

Genomic adaptations for vertical ecological niche diversification in Asian tree frogs

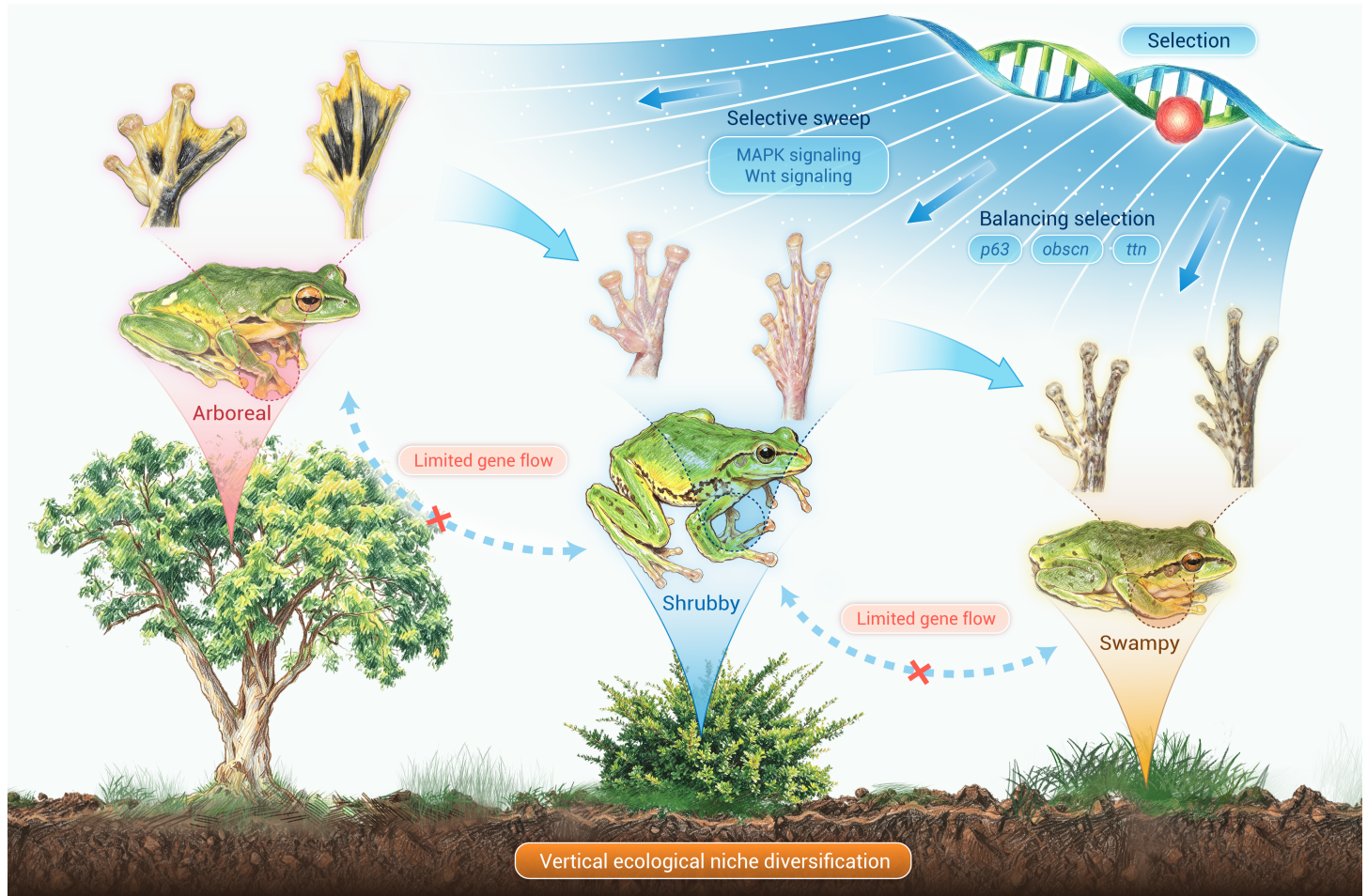
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GRAPHICAL ABSTRACT



PUBLIC SUMMARY

- Webbing morphology of Asian tree frogs is strongly correlated with their vertical niche position.
- Limited gene flow across distinct vertical strata reinforces speciation along the vertical ecological gradient.
- Positive and balancing selection on functionally distinct genes drive the evolution of webbing morphology.

Genomic adaptations for vertical ecological niche diversification in Asian tree frogs

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Species occupying heterogeneous ecological niches often exhibit pronounced phenotypic and genomic divergence, yet the mechanisms driving ecological differentiation remain unclear. Asian tree frogs (*Rhacophorus sensu lato*) span a vertical gradient from swamp, shrub, to arboreal habitats, providing an ideal system to investigate the evolutionary mechanisms underlying niche specialization. Here, we quantified interdigital webbing morphology in 630 individuals and generated genome-wide SNP data for 161 individuals representing 29 species. Our results show that webbing morphology is strongly correlated with vertical niche position. Phylogenomic analyses resolved ambiguous relationships and revealed a trend toward webbing reduction. Based on the clear phylogenetic relationships, introgression among distinct niches was inferred. Introgression was limited primarily between adjacent niche pairs (shrubby-swampy), while nearly complete absence of haplotype sharing across distinct niches indicates strong ecological barriers to gene flow. Selective sweep mapping identified 760 candidate genes exhibiting selection signals between arboreal and swampy groups, enriched in a wide range of pathways related to skeletal development (such as MAPK signaling) and cytoskeletal organization (such as Wnt signaling)—consistent with climbing and gliding adaptations. In contrast, long-term balancing selection preserved ancient polymorphisms to maintain functional allelic variation in genes associated with epithelial developmental plasticity (*p63*) and muscle architecture (*obscn*, *ttn*), with transposable elements potentially modulating regulatory variation. Our findings demonstrate that contrasting signatures of positive and balancing selection on functionally distinct genes drive the evolution of webbing morphology and locomotor traits, and limited gene flow across adjacent niches prevents homogenization, which together may contribute to adaptive diversification along vertical gradients.

INTRODUCTION

Ecological heterogeneity exerts complex selective pressures that drive phenotypic and genomic differentiation, ultimately leading to population divergence and subsequent speciation.¹ These ecological interactions generate phenotype-environment correlations that can form barriers to gene flow between species and reinforce ecological divergent selection.² Under persistent selective pressures within distinct habitats, adaptive divergence occurs at both morphological and genomic levels,^{3–6} guiding populations toward trait combinations that optimize fitness under local ecological constraints.² Most fitness-related traits are quantitative and polygenic, influenced by pleiotropic interactions. Therefore, understanding the emergence of ecologically relevant phenotypes requires integration of evolutionary dynamics with trait-based and genomic analyses.⁷ Differentiation across ecological gradients may result from directional selection, balancing selection, genetic drift, or combinations thereof.⁸ Clarifying the dominant forces requires linking theoretical mechanisms with empirical research to elucidate the evolutionary dynamics of ecological niche divergence.^{9,10}

Vertical stratification fosters species coexistence by partitioning habitats along structural gradients, imposing distinct selective pressures that drive

phenotypic diversification.^{11,12} This ecological segregation creates discrete adaptive zones, each with unique biomechanical and physiological demands. Arboreal snakes, for instance, have evolved elongated tails and more slender bodies to mitigate gravitational load and enhance stability in arboreal environments.^{13–15} Within Viperids, this arboreal adaptation manifests as constrained morphological evolution toward intermediate body size coupled with tail elongation, reflecting specific cardiovascular and mechanical solutions to life in trees.¹⁴ Similarly, in *Anolis* lizards, locomotor performance varies systematically with habitat height, and corresponding modifications in toe pads and feet enhance movement efficiency on arboreal substrates.^{16–18} Such convergent patterns across distantly related taxa underscore the power of natural selection in shaping phenotypic diversification. At a broader taxonomic scale, amphibians exhibit analogous patterns of stratum-specific adaptation. Global amphibian assemblages display pronounced latitudinal gradients in vertical strategies: arboreality peaks in tropical rainforests where dense vegetation and climatic stability prevail, whereas fossorial lifestyles dominate in arid and highly seasonal regions.¹¹ This global gradient in vertical strategies suggests that ecological opportunity and environmental predictability play key roles in niche evolution. However, while these patterns of association between phenotype and environment are well documented, the underlying evolutionary mechanisms remain poorly understood. Fundamental questions persist regarding the relative contributions of directional selection, including selective sweeps on key traits, versus balancing selection that maintains functional allelic variation, as well as the role of stochastic genetic drift in shaping patterns of vertical niche diversification.

