Comparing Neutral and Adaptive Genetic Variation in Shaping Phenotypic Diversity of the Polymorphic Bush Cricket *Isophya rizeensis* Along an Altitudinal Gradient

by

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[To my lovely wife]

ABSTRACT

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Ufuk Topalan

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The accelerating impacts of global environmental and climatic change present significant threats to biodiversity, emphasizing the need to understand how natural populations adapt to environmental gradients. *Isophya rizeensis*, a univoltine bush cricket endemic to Turkey's Fırtına Valley, offers a compelling model for studying local adaptation, owing to its striking altitudinal color polymorphism in males. Lower-altitude populations predominantly exhibit black morphs, while higher altitudes are characterized by pale-green individuals, a pattern suggesting adaptation to microclimatic conditions rather than classical thermal melanism. Using RAD-seq data from 71 individuals across 10 populations, we identified 92,048 high-confidence polymorphic loci, including 1,113 putatively adaptive loci. Genetic analyses revealed significant differentiation driven by altitude, with Mantel tests confirming isolation by distance and selection. Mid-altitude populations exhibited higher genetic diversity, while extreme-altitude populations showed reduced diversity and fixation of adaptive loci, highlighting the dual influence of gene flow and selection. Genome-wide association studies identified 42 loci significantly associated with altitude, supporting the role of disruptive selection in shaping genetic variation and maintaining polymorphism along the gradient. Discriminant analysis of principal components (DAPC) revealed strong genetic differentiation between color morphs, with three major loci contributing to this separation. These loci exhibited bimodal genotype distributions, indicating the existence of distinct genetic groups linked to color morphs and their altitudinal distribution. This study underscores how environmental pressures shape adaptive and neutral genetic variation, maintaining diversity despite high connectivity. Our findings highlight the interplay between gene flow, selection, and local adaptation, providing valuable insights into the mechanisms driving biodiversity in montane ecosystems and informing conservation strategies in the face of climate change.

ÖZETÇE

Yükseklik Gradyanı Boyunca Polimorfik Çalı Çekirgesi *Isophya rizeensis*'te Fenotipik Çeşitliliğin Şekillenmesinde Nötral ve Adaptif Genetik Varyasyonun Karşılaştırılması

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Küresel çevresel ve iklimsel değişimin hızlanan etkileri, biyolojik çeşitlilik için önemli tehditler oluşturmaktadır ve doğal popülasyonların çevresel değişimlere nasıl uyum sağladığını anlama ihtiyacını arttırmaktadır. Türkiye'nin Fırtına Vadisi'ne özgü çalı çekirgesi olan *Isophya rizeensis*, erkeklerde çarpıcı yüksekliğe bağlı renk polimorfizmi göstermekte ve yerel adaptasyonu incelemek için çok uygun bir model sunmaktadır. Düşük rakım popülasyonları ağırlıklı olarak siyah morflardan oluşurken, yüksek rakımlardaki bireyler, klasik termal melanizmden yerine, mikro iklim koşullarına adaptasyonu gösteren bir model olan soluk yeşil bireylerle karakterize edilmiştir. 11 popülasyondaki 71 bireyden alınan RAD-seq verilerini kullanarak, 1.113 adaptif lokus dahil olmak üzere 92.048 yüksek güvenilirlikli polimorfik lokus belirledim. Genetik analizler, yükseklik tarafından sürülen önemli farklılaşmayı ortaya koydu ve Mantel testleri mesafe ve seçilim yoluyla izolasyonu doğruladı. Orta rakımlardaki popülasyonlar daha yüksek genetik çeşitlilik sergilerken, ekstrem yüksekliklerdeki popülasyonlar daha az çeşitlilik ve adaptif lokusların sabitlendiğini gösterdi ve bu durum gen akışı ve seçilimin ikili etkisini vurguladı. Genom çapında ilişki çalışmaları, yükseklik ile önemli ölçüde ilişkili 42 lokus tanımladı ve genetik çeşitliliği şekillendirmede ve yükseklik boyunca polimorfizmi sürdürmede yıkıcı seçilimin rolünü destekledi. Ana bileşenlerin ayırıcı analizi (DAPC), renk morfları arasında güçlü genetik farklılaşmayı ortaya koydu ve üç ana lokus bu ayrıma katkıda bulundu. Bu lokuslar, renk morflarına ve bunların yükseklik dağılımlarına bağlı farklı genetik grupların varlığını gösteren bimodal genotip dağılımları sergiledi. Bu çalışma, çevresel değişimin adaptif ve nötral genetik çeşitliliği nasıl şekillendirdiğini ve yüksek bağlantıya rağmen çeşitliliği nasıl koruduğunu vurgulamaktadır. Bulgularımız, gen akışı, seçilim ve yerel adaptasyon arasındaki etkileşimi vurgulayarak dağlık ekosistemlerde biyoçeşitliliği yönlendiren mekanizmalara ilişkin değerli bilgiler sağlıyor ve iklim değişikliği karşısında koruma stratejilerine katkı sağlıyor.

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ABBREVIATIONS

ANGSD	Analysis of Next Generation Sequence
DNA	Deoxyribonucleic acid
SNP	Single Nucleotide Polymorphism
RAD	Restriction-site Associated
DAPC	Discriminant Analysis of Principal Components
Fst	Fixation Index
PCA sNMF	Principal Component Analysis Sparse Nonnegative Matrix Factorization

CHAPTER 1:
INTRODUCTION

1.1 Adaptations and Climate change

The accelerating impacts of global environmental and climate change pose an unprecedented threat to biodiversity worldwide, with species facing challenges such as habitat loss, altered resource availability, and shifting climatic conditions (Thomas et al., 2004; Malcolm et al., 2006; Kearney et al., 2009; Harvey et al., 2020). Unexpected rates of extinction are being caused by these changes, especially for species with restricted dispersion capabilities or constrained ecological niches. Understanding how species react to these environmental changes is crucial for guiding conservation efforts and forecasting future biodiversity results in order to reduce these threats. (Schilthuizen & Kellermann, 2013; Kingsolver & Buckley, 2017; Kellermann & Heerwaarden, 2019; Waldvogel et al., 2020).

Adaptive responses to environmental change can occur across different timescales, ranging from phenotypic plasticity that allows immediate adjustments to environmental stressors, to long-term genetic changes driven by natural selection (Merilä & Hendry, 2014; Kelly, 2019). To promote the long-term conservation of biodiversity, it is essential to investigate the evolutionary mechanisms that enable species to survive in changing habitats (Feiner et al., 2021). This research is particularly critical given that the pace of anthropogenic climate change often outstrips the ability of many species to adapt, highlighting the urgent need for adaptive management strategies.

One of the most effective ways to search adaptive responses is by examining phenotypic and genetic variation along environmental gradients, such as altitudinal or latitudinal ranges, which often serve as natural laboratories for studying evolutionary processes (Wogan & Wang, 2017; Kelly, 2019). These gradients give important information on how environmental factors and genetic diversity interact, as well as insights into the selective forces influencing local adaptations. By analyzing how populations are adapted across these gradients, researchers can identify important traits and genetic loci associated with survival in different environments, contributing to a better understanding of the evolutionary and adaptive basis of biodiversity (Merilä & Hendry, 2014; Waldvogel et al., 2020). As global climate conditions continue to shift, such studies are critical for identifying populations that may act as sources of genetic diversity, those

at greatest risk of extinction, and the adaptive potential of species to cope with future challenges. By linking ecological, genetic, and evolutionary perspectives, this research forms the foundation for evidence-based conservation strategies that align with the realities of climate change.

1.2 Clinal variation and color polymorphism in ectotherms.

Many studies investigating the evolutionary responses of organisms to climatic or environmental change have focused on species or populations exhibiting clinal variation (Storfer et al., 2018). Clinal variation refers to the gradual change in specific traits (e.g., allele frequency, body size, coloration, behavior) across environmental or geographic gradients (Endler, 1977). Altitudinal clines, in particular, provide ideal systems for studying spatially varying selective pressures, as they capture high spatial and phenotypic heterogeneity over short geographic distances (Chown & Klok, 2003; Flatt, 2016). For instance, clinal variations in thermoregulatory traits among montane species have been used to uncover physiological adaptations to climate and temperature changes, as well as their genetic underpinnings (Moritz et al., 2008; Zamora-Camacho et al., 2014, 2016; Verheyen et al., 2019; Lymburner & Blouin-Demers, 2020). Research on mammals and amphibians has identified numerous genetic regions associated with adaptation to climatic change (Bonin et al., 2006; Guo et al., 2016). Moreover, studies combining altitudinal clines with genotype-environment association analyses have discovered genetic regions linked to specific climatic or environmental parameters (Hancock et al., 2011; Slatyer et al., 2014; Hornoy et al., 2015; Pluess et al., 2016). These integrated approaches have significantly advanced our understanding of how environmental factors influence genetic regions and how selection drives adaptation to varying conditions.

One particularly well-studied natural cline is color variation in invertebrates, especially insects, which provides insights into the evolutionary and adaptive responses of organisms to environmental changes (Ellers & Boggs, 2002; Zeuss et al., 2014; Bishop et al., 2016). Adaptive color polymorphism, wherein populations with alternative color morphs occupy distinct ecological niches, has been associated with broader resource use, greater stability, expanded distribution capacity, and higher resilience to environmental changes and local extinctions compared to less variable species (Forsman et al., 2008; Wennersten & Forsman, 2009; Kozlov et al., 2021). Such findings underscore the importance of studying geographic variation in coloration and its genetic basis, as this

knowledge not only informs conservation efforts (Forsman, 2016) but also enhances understanding of how evolutionary and ecological processes drive population differentiation and speciation (McLean & Stuart-Fox, 2014).

Color polymorphism in insects often correlates with temperature variation, with darker individuals warming faster and achieving higher equilibrium temperatures in cold conditions, a phenomenon known as thermal melanism (Clusella-Trullas & Nielsen, 2020). However, recent studies indicate that insect color variation may also relate to other environmental factors, such as habitat and vegetation adaptation (Karlsson et al., 2008; Forsman, 2011; Valverde & Schielzeth, 2015; Dieker et al., 2018; Goodman, 2021). This is supported by genome-wide scans revealing that pigmentation and melanin accumulation, especially in Drosophila, are influenced by well-characterized molecular mechanisms involving dopamine-related genes and their interactions with ebony, black, aaNAT, tan, and yellow (Wright, 1987; Wittkopp et al., 2003; Wittkopp & Beldade, 2009). Yet, in other species, such as ladybirds (Harmonia axyridis), stick insects, and grasshoppers (Tetrix undulata), the genetic basis of color polymorphism often diverges, reflecting species-specific adaptations to environmental pressures, with limited connections to known melanin pathways (Michie et al., 2010; Bastide et al., 2016; Comeault et al., 2015, 2016; Forsman et al., 2002).

1.3 Isophya rizeensis as a model organism

Isophya rizeensis Sevgili, 2003 (Orthoptera: Tettigoniidae: Phaneropterinae: Barbitistini) is a univoltine (single generation per year) bush cricket species endemic to the Fırtına Valley and its surroundings within the Kaçkar Mountains National Park in Türkiye's Eastern Black Sea region. The species is active between May and August (Sevgili, 2003; Sağlam & Çağlar, 2005). Despite its geographically restricted range, *I. rizeensis* occupies a wide altitudinal gradient, ranging from 350 to 2500 meters within the Fırtına Valley (Sağlam & Çağlar, 2005).

One of the most striking characteristics of *I. rizeensis* is the high variability males show in dorsal coloration, which is strongly correlated with altitude. Studies have shown that melanism in males decreases with increasing altitude, dividing individuals into two morphs: dark black individuals predominantly found between 350 and 1000 meters, and pale green individuals more common between 1000 and 2500 meters (Çağlar et al., 2013).

Interestingly, this distribution pattern conflicts with the thermal melanism hypothesis, as darker individuals are associated with relatively warmer environments, while paler individuals are found in cooler habitats. Ecological and physiological studies aiming to understand the processes driving these variations in males have revealed that darker individuals warm faster and achieve higher body temperatures compared to their pale green counterparts (Kuyucu et al., 2016). According to these results, the observed variation in color is likely an adaptation to overheating at higher altitudes due to increased solar radiation. In subalpine habitats, where temperatures are highly variable, vegetation cover is sparse, and surface temperatures can exceed 40°C due to direct solar radiation, pale individuals may have a survival advantage. Their slower heating rates could help them avoid lethal temperatures in these conditions (Kuyucu et al., 2016).

Despite many studies on color polymorphism in crickets, research on its genetic underpinnings remains limited. Quantitative genetic approaches have shown that both genetic and environmental factors contribute to total phenotypic variation (Forsman et al., 2002; Ahnesjö & Forsman, 2006; Forsman, 2011; Roff & Fairbairn, 2013). Recent work suggests a strong genetic basis for black/green color polymorphism in crickets, potentially controlled by a few dominant genes, though their connections to known melanin pathways remain unclear (Schielzeth & Dieker, 2020; O'Connor et al., 2021; Winter et al., 2021).

Collectively, these findings position I. rizeensis as an exceptional model for studying evolutionary responses to environmental changes, particularly in understanding the genetic mechanisms underlying adaptations to spatially and temporally variable conditions. Its distinct altitudinal color polymorphism and environmental adaptations provide a unique opportunity to unravel how species balance complex ecological pressures through genetic and phenotypic diversity.

Figure 1.1: *Isophya rizeensis* individuals from 800 meters (A) and 1900 meters (B)

1.4 Next Generation Sequencing and RAD sequencing

Recent advancements in next-generation sequencing (NGS) technologies have revolutionized genomic research, enabling the rapid and cost-effective generation of large-scale genetic data across diverse organisms (Metzker, 2009; Goodwin et al., 2016). NGS has the potential to produce enormous amounts of data without requiring a reference genome, in contrast to traditional moleculer techniques that frequently required prior genomic knowledge and were restricted to a few genetic markers. Because non-model organisms frequently do not have annotated genomes, NGS is especially advantageous for studying them (Baird et al., 2008; Helyar et al., 2011). Researchers may now analyze genetic diversity, population structure, and evolutionary processes with unprecedented resolution because of the ability to sequence millions of DNA fragments in simultaneously. NGS has significantly improved our understanding of population differentiation, gene flow, local adaptation, and speciation by making it possible to analyze hundreds to thousands of loci throughout the genome (Ellegren, 2014; Ekblom & Galindo, 2011). Moreover, these tools have transformed the field of conservation genomics, allowing for more specific evaluations of genetic health and connectivity in threatened populations (Allendorf et al., 2010).

