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A ring-species or a ring of species? Phylogenetic relationship between two treefrog species around the Yellow Sea: *Dryophytes suweonensis* and *D. immaculatus*

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Phylogenetic patterns due to glaciation are still understudied in North East Asia (NEA). Furthermore, the effects of the Last Glacial Maximum on phylogenetic patterns are less explicit in NEA than for other regions of the northern hemisphere due to topographically homogenous landscapes in general. Here, we aim to assess the phylogenetic status of the Dryophytes suweonensis and D. immaculatus treefrog clades. We used concatenated partial mitochondrial 12S and 16S gene fragments, with a combined length of 678 bp for D. suweonensis (n = 32) and D. immaculatus (n = 5), collected from the Republic of Korea and downloaded from GenBank (originating from the People's Republic of China). Dryophytes suweonensis formed an apparently monophyletic clade whereas D. immaculatus was divided in two clades. Our results also demonstrated the continuous genetic variation through haplotypes forming a ring around the Yellow Sea. It is therefore difficult to conclude on either a ring-species or a ring of species around the shallow Yellow Sea, which acted as a land-bridge several times during recent geological times. We recommend the use of other data such as call characteristics and morphology to determine the species or sub-species status of these two clades.

Key words: Dryophytes suweonensis, Dryophytes immaculatus, species divergence, *Hylidae*, Yellow Sea

INTRODUCTION

The impact of glacial cycles on speciation events in Europe and North America has been clearly demonstrated for a number of species (Hewitt, 2000; Knowles, 2001; Veith et al., 2003; Avise, 2007). For instance, the *Hyla arborea* complex, like many other species (Hewitt, 2000), survived glaciation cycles through the use of peninsular refugia in southern Europe (Stöck et al., 2008; Stöck et al., 2012). However, this kind of study is generally missing in North East Asia, here limited to the south by the Yangtze River and around 100° west by the monsoon weather (Wang & Lin, 2002; Lee et al., 2005), where the continuous mainland landscape was not as conducive to speciation events as those in other regions of the northern hemisphere. The major impermeable geographical barrier to gene flow on the East Asian mainland is the Himalayan range to the south and the adjacent southern edge of the Tibetan Plateau. These topographical patterns delimit a southern edge of species distribution, as visible for instance in *Bufo gargarizans* (Yan et al., 2013, Borzée et al., 2017c), *Rana chensinensis* (Kuzmin et al., 2004b) and *Pelophylax nigromaculatus* (Kuzmin et al., 2004a). However, glaciation cycles did influence speciation events for peninsular populations, as demonstrated in the split between *B. gargarizans* and *B. bankorensis* (Chen et al., 2013; Yu et al., 2014), and

Hyla chinensis and *H. simplex* (Hua et al., 2009).

Due to the sea level rise and recession, the Korean peninsula was alternatively isolated and linked to the Japanese Archipelago and the Chinese mainland. During glacial oscillations, the peninsula became totally isolated and acted as an isolated refugium during the last glacial maximum (LGM), even for good dispersers such as racoon dogs (*Nyctereutes procyonoides*; Kim et al., 2013). The Korean Peninsula was not covered by glaciers but was colder and drier during glacial periods (Kong, 2000; Yi & Kim, 2010), and acted as one of the two refugia during the late Pleistocene, together with current central China. For instance, the water frog *Pelophylax nigromaculatus* (Zhang et al., 2008) and the clawed salamander *Onychodactylus koreanus* (previously assigned to *O. fischeri*; Poyarkov et al., 2012) benefitted from these refugia (Yoshikawa et al., 2008). The Korean peninsula itself is divided by non-crossable landscape elements, leading to genetic diversification within *P. chosenicus* (Min et al., 2008), *Hynobius* spp. (Baek et al., 2011; Min et al., 2016), and *Dryophytes japonicus* (Dufresnes et al., 2016).

Glaciation cycles lead to species specific speciation scenarios, with some clear examples of segregated species separated by the Yellow Sea, such as *P. chosenicus* in Korea and *P. plancyi* in China (Liu et al., 2010). However, the same climatic variations also resulted in repeated

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Table 1. Specimen information used in this study. For each specimen, species identity, GenBank accession numbers for each partition, specimens with voucher code (if available), and locality are included. *indicates an update from *Hyla* to *Dryophytes* (Duellman et al., 2016), independently of the name present on the GenBank upload. RoK stands for Republic of Korea.