Asian tree frogs (*Rhacophorus sensu lato*), currently including three recognized genera: *Rhacophorus*, *Leptomantis*, and *Zhangixalus*,¹⁹ represent a compelling example of adaptive radiation across vertical niche space. Broadly distributed across South and Southeast Asia, these anurans have diversified across a range of microhabitats, from swampy and shrubby layers to the upper arboreal strata, giving rise to derived morphological features and extensive ecological specialization.²⁰ While each genus encompasses species occupying multiple vertical zones, individual species tend to specialize within specific microhabitats, driving the evolution of habitat-associated phenotypic traits. Slender limbs combined with strong grasping abilities prevent falling and facilitate vertical movement, while a bony knob on the third metacarpal enhances grip during vertical climbing.²¹ Quantitative assessments have further revealed that arboreal taxa possess significantly elongated forelimbs and hands, traits associated with improved feeding efficiency and balance maintenance in arboreal life.²² Beyond morphology, ecophysiological studies underscore genus-specific specializations that correspond to distinct microhabitat use. For instance, *Rhacophorus* species exhibit dermal modifications that enhance cutaneous respiration, while *Zhangixalus* species display osteological specializations in the pectoral girdle that optimize force transmission during substrate attachment.^{23,24} Such trait divergence reflects adaptive radiation driven by niche partitioning, with each lineage evolving specialized phenotypes to exploit distinct arboreal microhabitats.²⁵

Specially, interdigital webbing represents a key morphological innovation in rhacophorid tree frogs, exhibiting pronounced variation across taxa—from

extensive, fully developed membranes to markedly reduced or vestigial structures.²⁶ This variation corresponds closely to ecological specialization: arboreal species such as *R. kio* possess fully expanded interdigital webbing adapted for gliding locomotion, whereas swamp-dwelling species like *Z. minimus* retain only rudimentary hand webbing and partial foot membranes limited to approximately one-third of toe length. The functional significance of this trait is well established in other amphibian lineages—interdigital webbing enhances arboreal performance in tropical salamanders—suggesting that it similarly contributes to survival and habitat specialization in tree frogs.²⁷ Recent developmental investigations in *R. kio* have uncovered the molecular underpinnings of webbing variation. Differential expression of Wnt signaling components and angiogenesis-associated genes influences both interdigital membrane expansion and toe-pad morphogenesis.³ Specifically, *TCF7L1* and *TCF7L2* act as key regulators of limb morphogenesis, while vascular remodeling genes such as *Hes5* and *Ednrb* contribute to webbing formation and oxygen delivery required for sustained climbing activity. Despite these emerging insights into the developmental basis of webbing, the evolutionary and genomic mechanisms driving its divergence across ecological contexts remain largely unexplored.

Based on the above observations, we hypothesize that variation in webbing morphology among Asian tree frogs is correlated with vertical ecological niche diversification. To test this hypothesis, we quantitatively characterized interdigital webbing morphology across a representative set of tree frog species occupying a microhabitat gradient spanning swamps, shrubs, and arboreal strata. In parallel, we generated genome-wide single-nucleotide polymorphism (SNP) datasets using high-throughput sequencing for representative Asian tree frog species. This integrative approach was designed to address three central questions: (1) Does webbing morphology constitute an adaptive trait correlated with vertical niche diversification? We evaluated this through statistical correlation between webbing metrics and microhabitat position. (2) What is the evolutionary pattern of webbing traits and vertical niche usage across the phylogeny? We assessed this through phylogenetic reconstruction, ancestral state estimation, and population genetic analyses to elucidate the evolutionary trajectory of morphological and ecological divergence. (3) What is the potential genetic basis underlying adaptive divergence in webbing morphology? We addressed this by identifying candidate genes under recent selection sweep and long-term balancing selection, thereby inferring possible molecular pathways associated with phenotypic adaptation to vertical microhabitat zones. The accumulation of genetic differences will contribute to significant population divergence and drive speciation.

MATERIALS AND METHODS

Measurements of web-to-space ratio

A total of 630 individuals representing 49 Asian tree frog species (18 *Rhacophorus*, five *Leptomantis* species, and 26 *Zhangixalus* species) and three outgroup species (*Feihyla fuhua*, *Polypedates mutus* and *Kurixalus odontotarsus*) were collected to measure the web-to-space ratio (the ratio of interdigital webbing area to interdigital space area) of both hands and feet (Table S1). Only adult males were included to control for sexual dimorphism. Each specimen was photographed for measuring the size of the webbing and the whole space between each pair of fingers on the left side (Figure S1) using ImageJ v1.42.²⁸ The area of interdigital webbing and the corresponding interdigital space in a full hand or foot was summed for the calculation of web-to-space ratio. To standardize the measured data, the web-to-space ratio was defined as the total webbing area divided by the total interdigital space area, separately for hands and feet.

Niche definition and principal component analysis (PCA) of webbing morphology in relation to ecological niches

The Asian tree frogs were divided into arboreal, shrubby or swampy groups according to their ecological niches based on the primary microhabitat that a species occupies the most time throughout its life history (see details in Tables S1 & S2). Swampy species inhabit the lower layer, usually dwelling by water and within grass. Shrubby species occupy the middle layer, typically found in shrubs. Arboreal species reside in the upper layer, usually perching on tree branches and canopies of tall trees. Geographic distributions and sampling sites are shown in Figure 1A, generated using QGIS Desktop 3.28.8

(<http://qgis.osgeo.org>), with species abbreviations and geographic coordinates (latitude/longitude) listed in Table S2. Web-to-space ratios for each ecological group were visualized using boxplots in R. Principal component analysis (PCA) was conducted using the “prcomp” function in R, based on web-to-space ratio data of each interdigital webbing from both hands and feet. Confidence ellipses were set to 95% to visualize group-level variation.

DNA sample collection and genomics sequencing

A total of 161 individuals representing 29 species were collected, including 20 *Zhangixalus* species, six *Rhacophorus* species, and three outgroup taxa (*Feihyla fuhua*, *Polypedates mutus* and *Kurixalus odontotarsus*) (Table S2). Since *Leptomantis* has few species and is entirely distributed in Maritime Southeast Asia, outside China, genomic samples could not be obtained. Genomic DNA was extracted from liver or muscle tissues preserved in 95% ethanol. DNA fragmentation, library construction, and sequencing were carried out by Annoroad Gene Technology (Beijing) Co., Ltd. (Beijing, China). For each individual, 0.2–3.0 µg of DNA was randomly fragmented by sonication to generate a short-insert library (length < 500 bp). Library preparation followed standard Illumina protocols, and sequencing was based on a DNBseq-T7 platform. Each sample was sequenced to a target depth of 15× relative to the *Zhangixalus dugritei* reference genome.

Quality control, single nucleotide polymorphism (SNP) calling and phylogenetic analysis

Raw sequencing reads were quality controlled using Fastp v0.21.0²⁹ with low-quality reads removed (Table S3), yielding a total of 10.3 Tb clean reads. Clean reads from each individual were mapped to the *Z. dugritei* genome assembly³⁰ as a reference using BWA-MEM v0.7.17-r1188³¹ with default parameters. Samtools v1.9^{32,33} was employed to mark duplicates and to sort the output alignment reads. Subsequently, Genome Analysis Toolkit (GATK) v4.1.9.0³⁴ was utilized for local rearranging of reads to improve the quality of alignments in regions surrounding potential indels.

For SNP calling, low-quality alignments were filtered using the multi-way pileup method (mpileup) in BCFtools v1.9³⁵ based on the following criteria: mapping quality score less than 20; base quality less than 20; multiple best hits and adjusted mapping quality less than 50. The method of “call” in BCFtools was employed to detect SNP and indel variants. Finally, a total of 179,810,217 high-confidence SNPs were retained after removing sites with minor allele frequency less than 0.01; missing data over 0.75; quality score below 30 and coverage depth less than 4 or average depth over 60.

For phylogenetic inference, linkage disequilibrium pruning was applied to the final VCF using PLINK v1.9³⁶ with the parameter “-indep-pairwise 50 10 0.2” to remove correlated loci. The filtered VCF was converted to PHYLIP format using vcf2phylip v2.6 (<https://github.com/edgardomortiz/vcf2phylip>). Alignments were further refined with trimAl v1.4.rev15³⁷ using the “-gt 1” option to exclude gap-containing sites, producing a final concatenated alignment of 131,098 bp.

