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BIODIVERSITY RESEARCH

Global and endemic Asian lineages of the emerging pathogenic fungus *Batrachochytrium dendrobatidis* widely infect amphibians in China

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ABSTRACT

Aim Panzootic chytridiomycosis caused by the chytrid fungus *Batrachochytrium dendrobatidis* (*Bd*) is the proximate cause of rapid amphibian declines across diverse biomes. While the origin of *Bd* remains unclear, increasingly the global trade in amphibians is associated with the spread of the infection. Global samples of *Bd* genotypes from previously unsampled regions are essential to test this hypothesis. In this paper, we present a study of the prevalence and phylogeny of *Bd* in both invasive and native amphibian species in markets and in the wild in ten provinces of China.

Location China.

Method We used a nested PCR assay to amplify the ribosomal internal transcribed spacer region of *Bd* followed by sequencing.

Result Our results showed 246 of 2734 amphibians testing positive for *Bd*, with 157 positive samples in the wild (7.6%) and 89 in markets (13.5%). 30 haplotypes of *Bd* were identified, including 20 first detections. Introduced *Lithobates catesbeianus* had the highest prevalence of infection and the largest number of *Bd* haplotypes in both the wild and markets. Phylogenetic analysis based on 73 haplotypes (57 from Asia and 16 from other continents) showed that a unique, well-supported, basal haplotype is present in Asia. Phylogeographical analyses revealed that some geographical structure exists amongst a subset of global haplotypes.

Main conclusions Strains of the basal haplotype infected *Babina pleuraden*, an amphibian that is endemic to China, and *Andrias japonicus*, endemic to Japan, showing that Southeast Asia harbours a novel endemic lineage of amphibian-associated *Bd*. Our data suggest that *Bd* in Asia pre-dates the expansion of a globalized lineage of *Bd*, a finding that is indicative of a broader association of amphibians and chytrids than has previously been recognized. More genetic data from *Bd* isolates are needed to reveal the phylogenetic relationship of *Bd* in China compared to that found elsewhere.

Keywords
Amphibian trade, *Batrachochytrium dendrobatidis*, chytridiomycosis, invasive species, ITS, phylogeny.

INTRODUCTION

Amphibian chytridiomycosis, caused by the chytrid fungus *Batrachochytrium dendrobatidis* (*Bd*), is an emerging infection that has caused the rapid decline of more than 200 amphibian species across the world (Berger *et al.*, 1998; Lips, 1999; Skerratt *et al.*, 2007; Bielby *et al.*, 2008; Fisher *et al.*, 2009; Vredenburg *et al.*, 2010). *Bd* infects the keratinized epidermis

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of metamorphosed amphibians as well as the tooth rows and jaw sheaths of anuran larvae (Berger *et al.*, 1998; Longcore *et al.*, 1999; Daszak *et al.*, 2003). Mortality occurs when the pathogen disrupts cutaneous osmoregulatory function, leading to electrolyte imbalance in susceptible amphibians (Voyles *et al.*, 2009). Mass mortalities and declines of amphibian populations have been documented in North America, Central America, Europe and Australia, leading to extirpations of up to 40% of species in affected areas (Crawford *et al.*, 2010). Consequentially, *Bd* is now an internationally notifiable disease, and reporting is now obligatory for signatories of the World Organisation for Animal Health (OIE) legislation.

There has been much debate on the origin and mechanisms of spread of chytridiomycosis. Currently, consensus opinion holds that *Bd* is undergoing international spread and introduction into naïve regions and species (Skerratt *et al.*, 2007; Fisher *et al.*, 2009). Evidence supporting this viewpoint is derived from observations that (1) global isolates of *Bd* evince low phylogeographical diversity (James *et al.*, 2009); (2) wave-like *Bd*-associated declines of amphibians have been documented in Australia and South America suggestive of epizootic fronts (Lips *et al.*, 2008); (3) *Bd* is frequently found associated with globally traded invasive non-native amphibian species (Garner *et al.*, 2006; Fisher & Garner, 2007); and (4) epizootics and introductions of *Bd* are associated with a single, or a low diversity, of haplotypes (Walker *et al.*, 2008, 2010).

Three ancestral centres have been proposed for the spread of *Bd*: Africa, America or Asia. The '*Bd* out of Africa' hypothesis postulates that *Bd* has spread around the world through the trade in the clawed frog (*Xenopus laevis*, referred to hereafter as clawed frog) for pregnancy assays and scientific research (Weldon *et al.*, 2004; Fisher & Garner, 2007; Soto-Azat *et al.*, 2010). This hypothesis is based on the observation that the earliest cases of *Bd* infection were detected in a specimen of *Xenopus fraseri* collected from Cameroon in 1933 and specimens of clawed frog from Uganda in 1934 and South Africa in 1938 (Weldon *et al.*, 2004; Soto-Azat *et al.*, 2010). On the other hand, genotyping data show that a higher diversity of *Bd* strains occurs in North America relative to Africa, and there is an exclusive relationship between North American and European strains of *Bd* (James *et al.*, 2009). As the American bullfrogs (*Lithobates catesbeianaus*, referred to hereafter as bullfrogs) were found to manifest a higher diversity of *Bd* strains than do clawed frogs, this suggests that either North America is acting as a source of global *Bd* isolates, or is at least amplifying, and then exporting, the pathogen *via* the trade in bullfrogs (Garner *et al.*, 2006; Fisher & Garner, 2007; Morgan *et al.*, 2007; Fisher, 2009; Goka *et al.*, 2009; James *et al.*, 2009).