Taxon	GenBank No.		Specimen voucher no./isolate no.	Locality
	12S	**16S**		
Hyla arborea	DQ055835	DQ055814	-	Croatia, Kamesnica, Donja Korita
H. chinensis	KP742571	KP742700	IOZCAS4796	China: Zhaowu, Fujian
*Dryophytes femoralis**	DQ055838	DQ055819	MVZ137344	USA: Emanuel, Georgia
*D. immaculatus**	KP742584	KP742712	CIBLJT060811	China: Conghua, Guangdong
	KP742585	KP742713	CIBLJT060812	China: Conghua, Guangdong
	KP742586	KP742714	SCUM0606003	China: Jingzhou, Hubei
	KP742587	KP742715	SCUM0606004	China: Jingzhou, Hubei
	KP742588	KP742716	SZ100604	China: Sangzhi, Hunan
	KP742589	KP742717	CIBLJT070515	China: Conghua, Guangdong
*D. japonicus**	KP742597	KP742724	CIBLJT070602	China: Hailin, Heilongjiang
	KP742598	KP742725	CIBLJT070607	China: Shenyang, Liaoning
	KP742599	KP742726	ZISPRussia101001	Russia: Ussuriysk Distr.
	KP742600	KP742727	ZISPRussia101002	Russia: Ussuriysk Distr.
*D. suweonensis**	MG282190	MG282222	BHV383	Geumchon, RoK
	MG282191	MG282223	BHV387	Geumchon, RoK
	MG282192	MG282224	BHV390	Geumchon, RoK
	MG282193	MG282225	BHV391	Geumchon, RoK
	MG282194	MG282226	BHV393	Geumchon, RoK
	MG282195	MG282227	BHV394	Geumchon, RoK
	MG282196	MG282228	BHV396	Geumchon, RoK
	MG282197	MG282229	BHV397	Geumchon, RoK
	MG282198	MG282230	BHV398	Geumchon, RoK
	MG282199	MG282231	BHV399	Geumchon, RoK
	MG282200	MG282232	BHV400	Geumchon, RoK
	MG282201	MG282233	BHV403	Geumchon, RoK
	MG282255	MG282234	SUR12	Cheonan, RoK
	MG282212	MG282245	SUR32	Cheonan, RoK
	MG282213	MG282246	SUR33	Cheonan, RoK
	MG282214	MG282247	SUR34	Cheonan, RoK
	MG282215	MG282248	SUR35	Cheonan, RoK
	MG282216	MG282249	SUR36	Cheonan, RoK
	MG282217	MG282250	SUR38	Cheonan, RoK
	MG282218	MG282251	SUR39	Cheonan, RoK
	MG282219	MG282252	SUR40	Cheonan, RoK
	MG282220	MG282253	SUR43	Cheonan, RoK
	MG282221	MG282254	LTR1	Iksan, RoK
	MG282202	MG282235	LTR2	Iksan, RoK
	MG282203	MG282236	LTR3	Iksan, RoK
	MG282204	MG282237	LTR4	Iksan, RoK
	MG282205	MG282238	LTR5	Iksan, RoK
	MG282206	MG282239	LTR6	Iksan, RoK
	MG282207	MG282240	LTR8	Iksan, RoK
	MG282208	MG282241	LTR9	Iksan, RoK
	MG282209	MG282242	LTR10	Iksan, RoK
	MG282210	MG282243	LTR11	Iksan, RoK
Smilisca fodiens	AY819387	AY819519	MVZ132994	Mexico: Sonora
Pseudacris nigrita	AY819386	AY819518	MVZ145454	USA: North Carolina

gene exchanges over large geographic areas, such as for *B. gargarizans* in North East Asia (Borzée et al., 2017c). Intermediate and unresolved cases also exist, such as for the *D. suweonensis/immaculatus* complex (Li et al., 2015; Dufresnes et al., 2016; Borzée et al., 2017a), for which studies on clade divergence and relationship with the sister clade *D. japonicus* are not consistent (Riehl et al., 1995; Hill, 2009; Li et al., 2015; Dufresnes et al., 2016).

The origin of the Eurasian Hylinae (Rafinesque, 1815;

Smith et al., 2005; Faivovich et al., 2005; Hua et al., 2009; Wiens et al., 2005; Wiens et al., 2010) is consistent with the Savage (1973) hypothesis of a late Cenozoic invasion. The northern oriental Hylid populations belong to two main groups, namely "*H. arborea* group" (*sensu stricto* Anderson & Green, 1991) and "*D. japonicus* group" (*sensu stricto* Hua et al., 2009). In North East Asia, *D. japonicus*, *D. suweonensis* and *D. immaculatus* were recently moved from the genus *Hyla* to *Dryophytes* (Duellman et al.,

Figure 1. Ranges and sampling sites for Dryophytes suweonensis and D. immaculatus. The range based on data from AmphibiaChina (http://www.amphibiachina.org/) is drawn from the most external localities and in a way that avoids sharp angles in the polygon created. Map generated through ArcMap 10.5 (Environmental Systems Resource Institute, Redlands, California, USA), with Service Layer Credits & Sources to Esri, USGS, NOAA, DeLorme, USAS and NFS.