Among the various NGS-based approaches, restriction site-associated DNA sequencing (RAD-seq) has emerged as a particularly powerful method in population genetics and evolutionary biology (Davey et al., 2011). By using restriction enzymes to

cleave genomic DNA at particular recognition sites, RAD-seq generates a library of short segments that are next to these sites and are subsequently read. Even in species lacking a reference genome, this method makes it possible to find and genotype thousands of single nucleotide polymorphisms (SNPs) throughout the genome (Baird et al., 2008; Peterson et al., 2012). RAD-seq offers several advantages for population genomic studies. First, it is cost-effective compared to whole-genome sequencing, making it an accessible option for projects involving large numbers of individuals or species with large genomes (Andrews et al., 2016). Second, by focusing on a section of the genome, it reduces the computational challenge of processing whole-genome data while enabling high-throughput marker discovery. Third, by altering the restriction enzyme or sequencing depth, RAD-seq data may be adjusted to the degree of precision needed for the study, making it an adaptable tool for a range of studies (Catchen et al., 2013).

Additionally, by offering genome-wide information on polymorphic sites that may be utilized to pinpoint genetic loci linked to environmental factors, RAD-seq makes studying adaptations more useful. Studying local adaptation to ecological gradients like elevation temperature, and precipitation particularly is useful (Narum et al., 2013). Furthermore, to evaluate how geographic and environmental variables influence genetic diversity across spatial scales, RAD-seq data may be used with landscape genomic techniques. In summary, by offering high-resolution, reasonably priced genomic data for non-model species, NGS and RAD-seq have revolutionized the study of genetic diversity and evolutionary processes.

1.5 Objectives and Significance of the Study

The primary objective of this study is to investigate the genetic differentiation and diversity of *Isophya rizeensis* along its altitudinal gradient in the Fırtına Valley and to determine genetic variation associated with color polymorphism in this endemic bush cricket species. By addressing key gaps in our understanding of how spatial variation influences genetic and phenotypic variation, this research aims to improve our understanding of adaptive processes in montane ecosystems.

Specific Objectives:

1. **Population Structure and Genetic Diversity** ○ To analyze the population structure and genetic diversity of *I. rizeensis* across its altitudinal range using RAD sequencing data.
    - To compare genetic structure patterns in neutral and adaptive loci.
2. **Genetic Basis of Color Polymorphism** ○ To identify genetic loci associated with the black and green color morphs. ○ To evaluate the potential role of these loci in adaptation to altitudinal gradients.

1.5.1 Significance of the Study

Understanding the mechanisms driving adaptive traits is vital for predicting how species respond to rapid environmental changes. This study holds both theoretical and practical significance. By uncovering genetic variation associated with color polymorphism in *I. rizeensis*, it sheds light on the evolutionary processes shaping phenotypic diversity under spatially variable selection pressures.

The focus on a thermoregulatory trait that deviates from classical thermal melanism theory provides a unique opportunity to expand our understanding of ectothermic adaptation in diverse environmental conditions. Moreover, *I. rizeensis* represents a remarkable model for investigating the interplay between genetic and environmental factors in shaping adaptive traits, with broader implications for understanding adaptation and speciation in montane ecosystems.

In the context of changing climates, this research offers critical insights into the adaptive potential of *I. rizeensis*. Such knowledge is essential for developing effective strategies to conserve biodiversity in montane and other vulnerable ecosystems. By integrating ecological, genetic, and evolutionary perspectives, this study not only enhances our fundamental understanding of adaptation but also informs practical approaches to mitigate biodiversity loss amid global environmental challenges.

CHAPTER 2:

METHODS

2.1 Collection of Samples and DNA extraction

In this study, I utilized pre-existing RAD sequencing data from 96 *Isophya rizeensis* individuals sampled from 11 sub-populations along an altitudinal gradient in the Fırtına Valley **(Figure 2.2, Table 2.1)**. The specimens were originally collected between June and August 2006, as described in Çağlar et al. (2013). Paired-end RAD libraries were constructed using the PstI restriction enzyme, following the protocol detailed by Ali et al. (2016). Sequencing was performed at 10X coverage on an Illumina HiSeq4000 at the UC Davis Genome Center core facilities.

Figure 2.1 : Distribution area of *Isophya rizeensis* in Türkiye, limited to the northeastern Black Sea region.

2.2 De novo Reference Genome Assembly

Due to the absence of a reference genome for *I. rizeensis* or in a closely related species, we generated a de novo RAD sequence reference genome library from two individuals sourced from outside the Firtina Valley, following the protocol by **Sağlam et al. (2016)**. Initially, sequences were assigned to individual samples based on unique 8 bp barcodes. Reads that perfectly matched these barcodes, followed by the 8 bp restriction site sequence, were sorted into separate files. Afterward, the 16 bp identifier sequences were removed, and matching read pairs were grouped based on their headers and organized into individual files.

For de novo discovery and extension of RAD loci, we utilized custom PERL scripts, Novoalign alignment software, and the PRICE genome assembler (Ruby et al., 2013). We used two individuals from the CAYMY population of *I. rizeensis* to identify unique RADloci. In brief, 88 bp forward reads were trimmed to 80 bp from the 3' end, quality-filtered with a 20% threshold, and concatenated into a single FASTA file. This FASTA file was indexed using Novoalign and aligned against itself to produce a pairwise alignment map. The map included scores for both internal (within-individual) and external (betweenindividual) alignments (Miller et al. 2012). Distinct loci were determined using the following criteria: a maximum alignment score of 30 between sequences within a locus; alignments exceeding a score of 90 or perfect internal alignments were excluded; singleoccurrence sequences were ignored unless they perfectly matched a sequence occurring in multiple instances externally; and each locus had to include at least one sequence from two different individuals.

Identified RAD loci were then extended into longer contigs using PRICE. Paired-end read data were used to iteratively extend each RAD locus into contigs. For each locus, three input files were created: (1) a template file with the 80 bp RAD locus and its restriction site restored, (2) a forward read file with all exact matches to the RAD locus, and (3) a reverse read file with paired reads. To prevent upstream extension, a 100 bp poly-A sequence was added before the restriction site in the template file. These files were

processed in PRICE using the following parameters: 300 bp insert size, 95% minimum sequence identity, 40 nt minimum overlap length (-mol 40), and 80% sequence identity threshold (-mpi 80). PRICE was set to extend contigs in a single cycle (-nc 1) with a 100% target mode (-target 100 0). The resulting extended RAD contigs will then serve as a de novo partial genome reference for subsequent analyses.

2.3 Alignment and filtering

Raw reads were aligned to the constructed reference genome using the BWA-MEM algorithm (Li & Durbin, 2010; Li, 2013). Resulting SAM files were converted to coordinate-sorted BAM files using samtools (Danecek et al., 2021), and duplicate reads were marked with Picard tools (Picard Toolkit, 2019). Paralog regions were identified using the ngsParalog (https://github.com/tplinderoth/ngsParalog) and removed from subsequent analysis. In addition, we filtered out any individual with fewer than 100,000 alignments.

2.4 SNP discovery and genotyping

Polymorphic regions were identified using the ANGSD platform (Korneliussen et al., 2014), which is well-suited for non-model organisms and low-to-medium depth genome sequences (Fumagalli et al., 2013; Korneliussen et al., 2014; Fumagalli et al., 2014). SNP discovery was carried out by calculating genotype likelihoods (-GL 1), major and minor alleles (-doMajorMinor 1), minor allele frequencies (-doMaf 1), and generating BCF files (-doBcf 1). Polymorphic sites were filtered to include only those with a minor allele frequency (MAF) of 0.05 or higher (-minMaf 0.05). Additional filtering parameters included a minimum base quality score of 20 (-minQ 20), a minimum mapping quality of 10 (-minMapQ 10), and a posterior genotype probability threshold of 0.85 (-postCutoff 0.85). Only properly paired reads (reads with both mates mapped correctly; only_proper_pairs 1) were considered, and sites were retained only if the probability of them being polymorphic was statistically significant ($P < 10^{-12}$). Finally, we filtered out any site that had an average per individual read depth under 6X and was not present in at least 50% of individuals in each population.

2.5 Population Genetic Structure

Population structure was assessed using PCAngsd (version 1.02; Meisner & Albrechtsen, 2018), which computes principal components directly from genotype likelihoods. Beagle files containing these likelihoods were used to estimate the covariance matrix, and the percentage variance explained by each principal component was calculated. The first two principal components (PCs) were visualized using a custom R script. The same Beagle file was also used to investigate genetic admixture patterns within the dataset.

Admixture analyses were performed with ngsADMIX (Skotte et al., 2013; Meisner et al., 2018), a method that infers individual ancestry proportions without requiring phased data. To identify the most likely number of genetic clusters, I explored clustering parameters ranging from K=2 to K=5, running 10 replicates for each K value to ensure reliable results. The optimal number of clusters was determined using Evanno's method (Evanno et al., 2005), which calculates ΔK based on the rate of change in loglikelihood values across successive K values.

To validate and refine the clustering results, I employed Clumpak (Kopelman et al., 2015), which aggregates and visualizes outputs from multiple ngsADMIX runs. This approach confirmed K=2 as the most likely number of genetic clusters within the dataset (Figure 2.3). Admixture proportions were visualized using ggplot2 in R, and the geographic distribution of admixture was mapped with ArcGIS Pro (Esri, 2024), highlighting the spatial patterns of genetic diversity across populations.

2.6 Genetic Differentiation and Isolation By Distance (IBD)

To determine genetic differentiation, I calculated pairwise F_{ST} values between populations. Genome-wide Fst estimates were obtained using ANGSD, starting with the calculation of the joint site frequency spectrum (2D-SFS) for each pair of populations. The global weighted F_{ST} was then computed using the REALSFS module (Fig. A.3; Nielsen et al., 2012). To evaluate patterns of isolation by distance (IBD), I performed a Mantel test to assess the correlation between geographic and genetic distances. A Euclidean distance matrix between populations was generated using the Geographic Distance Matrix Generator v1.2.3 (Ersts, P. J., 2011) and used as the predictor variable, while the linearized pairwise F_{ST} matrix [F_{ST}/ (1- F_{ST})] served as the response variable.

IBD patterns were analyzed using Pearson and Spearman correlation Mantel tests with 999 permutations, implemented in R using the vegan package (Oksanen et al., 2001; Mantel, 1967).

2.7 Comparing Neutral and Adaptive Genetic Variation

To distinguish between putatively neutral and adaptive genetic variation, I used the pcadapt function in PCAngsd (Meisner & Albrechtsen, 2018) to identify outlier loci associated with population structure (Luu et al., 2017). Pcadapt applies the Mahalanobis distance (DD) statistic to perform a genome-wide selection scan across all significant K principal components. The resulting z-scores are converted to a test statistic that follows a chi-square (X^2) distribution with K degrees of freedom.

PCAngsd identified one significant principal component (K = 1) based on Velicier's minimum average partial (MAP) test (Shriner & Vaughan, 2011). To reduce the impact of inbreeding, I utilized the --inbreedSites and --inbreed options in PCAngsd to filter out SNPs that showed significant deviations from Hardy-Weinberg equilibrium (HWE) due to inbreeding. This filtering resulted in a final dataset of 82,976 SNPs, yielding a genome-wide significance threshold of 6.03×10^{-7} (α=0.05; 82,976 SNPs×1 principal component) for detecting outlier loci (Meisner et al., 2021).

After identifying adaptive and neutral loci, I quantified genetic diversity for each population separately for adaptive and neutral loci by calculating average pairwise nucleotide differences (Theay Pi, π) based on the global site frequency spectrum (SFS). To estimate the SFS for each population, I first calculated allele frequency likelihoods using ANGSD (-doSaf 1) and then derived the maximum likelihood estimate of the SFS with the realSFS module (saf2theta). The resulting SFS was used as a prior to calculate π for each RAD locus using the thetaStat module in ANGSD (-do_stat). Per-site π values for each RAD locus were obtained by dividing the statistic by the length of the loci, and genome-wide averages for both adaptive and neutral loci were calculated by averaging across all RAD loci within each population.

To compare genetic differentiation between populations and to examine patterns of isolation by distance for neutral and adaptive loci, I calculated pairwise F_{ST} values and performed a Mantel test between geographic and genetic distances, as described earlier.

2.8 Genetic Variation Associated with Altitude

To investigate genetic variation associated with altitude, I performed a genomewide association study (GWAS) based on genotype likelihoods using score statistics (doAsso 2) in ANGSD. The score test utilizes a generalized linear model framework, where posterior genotype probabilities serve as the dependent variable and altitude (yQuant) as the predictor variable, calculating likelihood scores for each SNP independently (Skotte et al., 2012).

Association mapping was conducted by identifying polymorphic sites (-SNP_pval 1e-06), inferring major and minor alleles (-doMajorMinor 1), and estimating allele frequencies (-doMaf 1). SNPs with a minor allele frequency below 5% (-minMaf 0.05) or fewer than ten observations for each genotype class (homozygous major, heterozygous, and homozygous minor; -minHigh 10) were excluded. Likelihood ratio test (LRT) scores for each SNP were converted to PPP-values, assuming a chi-square (X^2) distribution with one degree of freedom. A Bonferroni correction was applied to account for multiple testing, resulting in a genome-wide significance threshold of 1.07×10^{-7} (α=0.05; 468137 SNPs; LRT>28.25).

To explore how allele frequencies at significantly associated sites vary with altitude, I plotted the frequency of the reference allele (defined as the minor allele across the entire dataset) at each altitude. This approach highlights patterns of allele frequency changes associated with elevation, providing insight into altitude-driven genetic variation.

2.9 Genetic Differentiation Between Color Morphs

To investigate genetic variation associated with color morphs, I conducted a Discriminant Analysis of Principal Components (DAPC) using the R package adegenet (v2.1.1; Jombart, 2008; Jombart & Ahmed, 2011). Genotypes were called in ANGSD (doGeno 4) and exported as VCF/BCF files (-doBcf 1) using a uniform prior (-doPost 2) and a posterior probability cutoff of 85% (-postCutoff 0.85). The DAPC was performed by retaining all principal component axes that explained up to 80% of the variance, with color morph (dark vs. light) as the predefined grouping variable.

To identify SNPs contributing most strongly to differentiation between the color morphs, I analyzed the variable contributions of SNPs to the linear discriminant functions. These contributions were hierarchically clustered using the “Average” method in the snpzip function of adegenet, grouping SNPs into 'outlier' and 'non-outlier' categories.

To further explore genetic differentiation at the identified outlier loci, I categorized individuals based on their genotypes at these sites (heterozygous, homozygous for the minor allele, or homozygous for the major allele). Genotype distributions were visualized as a heatmap using ggplot2, highlighting the variation in genotypic states between the dark and light morphs.