More recently, Goka *et al.* (2009) studied the haplotypes and their distribution of *Bd* infecting invasive and native amphibians by sequencing the ribosomal DNA of *Bd* from Japan. This study showed that three *Bd* haplotypes from a native species (*Andrias japonicus*) were endemic to Japan and that *Bd* infection in museum specimens of *A. japonicus* could be dated to as early as 1902. These results suggest that *Bd* has a longer historical association with Asia than with Africa, raising the '*Bd* out of Asia' hypothesis (Fisher, 2009a; Goka *et al.*, 2009). A more recent study, based on a comparison of the pattern of prevalence in 15 countries across Asia alongside the predicted ecological suitability for *Bd* based on niche models, suggested that *Bd* is either newly emerged in Asia or prevented from becoming widely prevalent by unique biotic and abiotic factors (Swei *et al.*, 2011). However, no evidence for any of these models is definitive as the distribution and genetic diversity of *Bd* on amphibians across the world have not been adequately sampled (Fisher *et al.*, 2009b; Kilpatrick *et al.*, 2009), with especially poor sampling of *Bd* in Asia and Africa.

Information on the prevalence and phylogenetic relationships of *Bd* from mainland Asia is essential for understanding the origin and transmission of *Bd*. China is the home to 333 described amphibian species, including approximately 200 endemic species (IUCN 2011). Both bullfrogs and clawed frogs are widely traded in markets in China, and bullfrogs have invaded the wild in large numbers (Li *et al.*, 2006; Liu & Li, 2009). To date, five studies have assayed for *Bd* on amphibians from the field in China (Bai *et al.*, 2010; Wei *et al.*, 2010; Swei *et al.*, 2011; Lehtinen *et al.*, 2008; Ouellet *et al.*, 2005), but only one study has detected *Bd* infected bullfrogs in the wild and markets and on three native Chinese species in Yunnan Province (Bai *et al.*, 2010). In this study, we investigated the prevalence and phylogeny of *Bd* on both invasive and native amphibian species in markets and wild in China using spatial sampling, a nested PCR assay and sequencing. The nested PCR assay amplifies the internal transcribed spacer (ITS) region of the ribosomal DNA in *Bd* and has shown increased specificity (Gaertner *et al.*, 2009; Goka *et al.*, 2009) and sensitivity to *Bd* detection compared with quantitative TaqMan PCR (qPCR). The final products of the nested PCR are able to be subcloned and sequenced, and therefore, they provide a rapid and culture-independent tool for detecting variable and previously unknown DNA haplotypes of *Bd* (Goka *et al.*, 2009) compared to previous multilocus approaches (James *et al.*, 2009).

METHODS

Samples from wild amphibians

Sampling of amphibians was conducted at 112 wild sites on the mainland and islands of China from six provinces (Fig. 1). The climate in these provinces varies from a subtropical monsoon zone to the low-latitude monsoon zone of the Chinese mountain plateau (Chen, 1982). Of the six provinces, Yunnan and Sichuan are located in the mountains of south-western China, a region that is a recognized global biodiversity 'hotspot' (Myers *et al.*, 2000) with many endemic and endangered amphibian species.

We sampled amphibians at sites in lowland areas (elevation < 1500 m) during the spring of 2007–2010 before daily maximum temperatures exceeded 30 °C and in highland areas (most of Yunnan Province, elevation > 1500 m) during summer and autumn, where daily maximum temperatures rarely reach 30 °C year round. Each frog was handled with