Maximum-likelihood phylogenetic reconstruction was performed in IQ-TREE v1.6.5,³⁸ with node support evaluated using 1,000 bootstrap replicates and the SH-like approximate likelihood ratio test (SH-aLRT)³⁹ (Figure S2). Divergence times were estimated using MCMCTREE in PAML 4 package.⁴⁰ One individual per species was randomly selected for temporal analyses. Markov chain Monte Carlo (MCMC) sampling was conducted for 200,000 generations, with the initial 20,000 generations discarded as burn-in and sampling performed every two generations. The independent rates clock model (clock = 2) and HKY85 substitution model (model = 4) were applied, with the root age constrained to less than 60 million years. Three calibrated priors, which came from secondary calibration points in a previous phylogenetic study of rhacophorid frogs,⁴¹ were used as age constraints including 38.7–29.6 million years ago (Mya) for *Feihyla fuhua* and *Polypedates mutus*, 45.5–34.7 Mya for *Kurixalus odontotarsus* and Asian tree frogs, and 9.8–5.5 Mya for *R. norhayatae* and *R. rhodopus*.

Variance partitioning, ancestral character-state reconstruction and admixture clustering

To assess the relative contributions of ecological niche, genetic structure, and geographic location to variation in webbing morphology, distance-based

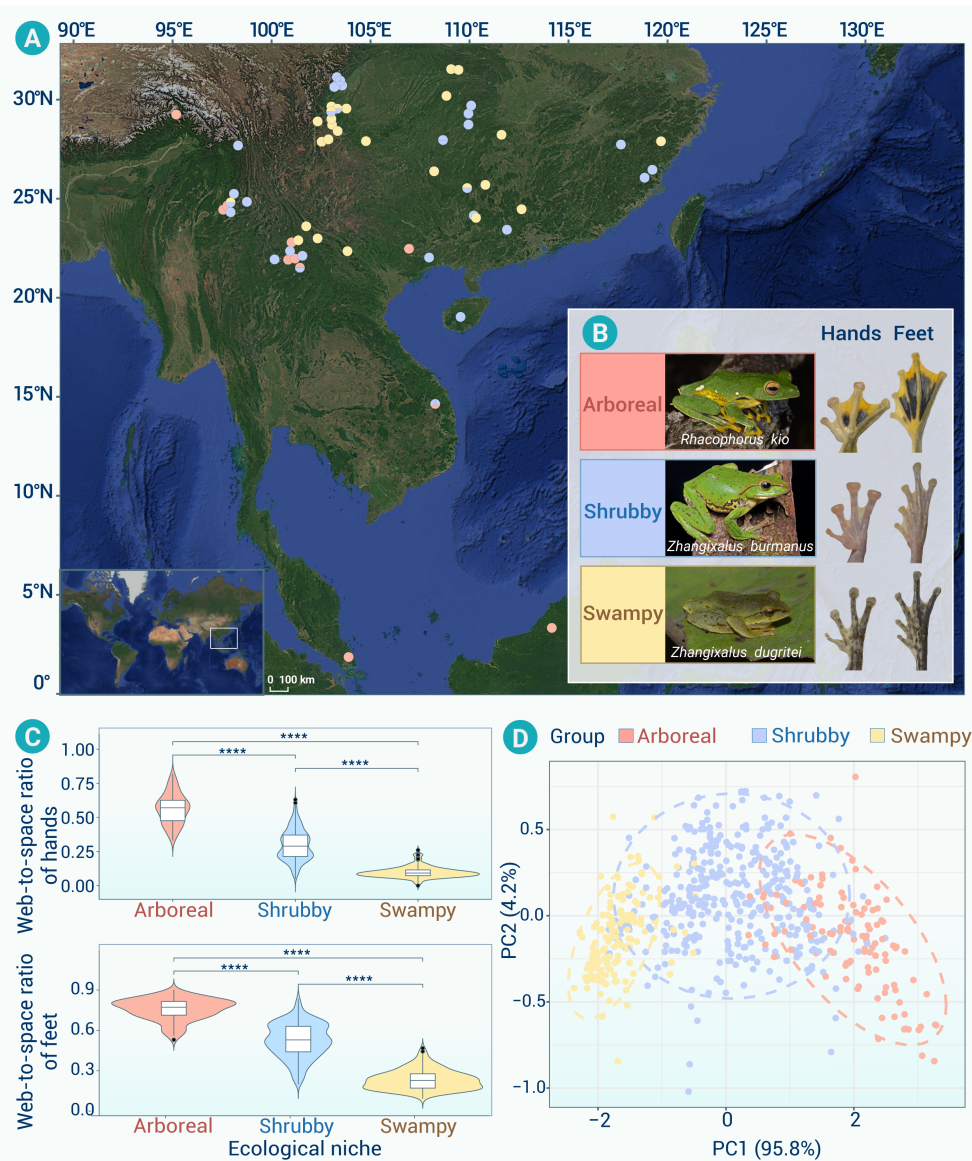


Figure 1. Geographic sampling and webbing morphological differentiation of *Rhacophorus sensu lato* species (A) Geographic distribution of sampling sites, with each specimen represented by a colored point. (B) The three niche-defined groups and their corresponding webbing morphologies. Arboreal groups marked in pink; shrubby group marked in light blue and swampy group marked in light yellow. (C) Variation in web-to-space ratio across ecological niches. The web-to-space ratio represents the ratio of interdigital webbing area to interdigital space area. Measurement protocols are described in Materials and Methods section and Figure S1. (D) Principal component analysis (PCA) of both hand and foot morphological traits. The ellipses represent the 95% confidence intervals for each ecological niche group.

between species can be calculated using f -branch statistic fb based on $f4$ admixture ratios. To detect inter-population introgression, we performed an additional Dsuite analysis involving three ecological niche populations, comprising individuals from arboreal ($n = 24$), shrubby ($n = 63$), and swampy ($n = 71$). Furthermore, detailed genome regions of potential introgression were further explored by calculating D-statistic with a sliding window of 50 kb and a step size of 10 kb. TreeMix⁵¹ was used to reconstruct gene flow among habitat-defined groups based on a maximum-likelihood framework, with the migration parameter set to $-k 200$.

Identification of genes by selective sweeps

Genomic regions exhibiting markedly reduced genetic diversity across populations were considered candidates for positive selection, consistent with signatures of selective sweeps.⁵² F_{ST} values and pairwise nucleotide diversity (π) values were generally used for genetic distances and differentiation degrees calculation between populations. We used F_{ST} and π to identify genes under selection pressure between the arboreal and swampy

redundancy analyses (db-RDA)⁴² and associated variance partitioning⁴³ were conducted using the “vegan” package in R.⁴⁴ Ancestral character-state reconstruction was performed in Mesquite 3.6⁴⁵ using maximum parsimony, which minimizes the total number of evolutionary transitions across the phylogeny. To ensure that individuals used for phylogenetic analysis were morphologically representative, PCA was conducted on phenotypic data from 105 sequenced individuals, excluding outgroup samples and those lacking morphological measurements. PCA was performed using the LEA v2.0 package in R,⁴⁶ with 95% confidence ellipses applied. The statistical significance of eigenvectors was assessed through the Tracy-Widom distribution test. Admixture clustering was inferred using ADMIXTURE v1.3.0,⁴⁷ with subpopulations (K) ranging from 1 to 26. The K value corresponding to the lowest cross-validation (CV) error was selected as the optimal model.

Shared haplotypes inference and introgression analyses

In order to understand the relationships among these Asian tree frogs, we employed two methods to infer the shared haplotypes and history of introgression. Identity-by-descent (IBD) blocks analysis was conducted to identify the shared haplotypes among species utilizing Refined IBD⁴⁸ on SNP data genotype-imputed with Beagle 5.2.⁴⁹ Dsuite⁵⁰ was applied using the Dtrios module to calculate Patterson's D (ABBA-BABA) statistic in order to estimate the history of introgression and secondary contact between Asian tree frog species. This test evaluates gene flow between P2 and P3 ($D > 0$) or P1 and P3 ($D < 0$) based on allele pattern asymmetries across four-taxon sets (P1, P2, P3, and outgroup). By combining with the phylogeny, excess allele-sharing

groups. Selection signals were identified by performing pixy⁵³ with a sliding window of 50 kb to calculate the F_{ST} values and π ratio ($\pi_{\text{arboreal}}/\pi_{\text{swampy}}$). Candidate regions were defined as those falling within the top 5% of F_{ST} values and either the top or bottom 5% of π ratio, following previously reported methods.⁵⁴ Genes located within these regions were annotated and subjected to Kyoto Encyclopedia of Genes and Genomes (KEGG) and gene ontology (GO) functional enrichment analysis using clusterProfiler v4.0.2.⁵⁵ The interaction networks among genes, KEGG pathways and GO terms were visualized using Cytoscape.⁵⁶

Detection of balancing selection, transposable elements (TEs) and topology weighting

To investigate ancient polymorphisms maintained by balancing selection, genotypes counting and format conversion were performed using glactools.⁵⁷ BetaScan⁵⁸ was subsequently employed with a maximum window size of 50 kb to calculate β values for each SNP across the genome. Previous reports revealed that the β scores of those in the top 1% or 0.1% of SNPs are considered to encompass most of the extreme β scores.^{58–60} To obtain the most accurate genes under balancing selection pressure, we collected the SNPs with β values of the extreme top 0.01% and overlapped their genomic coordinates with full-length gene regions. Transposable elements (TEs) were annotated in the reference genome using RepeatMasker (<http://www.repeat-masker.org>) with the default Repbase database. TE insertions intersecting significant SNPs were identified, and their genomic positions were mapped relative to gene structures. To investigate the impact of balancing selection on regional phylogenetic topology, topology weighting among populations

was estimated for both genome-wide and balancing selection-enriched regions using Twisst2.⁶¹ Output visualizations were generated with the accompanying R script provided by the developers. Significance was assessed by bootstrap resampling from the genome-wide regions, resampling the same number of windows as contained in each gene region, and repeating this 10,000 times.