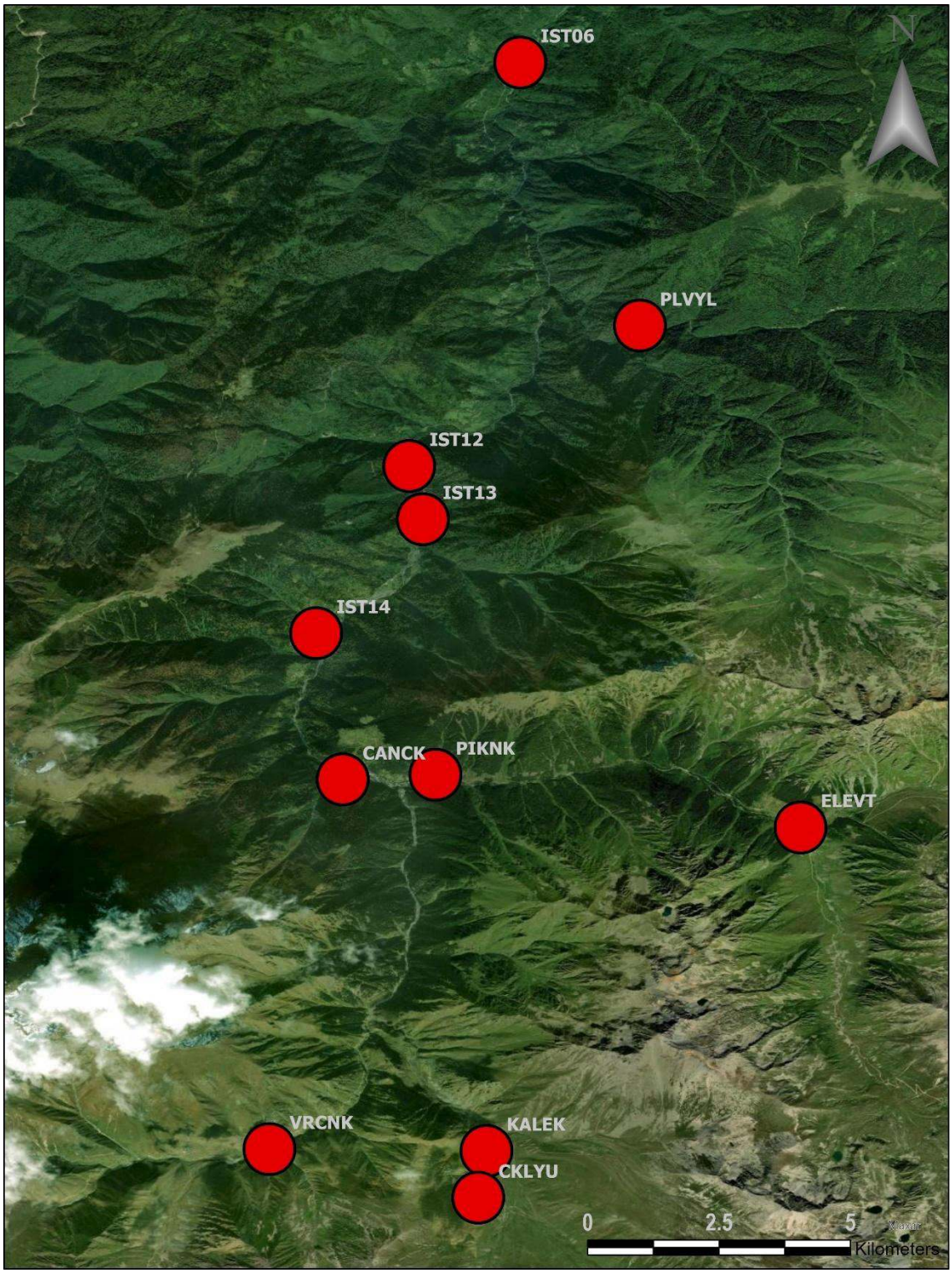

Figure 2.2: Collection sites of 11 populations of Isophya rizeensis within the Fırtına Valley.

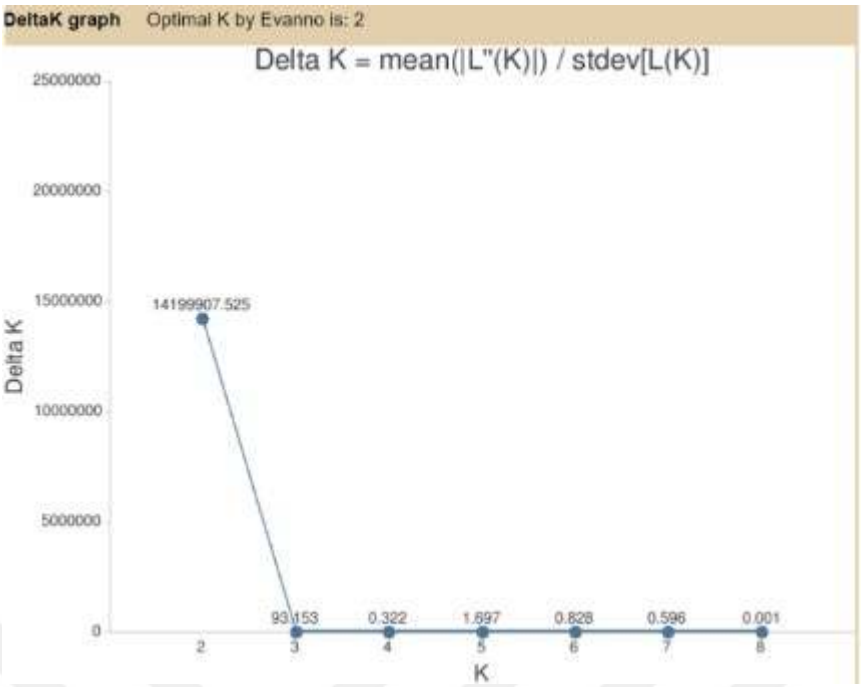

Figure 2.3: The most optimal K value by Evanno.

CHAPTER 3:
RESULTS

3.1 Reference RAD-loci assembly, alignment statistics and SNP discovery

Using our de novo reference assembly strategy, we identified 386,174 unique RAD loci, each 80 bp in length. After extending these loci with reverse reads, 224,187 loci were assembled into contigs ranging from 250 to 800 bp, with an average length of 324 bp. Raw read alignment to the de novo reference achieved mapping success rates of 73% to 83%, averaging 77%, with a mean individual read depth of approximately 5–6X. Detailed data on raw read counts, raw alignments, and alignments after filtering for proper pairs and removing PCR duplicates are provided in Table A1. Based on alignment statistics, we excluded individuals with fewer than 1,000,000 aligned reads after all filters, resulting in a final dataset of 71 individuals from 11 locations (Table 3.1). Across all collection sites, we identified 9,616 polymorphic loci and 92,048 high-confidence SNPs ($P < 10^{-12}$) with a minor allele frequency above 0.05.

Table 3- 1: Population names, geographic coordinates, altitudes, color morphs, and sample sizes of *Isophya rizeensis*.

Pop_name	Latitude	Longitude	Altitude (m)	Color	Number of Individuals
IST06	40.9856	40.9646	450	DARK	7
PLVYL	40.9406	40.9850	850	DARK	6
IST12	40.9166	40.9456	900	DARK	7

IST13	40.9075	40.9479	1000	DARK	7
IST14	40.8880	40.9297	1100	PALE	7
CANCK	40.8630	40.9342	1200	PALE	5
PIKNK	40.8638	40.9501	1300	PALE	5
ELEVT	40.8548	41.0125	1900	PALE	7
VRCNK	40.7998	40.9217	2000	PALE	7
KALEK	40.7995	40.9588	2100	PALE	7
CKLYU	40.7915	40.9574	2300	PALE	6

3.2 Population Genetic Structure

3.2.1 Principal Component Analysis

Principal Component Analysis (PCA), conducted using PCAngsd, revealed two distinct clusters along PC1, corresponding to dark- and pale-colored individuals sampled from lower and higher altitudes, respectively (Figure 3.1). However, both PC1 and PC2 accounted for only a small proportion of the total genetic variation (3.82% and 2.08%, respectively), suggesting limited overall genetic divergence between the groups. Additionally, a transitional zone was observed along the PC1 axis, consisting of individuals from intermediate altitudes (1100–1900 meters; IST14, CANCK, PIKNK, and ELEVT).

3.2.2 Admixture Analysis

Admixture analysis showed a distinct genetic pattern across populations, with Evanno's method (2005, Figure 2.3) indicating that the ideal K value was 2. Individuals from the first three localities, representing the lowest altitude populations, showed almost no genetic admixture with the second cluster, which predominantly consist of individuals from the highest altitude populations (Figure 3.2). This pattern shows the distinct separation of genetic clusters based on altitude. Furthermore, a transition zone was observed, comprising individuals with mixed ancestry from both clusters at mid altitudes (1100 -1900 meters). This observation is consistent with the results of the Principal

Component Analysis (PCA), which also indicated mixed genetic profiles in populations from mid-altitude ranges (Figure 3.1).

At K=3, the genetic differentiation between the lowest and highest altitude populations remained obvious. This further supports the hypothesis that color morphs contribute significantly to population structure and differentiation along altitudinal gradients. The transition zone, however, showed clear gene flow between populations, indicating ongoing genetic exchange despite environmental and morphological divergence (Figure 3.3). These findings underscore the combined influence of altitude and color morphs in shaping genetic differentiation and connectivity in *Isophya rizeensis* populations.

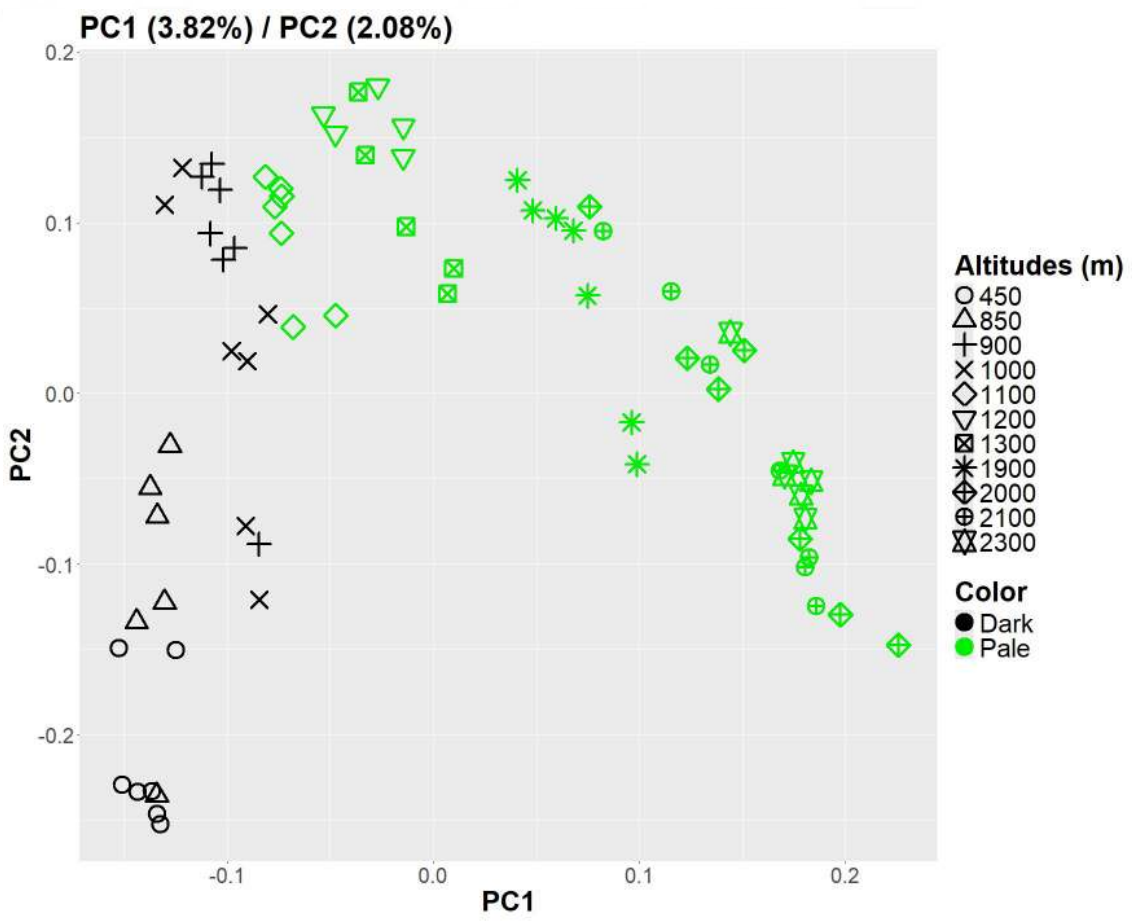

Figure 3.1: Distribution of individuals based on the first two components of the principal component analysis. Variance explained by each component is shown in parentheses. Dark individuals are represented by dark colors and pale-green individuals are represented by green colors. Populations are ordered by lowest to highest elevation.

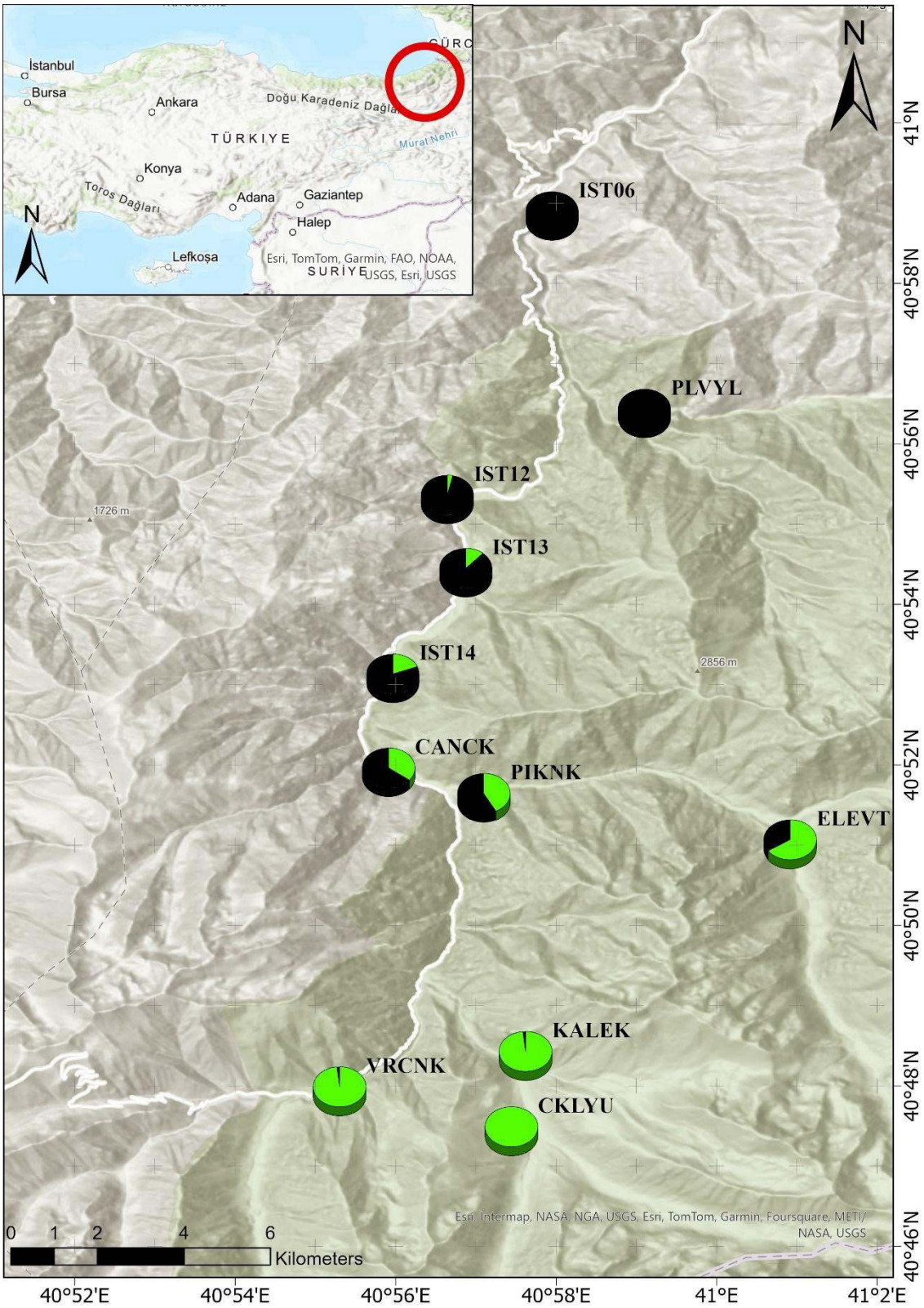
İstanbul
Bursa
Ankara
Doğu Karadeniz Dağları
TÜRKIYE
Murat Nehri
Konya
Toros Dağları
Adana
Gaziantep
Halep
Lefkoşa
SURİYE
Esri, TomTom, Garmin, FAO, NOAA, USGS, Esri, USGS
N
IST06
PLVYL
IST12
IST13
IST14
CANCK
PIKNK
ELEVT
KALEK
VRCNK
CKLYU
1726 m
2856 m
0 1 2 4 6
Kilometers
Esri, Intermap, NASA, NGA, USGS, Esri, TomTom, Garmin, Foursquare, METI/NASA, USGS
41°N
40°58'N
40°56'N
40°54'N
40°52'N
40°50'N
40°48'N
40°46'N
40°52'E
40°54'E
40°56'E
40°58'E
41°E
41°2'E