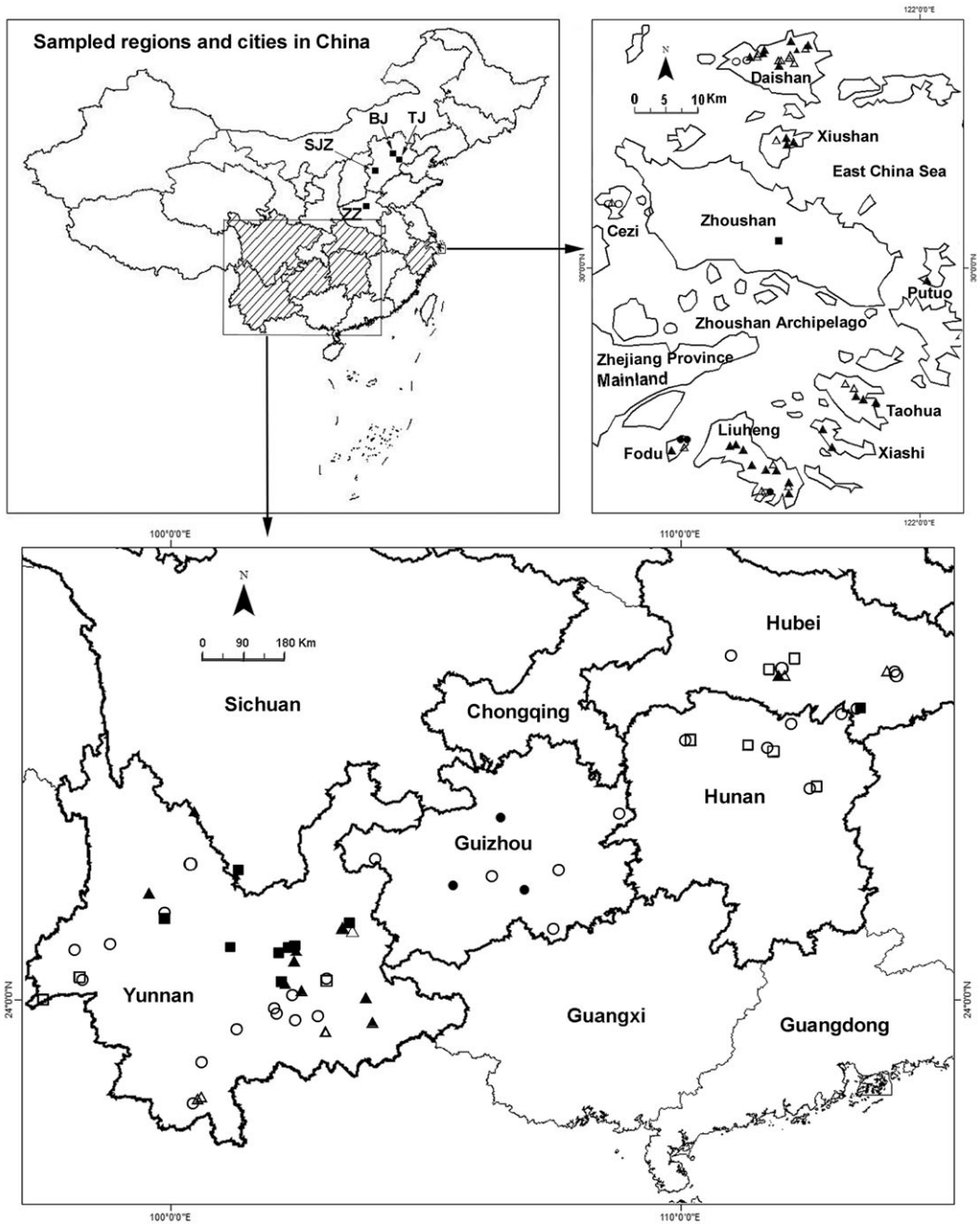

Figure 1 Map of sampling sites for *Batrachochytrium dendrobatidis* (*Bd*) in the studied regions, China. Open triangles indicate bullfrog invaded sites; open circles indicate uninvaded sites; open squares denote food markets or pet shops. The closed ones of these symbols indicate sites where *Bd* was detected. Some points are superimposed. See Table S1 for names of sites. BJ, Beijing; TJ, Tianjin; SJZ, Shijiazhuang; and ZZ, Zhengzhou.

disposable, non-powdered latex gloves, and the top third of the third toe of the right hind foot from each post-metamorphosed frog was clipped (Bai *et al.*, 2010). We disinfected all equipment, including boots and capture tools, before entering each site to prevent the spread of *Bd* among sites, then released the frog at its capture site. Bullfrog tadpoles were euthanized with ethyl ether, and the mouthparts excised from the dead tadpoles. Each tissue sample from toe clips and mouthparts was preserved separately in 70% EtOH in 2-ml screw-cap microcentrifuge tubes then stored at −20 °C in the laboratory. We determined whether a site had been invaded by bullfrogs or clawed frogs by surveying all accessible water bodies in each site for three consecutive nights with line transects (Li *et al.*, 2011a).

Samples from amphibians in pet shops and food markets

We sampled captive amphibians in six pet shops and 25 food markets in cities of eight provinces and two municipalities (Fig. 1). Samples collected from captivity were found piled up in string bags placed on ice at food markets, or in containers with long-standing waters at pet shops. This will increase the chance of transmitting *Bd* zoospores between them, so we generally sampled no more than ten frogs from each container of live amphibians in a food market or pet shop (Rowley *et al.*, 2007). The amphibians collected in markets in Zhengzou, Shijiazhuang, Tianjin and Beijing were sampled using sterile swabs during May 2010. Each of the following parts of an individual was firmly swabbed 10 times: the ventral surface, the frog's sides (from groin to armpit) and the undersides of the thighs and feet. The swabs were then placed into 2-ml microcentrifuge tubes and stored at −20 °C in the laboratory. Amphibians from other markets were sampled using toe clipping during 2008 and 2009.

Laboratory analysis

Batrachochytrium dendrobatidis DNA was extracted following the procedure of Goka *et al.* (2009). We amplified the *Bd* DNA using a nested PCR assay (Gaertner *et al.*, 2009; Goka *et al.*, 2009). The primers for the first amplification were ITS1f and ITS4. These primers target the conserved regions of the 28S and the 18S rRNA genes and amplify the 5.8S rRNA gene along with the flanking internal transcribed spacer (ITS) of all fungi (White *et al.*, 1990; Gaertner *et al.*, 2009). The amplified products from the first-round PCR were subsequently amplified in the second amplification step of nested PCR using primers Bd1a and Bd2a, which have shown high specificity for *Bd* (Annis *et al.*, 2004).

Our initial PCR procedure was optimized to achieve sensitivity for detecting DNA containing as little as 0.1 *Bd* zoospore standards. The final protocol was as follows: with 2 μl of each template DNA in a total reaction volume of 25 μl, the PCR reaction mix contained 10 × PCR buffer (200 mM Tris–HCl, pH 8.4, 200 mM KCl, 100 mM $(NH_4)_2SO_4$, 20 mM $MgSO_4$ and PCR enhancer), 0.2 mM of each dNTP, 1.25 units of *TransStart* Taq DNA polymerase (Beijing TransGen Biotech, Beijing, China) and 0.4 mM of each primer. The conditions for the first amplification included an initial denaturation for 5 min at 94 °C; 30 cycles of 30 s at 94 °C, 30 s at 59 °C and 1 min at 72 °C and a final extension for 10 min at 72 °C. The conditions for the second amplification were an initial denaturation for 5 min at 94 °C; 30 cycles of 30 s at 94 °C, 30 s at 65 °C and 30 s at 72 °C and a final extension for 7 min at 72 °C. For each amplification, sterilized distilled water and DNA containing 0.1 *Bd* zoospore standards were used as negative and positive controls, respectively. The PCR products (approximately 300-bp fragments) were then separated on agarose gel electrophoresis (2% agarose gels). Finally, each product of the positive samples was directly sequenced using primers Bd1a and Bd2a (Beijing Genomics Institute, Beijing, China).

Some sequencing chromatograms of amplicons could not be identified unambiguously. We cloned these amplicons using the pEASY-T1 Simple Cloning Vector in accordance with the manufacturer's protocol (Beijing TransGen Biotech) and sent for sequencing using the universal sequencing primers (Beijing Genomics Institute).