RESULTS

Geographical distribution and morphological divergence across vertical ecological niches

Arboreal species of Asian tree frogs are predominantly distributed throughout Southeast Asia, particularly in the western and southern margins of the Hengduan Mountains, where they occur in sympatry with selected shrubby and swampy taxa (Figure 1A). In contrast, shrubby and swampy species exhibit extensive sympatric distributions across South China and the adjacent ranges of Hengduan Mountains. Morphological analyses encompassing 630 individuals (Table S1) enabled quantitative assessment of interdigital webbing across three ecologically defined groups (Figures 1B & S1). Web-to-space ratios of both hands and feet followed a consistent ecological gradient: arboreal > shrubby > swampy (Figure 1C), with statistically significant differences observed among all groups (p -value < 0.0001). PCA of integrated hands and feet traits further resolved distinct morphological clustering among the three niche categories (Figure 1D). While partial overlap was observed between shrubby-arboreal and shrubby-swampy groups, arboreal and swampy species exhibited complete morphological separation.

Phylogeny-informed evolutionary dynamics of morphology-niche coupling

Variance partitioning revealed that webbing morphology and phylogenetic relationships showed strong correlation with vertical ecological niche diversification, independently explaining 26.8% and 26.0% of the variation, respectively, whereas geographic distribution contributed only 2.5% (Figure S3). Individuals used in re-sequencing and phylogenetic analyses were confirmed to be morphologically representative of the broader dataset (Figure S4). The phylogenetic topology and divergence estimate of these individuals (Figure S5) were consistent with previous phylogenetic reconstructions,^{19,41} supporting the robustness of the inferred species relationships. To probe further the evolutionary dynamics of webbing morphology among the three niche groups, we conducted ancestral character-state reconstruction of web-to-space ratios across the phylogeny in 161 re-sequenced individuals (Figures 2A & C). Both *Rhacophorus* and *Zhangixalus* exhibited evolutionary trajectories favoring webbing reduction. Several *Zhangixalus* lineages displayed clade-specific divergence. For instance, *Z. smaragdinus* and *Z. pachyproctus*—both arboreal—exhibited intermediate webbing: greater than that of shrubby species but reduced relative to other arboreal taxa. The swampy species *Z. chenfui* was positioned as an early-diverging lineage relative to shrubby species *Z. burmanus* and *Z. omeimontis*. To delineate the genetic origins and structural composition of Asian tree frogs, we conducted admixture analyses (Figures 2B & S6–S7). Several shrubby and arboreal taxa with close phylogenetic proximity shared highly similar genomic profiles, with analogous patterns observed between shrubby and swampy groups, mirroring trends in morphological clustering. It should be noted that these admixture analyses are exploratory and are not intended as formal inference of population structure.

Extensively shared haplotypes within populations and limited introgression across niche boundaries

To investigate the relationships among the species, we analyzed the patterns of shared IBD haplotypes. The results indicated extensively shared haplotypes prevalent within populations of the three niches (Figure 3). Among the shared IBD blocks between species (Table S4), 79.7% (3595) were detected within the swampy population, 13.5% (608) were detected within the shrubby population, and 6.8% (305) were detected within the arboreal population. The length of IBD haplotypes shared between species ranged from 1.5 Mb to 8.7 Mb, with a median of 2.2 Mb. However, only four blocks were detected across shrubby and swampy populations, and just one block was found across arboreal and shrubby populations. To further investigate intro-

gression and secondary contact among species from different vertical ecological niches, we calculated the excess allele-sharing between all the species pairs using f_b -branch statistic f_b (Figure 4). The statistic f_b was computed based on f_4 admixture ratios (Table S5) and in combination with the species phylogenetic relationships. The most significant introgression was detected between arboreal species *R. norhayatae* and swampy species *Z. dugritei*, which is consistent with our admixture analysis. *R. norhayatae* also exhibited excess allele-sharing across multiple groups and species, with significantly higher ABBA score than BABA score in all D-statistics (Table S5). This suggests that the observed introgression between *R. norhayatae* and other species likely reflects vertical inheritance from a common ancestor rather than recent gene flow. A second notable introgression signal was detected between the swampy species *Z. hongchibaensis* and several shrubby and swampy species located on the terminal branches of the phylogenetic tree. Approximately half of the D-statistics comparing *Z. hongchibaensis* with its phylogenetically close relatives showed ABBA scores lower than BABA scores, indicative of gene flow between swampy species and shrubby species. The Dsuite (Table S6) and TreeMix (Figure S8) analyses of the three ecological niche populations further supported this pattern, and revealed only one gene flow event between the shrubby (P2) and swampy (P3) populations. In addition, a weak D-statistic signal was detected between the arboreal (P2) and shrubby (P3) population. In total, 170 genes exhibited signatures of introgression (see Table S7) with significant D-statistic values among the three ecological niche populations.

Selective sweep mapping of genes associated with arboreal-swamp divergence

To understand the genomic divergence landscape induced by evolutionary forces, genomic regions under selection were identified between the morphologically distinct arboreal and swampy groups (Figure 5A) based on F_{ST} values (top 5%) and π values (top and bottom 5%). A total of 760 unique genes were located within regions exhibiting selection signals, including 668 under positive selection in arboreal species and 94 under positive selection in swampy species (two overlapping). KEGG and GO functional enrichment analyses identified 12 enriched KEGG pathways and 10 enriched GO terms encompassing 130 genes (Figures 5B–C & Table S8). Many of these pathways and terms were associated with cell proliferation, cell differentiation and apoptosis, including the p53 signaling, FGF signaling, MAPK signaling, Ras signaling, Hippo signaling and cell differentiation. Additional pathways and terms were related to cytoskeletal dynamics and cell adhesion, such as Wnt signaling, tight junction, axon guidance, extracellular matrix organization, and epithelial tube branching morphogenesis. Notably, 32 putative copies of *prkcd*, which were identified via genome annotation, were found to be under selection and co-enriched across multiple pathways and terms. Several enriched categories also involved vascular formation, function, and regulation, including the VEGF and Endothelin signaling pathways and angiogenesis. Within regions showing significant selection signals, eight genes associated with the MAPK signaling pathway were identified, including *mapt*, *max*, *fgf18*, *mapk8ip3*, *taok2*, *raf1*, *cacna1e* and *mkkn1* (Figures 6A–B).

Genes and putative regulatory factors under long-term balancing selection

Ancient polymorphisms across species can be maintained by long-term balancing selection.^{62,63} Strong balancing selection signals spanning entire gene regions were detected for 11 genes (Figure 7A & Table S9). Among these, coding-region signals were detected in nine genes, including *mecom-a*, *col11a1*, *col12a1*, *nrip2*, *vcn*, *zfhx4*, *col2a1*, *obscn* and *ttn*. Additionally, 40 TE regions associated with nine genes exhibited significant balancing selection signatures (Table S10), comprising 21 DNA repeat elements, 15 long interspersed nuclear elements (LINEs), two long terminal repeats (LTRs) and two unknown TEs. A 25 kb interval spanning 77.65–77.90 Mb on chromosome 6 showed a particularly strong selection signal and contained two giant protein genes (Figure 7B), *obscn* and *ttn*. Multiple loci on chromosome 4 also displayed elevated β values (Figure 7A), including regions containing the tumor protein gene, *p63*. Topology weighting analysis revealed contrasting phylogenetic signals between genome-wide patterns and loci under balancing selection (Figure 7C). Genome-wide data predominantly supported topology