Figure 3.2: Locations of the populations and the accumulated admix proportion for two genetic clusters. The dark color indicates the first cluster, and the green color represents the second cluster Altitude in the study area increases progressively from the topmost location (450 meters) to the bottommost location (2300 meters).

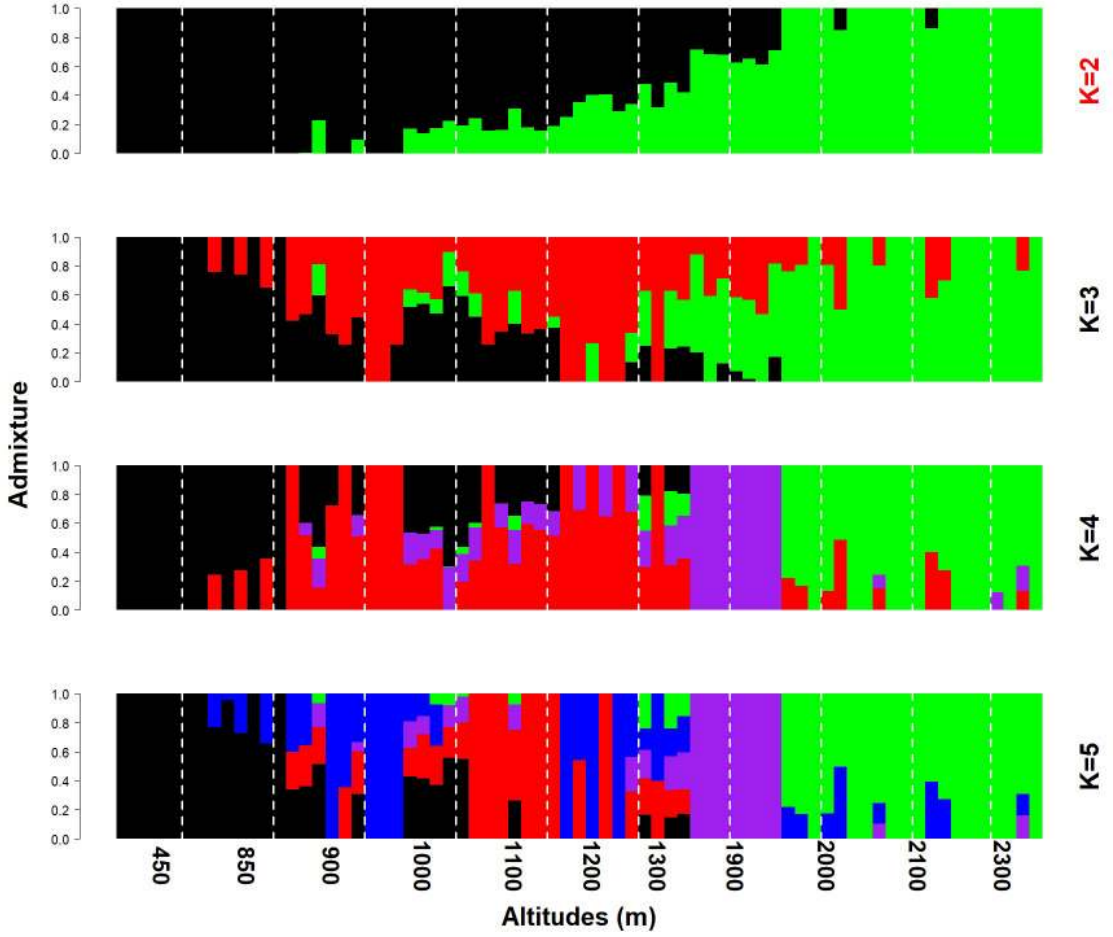

Figure 3.3: Admixture proportions of 11 populations with the K values ranges from 2 to 5.

3.3 Genetic Differentiation and Isolation by Distance (IBD)

Pairwise F_{ST} comparisons revealed relatively low genetic differentiation along the altitudinal gradient and between populations (<0.05), indicating substantial genetic connectivity across the landscape (Figure 3.4). Despite the overall low divergence, relative genetic differentiation correlated with variation in coloration. For instance, populations dominated by black-colored individuals, such as IST06-PLVYL and IST12IST13, exhibited higher genetic similarity to each other compared to green-pale populations like CANCK, VRCNK, and CKLYU (Figure 3.4). Additionally, a Mantel test revealed a significant relationship between linearized F_{ST} and geographic distance for both Pearson

(r = 0.805, p = 0.001) and Spearman (r = 0.8242, p = 0.001) correlation tests, indicating a clear pattern of isolation by distance (Figure 3.5).

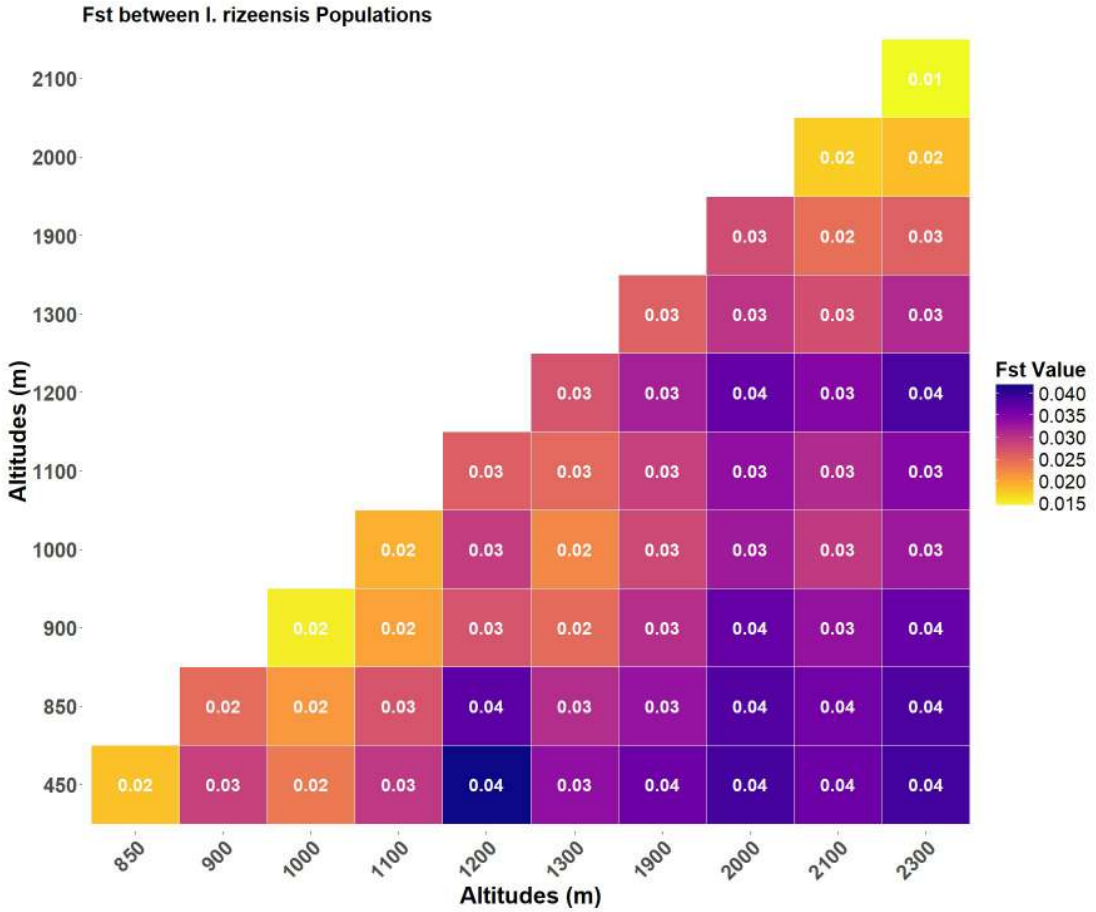

Figure 3.4: Genetic divergence is estimated by pairwise Fst values, shown as heatmap plots. Pairwise comparisons correspond to each box, and values represent the Fst values.

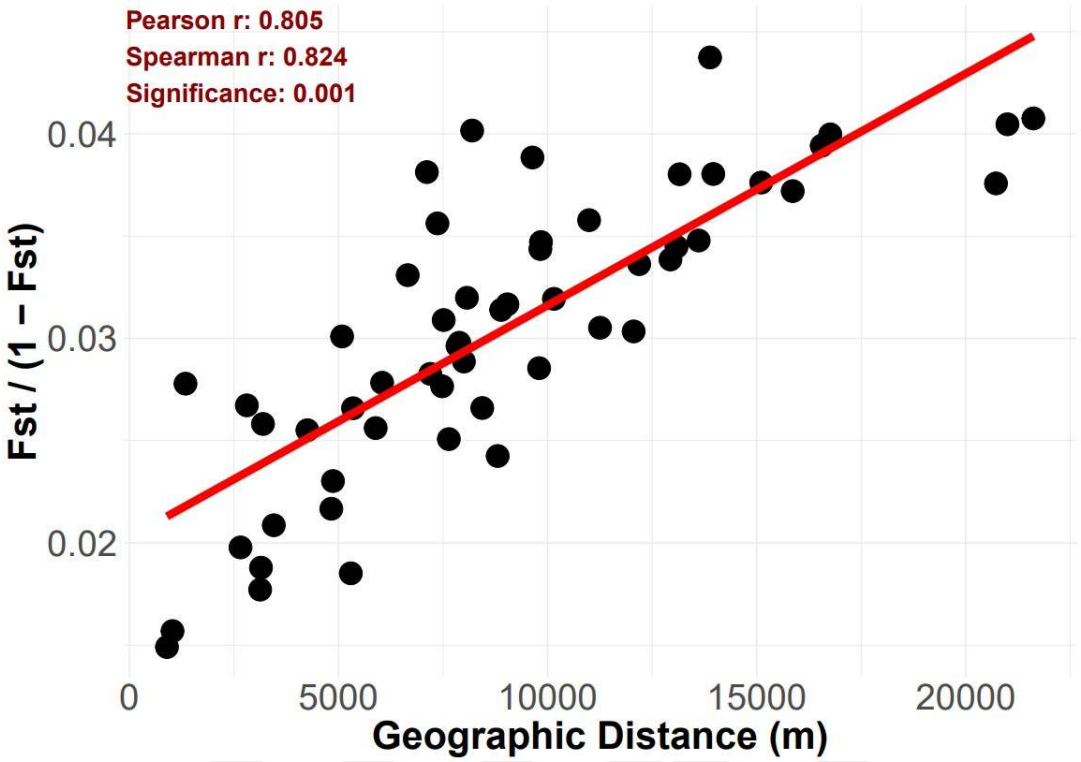

Figure 3.5: The average genomic differentiation is plotted against the estimated geographic distance on a pairwise level. Results of the Spearman and Pearson Mantel statistics are noted in the lower right corner of the graph. A trendline is drawn in red.

3.4 Comparing Neutral and Adaptive Genetic Variation

PCAdapt detected 1,113 putatively adaptive loci and 81,863 neutral loci. Mean genetic diversity was highest at mid altitudes (IST14, CANCK, PIKNK) and fell off at both low and high altitudes for both putatively adaptive and neutral loci (Fig. 3.6). However, the comparison of diversity at neutral and adaptive loci revealed distinct patterns along the altitudinal gradient. Populations at mid-altitudes showed relatively similar diversity values for both neutral and adaptive loci, indicating homogeneity in these measures. However, populations at low and high altitudes displayed marked differences between neutral and adaptive loci. Specifically, adaptive loci at these altitudinal extremes exhibited significantly reduced diversity compared to neutral loci, accompanied by an increase in genetic divergence (Fig. 3.6).

To compare patterns of genetic divergence between populations in relation to adaptive and neutral variation along the altitudinal gradient, I conducted separate Mantel tests to evaluate the correlation between geographic distance and linearized F_{ST} values for both putatively adaptive and neutral loci. The results revealed that genetic divergence increased with geographic distance for both types of loci; however, the slope of this relationship was significantly steeper for adaptive loci (Fig. 3.7). These findings align with the observed patterns of genetic diversity along the gradient, where adaptive loci exhibited greater divergence and signs of allelic fixation at high and low altitudes. This suggests that adaptive processes might be playing a critical role in driving genetic differentiation at both extremes of the altitudinal gradient.