Phylogenetic analysis of the ITS gene

Sequences were aligned using Clustal_X (Thompson *et al.*, 1997) with the default settings, followed by manual adjustments with BioEdit 7.0.0 (Hall, 1999). The number of haplotypes were calculated using DnaSP 5.10 (Rozas *et al.*, 2003). To compare the genetic variation between China and the other countries, 26 haplotypes from Japan and 16 haplotypes from 51 sequences of the ITS1-5.8S-ITS2 region of *Bd* previously detected from continents other than Asia (Federici *et al.*, 2009; Rodriguez & Hirt, 2009) were added to the dataset. The seven non-*Bd* outgroup sequences were selected according to Goka *et al.* (2009). Haplotypes from China, Japan and the other countries were aligned with the outgroup sequences and modified manually to minimize the number of insertions and deletions. Aligned gaps were treated as presence/absence characters applying the single indel coding method described by Simmons & Ochoterena (2000) using the program GapCoder (Simmons & Ochoterena, 2000; Young & Healy, 2003).

Phylogenetic relationships between the ITS haplotypes were subsequently inferred using paup 4.0b10 (Swofford, 2002) by maximum parsimony. All characters were weighted equally, and tree reconstruction was conducted using two-part heuristic searches. Initially, 1000 random sequence additions were performed, holding 10 trees at each step with the tree bisection–reconnection (TBR) branch-swapping option. The trees saved in this search were then swapped for completion in a second search using TBR. A strict consensus tree was generated from the final saved trees. Support for individual nodes was assessed using bootstrap analyses with 1000 pseudoreplications performing 10 random sequence additions, TBR and MULTREES; 10 trees were held during each step of sequence addition.

Haplotype network estimation and phylogeographical analyses

Phylogenetic networks are considered to be more appropriate over phylogenetic trees to represent relationships between genes sampled from individuals within a species (Clement *et al.*, 2000; see example Lee-Yaw *et al.*, 2008). Therefore, a parsimony network was constructed for haplotypes of *Bd* identified in the field in China, then based on the network, we analysed the phylogeographical pattern of the ITS gene for *Bd* populations using nested clade phylogeographical analysis (NCPA). Both of these analyses were conducted using aneca v.

1.2 (Clement *et al.*, 2000; Posada *et al.*, 2000; Panchal, 2007). A *Bd* population was defined as the assemble of *Bd* on amphibian individuals separated from each other by no more than 10 km (Smith & Green, 2005). We treated *Bd* on each of seven islands in the Zhoushan Archipelago as a population because amphibians in the field are not able to disperse between islands due to the fact that sea water is harmful to amphibians (Li *et al.*, 2006).

The correlation between geographical and genetic differentiation (F_{ST}) was assessed through Mantel test using Arlequin v 3.5 (Excoffier & Lischer, 2010). The Pearson product–moment correlations of environment parameters (precipitation of driest quarter ($Prec_{dry}$), precipitation of wettest quarter ($Prec_{wet}$), maximum temperature of warmest month (T_{max}) and altitude) with haplotype diversity and nucleoside diversity (Excoffier & Lischer, 2010) of *Bd* populations were analysed using spss 17.0 (SPSS Inc., Chicago, IL, USA).

RESULTS

ITS DNA haplotypes

A total of 246 of 2734 samples (9.0%) tested positive for *Bd* by nested PCR. Of the 246 samples, PCR products of 180 samples were successfully sequenced directly. The remaining 66 samples could not be sequenced directly and were therefore cloned and then sequenced. Out of the 66 samples, 26 individuals carried one haplotype, 30 individuals carried two haplotypes, seven individuals carried three haplotypes and three individuals carried four haplotypes (Tables 1 and S1).

A total of 30 haplotypes were identified from the 246 positive samples. These were numbered from CN1 to CN30 and registered in GenBank with the accession numbers JN870740-JN870769. These haplotypes varied from 247 to 264 bp in length after excluding indels inserted by the alignment. The total length including indels was 321 bp. Most genetic variation was found in the ITS1 region, and only a small number of polymorphisms were found in the 5.8S rDNA region. Only 20 bp in the ITS2 region was available for analysis, and no polymorphisms were observed.

Forty three global haplotypes were identified from 10 countries except China (Table S2), including 26 haplotypes (indicated by A–Z) from Japan (Goka *et al.*, 2009) and 17 haplotypes (numbered as AE1∼AE17) for 53 sequences from continents other than Asia. Of the 30 haplotypes from our study, 20 were unique to China and another 10 haplotypes were shared between China and other countries. Two haplotypes (CN14 and CN18) were common to China, Japan and other countries outside of Asia; four haplotypes were shared between China and the other countries outside of Asia, and four were only shared between China and Japan (Table S2).

Prevalence of *Bd* infecting amphibians in the field

In total, 2075 individuals from 19 native species and the alien bullfrog were collected across 112 sites in field. 157 sampled frogs of eight native species and the alien bullfrog were tested positive for *Bd* infection, which were distributed across forty-five (45/112 = 40.2%) sites (Table S3).

All villages where sites were sampled had bullfrog farms. Bullfrogs had invaded 64 sites (Table S3). The prevalence of *Bd* infections on bullfrogs was significantly higher than native amphibian individuals in positive populations with sample size > 10 individuals ($X^2 = 52.347$, df = 1, $P < 0.001$). A total of 27 haplotypes were identified from 203 nucleotide sequences obtained from wild populations for both bullfrogs and native amphibians. Of the 203 sequences, 120 (59.1%) belonged to the haplotype CN18, followed by CN2 (19 sequences) and CN12 (13 sequences). There was no difference in the frequencies of these three haplotypes between invasive bullfrogs and native amphibians ($X^2 = 1.528$, df = 2, $P = 0.466$). Other haplotypes accounted for a small proportion of the sequences with fewer than ten sequences per haplotype.

The prevalence of *Bd* infecting amphibians in pet shops and food markets

A total of 89 individuals of 659 samples of two alien species (bullfrogs and clawed frogs) and two native amphibian species in pet shops and food markets were found to be positive for *Bd* (Table S1), carrying 14 haplotypes. Three *Bd* haplotypes were unique to the food markets, and no haplotype was unique to the pet shops. CN18 (72/96 sequences) and CN12 (7/96 sequences) were the top two most widely distributed haplotypes.