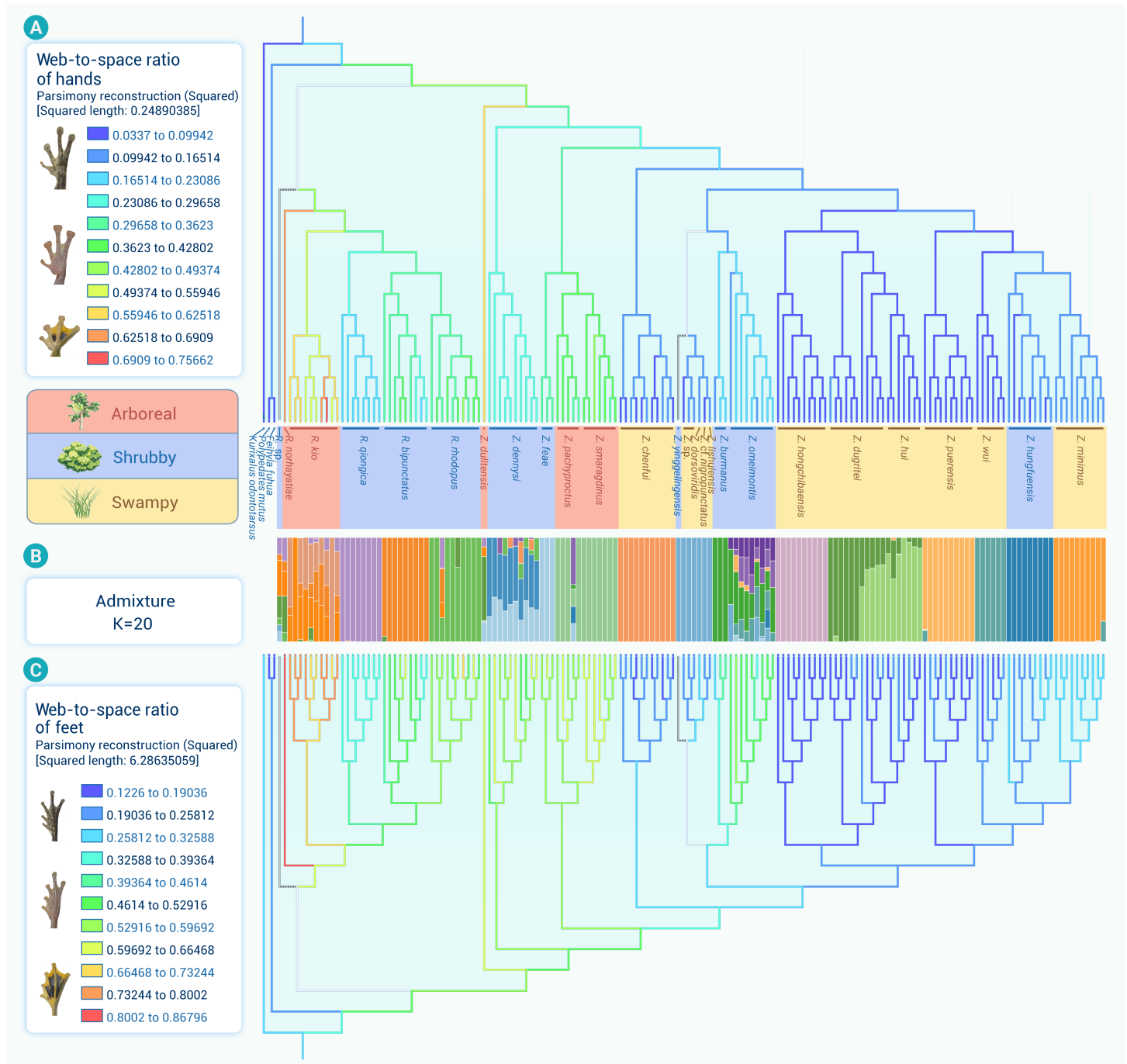


Figure 2. Morphological evolution and niche differentiation in Asian tree frogs (A) and (C) Ancestral character-state reconstruction of interdigital webbing across the phylogeny. Arboreal groups marked in pink; shrubby group marked in light yellow and swampy group marked in light yellow. Branches are color-coded by web-to-space ratio from red (complete webbing) to green (intermediate webbing) and to blue (reduced webbing). (B) Admixture analysis of population structure in Asian tree frogs. Subpopulation number was set to K = 20, which yielded the lowest cross-validation (CV) error. The other admixture analysis and the CV error line chart from K = 2 to 26 were plotted in Figures S6-S7.

1, consistent with inferred gene flow structure (Figure S8). In contrast, most balancing selection-enriched genes (8 of 11, 72.7%) favored topology 2, indicating closer genetic affinity between shrubby and swampy groups. Six of these genes (*mecom-a*, *col11a1*, *col12a1*, *vcan*, *col2a1* and *auts2*) showed significantly higher average weighting for topology 2 than the genome-wide average, whereas the remaining two (*npr2* and *p63*) exhibited significantly higher weighting for topology 3 and significantly lower weighting for topology 1 (Table S11).

DISCUSSION

Phylogenetic resolution and webbing-niche correlation in Asian tree frogs

The phylogenomic topology reconstructed from genome-wide SNP re-

sequencing data largely corroborates previously published frameworks for the Asian tree frogs clade,^{19,41,64,65} while offering enhanced resolution for historically ambiguous relationships. Notably, the phylogenetic placement of *Z. yinggelingensis*, which was previously inferred from limited mitochondrial and nuclear markers,⁶⁶ was confidently resolved. Divergence between the sister lineages *Zhangixalus* and *Rhacophorus* was estimated at 32.21–45.31 Mya, somewhat earlier than previous estimates.⁴¹ It should be noted, however, that the outgroups employed in this study, while belonging to the family Rhacophoridae, diverged relatively recently from the ingroup taxa. This phylogenetic proximity may have resulted in branch length compression within *Rhacophorus* (Figure S2) and introduced potential calibration biases. Broader taxon sampling encompassing more deeply divergent outgroups will be necessary to address this limitation in future divergence-time estimates.

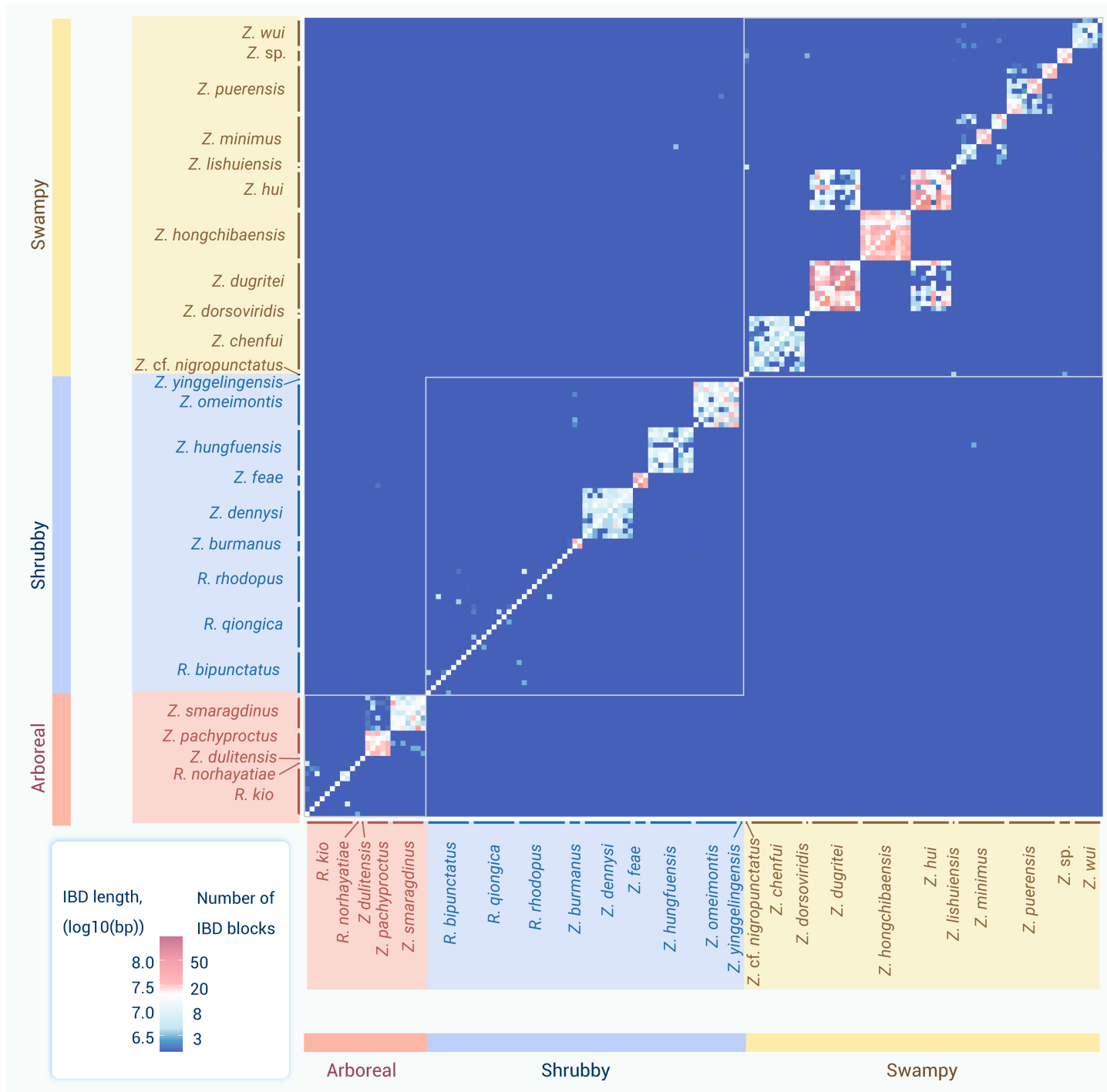


Figure 3. Shared haplotype among individuals within the genus Asian tree frogs Pairwise comparisons of total identity-by-descent (IBD) segment length and IBD block count were shown on either side of the diagonal. Arboreal groups marked in pink; shrubby group marked in light blue and swampy group marked in light yellow.