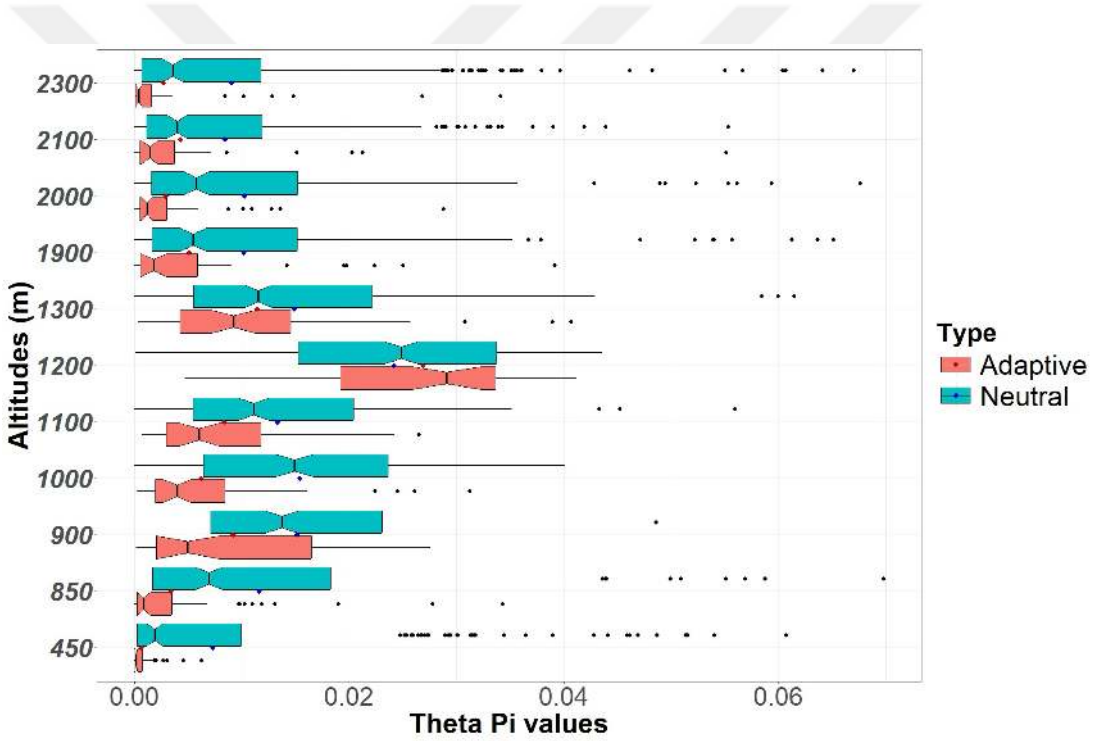

Figure 3.6: Comparison of genetic diversity of neutral and putatively adaptive variation along an altitudinal gradient for *I. rizeensis*.

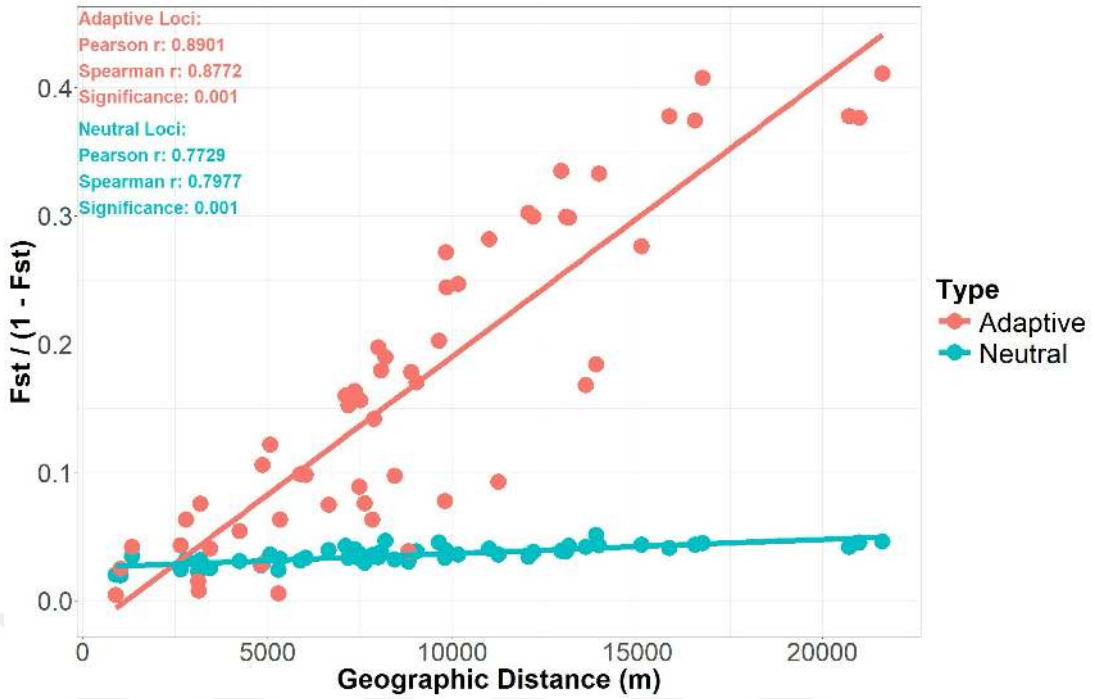

Figure 3.7: Comparison of genetic differentiation of neutral and putatively adaptive variation along an altitudinal gradient for *I. rizeensis*. Results of the Spearman and Pearson Mantel statistics are noted in the top left corner of the plot. Trendline for both loci classes indicated as red (adaptive) and blue (neutral).

3.5 Association mapping of altitudinal variation

Genome-wide association studies using altitude as a quantitative variable identified 42 loci significantly associated with genetic variation along the altitudinal gradient (LRT > 28.25, Table 3-2). Allele frequency changes at these loci across the altitudinal gradient revealed two distinct patterns. At some loci, frequency of the reference allele (defined as the minor allele in the full dataset) was elevated in populations at low altitudes (Fig. 3.8), while at other loci, the reference allele frequency was elevated at high altitudes (Fig. 3.9). This opposing pattern suggests that different loci may be under selection at the extremes of the gradient. Such divergent allele frequency shifts are consistent with the action of disruptive selection, potentially playing a key role in shaping genetic variation and promoting local adaptation across the altitudinal gradient.

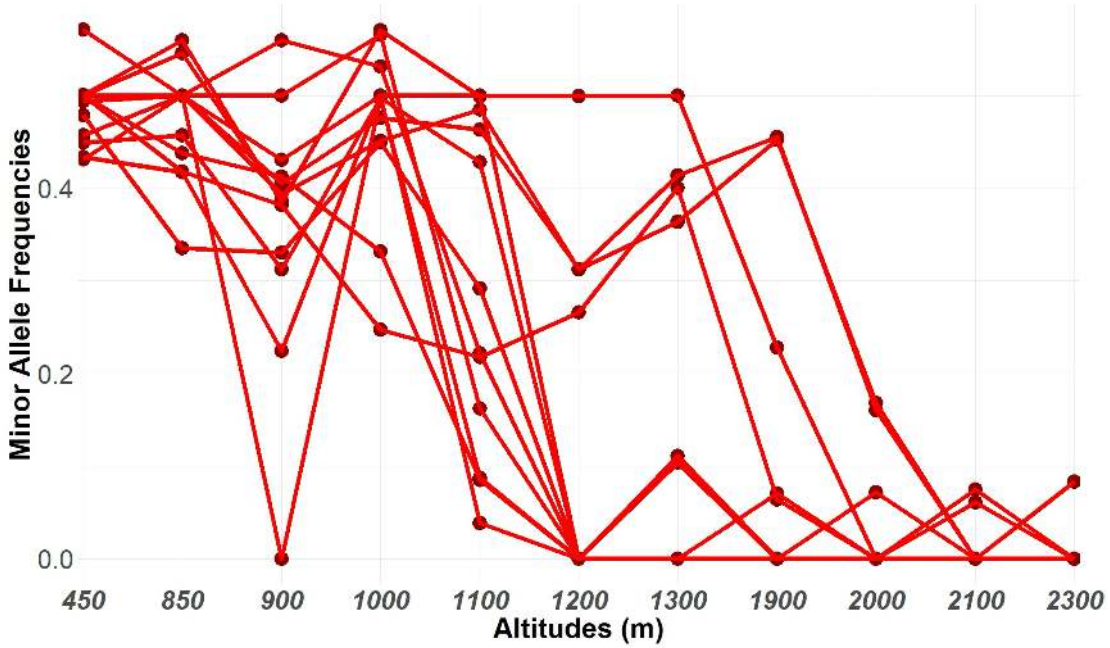

Figure 3.8: Minor allele frequency changes across 12 loci show a decreasing trend with increasing altitude. Each point along a line represents the same locus across different altitudinal populations. The y-axis indicates minor allele frequency, while the x-axis represents populations ordered by altitude.

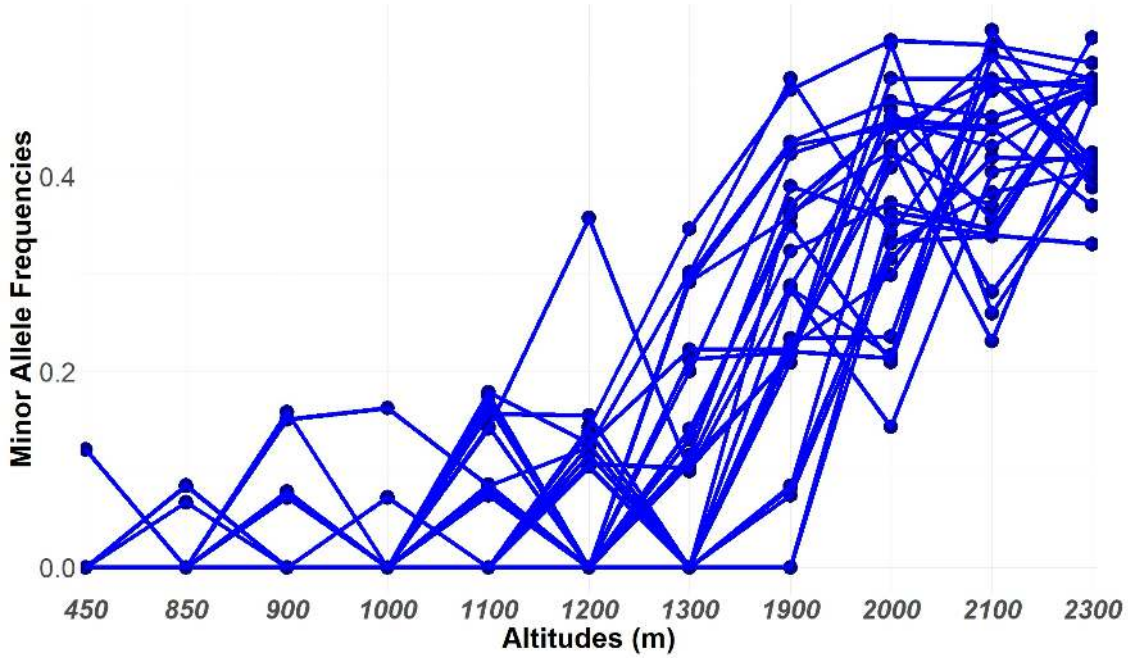

Figure 3.9: Minor allele frequency changes across 30 loci show an increasing trend with increasing altitude. Each point along a line represents the same locus across different

altitudinal populations. The y-axis indicates minor allele frequency, while the x-axis represents populations ordered by altitude.

Table 3-2: Genome-wide association results using altitude as a quantitative variable, identifying 42 loci significantly associated with genetic variation along the altitudinal gradient in *Isophya rizeensis.* Columns represent the locus identifier (Chromosome), nucleotide Position, Major and Minor alleles, Minor allele Frequency, sample size (N), likelihood ratio test statistic (LRT), and genotype counts for homozygous wild type (high_WT), heterozygous (HE), and homozygous alternative (HO) genotypes.

Chromosome	Position	Major	Minor	Frequency	N	LRT	high_WT/HE/HO
R024672	18	G	A	0.270594	71	34.494475	28/30/2
R024672	35	G	A	0.225487	71	31.35841	30/27/0
R024672	53	T	C	0.260476	71	40.341002	30/29/2
R049758	202	C	A	0.225285	71	32.766915	36/28/0
R056201	76	C	A	0.181986	71	29.389487	36/20/0
R065322	41	A	G	0.186472	71	30.172101	38/23/0
R070847	35	C	A	0.173243	71	28.544634	40/22/0
R072477	29	A	C	0.343051	71	28.360519	11/41/0
R072477	49	G	A	0.346316	71	29.434433	14/40/1
R075206	18	T	C	0.117529	71	31.9856	51/15/0
R075206	42	C	T	0.108761	71	31.810491	54/14/0
R106851	52	C	G	0.135365	71	32.153199	49/15/0
R106851	58	G	A	0.141	71	33.466291	50/18/0
R106851	79	G	A	0.142361	71	29.341852	48/19/0
R108022	64	T	A	0.228723	71	29.840657	34/29/0
R112328	80	C	T	0.131155	71	32.039096	52/17/0
R118398	69	A	G	0.182765	71	29.192555	38/22/0
R126146	82	C	T	0.123794	71	31.050146	48/15/0
R138588	36	G	T	0.148793	71	37.139785	47/17/0
R160254	68	C	A	0.111066	71	29.641676	49/14/0
R160254	69	C	A	0.112441	71	29.386419	48/14/0
R170442	103	C	A	0.160506	71	37.159325	46/19/0
R182550	54	G	A	0.215541	71	29.328153	38/25/0
R199428	67	G	A	0.119792	71	28.739974	50/15/0
R242332	26	A	G	0.328726	71	47.937508	20/45/0
R249864	82	C	T	0.161864	71	29.237197	46/20/0
R251367	38	T	C	0.171165	71	29.883969	38/20/0
R253129	55	C	A	0.2302	71	40.15776	36/27/0
R262745	81	C	A	0.143476	71	32.873732	50/16/0
R270196	59	A	G	0.179943	71	40.308224	42/22/0
R280198	164	T	A	0.249824	71	31.275437	30/31/0
R314063	61	G	A	0.23729	71	46.064349	31/27/0
R316726	66	A	T	0.176149	71	28.984722	45/17/0
R317285	24	C	G	0.226245	71	42.964859	32/27/0
R317285	36	G	C	0.217797	71	40.878552	33/26/0
R317285	47	A	T	0.203874	71	40.66285	34/24/0
R317285	78	G	T	0.208516	71	40.951667	35/26/0
R317285	79	C	G	0.21088	71	40.849	33/26/0
R350379	40	C	T	0.149595	71	28.308667	46/19/0
R379515	81	T	G	0.140631	71	33.096485	49/17/0
R382423	70	T	A	0.193679	71	30.503884	30/23/0
R383457	51	C	A	0.14941	71	35.639803	49/18/0

3.6 Genetic Discrimination between Color Morphs

Discriminant analysis of principal components (DAPC) based on dark and pale color morphs successfully separated individuals into two distinct groups along the first discriminant function (DF1), with high posterior probabilities of assignment (0.97; Fig. 3.10). Pale individuals were classified with perfect accuracy (posterior assignment success

= 1.0), while 92.6% of dark individuals were correctly assigned, with two individuals misclassified as pale (Fig. 3.11).

Analysis of loadings across DF1 identified three loci with major effects that significantly contributed to genetic differentiation between the two color morphs (Fig. 3.12). Genotype analysis at these loci revealed two clear genotypic blocks corresponding to color morphs: pale individuals were nearly fixed and homozygous for the major allele, whereas dark individuals were predominantly heterozygous (Fig. 3.13). Moreover, all three major effect loci identified in the DAPC analysis were also detected to be significantly associated with altitude (Table 3-2). This overlap indicates that maintenance of color polymorphism is also linked to altitudinal variation.

