orthologs with MUSCLE v3.8.31 (ref. 24) for 50% identical sequences in the smallest contiguous sequence, and then concatenated alignments. In total, we aligned 4.1 MB of transcripts for each chytrid. From greatest to smallest in nucleotide similarity of core orthologs, Bsal and Bd were 66% identical, Bsal and Bd were each 59% identical to Hp and Sp was 53% identical with Bd, Bsal and Hp. ProtTest v3.4 (ref. 53) was used to determine the best-fitting amino acid transition model. For Bsal and Bsd, we used SecretomeP60 (NN-score 206). Finally, one of the CBM18 genes in Sp had 3 DE CBM18s, and Hp had none. Another group of CBM18 domains was observed from chitinase-like and was previously described as tyrosinase-like (TL). Bd had 5 (previously 3 were reported)60.

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subsets were compared against the remaining set. For the enrichment tests, and identification of differentially expressed immune genes, we joined those genes that were significantly up or downregulated in Bd relative to Bsal with those up or downregulated in Bsal relative to control, and did the same for Bd (that is, merged up or down in Bsal relative to Bd with up or down in Bsal relative control).

Overall, Bd genes appear downregulated in vivo (5,352/8,249; 67%; Supplementary Fig. 8d, Supplementary Data 1); however, this is probably affected by the lower sensitivity of the in vivo samples compared to in vitro. The majority of Bd G1M36s are upregulated in vivo (16/25; 64%) (Fig. 2b), five of which (20%) were significantly upregulated—while none were significantly downregulated. Of these, 613 genes were significantly downregulated, while 447 were significantly upregulated (5,468/9,372; 56.5%; Supplementary Fig. 8b, Supplementary Data 1). Much like Bd, 8/10 Bsal CRNs were downregulated, of which 8/10 were significantly downregulated. Similarly, the majority of Bd G1M36s are upregulated in vivo (22/41; 54%). However, unlike Bd, eight (19%) were significantly downregulated in vivo, while 11 (27%) were significantly upregulated.

This comparison suggests a more complex regulation of this subcell of protease in Bsal. Bsal G2M36s are mostly upregulated in vivo (26/36; 72%), 4 of which are significant—while none are significantly downregulated in vivo. A small number of M36s did not cluster with G1M36s or G2M36s, and included those also found in Zp. These data were deposited at GenBank under Accession GESS00000000, and RNA-Seq data from T. wexnianensis is deposited at GenBank under accession GESC00000000.

We found substantial differences in host transcriptional response to Bd and Bsal (Supplementary Data 2). The largest category of genes differentially expressed in T. wexnianensis relative to control (that is, merged up or down in vivo) is host immune genes (1,930/5,830; 32.9%), 16/25 of which were significantly upregulated during Bd infection and 3 downregulated during Bsal infection. Most of the genes were upregulated (1,080; 56.5%) compared with 96 for Bd. Tw also had a large number of downregulated genes during Bsal infection (n = 106) compared with only 12 for Bd. Only two GO terms were enriched, including Immune system processes and extracellular region (Fisher exact test with q-value multiple correction), both from Tw genes upregulated during Bsal infection (q = 3.89e-07 and 5.15e-07, respectively, Supplementary Data 3). We found that a large number of immune-related genes, including Antimicrobial peptides, B-cell related protein, interferons, macrophage proteins, MHCs, NF-KB and Toll-like receptors were differentially upregulated in Bd-infected salamanders (Fig. 5c, Supplementary Data 4), but with no differential expression found in Bsal infected animals. We also found a number of immune genes that were overwhelmingly differentially expressed by Bd infected animals, but also present in low numbers during Bsal infections (Fig. 5b). These included cytokines, immunoglobulins, interleukins and Tumour Necrosis Factors (Supplementary Data 4). In addition to inflammatory factors, we saw a number of metalloprotease inhibitors. Finally, a large number of transporters were differentially expressed during Bd infection, including nine downregulated and five upregulated aquaporins, which have been described as ‘the plumbing system for cells’ (3). By comparison, only one (sodium/citrate cotransporter) was upregulated by animals infected by Bsal. Keratin was also differentially expressed, including three upregulated during Bd infection and 3 downregulated during Bsal infection.

**Data availability**
The data reported in this paper are outlined in Supplementary Information files and the raw sequences are deposited at GenBank under Bioproject PRJNA326253 for *B. dendrobatidis* and Bioprojects PRJNA326249 and PRJNA311566 for *B. salamandrivorans*. The genome assemblies and annotations are deposited at GenBank under Bioprojects PRJNA31653 for *B. dendrobatidis* and PRJNA311566 for *B. salamandrivorans*. The RNA-Seq dataset, B. salamandrivorans T. wexnianensis is deposited at GenBank under accession GESC00000000, and RNA-Seq data from T. wexnianensis is deposited at GenBank under Bioproject PRJNA300849.

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