Additionally, the use of a single reference genome raises the possibility of reference bias, which may impact SNP calling and consequently affect phylogenetic inference. Using a whole genome assembly for each species in future studies could be a valuable improvement to reduce this bias.

Our results further revealed that webbing morphology in Asian tree frogs exhibits a strong and quantifiable association with ecological niche, consistent with analogous ecological patterns documented in other anuran lineages.⁶⁷ Morphological variation across arboreal, shrubby, and swampy groups formed a continuous gradient rather than discrete partitions (Figures 1C-D), suggesting an underlying evolutionary trajectory. Variance partitioning revealed that webbing morphology and phylogeny contributed most and equally to vertical niche diversification, whereas geographic distribution contributed little. This highlights that phylogenetic influence and accu-

mulated genetic mutations also play key roles in niche expansion and differentiation in Asian tree frogs. Previous biogeographic reconstruction has traced the ancestral origin of Asian tree frogs to Southeast Asia, with progressive radiation into India and East Asia.⁶⁴ This spatial pattern supports a hypothesis wherein arboreal ancestors originated from Southeast Asia and subsequently expanded into the western and southern margins of the Hengduan Mountains, where arboreal and shrubby forms now occur sympatrically. Continued eastward expansion into South China likely facilitated further ecological transitions in response to local microhabitat availability, culminating in the sympatric distributions of shrub-adapted and swamp-adapted lineages. Ancestral character-state reconstruction revealed a phylogenetically supported trend toward reduction in web-to-space ratio (Figures 2A & C), providing evolutionary context for observed morphological differentiation

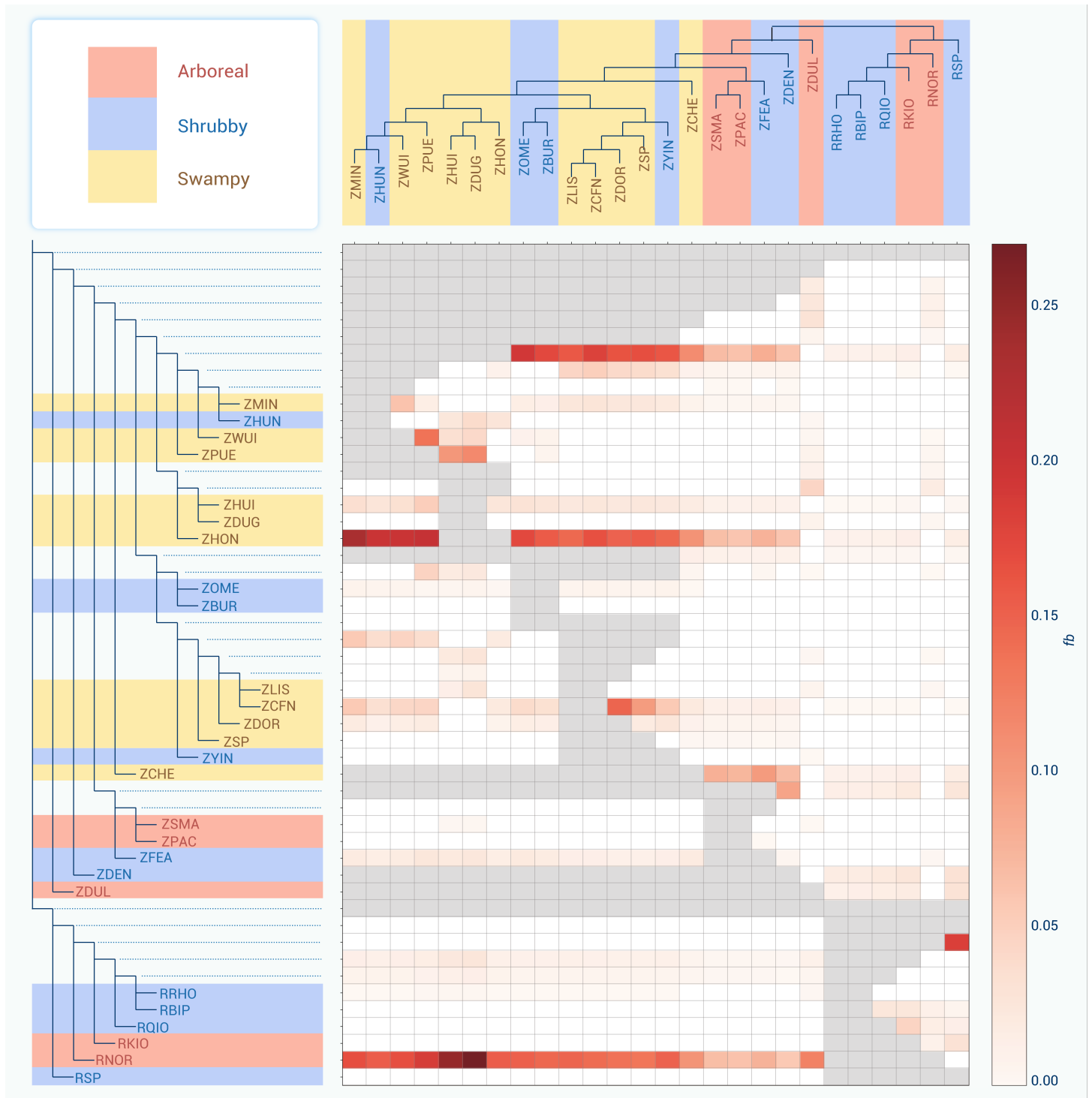


Figure 4. Excess allele-sharing pattern between Asian tree frog species based on f-branch statistic fb Arboreal species marked in pink; shrubby species marked in light blue and swampy species marked in light yellow. The intensity of red in each cell indicates the degree of excess allele-sharing between the corresponding tree tip and each tip or node. Species name abbreviations were provided in Table S2.

across vertical strata.

Shared polymorphism and gene flow in vertical niche diversification

The combination of IBD haplotype sharing patterns and f-branch statistics provides powerful evidence for disentangling the complex history of gene flow and isolation during vertical niche diversification. These two lines of evidence offer complementary perspectives: IBD haplotypes capture the genomic footprint of relatively recent shared ancestry, while f-branch statistics detect and quantify historical introgression events across a phylogeny.^{68,69}

Our f-branch (fb) analysis revealed the most significant introgression

signal was detected between the arboreal species *R. norhayatae* and the swampy species *Z. dugritei*, yet closer examination of D-statistic patterns—specifically, the ABBA/BABA ratios across multiple test configurations—argues strongly against recent gene flow.⁷⁰ Instead, this signal stems from vertical inheritance from their common ancestor, where the underlying mechanism is incomplete lineage sorting (ILS). This interpretation is supported by the observation that *R. norhayatae* exhibited elevated allele sharing with multiple distantly related lineages, a pattern more consistent with retained ancestral polymorphism than with episodic introgression. In contrast, a second major introgression signal—between the swampy species *Z. hongchibaensis* and multiple shrubby and swampy species—showed a