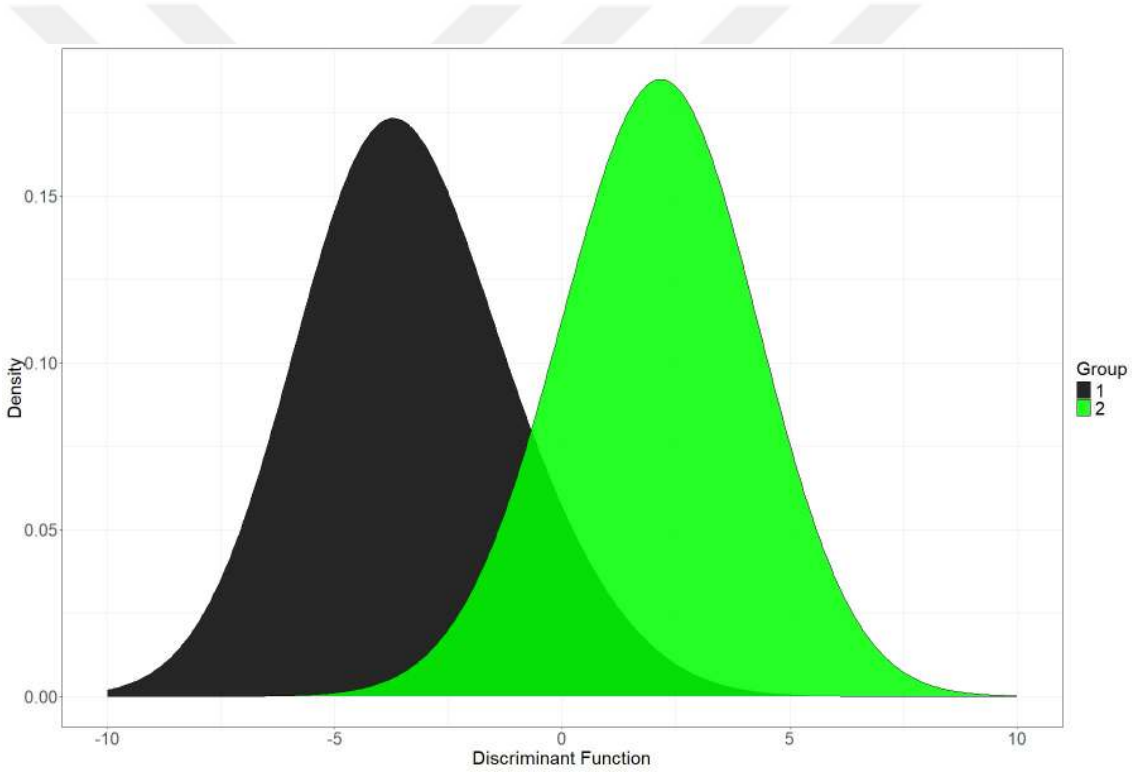

Figure 3.10: The density plot shows Discriminant analysis for partitioning genetic variation between color morphs in *I. rizeensis*. Dark color represents Group 1, which contains dark-color individuals, and green color represents Group 2, which contains green-color individuals.

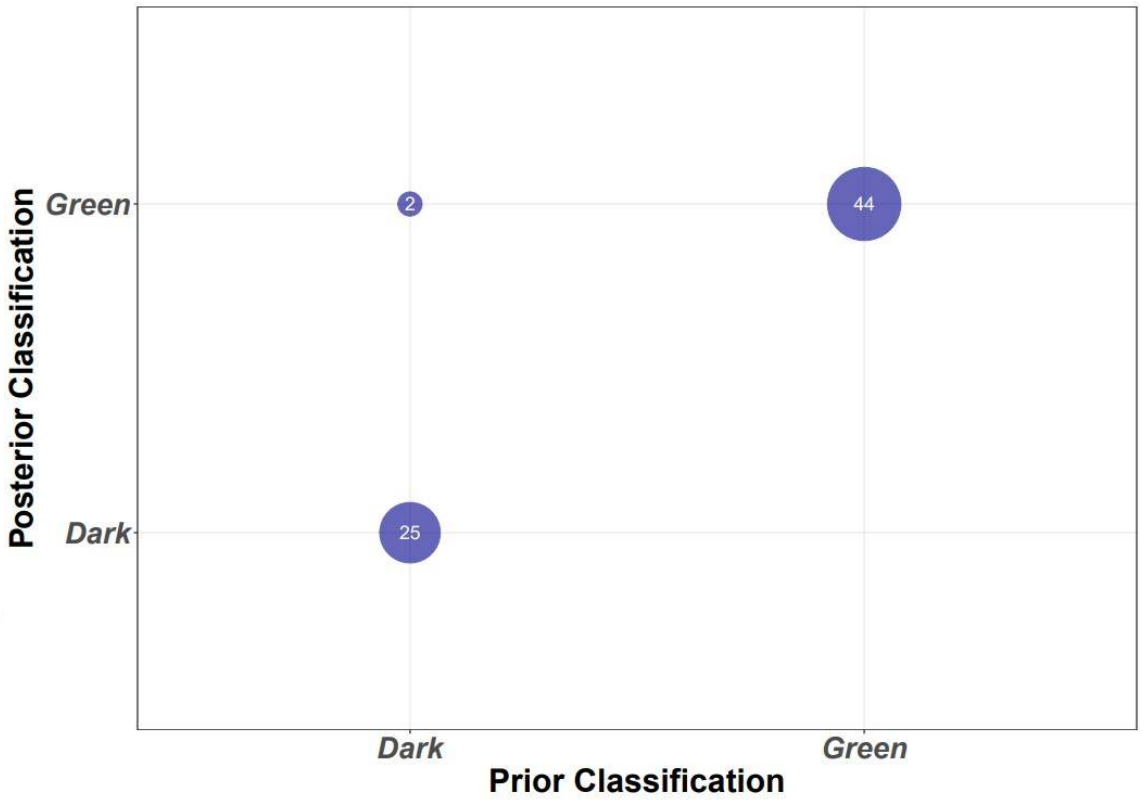

Figure 3.11: Bubble plot represents DAPC analysis, assignment probabilities (x axis prior information and y axis posterior classifications, 97% successful classification.)

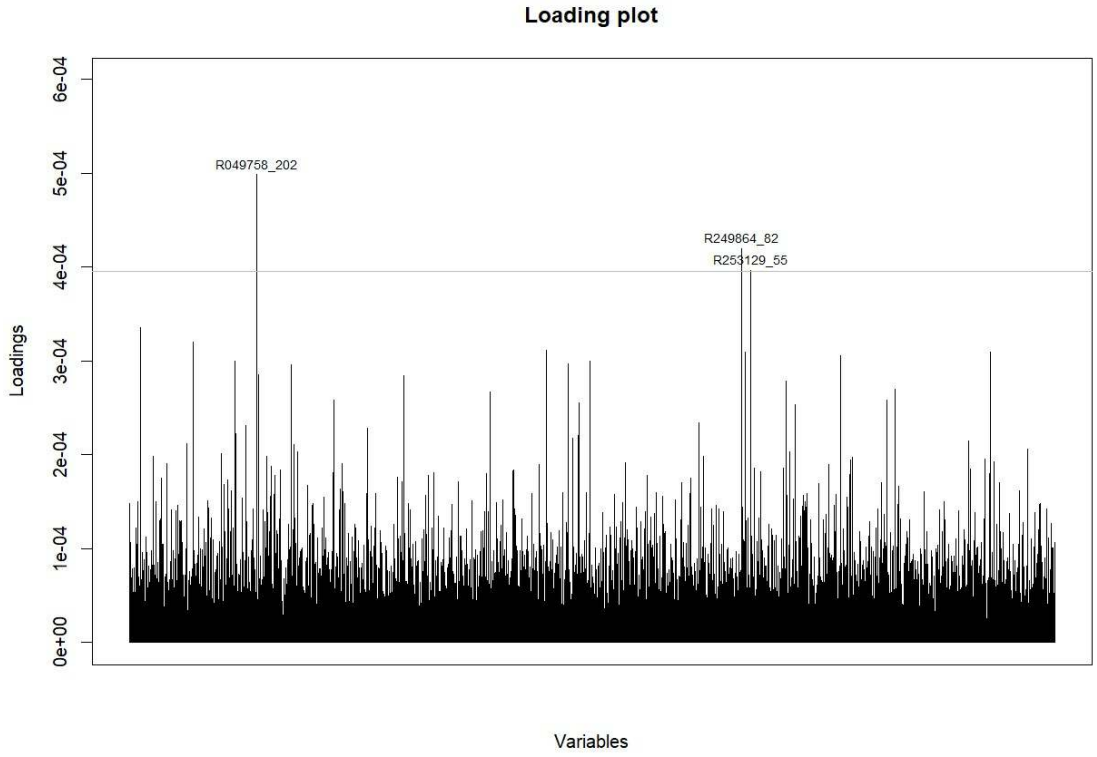

Figure 3.12: The loading plot of contributions of loci to the DAPC. A hierarchical clustering analysis using the average clustering method was performed on the distance matrix to separate loci into those that contribute to group divergence and those that do not. The threshold is represented as a black horizontal line.

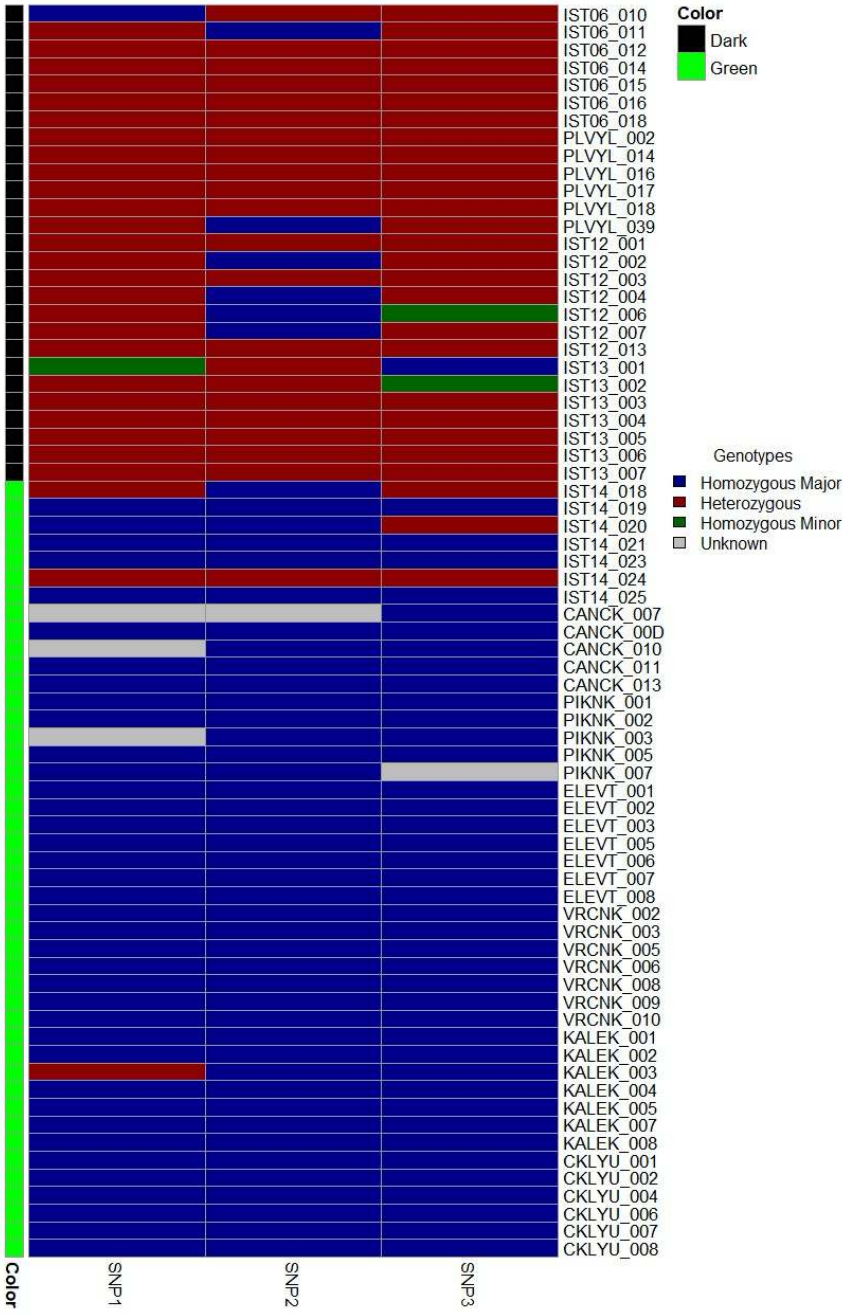

Figure 3.13: Genotypes of 3 significant outliers detected with two different approaches and their changes among different subpopulations within different altitudes. Color of dark and green represent the body colors of *I. rizeensis*. Individuals' names are on the right y-axis of the plot.

CHAPTER 4:

DISCUSSION

Global biodiversity is under unprecedented threat due to the accelerating impacts of climate change and environmental degradation, particularly for species with restricted ranges or specialized ecological requirements (Thomas et al., 2004; Malcolm et al., 2006; Harvey et al., 2020). Predicting future biodiversity outcomes and designing effective conservation strategies require a thorough understanding of how species respond to environmental changes (Schilthuizen & Kellermann, 2013; Kingsolver & Buckley, 2017; Kellermann & Heerwaarden, 2019; Waldvogel et al., 2020).

Research into the genetic and phenotypic processes that enable species to survive in changing environments is critical for developing adaptive management strategies that can promote long-term biodiversity conservation (Merilä & Hendry, 2014; Kelly, 2019; Feiner et al., 2021). Studying phenotypic and genetic variation along environmental gradients, such as altitude, offers a powerful approach for investigating adaptive responses. These natural laboratories provide insights into the evolutionary processes shaping population differentiation, local adaptation, and the genetic mechanisms underlying key traits (Wogan & Wang, 2017; Kelly, 2019).

This study represents the first comprehensive examination of the genetic basis of color polymorphism in *I. rizeensis*, an endemic bush cricket, along an altitudinal gradient. By analyzing associations between genetic architecture, color morphs, and altitude, this research sheds light on how populations of this species adapt to varying environmental conditions along a cline. The findings highlight the role of altitude as a selective force, influencing loci linked to color polymorphism and driving genetic differentiation.

This work not only advances our understanding of the genetic mechanisms underlying adaptation in *I. rizeensis* but also serves as a valuable case study for exploring how montane species adapt to climatic and ecological gradients. By integrating genetic, phenotypic, and environmental perspectives, this research provides critical insights into

the evolutionary dynamics of adaptation and informs conservation strategies for species facing environmental challenges in rapidly changing ecosystems.