The Phylogeny of ITS DNA haplotypes

The aligned sequences resulted in a matrix of 321 characters, but after being coded by GapCoder, a final data matrix of 381 characters was created, of which 126 were potentially parsimony informative. The phylogenetic analyses for the 73 haplotypes and seven outgroup taxa generated 4796 most parsimonious trees of 308 steps with consistency index (CI) = 0:82, retention index (RI) = 0:92 and rescaled consistency index (RC) = 0.76.

Phylogenetic reconstruction resulted in two major clades (Fig. 2), one of which formed an early diverging (basal) lineage that contained four haplotypes, B, J and K haplotypes from Japan and CN30 from China, with a bootstrap support of 99%. The Chinese haplotype CN30 had the same sequence information as the Japanese haplotype B. CN30 was found infecting *Babina pleuraden* endemic to Yunnan Province of China, while the haplotype B infected *A. japonicus* endemic to Japan. The second major clade included all other haplotypes and manifested little internal bootstrap support, suggesting a relatively high degree of similarity between isolates. Most of the haplotypes from different countries were generally scattered evenly through the clade and showed no evidence for a phylogeographical signal.

Table 1 The prevalence and haplotypes of *Batrachochytrium dendrobatidis* in wild amphibian populations in six provinces of China.

Province	Species	No. of samples	Prevalence (%) (95% CI)	Haplotype CN(*n*)
Zhejiang	*Lithobates catesbeianaus (L)*	12	67 (35–89)	2(3), 15(1), 18(7)
	Lithobates catesbeianaus	173	30 (23–38)	2(4), 3(1), 9(3), 10(3), 12(8), 13(2), 14(2), 15(3), 18(41), 25(2)
	Pelophylax nigromaculatus	112	5 (2–12)	2(4), 10(1), 18(7), 28(1)
	Bufo gargarizans	104	4 (1–10)	2(1), 18(2), 23(1), 25(1)
	Fejervarya limnocharis	138	4 (1–9)	5(1), 18(3), 24(1)
	Hyla chinensis	1	100 (5–100)	12(1)
	Pelophylax plancyi	26	0 (0–16)	
	Rana zhenhaiensis	20	0 (0–20)	
Hubei	*Pelophylax nigromaculatus*	53	2 (0–11)	18(1)
	Fejervarya limnocharis	139	0 (0–5)	
	Pelophylax hubeiensis	55	0 (0–8)	
	Bufo melanostictus	16	0 (0–24)	
	Microhyla ornata	4	0 (0–6)	
Hunan	*Fejervarya limnocharis*	115	0 (0–4)	
	Pelophylax nigromaculatus	34	0 (0–13)	
	Pelophylax hubeiensis	19	0 (0–21)	
	Duttaphrynus melanostictus	12	0 (0–30)	
	Rana chaochiaoensis	10	0 (0–34)	
	Microhyla ornata	8	0 (0–40)	
	Hoplobatrachus tigerinus	5	0 (0–54)	
	Pelophylax plancyi	1	0 (0–95)	
Guizhou	*Fejervarya limnocharis*	90	7 (3–14)	2(2), 8(1), 14(1), 13(1), 18(6), 25(1)
	Pelophylax nigromaculatus	29	3 (0–20)	18(1)
	Hylarana guentheri	33	0 (0–13)	
	Odorrana andersonii	16	0 (0–24)	
	Microhyla heymonsi	14	0 (0–27)	
	Microhyla ornata	13	0 (0–28)	
	Duttaphrynus melanostictus	1	0 (0–95)	
Sichuan	*Pelophylax nigromaculatus*	16	13 (2–40)	12(1), 18(1)
	Lithobates catesbeianaus	7	0 (0–44)	
Yunnan	*Hyla annectans*	33	21 (10–40)	12(1), 15(1), 18(7)
	Rana chaochiaoensis	61	13 (6–25)	2(1), 18(6), 20(1), 22(1)
	Lithobates catesbeianaus	316	13 (10–18)	1(1), 2(2), 4(1), 7(1), 9(1), 12(1), 13(1), 15(2), 16(2), 18(30), 19(2), 21(2), 22(1)
	Odorrana andersonii	33	9 (2–25)	16(1), 18(2)
	Lithobates catesbeianaus (L)	40	8 (2–21)	2(1), 10(1), 17(1), 18(2)
	Fejervarya limnocharis	36	6 (1–20)	2(1), 12(1), 18(2)
	Babina pleuraden	131	5 (2–10)	18(2), 26(1), 29(1), 30(2)
	Microhyla ornata	42	0 (0–10)	
	Duttaphrynus melanostictus	41	0 (0–11)	
	Bombina maxima	28	0 (0–15)	
	Kaloula verrucosa	13	0 (0–28)	
	Pelophylax nigromaculatus	12	0 (0–30)	
	Polypedates megacephalus	11	0 (0–32)	
	Kaloula pulchra	2	0 (0–80)	

L = larvae.

Haplotype network estimation and phylogeographical analyses

The primary haplotype network for 21 *Bd* populations (Table S4) was composed of two separate parsimony subnetworks and a standalone haplotype (CN30), corresponding to the major clade of the phylogenetic tree and the basal clade, respectively (Figs 2 and 3). Only four clades (clade 1-6, 2-7, 3-1 and 4-3) showed an association with the geographical location (Table. 2). The inference key suggested that restricted gene flow was responsible for the significant geographical association