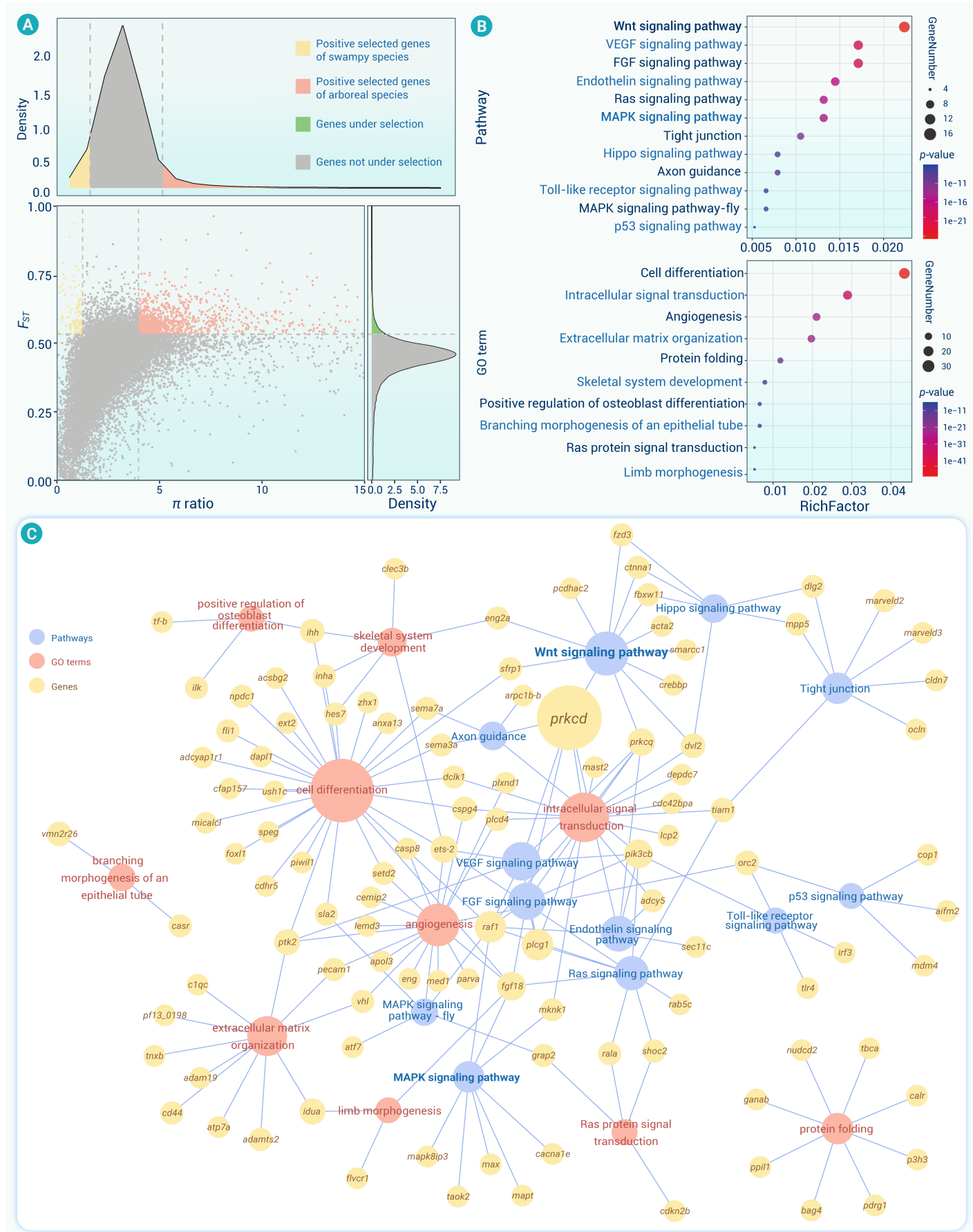


Figure 5. Genomic regions and candidate genes under selection between arboreal and swampy groups (A) Genome-wide distribution of F_{ST} values and π ratio by comparing the arboreal and swampy groups using 50 kb sliding window. (B) KEGG and GO enrichment of genes under selection. (C) Network representation of enriched KEGG pathways (light blue), GO terms (pink) and associated genes (light yellow).

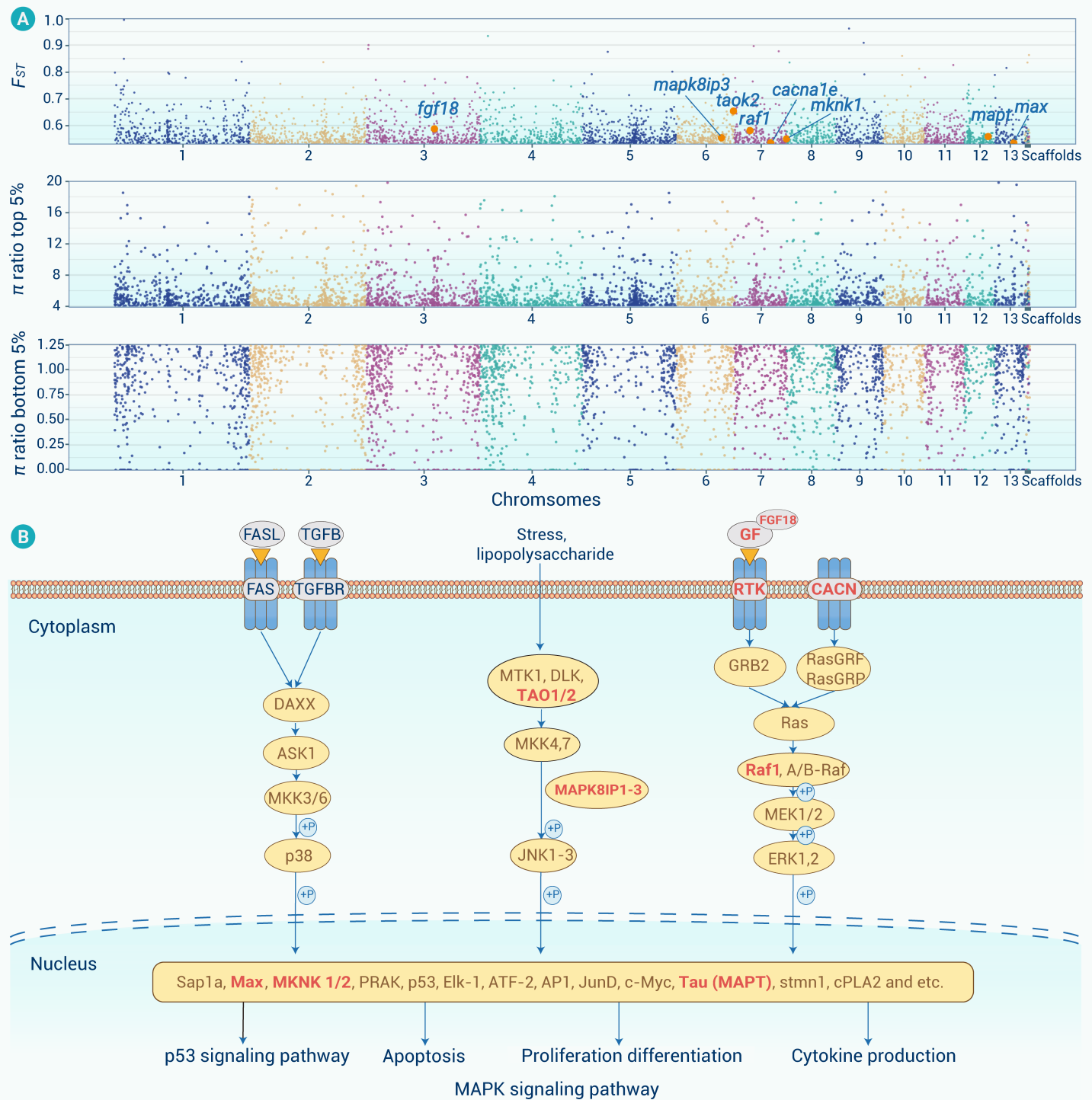


Figure 6. Genes under selection pressure in the MAPK signaling pathway (A) Manhattan plot of F_{ST} values and π ratio across chromosomes. Genes within the MAPK signaling pathway under selection are highlighted in orange. (B) Selected genes (red) mapped onto the three canonical MAPK cascades: extracellular signal-related kinases (ERK), Jun amino-terminal kinases (JNK) and p38 mitogen-activated protein kinases (p38-MAPK). These cascades regulate key cellular processes including proliferation, differentiation, cytokine production and apoptosis.

different signature. Approximately half of the D-statistics comparing *Z. hongchibaensis* with its close relatives yielded ABBA scores lower than BABA scores, a pattern indicative of genuine gene flow rather than ancestral structure. This signal was further corroborated by Dsuite⁵⁰ and TreeMix analyses, which consistently identified a single significant gene flow event connecting shrubby and swampy populations. These complementary approaches thus converge on a model in which gene flow has been primarily restricted to adjacent niche pairs, with the swampy-shrubby interface representing the most permeable ecological boundary. Notably, however, gene flow resulting

from these adjacent pairs was insufficient to prevent ongoing vertical ecological niche differentiation. This finding echoed theoretical predictions that even substantial gene flow can be overcome by strong disruptive selection when ecological niches are sufficiently distinct.⁷¹ Similar patterns have been documented in other biological systems: in the African malaria mosquito *Anopheles gambiae*, sympatric molecular forms maintain differentiation despite ongoing gene flow, with genomic divergence concentrated in regions under ecological selection.⁷²