2016). The *Hyla* clade expanded from Northern America through the Bering pass (Anderson & Green, 1991; Borkin, 1999; Duellman, 2001) 28 to 23 mya (Smith et al., 2005) and diverged between 22 to 18 mya into the Asian and European clades (Riehl et al., 1995; Smith et al., 2005). The *Dryophytes* clade, comprising *D. japonicus*, *D. stepheni* (Dufresnes et al., 2016), *D. suweonensis* (Kuramoto, 1984; Riehl et al., 1995) and *D. immaculatus* (Hua et al., 2009), reached Asia during a second expansion by the "*D. eximia* taxa" (Anderson & Green, 1991; Borkin, 1999; Faivovich et al., 2005) from the American continent (Hua et al., 2009) between 18.9 and 18.1 mya (Smith et al., 2005). Divergence between species of the '*D. japonicus* group' started 24 mya (Riehl et al., 1995), resulting in the split between *D. japonicus* and *D. immaculatus* 14 mya (Hill,

2009), and the divergence between *D. japonicus* and *D. suweonensis* between 6.4 and 5.1 mya (Li et al., 2015; Dufresnes et al., 2016).

The *Dryophytes* and *Hyla* Asian genera are largely parapatric (Hua et al., 2009), with an area of overlap from 28 to 33° N latitude (Hoffmann, 2001; Hua et al., 2009). This contact zone may have led to potential competition, such as between *H. chinensis*, occurring together with *D. immaculatus* (Anderson & Green, 1991; Hua et al., 2009). Other ambiguities have been noted, such as the homoplasy of calls properties between *D. japonicus* and *H. hallowelli* (Kuramoto, 1980), while *D. japonicus* and *D. suweonensis* calls are distinct despite their phylogenetic promiscuity (Riehl et al., 1995; Faivovich et al., 2005; Hua et al., 2009).

Since Gunther (1958), and Kuramoto (1980), two

clades of treefrogs have been identified on the Korean peninsula: *D. japonicus* and *D. suweonensis* (Yang & Park, 1988; Yang et al., 1997; Lee et al., 1999; Duellman et al., 2016; Dufresnes et al., 2016). On the opposite side of the Yellow Sea, the Chinese mainland is populated by *D. japonicus* and *D. immaculatus*. Here, we hypothesize that the *D. suweonensis/immaculatus* complex is composed of two divergent species at the extremities of their ranges, but less clearly differentiated when in proximity.

MATERIAL AND METHODS

Species description
Dryophytes suweonensis populations have been recorded as declining throughout the last decade (IUCN, 2017) and are today restricted to a narrow land strip between the Yellow Sea and the western edge of the Baekdudae Range (Roh et al., 2014; Borzée et al., 2016b; Borzée et al., 2017b). This spatial distribution in relation with urban development lead to the isolation of populations and poses a threat to the survival of the species in the long term. *Dryophytes immaculatus* is described as a lowland species from the Chinese mainland (Xie, 2017; AmphibiaChina, 2018), with a much broader range of habitats than *D. suweonensis*, and expected to be more tolerant to habitat modification (Xie, 2017).

Field sampling
Field sampling was conducted over three localities, encompassing the totality of the known range of *D. suweonensis* (Borzée & Jang, 2015; Borzée et al., 2016b; Fig. 1). This was necessary as *D. suweonensis* populations are fragmented due to landscape barriers such as Metropolitan Seoul and the Geum River (Borzée et al., 2015a; Borzée et al., 2017b). The three localities were Geumchon (North of Seoul, *n* = 12, annotated as BHV on subsequent tables and figures), Cheonan (South of Seoul, *n* = 10, annotated SUR), and Iksan, a non-connected population on the southern edge of the species' range (*n* = 10, annotated LTR). A minimum distance of at least 90 km separates the adjacent localities. The samples at the two northern localities, Geumchon and Cheonan, were collected in 2013 (Table 1) under the Ministerial authorisation number 2013-16, while the Iksan locality was sampled in 2014 under the permits 2014-04, 2014-08 and 2014-20. Due to the endangered status of the species, we followed the strict ethical regulation dictated by the permits.