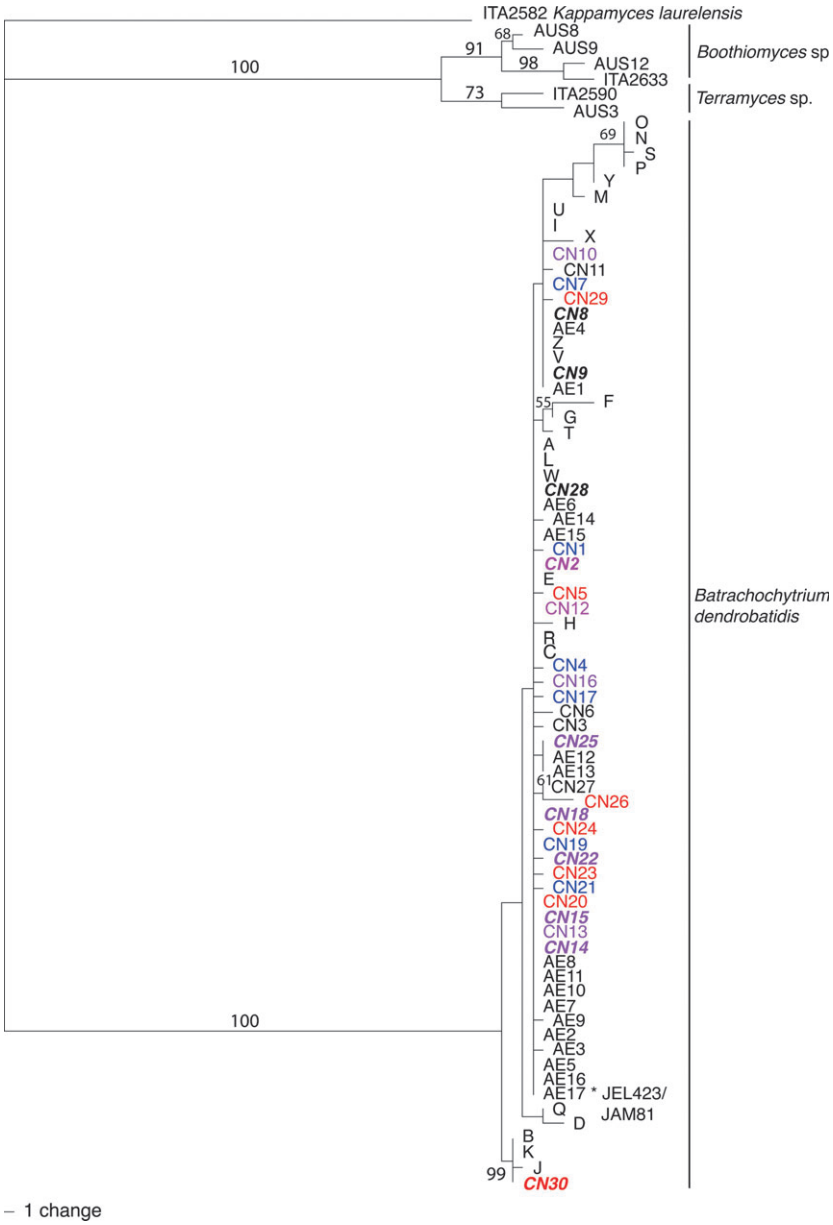

Figure 2 The strict consensus tree from phylogenetic analyses for the ITS haplotypes of *Batrachochytrium dendrobatidis* (*Bd*) using the maximum parsimony analysis. The numbers above the branches are the parsimony bootstrap support value > 50%. The CN prefix indicates haplotypes identified in China; alphabet characters indicate haplotypes identified in Japan; and the AE prefix indicates haplotypes identified in the other countries (see Table S2). Red haplotypes are unique to wild Chinese native amphibians, blue haplotypes are unique to wild *Lithobates catesbeianaus* and purple haplotypes are shared by both wild Chinese native amphibians and *L. catesbeianaus*. The bold italic haplotypes are shared by different countries. CN22 has the same sequence information as that of *Bd* used as a standard in this study, which was collected from Spain. * = ITS haplotypes from the genome-sequenced isolates JAM81 and JEL423.

within clade 4-3. The geographical pattern of clade 2-7 was compatible with allopatric fragmentation.

Mantel test indicated a poor correspondence between the matrix of pairwise genetic (F_{ST}) and geographical distances (log geographical distance in km) ($r = -0.0671$, $P = 0.974$). None of haplotype diversity (Pearson correlation test, $r = -0.063$, $P = 0.79$; $r = 0.143$, $P = 0.54$; $r = 0.309$, $P = 0.17$; $r = -0.099$, $P = 0.67$ for $Prec_{dry}$, $Prec_{wet}$, T_{max} and altitude, respectively) and nucleotide diversity ($r = -0.021$, $P = 0.93$; $r = -0.006$, $P = 0.98$; $r = 0.067$, $P = 0.77$; $r = 0.020$, $P = 0.93$ for $Prec_{dry}$, $Prec_{wet}$, T_{max} and altitude, respectively) was correlated with any environment parameters among *Bd* populations (Table S4).

DISCUSSION

The results of this study confirm the findings by Goka *et al.* (2009) by showing that China and Japan contain a previously unrecognized lineage that is endemic to this region. To date, *Bd* has now been recognized infecting nine countries in Asia: China, Japan, South Korea, Kyrgyzstan, Laos, Malaysia, Sri Lanka, Philippines and Indonesia (Swei *et al.*, 2011; also see at http://www.Bd-maps.net). The novel basal *Bd* lineage containing the Chinese haplotype CN30 (= Japan B) was originally found infecting *A. japonicus,* a species of giant salamander endemic to the Japanese archipelago (Goka *et al.*, 2009). However, we have now shown that this basal lineage also infects *B. pleuraden,* a species of ranid frog endemic to China. This haplotype is unlikely to have been introduced into China from Japan (or *vice versa*) by exotic traded species as none of the animals in the food markets or pet shops carried the haplotype. Furthermore, there are no records of trade in *A. japonicus* or *B. pleuraden* between these countries, and *B. pleuraden* has rarely been hunted in the field or traded in markets in Yunnan Province of China (Yang *et al.*, 1991).

Table 2 Results of the nested geographical analysis for the clades with significant geographical associations.