4.1 Population Structure and Genetic Connectivity

Our findings revealed clear genetic structuring along the altitudinal gradient in *I. rizeensis*, with distinct genetic clusters observed at high and low altitudes and a transitional zone at mid-elevations. This structure, as indicated by PCA and admixture analyses, reflects the influence of altitude on genetic differentiation. Admixture analysis identified two primary genetic clusters, with individuals from mid-altitudes showing mixed ancestry, highlighting the transitional nature of these populations (Figs. 3.1–3.4).

Despite the genetic structuring, pairwise F_{ST} values (<0.05) indicate substantial genetic connectivity across populations. This suggests that while gene flow reduces absolute isolation, localized selection pressures at different altitudes drive divergence at adaptive loci (Tigano & Friesen, 2016; Wang & Bradburd, 2014). The significant Mantel test results (Pearson r=0.805, p=0.001) confirm a pattern of isolation by distance, but the overall weak genetic differentiation implies that migration continues to play a significant role in maintaining connectivity along the gradient. Similar patterns of partial genetic isolation have been reported in other mountain-dwelling species, where gene flow coexists with local adaptation to altitude-specific environmental pressures (Orsini et al., 2013; Sexton et al., 2014).

The association between genetic clusters and color morphs underscores the role of altitude in shaping population differentiation. Black morphs dominate low-altitude populations, while pale morphs are prevalent at higher altitudes, a distribution pattern consistent with local adaptation to environmental variables such as temperature, vegetation, and UV radiation. This correlation suggests that color polymorphism in *I. rizeensis* is an adaptive trait influenced by selective forces operating along the altitudinal gradient. Similar associations have been documented in other orthopterans and insects, where color polymorphism reflects responses to ecological selection pressures (Ahnesjö & Forsman, 2006; Sandoval, 1994).

Although the presence of gene flow in the transitional zone suggests that genetic differentiation is not absolute, the observed structure indicates that selective pressures may maintain genetic divergence in key traits such as color morphs. This aligns with findings from other species inhabiting environmental gradients, where local adaptation to

microclimatic conditions reinforces genetic differentiation even in the face of substantial migration (Nosil, 2012; Wang & Bradburd, 2014). The results highlight the interplay between gene flow and selection, emphasizing the complex dynamics shaping genetic structure and connectivity in *I. rizeensis*.

4.2 Adaptive and Neutral Variation

Analysis of adaptive and neutral loci in *I. rizeensis* revealed distinct patterns of genetic diversity and differentiation along the altitudinal gradient. A total of 1,113 adaptive loci and 81,863 neutral loci were identified, highlighting the contrasting influences of selection and drift across populations. Neutral loci exhibited relatively uniform diversity across populations, suggesting minimal influence of selection, while adaptive loci showed significant reductions in diversity and increased divergence at the altitudinal extremes. This pattern is consistent with strong selection pressures driving local adaptation in contrasting habitats, as predicted by clinal selection theory (Savolainen et al., 2007).

At mid-altitudes, populations maintained higher levels of genetic diversity for both adaptive and neutral loci, likely due to gene flow and the mixing of alleles from high- and low-altitude populations (Endler, 1977; Savolainen et al., 2007). In contrast, high and low altitude populations exhibited reduced diversity and increased divergence at adaptive loci, suggesting selective forces promoting the fixation of alleles suited to specific environmental conditions. This divergence in adaptive loci underscores the role of local adaptation in shaping genetic variation, where traits advantageous for survival and reproduction are selected for under the unique pressures of lowland and highland environments (Hereford, 2009; Tigano & Friesen, 2016).

The steeper slope of genetic divergence with distance for adaptive loci compared to neutral loci further supports the hypothesis of isolation by environment (IBE). While geographic distance explains some of the genetic structure, adaptive responses to environmental pressures—such as temperature, humidity, and vegetation—drive additional differentiation, particularly at altitudinal extremes (Hermisson & Pennings, 2005). This dual influence of isolation by distance (IBD) and IBE highlights the complexity of genetic differentiation in montane environments.

These findings align with studies in other taxa showing that selection can lead to the fixation of adaptive polymorphisms even in the presence of gene flow (Hoffmann & Sgrò, 2011; Wakamiya et al., 2023). For *I. rizeensis*, adaptive loci likely confer advantages in specific habitats, facilitating survival in diverse environmental conditions along the altitudinal gradient. This trend is further supported by association studies, which show allele frequency shifts at adaptive loci consistent with selection at high and low elevations (Fig. 3.8–9).

Overall, these results underscore the critical role of adaptive loci in the evolutionary response of *I. rizeensis* to environmental variation, highlighting the importance of selection in maintaining genetic diversity and promoting local adaptation across ecological gradients.

4.3 Disruptive Selection Along the Latitudinal Gradient

Genome-wide association studies identified 42 loci significantly associated with altitude, with allele frequency shifts at these loci showing distinct patterns at high and low altitudes. These opposing allele frequencies suggest the action of disruptive selection, where different loci are under selection at each end of the gradient, promoting local adaptation to contrasting environmental conditions (Frei et al., 2013; Schluter, 2000). Disruptive selection, which favors extreme phenotypes over intermediate ones, likely maintains genetic variation across populations by favoring divergent alleles suited to high- or low-altitude habitats (Anderson et al., 2013; Zhao et al., 2021). This form of selection drives genetic divergence by promoting distinct allele frequencies at high and low altitudes, where populations encounter different selective pressures, such as varying vegetation, temperatures, humidity, and UV exposure (Frei et al., 2013; Haider et al., 2012).

In this study, disruptive selection appears to drive adaptation by maintaining genetic polymorphism across elevations, with a near-fixation of specific alleles at the extremes of the gradient, reflecting the specialized adaptations required for survival in these contrasting environments. The loss of specific alleles at one end of the gradient and their predominance at the other suggests that environmental pressures, acting as ecological filters, select for genetic variants that optimize fitness under local conditions (Nosil, 2012; Yeaman & Whitlock, 2011). This pattern is consistent with findings in alpine

and montane species, where disruptive selection often maintains genetic diversity by favoring locally adaptive alleles suited to specific altitudinal ranges (Chiou et al., 2022; Niu et al., 2024; Anderson et al., 2013).

As disruptive selection promotes genetic differentiation across ecological zones, populations at extreme altitudes may become genetically divergent, while populations at mid-elevations exhibit a mix of alleles from both ends of the gradient, enabling them to cope with a broader range of environmental conditions. This process illustrates how disruptive selection can preserve high levels of genetic diversity within species (Schluter, 2000; Freeland, 2020; Zhao et al., 2021).

4.4 Genetic Discrimination of Color Polymorphism

Discriminant analysis of principal components (DAPC) confirmed strong genetic differentiation between dark and pale color morphs, with three loci showing significant contributions to this separation. A strong genetic separation was observed in DAPC analysis when two groups (dark and green-pale color morphs) were set as priori clusters (Fig. 3.10-11). This suggests that environmental factors related to altitude are crucial in preserving this differentiation. Genotypic analysis revealed that pale morphs were nearly fixed for the major allele, while dark morphs were predominantly heterozygous at these loci. The observed bimodal genotype distribution at these loci suggests the existence of two discrete genetic classes within *I. rizeensis* populations, corresponding to the altitudedriven color morphs. Bimodal distributions in genetic data often indicate selective pressures promoting the maintenance of distinct phenotypic classes across environmental gradients, a phenomenon observed in other species with polymorphic traits linked to environmental factors (Lee & Mitchell-Olds, 2012; Haldane, 1948; Lande, 1980).

The overlap between loci identified in the DAPC analysis and those detected in association mapping with altitude further supports the idea that these genetic regions are under selection in response to altitudinal variation, in addition to differentiating color variants. These loci likely play a key role in the adaptive responses along the altitudinal gradient. The varying allele frequencies observed at mid-altitudes (1100–1900 meters) in the minor allele frequency (MAF) plots (Fig. 3.8-9) also suggest that other ecological factors—such as vegetation, temperature, humidity, and UV radiation—may drive

adaptive variation in these loci, beyond just altitude (Mullen & Hoekstra, 2008; Villoutreix et al., 2023).

It seems that both genetic differentiation and selection forces enable the persistence of color polymorphism in *I. rizeensis* across a broad altitudinal range. This pattern aligns with findings in other taxa, where discrete color morphs are maintained through local adaptation to contrasting environmental conditions, such as those along elevational gradients (Mullen & Hoekstra, 2008; Villoutreix et al., 2023). In such cases, color polymorphism often represents a balance between gene flow and selection, where environmental differences enforce the existence of multiple morphs within a population.

CHAPTER 5:
CONCLUSION AND FUTURE DIRECTIONS

This study highlights the complex interplay between gene flow, selection, and local adaptation in shaping the genetic and phenotypic diversity of *I. rizeensis*. While high connectivity across populations facilitates gene flow, divergent selection pressures at high and low altitudes continue to shape genetic and phenotypic traits, maintaining distinct color morphs and adaptations suited to different environmental conditions. These findings strongly suggest that a combination of environmental factors, including vegetation, temperature, humidity, and UV radiation along the altitudinal gradient, contribute to the evolution and maintenance of color polymorphism in this species. By examining how local adaptation influences genetic differentiation, this study provides important insights into the processes that allow species to adapt to altitudinal gradients, contributing to a broader understanding of the mechanisms underlying biodiversity in montane ecosystems and their implications for conservation

Future research should investigate the ecological and physiological functions of the identified adaptive loci, particularly their role in survival and adaptation across various environmental conditions. Further exploration into the adaptive significance of color morphs will be crucial for informing conservation strategies that address the challenges posed by climate change and habitat alteration.

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APPENDIX A

APPENDIX

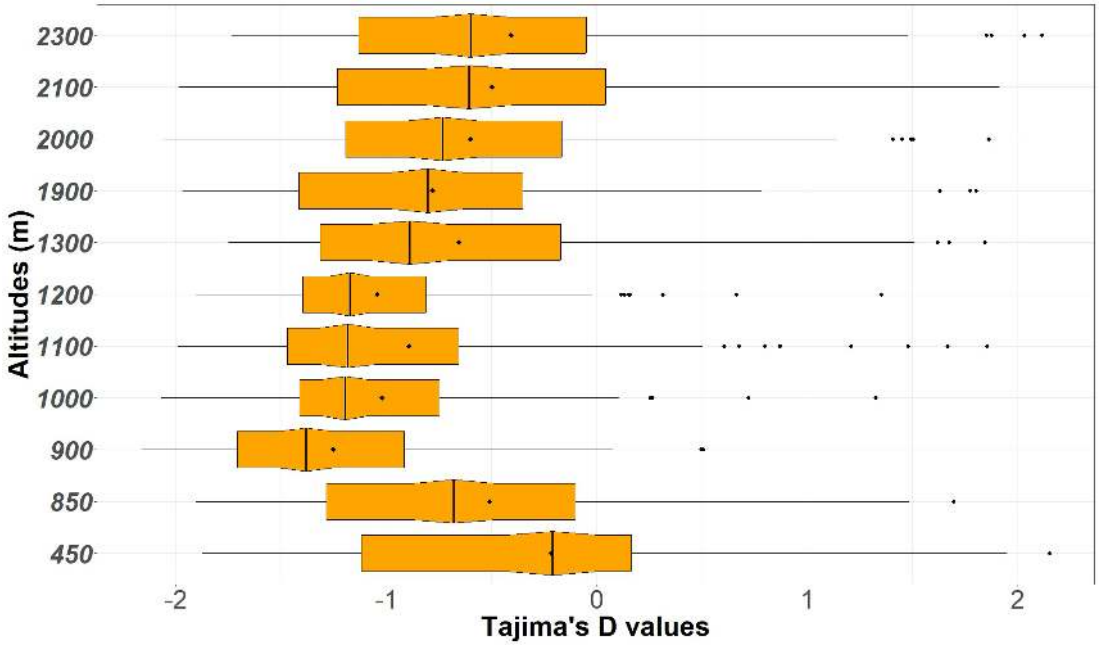

Figure A. 1: Overall Tajima's D values for *Isophya rizeensis* populations

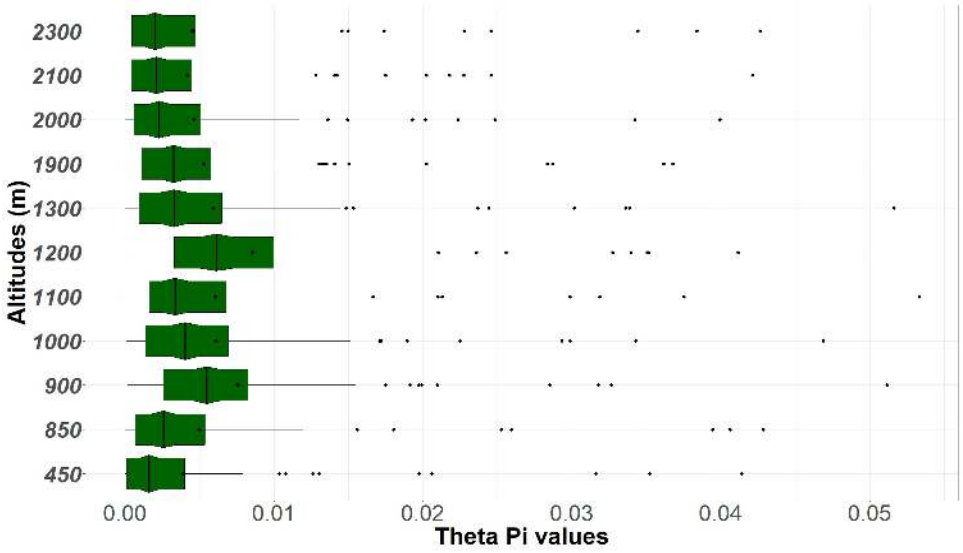

Figure A. 2: Overall Theta Pi Values for *Isophya rizeensis* populations.

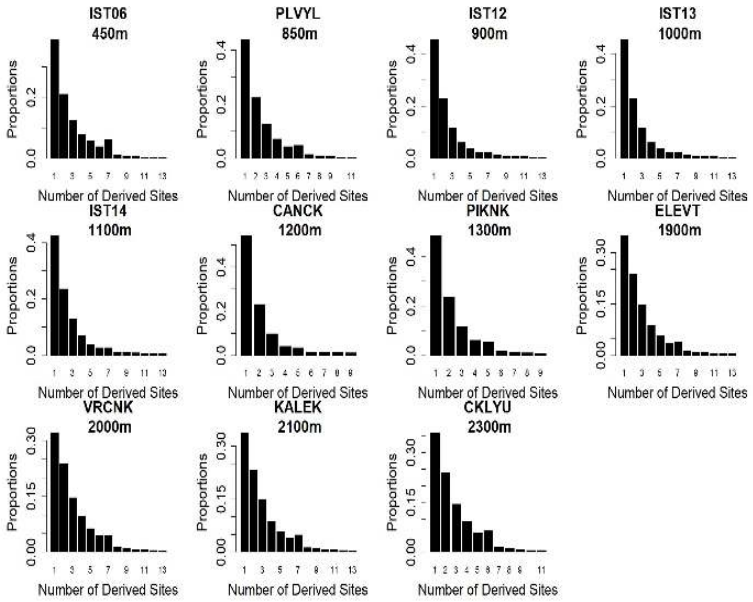

Figure A. 3: Unfolded SFS barplots for all populations after paralogs elimination.