Each locality was sampled once only to prevent pseudo-replication (Table 1). Localities were presumed to be independent of each other, as the dispersion range of most amphibian species is below 10 km per year (Smith & Green, 2005), and that of hylids not over more than a couple kilometres per season (Vos et al., 2000; Smith & Green, 2005; Arens et al., 2006). Genetic material was acquired through oral (= buccal) swabs (Broquet et al., 2007), frozen within 24 h at -20°C.

Molecular work
Mitochondrial DNA was extracted with the Enzynomic® Genomic DNA Extraction Kit (Tissue; Genomics Extraction Kit protocol V2013-1; Seoul, Korea) for Geumchon and Cheonan samples and the Qiagen DNeasy Blood and Tissue Kit (Qiagen, Hilden, Germany) for Iksan samples, following the instructions of the manufacturer.

Because *D. suweonensis* and *D. japonicus* are difficult to identify based on morphology only (Borzée et al., 2013), each individual was identified as belonging to *D. suweonensis* based on mtDNA Cytochrome c oxidase subunit I (COI) sequencing, following the protocol developed by Jang et al. (2011). These sequences were not used for further analysis as not sequenced for *D. immaculatus*. Then, as sequences of *D. immaculatus* were available from GenBank for partial mitochondrial ribosomal 12S and 16S genes (accession numbers KP742584 through KP742589 for 12S, and KP742712 through KP742716 for 16S, Table 1), we sequenced all individuals for part of these genes. For 12S, new primers were designed with Geneious v.9.1.6 (http://www.geneious.com, Kearse et al., 2012; plug-in Primer3 v.2.3.4) based on *D. immaculatus* sequences from GenBank: DIS-12S-F: CCC AAG ACA CCT AGC TAC GC; and reverse DIS-12S-R: TGG CTT TGA AGA GGG TGA CG. For 16S, the primers used were: 16SA-L CGC CTG TTT ATC AAA AAC AT and 16SB-H CCG GTC TGA ACT CAG ATC ACG T (Vences et al., 2005; Jeong et al., 2013), completed by specifically designed primers overlapping with the 16SA and 16SB primer set, also designed with the Geneious plug-in Primer3 (Koressaar & Remm, 2007; Untergasser et al., 2012): DIS-16S-F: GTA AGG GCC CCA ACG TAG TC and DIS-16S-R: AGG GAT GCT GTA GTT AGG GGT.

PCR reactions using published primers 16SA-L and 16SB-H were run following the protocols by Jeong et al. (2013). All newly designed primers were used in PCRs with each reaction containing 14.5 µL of distilled water, 2.0 µL of (10x) buffer, 1.6 µL of dNTPs (final concentration of 0.07 mM), 0.4 µL of each primer, 0.1 µL of Takara Taq polymerase and 1 µL of DNA. The thermocycler (SimpliAmp Thermal Cycler; Applied Biosystems by Life Technologies; Carlsbad, California, United States) was programmed to 94 °C for 5 min, followed by 35 cycles at 94 °C for 30 s, 58 °C for 30 s and 72 °C for 1 min, with a terminal elongation at 72 °C for 5 min (12S) and 10 min (16S). PCR products were then run on a 1.5 % agarose gel during a 12 min electrophoresis, and pre-stained with MaestroSafe dye (Maestrogen; Las Vegas, Nevada, USA). PCR products were subsequently cleaned-up with the PCR Clean-Up Kit (LaboPass PCR, PCR Purification Kit; Cat. No. CMR0112; Cosmogenetech; Seoul, Republic of Korea) and sent to Macrogen Inc. (Seoul, Republic of Korea) for direct sequencing with both forward and reverse primers on an ABI PRISM 3100 automatic sequencer (Applied Biosystem Inc., USA).

Molecular analysis
The partially sequenced mitochondrial 12S and 16S ribosomal RNA genes, 628 bp and 485 bp, respectively, were proofread, edited, assembled, and concatenated with Geneious v9.1.6 (Biomatters Limited, Auckland, New Zealand). Despite the 16S sequences being originally 485 bp long, only 50 bp were used in the analysis, due to short overlap with the selected reference sequences

for *D. immaculatus*. Details on specimen vouchers, GenBank accession codes, and sampling sites of the all sequences used for this study are listed in Table 1. The alignment was performed using MUSCLE (Edgar, 2004), implemented through the Geneious plug-in, with a maximum of 10 iterations following default parameters, and further revised manually when needed. The final alignment had a sequence length of 677 bp, and the new sequences were deposited in GenBank (accession number for 12S: MG282222 to MG282255, and 16S: MG282190 to MG282212).

Estimation of genetic variables

We computed the haplotype diversity, Fu's F (Fu, 1997) and Tajima's D test for neutrality (Tajima, 1989) to infer whether the genes chosen, although slow evolving, were under selection or at mutation-drift equilibrium. The software DnaSP was used to estimate these three indices (Librado & Rozas, 2009).