While f-branch statistics illuminate historical introgression, IBD haplotype



Figure 7. Genomic regions and genes under long-term balancing selection in Asian tree frogs (A) Manhattan plot of β statistics across chromosomes, with orange points indicating genes under selection. Red line denotes extreme top 0.01% threshold. (B) Strong signals of balancing selection at the *obsn* and *ttn* loci within the 77.65–77.90 Mb region on chromosome 6. (C) Topology weighting of three alternative phylogenetic configurations (Topo1, Topo2, and Topo3) evaluated at both genome-wide and gene-specific levels across the three populations.

sharing patterns provide a window into more recent demographic processes and ongoing barriers to gene flow.⁶⁹ Shared IBD blocks between species were overwhelmingly concentrated within the swampy population (79.7%), with substantially lower proportions in shrubby (13.5%) and arboreal (6.8%) populations. This distribution suggests that while all three niche types share a common ancestral gene pool, contemporary gene flow has been severely restricted, particularly for the arboreal niche. Critically, the near-complete absence of shared IBD haplotypes across niches stands in stark contrast to the extensive sharing observed within niches, indicating that current gene flow barriers are both strong and ecologically structured. This suggests that the transition to the arboreal niche—which likely involved more extreme morphological and physiological adaptations, including specialized webbing for gliding or enhanced climbing—imposed stronger disruptive selection and faster accumulation of reproductive isolation. Reproductive isolation in this system may have a multi-locus genetic basis. The length distribution of cross-niche IBD blocks (1.5–8.7 Mb, median 2.2 Mb) is consistent with the decay of ancestral haplotypes following reproductive isolation. Similar patterns have been observed in diverse taxa, like cichlids,⁷³ where the transition from shared polymorphism to lineage-specific variation accompanies the progression of speciation.⁷⁴

The geographic distribution of our study species—with arboreal, shrubby, and swampy populations occurring in sympatry or parapatry—raises the question of whether divergence occurred in the face of gene flow. In fact, the concept of “heteropatric speciation” has been proposed precisely for such cases: organisms whose ranges overlap but occupy ecological niches

distinct enough to prevent frequent hybridization, representing a behavioral rather than geographic barrier to gene flow.⁷⁵ The blind mole rat system provides a compelling parallel to our findings: populations inhabiting different soil types separated by just meters of easily dug dirt showed clear genetic differentiation and very low gene flow, despite the absence of any physical barrier.⁷⁶ This demonstrates that sharp ecological boundaries can generate reproductive isolation even in the face of potential contact.

Genomic insights into vertical niche specialization

Our results reveal that recent selective sweeps and long-term balancing selection have played distinct but complementary roles in driving local adaptation and ecological diversification in Asian tree frogs.

Selective sweeps usually rapidly fix beneficial mutations in response to novel environmental pressures, serving as a hallmark of local adaptation.⁷⁷ In arboreal Asian tree frogs, we identified pronounced sweep signals in genes and pathways linked to climbing, adhering, and gliding. The MAPK/ERK cascade may emerge as a central regulatory hub under selection,^{78,79} with five sweep genes—*fgf18*, *cacna1e*, *raf1*, *mapt*, and *max*—directly involved in skeletal morphogenesis and epidermal proliferation.^{80–83} Among these, *fgf18* plays a critical role in limb development,^{84,85} raising the hypothesis that its selection may contribute to expanded interdigital webbing—a key innovation serving dual functions: enhanced wet adhesion on unstable substrates^{3,21,24,86–88} and aerodynamic control during gliding.^{24,89–91} Muscle-related genes also exhibited sweep signatures: *cacna1e* mediates excitation-contraction coupling,⁹² and may serve as a candidate gene for enhancing

limb strength for sustained climbing.^{22,93} Vascularization gene *pik3cb*, associated with vessel density regulation,⁹⁴ may link to webbed tissue expansion.⁹⁵ Additionally, cytoskeletal genes *ctnna1* and *pcdhac2* within the Wnt pathway contribute to toe-pad epithelial reorganization, enhancing adhesion during vertical movement.^{3,96} While selective sweeps failed to recover the specific candidate genes *TCF7L1*, *TCF7L2*, *Hes5*, and *Ednrb* previously identified in *R. kio*,³ we did detect another Hes family member, *Hes7*, in this study. Considering the inclusion of multiple arboreal species in this study, this finding points to possible species-specific differences in the genetic targets of selection within the Hes family.

In contrast to the fixing effect of sweeps, balancing selection maintains genetic diversity by preserving advantageous polymorphisms over evolutionary timescales,^{62,97} enabling phenotypic flexibility in variable environments.⁹⁸ In Asian tree frogs, balancing selection preserved ancient polymorphisms, potentially contributing to developmental plasticity. Gene *p63*, a master regulator of epithelial differentiation,⁹⁹ may allow variable epidermal remodeling in webbed structures across fluctuating microhabitat conditions. Remarkably, numerous balancing selection signals localized to transposable element (TE) regions, including exonic segments of muscle genes *obscn* and *ttn*. TEs often serve as sources of regulatory variation co-opted for adaptation,^{100,101} potentially modulating gene expression to buffer against varying mechanical demands without fixing a single optimal allele.¹⁰²

Our results further demonstrate that, despite the detection of only limited introgression events, microhabitat adaptation can still be driven by selective sweeps. Furthermore, both directional selection and balancing selection likely contribute to vertical niche adaptation, potentially operating at different temporal or functional scales. Genetic drift could also contribute, as reflected by the random fixation of alleles introduced via introgression. Topological weighting analysis revealed a marked discrepancy between genome-wide phylogenetic relationships and the structure inferred from loci under balancing selection. This suggests that long-term balancing selection can obscure divergence signals, maintain ancient polymorphisms, and may promote the sharing of adaptive variants across ecological boundaries, even in the face of ongoing differentiation.^{3,103,104} Moving forward, functional validation through gene editing and expression analyses will be essential to establish causal links between candidate genes and trait evolution. In parallel, experimental investigation of TE-associated regulatory variation and expanded population sampling across closely related lineages will help clarify the evolutionary mechanisms underlying phenotypic innovation during adaptive radiation.

CONCLUSION

In this study, our results reveal a strong correlation between webbing morphology and vertical ecological niches in the Asian tree frogs. Genomic adaptations, including selective sweeps and balancing selection on functionally distinct genes, jointly shape phenotypic innovation, while limited gene flow across adjacent niche boundaries prevents homogenization and reinforces speciation. Together, these forces ultimately drive vertical ecological niche diversification.

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AUTHOR CONTRIBUTIONS

Ruihan Li: Investigation, Data curation, Formal analysis, Visualization, Writing – original draft, Funding acquisition. **Jin-Long Ren:** Supervision, Resources, Funding acquisition. **Ji-Shan Wang:** Resources, Writing – review & editing. **Chaochao Yan:** Data curation, Software, Methodology. **Wei Wu:** Supervision, Funding acquisition. **Zhi-Tong Lyu:** Visualization, Funding acquisition. **Mao-Liang Li:** Writing – original draft. **Junjie Huang:** Investigation. **Tao Xue:** Investigation, Validation. **Jia-Tang Li:** Supervision, Project administration, Funding acquisition. **Dechun Jiang:** Conceptualization, Supervision, Project administration, Funding acquisition, Writing – review & editing. All authors contributed to the manuscript and approved the final version.

DECLARATION OF INTERESTS

The authors declare no competing interests.

ETHICAL STATEMENT AND PATIENT CONSENT

All collections were conducted in accordance with animal use protocols approved by the Experimental Animal Ethics Committee of Chengdu Institute of Biology, Chinese Academy of Sciences (CIBDWLL2024011).

DATA AND CODE AVAILABILITY

The raw sequencing data reported in this paper have been deposited in the Genome Sequence Archive¹⁰⁵ at the National Genomics Data Center (NGDC)¹⁰⁶ China National Center for Bioinformation/Beijing Institute of Genomics, Chinese Academy of Sciences (GSA: CRA016237) that are publicly accessible at <https://ngdc.cncb.ac.cn/gsa>, and Sequence Read Archive (SRA Project ID: PRJNA1155233). The measured interdigital webbing morphology data were included in the Supplementary Materials. The custom analytical code used in this study is available in GitHub repository: <https://github.com/liruihanguo/lrhscripts>.

SUPPLEMENTAL INFORMATION

It can be found online at <https://doi.org/10.59717/j.xinn-life.2026.100243>.