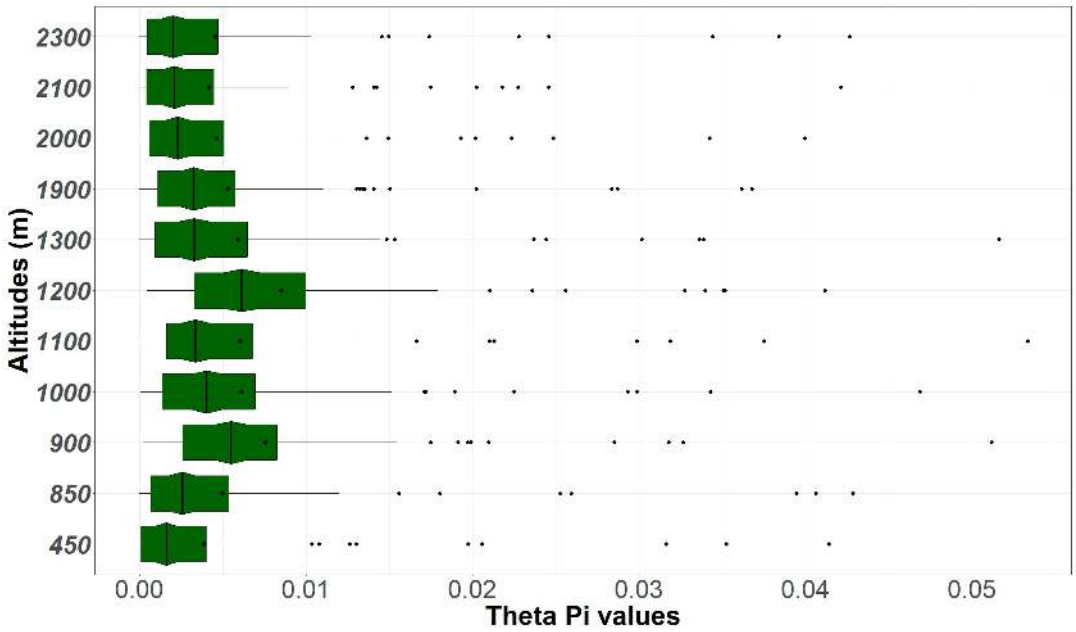

Figure A. 4: Overall Watterson's Theta values for *Isophya rizeensis* populations.

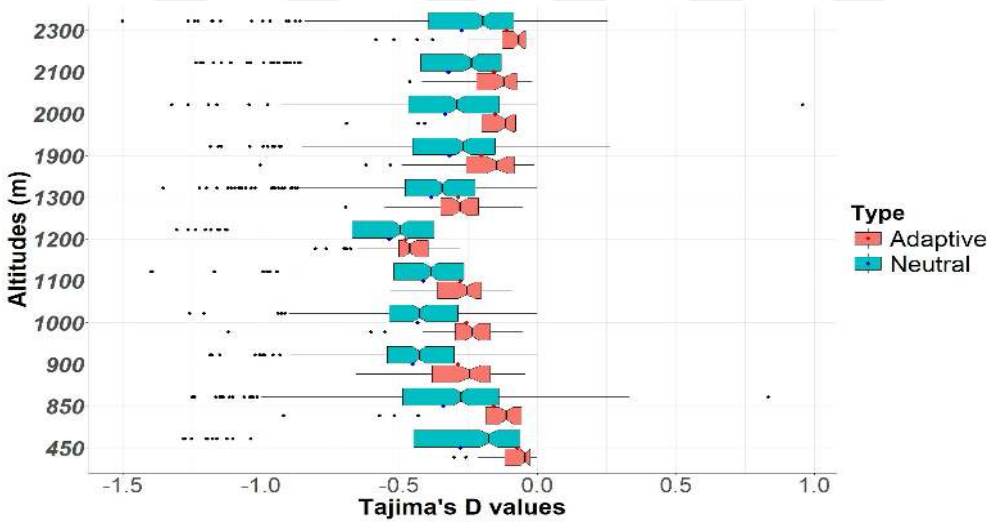

Figure A. 5: Tajima's D values for putatively adaptive and neutral loci.

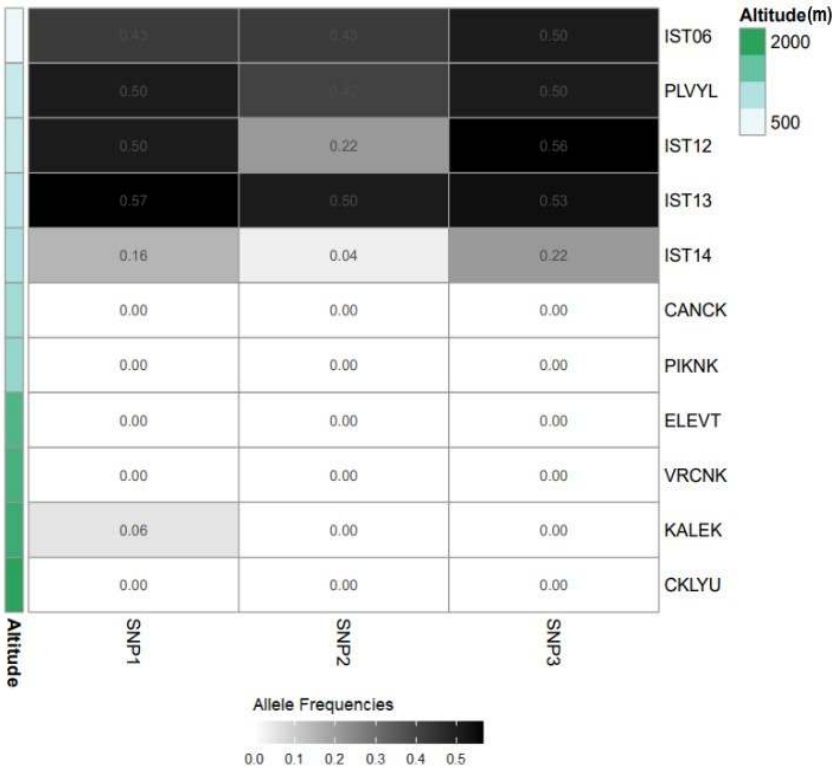

Figure A. 6: Minor Allele Frequency changes for 3 significant loci across populations.

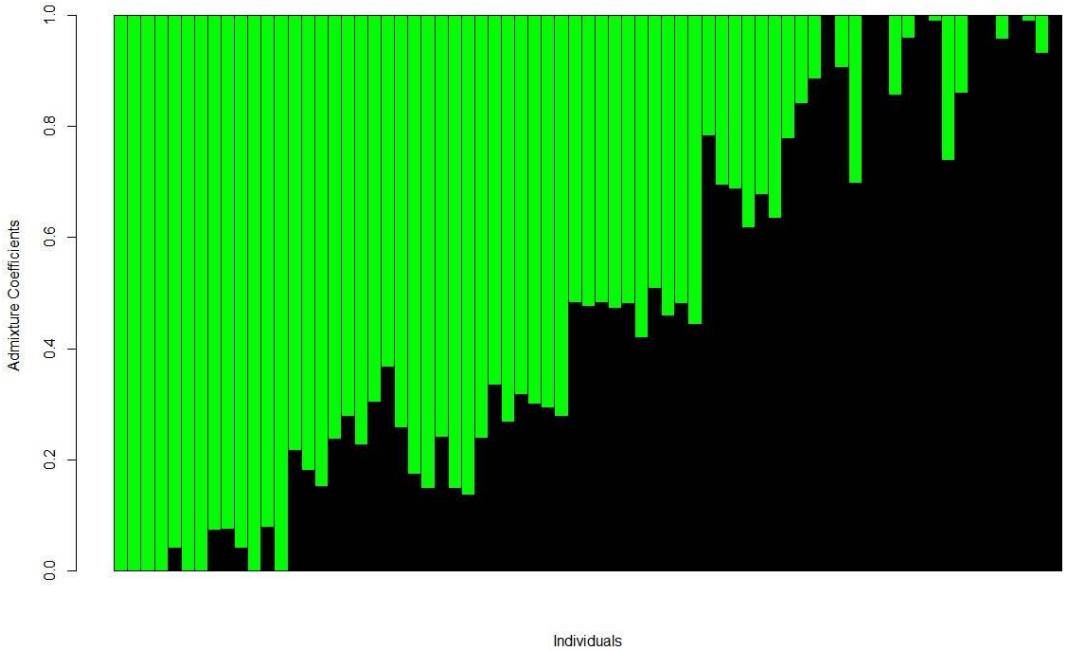

Figure A. 7: sNMF results for the best K value (K = 2) are consistent with the admixture analysis.

Table A- 1: Summary of Read Mapping Statistics for Individual Samples

Individual	Total_Reads	Mapped_reads	Paired&Mated	Properly_Aligned	Percent_Aligned
CANCK_007	1880294	1457202	1394817	1212354	77
CANCK_00D	3713753	2872027	2751031	2404263	77
CANCK_010	4135115	3166137	3022983	2615446	76
CANCK_011	4612920	3526001	3381866	2987618	76

CANCK_013	2277848	1746437	1665093	1434526	76
CAYMY_002	13200887	10515887	10001028	8466644	79
CAYMY_004	18003387	14233575	13534679	11462426	79
CIMLY_001	9487174	7187103	6770389	5580035	75
CIMLY_003	8152847	6135283	5780805	4801693	75
CKLYU_001	10299538	7884366	7480795	6334991	76
CKLYU_002	10733082	8277139	7831688	6549889	77
CKLYU_004	12319539	9517809	9009467	7552610	77
CKLYU_006	9480774	7296626	6898170	5745462	76
CKLYU_007	7163626	5628767	5372965	4622561	78
CKLYU_008	10799409	8507187	8103852	6914081	78
CKLYU_010	24236	15408	14714	13148	63
ELEVT_001	13384596	10270600	9794359	8389109	76
ELEVT_002	6424299	4969309	4758912	4135826	77
ELEVT_003	8368249	6381673	6077200	5196714	76
ELEVT_005	5929210	4525720	4324083	3733509	76
ELEVT_006	5947741	4576900	4359669	3733874	76
ELEVT_007	5137387	3986262	3808410	3278853	77
ELEVT_008	14132519	11007028	10462548	8829452	77
IST06_010	17699665	13651474	12863816	10620563	77
IST06_011	26976950	19884407	18638649	15163675	73
IST06_012	16245005	12497863	11751688	9643402	76
IST06_014	17430558	13399937	12622347	10408325	76
IST06_015	10442391	7743033	7285646	5973732	74
IST06_016	16715690	12897325	12147188	9975883	77
IST06_018	10848723	8381111	7909968	6541399	77
IST09_001	1905412	1481381	1418557	1235616	77
IST09_003	51296	32929	31789	30252	64
IST09_004	195694	136708	131563	119659	69
IST09_005	399052	290551	279876	250166	72
IST09_007	600558	454875	435245	380888	75
IST09_008	141873	70999	68432	66136	50
IST09_010	119504	66005	63284	59106	55

IST11_001	220397	142852	138788	137096	64
IST11_004	1780158	1174046	1137985	1123965	65
IST11_006	330057	214159	207619	203584	64
IST11_008	418780	275659	267047	262100	65
IST11_010	1620630	1069259	1034191	1015395	65
IST11_015	939309	620746	601631	582729	66
IST11_020	246805	160036	154854	148380	64
IST12_001	4271052	3340688	3207430	2830601	78
IST12_002	4997360	3916977	3755619	3282121	78
IST12_003	11929527	9285710	8879229	7717793	77
IST12_004	3955474	3090713	2965469	2602165	78
IST12_006	3321161	2579615	2488134	2229783	77
IST12_007	4695955	3665321	3524147	3111008	78
IST12_013	2804691	2216449	2138677	1906883	79
IST13_001	2226307	1752410	1674746	1443594	78
IST13_002	3504015	2645249	2534252	2236277	75
IST13_003	7221678	5677734	5423570	4687285	78
IST13_004	7692415	6086540	5836503	5068738	79
IST13_005	6230093	4789041	4538951	3815729	76
IST13_006	16455730	12750280	12113527	10309764	77
IST13_007	10117429	7739327	7398278	6442066	76
IST14_018	8218523	6407026	6125430	5298815	77
IST14_019	4019398	3090422	2942185	2527816	76
IST14_020	5126165	3981817	3815789	3331962	77
IST14_021	7856076	6065814	5778016	4954806	77
IST14_023	4887089	3765392	3577413	3043257	77
IST14_024	4877501	3579320	3413060	2946215	73
IST14_025	5717340	4412226	4193031	3559989	77
KALEK_001	7970825	6252403	5935582	5010067	78
KALEK_002	10546009	8090899	7648716	6399616	76
KALEK_003	14096848	10898078	10298291	8553754	77
KALEK_004	13550051	10481954	9889982	8195928	77
KALEK_005	4476058	3526383	3366569	2895995	78
KALEK_007	6228353	4868267	4622842	3906316	78

KALEK_008	15403968	11878531	11193509	9241305	77
OVITY_002	6946338	5317356	5042193	4272332	76
OVITY_003	5517605	4232881	4013158	3396365	76
OVITY_005	3491749	2673787	2541496	2170852	76
PIKNK_001	4069761	3183373	3028623	2572275	78
PIKNK_002	7487941	5883319	5597231	4739650	78
PIKNK_003	3325238	2586887	2471425	2132175	77
PIKNK_004	64233	38493	36924	33463	59
PIKNK_005	7536536	5974430	5696328	4856381	79
PIKNK_006	736149	581574	558099	486683	79
PIKNK_007	8548980	6971746	6703654	5812480	81
PLVYL_002	6976605	5418660	5175505	4470602	77
PLVYL_003	19612190	1898088	1743146	1547493	9
PLVYL_014	9449402	7177658	6771970	5591035	75
PLVYL_016	7391430	5734330	5436938	4558937	77
PLVYL_017	10828819	8241483	7770952	6423011	76
PLVYL_018	6534559	5114747	4882470	4191993	78
PLVYL_039	16229237	12395271	11703801	9714141	76
VRCNK_002	8404877	6483918	6120179	5048115	77
VRCNK_003	8398520	6542944	6195922	5179307	77
VRCNK_005	22161984	17123696	16114630	13222280	77
VRCNK_006	7729703	5983161	5649487	4689242	77
VRCNK_008	3536717	2746294	2609601	2217975	77
VRCNK_009	14897065	11322775	10675696	8826062	76
VRCNK_010	15177279	11592817	10884049	8835145	76