In order to refine the relationships between and within the two clades, we created a haplotype network in TCS (Clement et al., 2000) with a fix connection limit at 500 steps (estimated) for the concatenated partial 12S and 16S, with all other parameters set as defaults (Clement et al., 2000). PopART (Population Analysis with Reticulate Trees; http://popart.otago.ac.nz) was used to visualise the haplotype network.

Construction of phylogenetic tree

Three types of phylogenetic trees were constructed to resolve the phylogenetic relationship between the two clades. We first employed jModelTest 2.1.10 (Darriba et al., 2012; Guindon & Gascuel, 2003) to estimate the best-fitting substitution model of the concatenated 12S and 16S rRNA genes (Huelsenbeck & Ronquist, 2001; Ronquist & Huelsenbeck, 2003). We chose the General Time Reversible model with the addition of invariant sites and gamma distribution of rates across sites (GTR+I+G) for the reconstruction of the phylogeny (AIC data not shown).

We then created a PhyML tree with the PHYML plugin in Geneious (Guindon & Gascuel, 2003), as it implemented a fast and accurate heuristic for estimating maximum likelihood phylogenies. We applied the GTR substitution model with 50,000 bootstraps and all other variables as default. We also added *Pseudacris nigrita* and *Smilica fodiens* as outgroups to the analysis (GenBank accession numbers in Table 1), such as suggested by Li et al. (2015).

We then constructed two phylogenetic trees to clarify the relationship between the two species. We first ran a RAxML tree (Geneious plugin; RAxML 7.2.8) with the model selected with 50 000 bootstrap replicates and all other variables set as default. *Pseudacris nigrita* and *S. fodiens* were also integrated as outgroups to the analysis. We finally ran a Bayesian Inference phylogenetic analysis with a single Markov Chain Monte Carlo (MCMC) analysis of 1.1 million iterations, each with 4 heated chains on MrBayes 3.2.6 (Huelsenbeck & Ronquist, 2001; Ronquist & Huelsenbeck, 2003). One tree was saved every 200 generations, and prior distribution default parameters were used. The final 50% majority rule consensus tree

was saved. For this model, 18 species extracted from Li et al. (2015) were used as outgroups, and we added four *Dryophytes japonicus* individuals to the analysis (all GenBank accession numbers in Table 1).

Osteological comparison

In order to further assess variations between the two clades, we conducted a meta-analysis on osteological information extracted from the literature. Fei et al. (2012) and AmphibiaChina (2018) provided information on the snout-vent-length (SVL) and head morphology, that was compared with the data extracted from Borzée et al. (2013). Besides, Dang et al. (2017) described the bone structure of hands for several anurans in China, while Kim et al. (2017) described the bone structure of *D. suweonensis* in the South Korea. We extracted the corresponding pictures (Fig. 1 and Fig. 2 respectively), enlarged them to 300 % and measured the length of metacarpals and phalanges for fingers 2, 3 and 4. Because scale bars were not provided for one of the figures, and because metacarpals are known to be a phylogenetically informative feature (Dang et al., 2017), we created a ratio by dividing the length of phalanges by that of the metacarpal of the same finger. We then compared the ratios obtained for the two species.
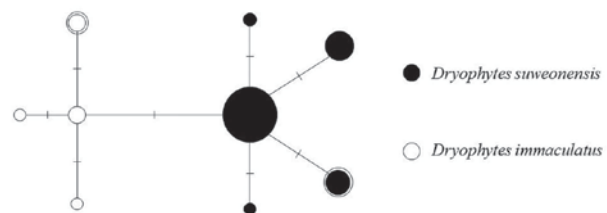

Figure 2. Haplotype network constructed with PopART overlapped on sampling localities for *D. immaculatus* (open circles) and *D. suweonensis* (solid circles). The population-specific haplotype found in Geumchon, north of Seoul for *D. suweonensis*, and Conghua, southernmost locality in China for *D. immaculatus*, are circled. The size of markers is representative of sample size (1 ≤ n ≤ 22).