	Position	*Dc*	*P*	*Dn*	*P*
Clade 1-6					
CN22	Tip	0.00	ns	822.93	ns
CN20	Tip	0.00	ns	822.93	ns
CN24	Tip	0.00	ns	1178.27	0.0231^{L}
CN9	Tip	937.63	ns	943.67	ns
CN25	Tip	803.82	ns	770.56	ns
CN23	Tip	0.00	ns	1178.27	ns
CN19	Tip	30.41	ns	863.33	ns
CN17	Tip	0.00	ns	822.92	ns
CN21	Tip	0.00	ns	822.92	ns
CN18	Interior	878.25	ns	885.33	ns
I–T		487.89	ns	3.24	ns
1-2-11-17 NO: IO					
Clade 2-7					
1-10	Tip	64.92	ns	789.78	0.0306^{S}
1-9	Interior	582.72	ns	627.24	ns
I–T		517.80	ns	162.54	ns
1-19 NO: AF					
Clade 3-1					
2-1	Tip	991.06	ns	990.51	ns
2-2	Tip	835.18	ns	848.30	0.0306^{S}
1-2 IO					
Clade 4-3					
3-2	Tip	809.85	ns	778.75	ns
3-4	Tip	650.93	ns	668.58	ns
3-3	Interior	894.77	ns	894.92	ns
I–T		189.05	ns	188.35	0.0354^{L}
1-2-11-17-4 NO: IBD					

Group designations correspond to those in Fig. 3. Probability values for significantly larger (L) or smaller (S) than expected based on the null hypothesis of no geographical association are based on 10,000 permutations. IO is inconclusive outcome, AF is allopatric fragmentation, IBD is restricted gene flow with isolation by distance (restricted dispersal by distance in non-sexual species).

We favour the hypothesis that this haplotype of *Bd* belongs to a lineage that has a unique association with Asian amphibians and has significantly diversified from the lineage that is associated with the global epizootic (James *et al.*, 2009). The fact that this lineage has now been recovered from two unrelated species (one Anuran and one Caudate) contradicts the hypothesis that the basal lineage is specific to Giant Salamanders (*Andrias*), and rather suggests that it is geographically widespread across Asia, and associated with multiple species of amphibian. However, a wider sampling of Chinese native amphibians, including the Chinese Giant Salamander *A. davidianus*, is necessary to confirm this observation.

The international trade in bullfrogs, clawed frogs and other species has been suggested as vectoring *Bd* across the globe (Weldon *et al.*, 2004; Garner *et al.*, 2006; Fisher & Garner, 2007). In pet shops in Japan, Goka *et al.* (2009) detected four haplotypes (A = CN18 = AE5, C = CN14, L and V, Table S2) that infected *Ceratophrys cornuta* imported from Suriname, *Ceratophrys ornata* and *Lepidobatrachus laevis* from Argentina and Brazil, and *X. laevis* from South Africa, suggesting that these four haplotypes could be widely transmitted by trading amphibians across countries. China has one of the largest international amphibian trades in the world (Gratwicke *et al.*, 2010). Although *Bd* was found not to be present on wild or imported amphibians in Hong Kong (Rowley *et al.*, 2007), *Bd* haplotypes may have been spread from other countries to mainland China through other ports. Both bullfrogs and clawed frogs have been widely introduced into China, and bullfrogs are now invasive in many regions of the country (Li *et al.*, 2006; Liu & Li, 2009); it is thus possible that these species vectored the global epizootic lineage of *Bd* into China when they were introduced. Several lines of evidence support this hypothesis: The bullfrogs that we sampled exhibited a high prevalence of infection and carried a large number of *Bd* haplotypes both in the wild and in Chinese food markets, a result that is consistent with the findings in Japan (Goka *et al.*, 2009). Wild bullfrogs (and some native amphibians such as *Pelophylax nigromaculatus*) have been heavily hunted for food in markets in China (Li *et al.*, 2006; Liu & Li, 2009), which may increase the chance of vectoring *Bd* from the wild to food markets. Therefore, bullfrogs may exert a key role in spreading *Bd* among bullfrog farms, food markets and the wild in China. No phylogeographical structure found in the total cladogram (Table 2) indicated a high level of gene flow among *Bd* populations. This arose possibly because bullfrogs have widely invaded native amphibian communities in China, which might transmit *Bd* lineages among different amphibian communities and have blurred the phylogeographical structure of the *Bd* populations.

Species distribution models predict high *Bd* suitability in Southwest China but low *Bd* suitability in Hubei and Hunan provinces (Rödder *et al.*, 2010; Swei *et al.*, 2011). High occurrence of *Bd* infection on amphibians in Yunnan, Guizhou and Sichuan provinces of the Southwest (14/38 localities) but the low prevalence of *Bd* infection in Hubei Province (1/14 localities), and our failure to detect *Bd* in Hunan Province (eight localities), provides support for these predictions. These results are strikingly different from Swei *et al.*'s recent surveying results in China (Swei *et al.*, 2011). They did not detect *Bd* infection on 256 individuals sampled from 121 localities in Yunnan and Guizhou provinces of Southwest China (Swei *et al.*, 2011).

In common with findings from other countries in Asia (Goka *et al.*, 2009; Swei *et al.*, 2011), the prevalence (3.4%) of *Bd* on amphibians was low in China. As we used toe clips for the field sampling, which may not be so effective as swabs for *Bd* detection (Puschendorf & Bolanos, 2006; Hyatt *et al.*, 2007), the prevalence of *Bd* in China may be likely to be higher than detected in this study. However, even taking this into account, the prevalence of *Bd* in Asia appears to be less than that seen in other infected continents (Fisher *et al.*, 2009b).