RESULTS

Our results highlight the differences, and divergence albeit potentially recent, between the *D. suweonensis* and *D. immaculatus* clades. *Dryophytes suweonensis* formed a monophyletic clade in both Maximum Likelihood and Bayesian Inference phylogenies. The absence of shared haplotypes at the extremes of the ranges for both species implies the two species are distinct in terms of sequence structure for the concatenated 12S and 16S rRNA. Furthermore, the range of the two species, *D. suweonensis* extracted from Borzée et al., 2017b and *D. immaculatus* extracted both from the IUCN (Xie, 2017) and AmphibiaChina (2018), show the absence of overlap in distribution. Additionally, the published ranges for *D. immaculatus* are not properly overlapping, and sequences for one of the individuals harvested from
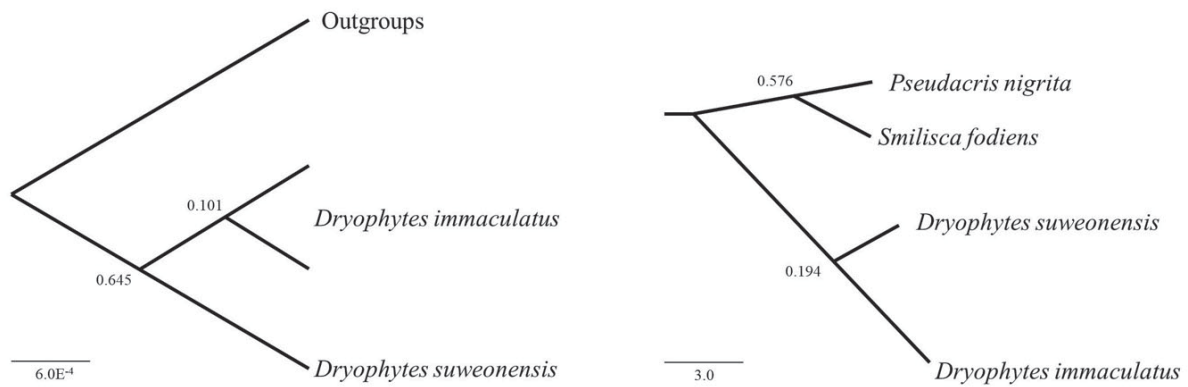
Figure 3. Simplified Maximum Likelihood Phylogenetic tree built with the PHYML plugin in Geneious (A) highlighting the segregation between the two *Dryophytes* clades and supporting the monophyly of *D. suweonensis*. Simplified Maximum Likelihood Phylogenetic tree built with the RaxML plugin in Geneious (B), with both species presented as monophyletic. Branch distances represent nucleotide substitution rate and scale bar represents the number of changes per nucleotide position.
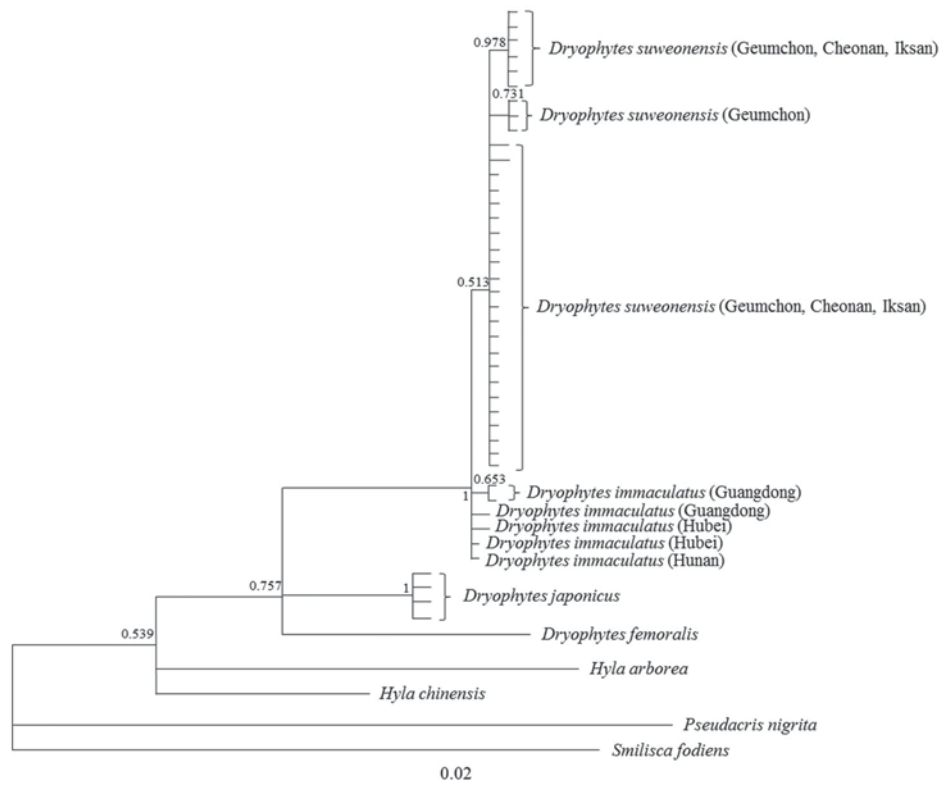

Figure 4. Bayesian Inference phylogenetic tree with a single Markov Chain Monte Carlo. This analysis also highlights the monophyly of *D. suweonensis*, despite some isolated branches. Branch distances represent nucleotide substitution rate and scale bar represents the number of changes per nucleotide position.

GenBank (isolate CIBLJT060812; Table 1) is about 800 km south of the southernmost point of the known species' range.

Estimation of genetic variables
The haplotype diversity calculated through DnaSP for *D. suweonensis* showed low genetic variability with only five haplotypes (*Hd* = 0.54, variance = 0.00812). Fu's F (F = -1.15, $p > 0.10$) and Tajima's D test for neutrality (D = - 0.99, $p > 0.10$) were not significant, thus not supporting any clear pattern of demographic dynamics.

The conflicting values of Fu's Fu and Tajima's D may indicate a low sample size or recent population reduction or bottleneck.

The haplotype network created (Fig. 2) highlights the non-overlap of common haplotypes between the two species, but also the same degree of variation (one nucleotide difference) between and among species. In addition, one of the *D. suweonensis* haplotypes is only found at the locality north of Seoul, while the isolated locality in Iksan does not display unique haplotypes. We found the same pattern for the southernmost locality for

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D. immaculatus, with all individuals from Conghua, the southernmost site, displaying the same haplotype (Fig. 2).

Phylogenetic analysis

The three models used with the phylogenetic analysis of the two concatenated mitochondrial genes were congruent for the monophyly of *D. suweonensis*, although variations were visible within the *D. immaculatus* clade, and the branching of the outgroups. The Maximum Likelihood tree constructed through PhyML highlighted the segregation between the two clades (Fig. 2A), and at the same time supported the non-monophyly of *D. immaculatus*. In contrast, the Maximum Likelihood tree constructed through RAxML suggested the early divergence between the two species, while both species were presented as monophyletic (Fig. 2B). The final Bayesian Inference analysis is congruent with both analyses on the monophyly of *D. suweonensis*, despite a posterior support of 0.512, and the phylogenetic relationships reflected by the RAxML analysis (Fig. 3).

Osteological comparison

The data provided by AmphibiaChina (2018) states that for *D. immaculatus*, male SVL is 31 mm, which is larger than *D. suweonensis* where male SVL is 293 ± 013 mm (Borzée et al., 2013). Oppositely, the cranial features extracted follow the same ratios, with the inter-nostril distance (IND) being lower than the distance between the anterior corner of the eyes (EAD), while being comparatively equal to the eye length (EL). No values are given for *D. immaculatus*, but the values for *D. suweonensis* are IND = 0.25 ± 0.02; EAD = 0.59 ± 0.03 and EL =: 0.24 ± 0.02. The ratios representative of the finger bone lengths are consistent between the two species. However, the ratios were higher for all phalanges for all fingers in *D. suweonensis*, indicating longer phalanges compared to metacarpals in *D. suweonensis* than in *D. immaculatus* (Table 2).

Table 2. Osteological comparison for the metacarpal and phalange bones of *Dryophytes suweonensis* and *D. immaculatus*, based on data extracted from Dang et al. (2017) and Kim et al. (2017). The values in the table are the ratios calculated from the length of the phalanges divided by the length of the metacarpal of the same finger.

Finger	*D. suweonensis*			*D. immaculatus*		
	2	3	4	2	3	4
Phalange 1	0.67	0.73	0.62	0.62	0.71	0.54
Phalange 2	0.35	0.61	0.60	0.11	0.58	0.59
Phalange 3		0.32	0.37		0.28	0.31

DISCUSSION

The results of our phylogenetic analyses are congruent in the monophyly of the *Dryophytes suweonensis* clade. However, and despite highlighting the absence of haplotype overlap between *D. suweonensis* and *D. immaculatus*, the haplotype network clearly shows the relatedness of the two clades, and the same genetic variation between and within clades. The phylogenetic analyses also highlight the low variation between the two clades, however, a generally similar level of variation is found between the closely related *Hyla molleri* & *H. orientalis* (Stöck et al., 2012) and there is an even lower variation between *H. intermedia* and *H. perrini*, a new cryptic species (Dufresnes et al., in review), previously called "clade N" by Canestrelli et al. (2007a, 2007b) and "new taxon 2" by Stöck et al. (2008, 2012). The pattern described here could very well be the one of a ring species, although more variable markers such as microsatellites, and additional sampling between the sites currently available, would be required to confirm such a claim. This pattern is consistent with the fact that landscape features usually are adequate predictors of genetic variations (reviewed by Storfer et al., 2007), such as seen here with the Yellow Sea creating a barrier between the two clades.