Consistent with other studies in Asia (Goka *et al.*, 2009; Swei *et al.*, 2011), we found that *Bd* infection on all individuals in the

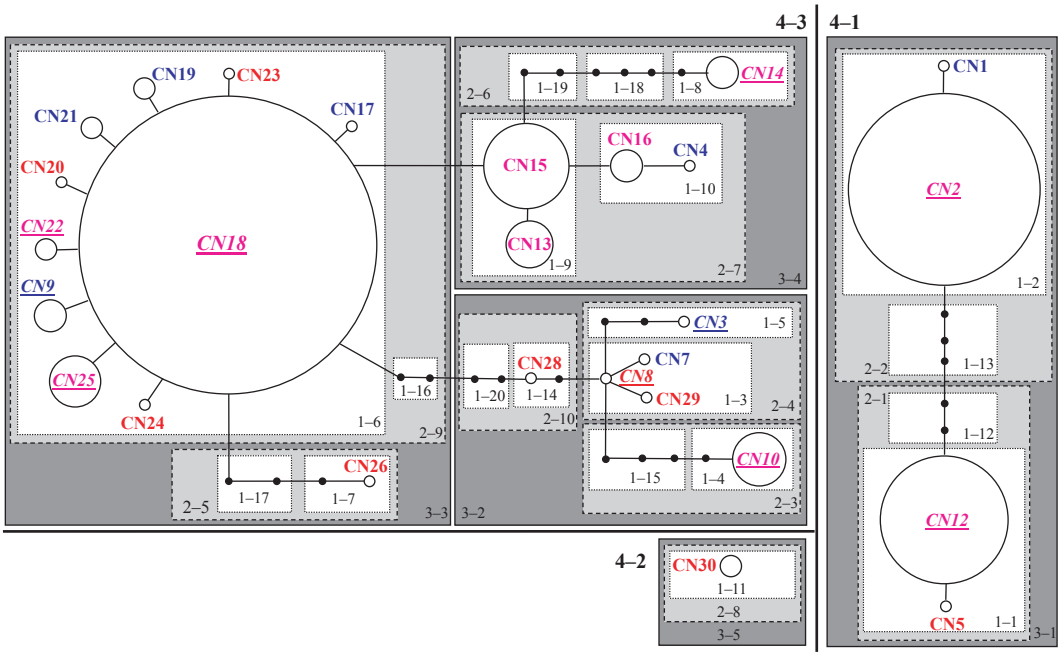

Figure 3 Haplotype networks and nested clade design for ITS haplotypes *Batrachochytrium dendrobatidis* identified in field amphibians in China. Circle size is proportional to the quantity of sequences per haplotype, except CN18, which will be too big to paint in a single page if drawn proportionally. Each line represents a single mutational change; unsampled haplotypes were represented by small black circles. Red haplotypes are unique to field Chinese native amphibians, blue haplotypes are unique to field *Lithobates catesbeianaus* and purple haplotypes are shared by both field Chinese native amphibians and *L. catesbeianaus*. The underlined italic haplotypes are those also detected in captive individuals.

field, pet shops and food markets in China showed no clinical signs of chytridiomycosis, and we did not find any dead amphibian individuals during our 4-year survey period. There have been no reports of mass mortalities of amphibians in China and other countries in Asia to date (Kusrini *et al.*, 2008; Yumi *et al.*, 2008; Yang *et al.*, 2009; Bai *et al.*, 2010; Wei *et al.*, 2010; Swei *et al.* 2011). The absence of clinical signs of chytridiomycosis, and the low prevalence of *Bd* infection in native amphibians in China, may be indicative of a history of co-evolution between native haplotypes of *Bd* and native amphibians. However, it is also possible that the strains of *Bd* in our study areas may not be so virulent to native amphibians in China as some strains in North America, Central America, Europe and Australia (Berger *et al.*, 2005), or that surveillance of amphibian populations has not been thorough enough to detect declines. In support of this latter point, native frog abundance and species richness have been observed to decline as a result of bullfrog invasions on the Zhoushan Archipelago, China, where there is a long-term study focus (Li *et al.*, 2006, 2011b; Wu *et al.*, 2006). The native frog species (*Fejervarya limnocharis*, *P. nigromaculata* and *Bufo gargarizans*) and the bullfrog on the islands all were found to have been infected with *Bd* (Table 1). The possible mechanisms for the threats posed by bullfrogs to native frogs include predation and competition, the introduction of virulent lineages of *Bd*, or a combination of these threats (Werner *et al.*, 1995; Daszak *et al.*, 2004; Hirai, 2004; Pearl *et al.*, 2004; Garner *et al.*, 2006; Wu *et al.*, 2006; Wang *et al.*, 2007). The exact reasons for the decline of native amphibians on the Zhoushan Archipelago remain unknown, and there remains a need to understand the role, if any, of *Bd* infection in the decline of native frog populations on the islands.

We only analysed a single locus in this study; therefore, the phylogenetic relationships among some *Bd* haplotypes could not be fully resolved (Morgan *et al.*, 2007). In this case, the phylogeny recovered from this locus and presented here should be viewed as preliminary. More genetic data from *Bd* isolates are needed to reveal the finer-scale phylogenetic relationship of global *Bd* haplotypes, as well as the potential for intragenomic heterogeneity within the multicopy ribosomal DNA array. Further, although the nested PCR used in this study was sensitive to detecting *Bd*, it was not able to provide quantitative information on the intensity of *Bd*, which is a predictor of lethal infection (Boyle *et al.*, 2004; Vredenburg *et al.*, 2010; Kinney *et al.*, 2011), and more research is needed to determine the distribution of zoospore loads of *Bd* infection on amphibians in China and its impact on their health.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Table S1 The prevalence and haplotypes of *Batrachochytrium dendrobatidis* on amphibians in food markets and pet shops in cities of China.

Table S2 A list of ITS-DNA sequence data of *Batrachochytrium dendrobatidis* already detected and registered. '=' indicates haplotypes with the same sequence information from different countries or regions.

Table S3 The prevalence and haplotypes of *Batrachochytrium dendrobatidis* in wild amphibian populations at different sites of China.

Table S4 Populations of *Batrachochytrium dendrobatidis* identified in field amphibians of China.

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BIOSKETCHES

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Author contribution: LYM and BCM conceived the study; LYM, LX and BCM collected the samples; BCM performed the experiment with the help of TWJG; BCM analysed the data with the help of MCF; BCM, LYM and MCF wrote the manuscript.

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