Despite being based on a single samples and averages for the two species, the osteological comparisons highlight differences between the two clades. While *D. suweonensis* is smaller than *D. immaculatus*, the bones of its hands are comparatively longer. This is likely related to variations in their breeding ecology: *D. suweonensis* calls while holding on leaf and vegetation, with the metacarpals and phalanges wrapped around the blade of the leaf (Borzée et al., 2016a). The breeding ecology of *D. immaculatus* has not been described, but based on these results, we expect differences between the two species. Li et al. (2015) and Dufresnes et al. (2016) recommended the synonymy of *D. suweonensis* (Kuramoto, 1980) and *D. immaculatus* (Boettger, 1888), under the name *D. immaculatus* as junior synonym. We, however, call for further analysis before this conclusion. Clearly, additional basic ecological and behavioural research on *D. immaculatus* is required before being able to answer the question, as even the range of the species is largely undescribed.

To better determine the status of species or subspecies of the two populations, we recommend a Total Evidence Analysis that would include call properties, morphometrics and genetic information, such as conducted for the phylogeny of *Dendropsophus elegans* (Forti et al., 2017). It has been suggested that intraspecific variation in call properties are not adequate to recover phylogenetic history due to selective factors related to sexual selection. However, inter-species phylogeny is accurately described by acoustic properties due to genetic isolation processes, in relation to species recognition and ecological pressure (Fonseca et al., 2008; Forti et al., 2017). Call variables are expected to be significantly different for species diverged for at least 6.5 million years (Forti et al., 2017). The alternation between periods of divergence and contact between *D. suweonensis* and *D. immaculatus* may have occurred repeatedly since the Triassic (Haq et al., 1987), with the repeated resurgence and decline of the Yellow Sea (Jingtai & Pinxian, 1980; Oba et al., 1991; Liu et al., 2009). Accordingly, the two clades may have been partially isolated, starting the process of speciation through isolation. Then, they were

brought back in contact during glacial maxima and the creation of the land-bridge over the Yellow Sea, before being isolated again. This is supported by the fact that the Korean Peninsula is known to have acted as a refugium for the ancestor of *Pelophylax* and *Onychodactylus* species (Zhang et al., 2008; Yoshikawa et al., 2008), and thus enabled the survival of clades that had previously colonised the area.

Alternatively, the two clades may be part of a ring-species, as individuals were not isolated in different refugia during LGM. This proposition is supported by the absence of ice sheets on the Korean peninsula circa 126,000 years ago (Kong, 2000; Walker et al., 2009; Yi & Kim, 2010), partially due to the presence of a shared monsoon regime because of the uplifting of the Himalayan range (An et al., 2001; Harris, 2006). This is also supported by the fact that the Korean peninsula was last connected to the Chinese mainland during the late Pleistocene (Millien-Parra & Jaeger, 1999). In this case, the clustering of individuals in a single clade implies the repeated gene flow between the Korean Peninsula and the Asian mainland. Besides, such clustering is supported by the shared paleo-river basin due to the southward flow of the Amur River, west of the Sakhalin Islands, due to the ice sheet blocking its current bed (Grosswald & Hughes, 2005). Furthermore, the paeleo-Yangtzae and paleo-Yellow rivers were closer than they are today and merged with the paleo-Han river (Ryu et al., 2008).

Within each of the Korean Peninsula and China, the north/south genetic structure was unexpected because of the continuous low plains allowing for easy dispersal of individuals, such as exemplified by *D. suweonensis* (Borzée et al., 2015b). This may indicate strong philopatry of *D. suweonensis*, leading to a larger genetic divergence than expected and population management for *Dryophytes* spp. needs to take into account the origin of individuals for introduction and translocations plans (Gascon, 2007). This is especially true if the individual *D. suweonensis* isolated on the phylogenetic tree (Fig. 3) are not monophyletic with the main *D. suweonensis* clade, due to hybridisation with *D. japonicus* (Borzée et al., 2015b).

We cannot confirm here the clade status of the two species, but we clearly highlight variations between the two clades. Thus, based on the genetic and osteological data available, and as the ranges of two species do not overlap and are consequently reproductively isolated, we recommend the preservation of the two clades at the species level until clear data becomes available. Considering the two species as the same entity would be a hastily made decision that would not be based on rigorous scientific knowledge. It would also decrease the amount of research conducted on these organisms as it would extend the range of the species, leading the Korean populations to a non-threated level, and resulting in the loss of interest, and research fund, generally attributed to endangered species.

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