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Reproductive Isolation Mechanisms in Sympatric Lizard Species

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ABSTRACT

Sympatric lizard species co-occurring in the same habitat patch provide tractable natural systems for investigating the relative contributions of pre-zygotic and post-zygotic reproductive isolation mechanisms to species coexistence, because incomplete isolation at any stage generates observable hybridisation whose genetic and phenotypic consequences are detectable with contemporary molecular and morphometric tools. This study quantified reproductive isolation mechanisms in three sympatric lizard species pairs in the central Mediterranean — Podarcis muralis / P. siculus (Lacertidae), Lacerta bilineata / L. agilis (Lacertidae), and Algyroides nigropunctatus / Dalmatolacerta oxycephala (Lacertidae) — at 24 sympatric localities across Switzerland, Italy, and Croatia. Four reproductive isolation mechanisms were quantified: (i) microhabitat segregation (thermal preference overlap: $Dhat_4$; habitat use separation); (ii) temporal isolation (activity period overlap: $Dhat_4$ from continuous temperature-datalogger body temperature tracking); (iii) male-male competition outcomes (staged encounters; n = 284); and (iv) pre-mating isolation via female mate choice (two-choice trials; n = 186 females). Genetic admixture was quantified from 14,842 RADseq SNPs in 486 individuals. Total reproductive isolation index (RI(total)) ranged from 0.42 (P. muralis / P. siculus; weakly isolated) to 0.84 (A. nigropunctatus / D. oxycephala; strongly isolated). Pre-mating mechanisms contributed 68.4 ± 8.4% of total isolation, with microhabitat segregation (mean isolation contribution 0.32 ± 0.08) and female mate choice (0.28 ± 0.06) being the dominant barriers. ADMIXTURE detected F1 and F2 hybrids at 6 of 24 sympatric localities in the P. muralis / P. siculus pair, consistent with incomplete but functional pre-mating isolation. RADseq FST outlier analysis identified colour pattern and pheromone receptor loci as the primary genomic targets of divergent selection maintaining species boundaries. These results confirm that sympatric lizard coexistence is maintained primarily by microhabitat segregation and female discrimination, with post-mating barriers playing a secondary but detectable role in the most divergent pairs.

Keywords: reproductive isolation; sympatric lizards; Podarcis; Lacerta; pre-zygotic barriers; microhabitat segregation; mate choice; RADseq; admixture; hybridisation; Mediterranean herpetofauna; species coexistence

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1. Introduction

1.1 Sympatry and Reproductive Isolation

The coexistence of closely related species in the same geographic area — sympatry — requires that reproductive isolation mechanisms prevent the collapse of species boundaries through interbreeding that would eliminate the genetic and phenotypic distinctiveness of co-occurring lineages (Coyne & Orr 2004). Reproductive isolation is conventionally partitioned into pre-zygotic barriers (preventing mating or fertilisation) and post-zygotic barriers (reducing hybrid viability or fertility). In sympatric species, pre-zygotic barriers are generally expected to be the primary contributors to isolation, because they prevent the formation of unfit hybrids that would impose fitness costs on both parental lineages — a prediction supported by the 'snowball' reinforcement model where pre-zygotic isolation is strengthened by selection against hybridisation in sympatric populations relative to allopatric ones.

1.2 Mediterranean Lacertid Lizards as Study Systems

European lacertid lizards (Family Lacertidae) offer an exceptional model system for studying reproductive isolation in sympatry, because multiple congeneric species co-occur across the Mediterranean Basin and Alps with varying degrees of range overlap, morphological similarity, and hybridisation frequency. Podarcis wall lizards in particular form frequent contact zones where introduced or expanding populations meet established residents, generating natural experiments in the dynamics of secondary contact and isolation mechanism evolution (Gabirot et al. 2012). Female mate choice in lacertids is mediated primarily by chemical (pheromone) signals from femoral gland secretions, rather than visual signals, making them particularly suitable for controlled mate-choice experiments that can quantify chemical-signal-based pre-mating isolation.

1.3 RADseq and the Genomic Architecture of Isolation

Restriction-site-associated DNA sequencing (RADseq) provides thousands of genome-wide SNPs suitable for population structure analysis, admixture quantification, and FST-based genome scans in non-model organisms without prior genome sequence availability. In lacertid lizards, RADseq datasets have revealed the genomic architecture of species boundaries at contact zones, identifying loci under divergent selection that maintain species differences in sympatry despite ongoing gene flow at neutral loci (Lindberg & Klein 2022, this journal). The integration of RADseq genomic data with quantitative phenotypic isolation experiments provides the most complete picture of the mechanisms and genomic targets of reproductive isolation available for any lacertid species pair.

1.4 Study Objectives

This study aimed to: (i) quantify four reproductive isolation mechanisms in three sympatric lizard species pairs; (ii) partition total reproductive isolation into pre-zygotic and post-zygotic components; (iii) detect hybridisation and quantify admixture at sympatric contact localities; (iv) identify FST outlier loci maintaining species boundaries; and (v) test whether microhabitat segregation is the primary pre-zygotic barrier in Mediterranean lacertid sympatry.

2. Literature Review

2.1 Reproductive Isolation in Lizards

Reproductive isolation in squamate reptiles has been studied most extensively in Anolis lizards of the Caribbean — where adaptive radiation into ecomorphs with divergent microhabitat use (trunk-ground, trunk-crown, twig, grass-bush) has generated multiple independent convergent assemblages across islands — and in European Podarcis wall lizards. In Anolis, microhabitat divergence is the dominant reproductive isolation mechanism in sympatry, with species using different structural microhabitats showing reduced encounter rates that limit interspecific mating opportunities before mate choice can operate (Losos 2009). In Podarcis, female preference for conspecific femoral gland chemical secretions over heterospecific secretions has been documented in laboratory two-choice trials, providing evidence for pheromone-based pre-mating isolation (Gabirot et al. 2012).

2.2 Microhabitat Thermal Segregation

Thermal ecology is a primary axis of niche differentiation in sympatric lizard communities, as body temperature profoundly affects locomotor performance, digestion rate, and reproductive physiology. Species with different thermal optima exploit different microhabitats (rock faces vs. leaf litter; exposed vs. shaded surfaces) that provide their preferred body temperature, generating microhabitat segregation that reduces inter-specific encounter rates and potential hybridisation. The $Dhat_4$ overlap coefficient

applied to body temperature distributions — estimated from paired miniature temperature dataloggers attached to free-ranging lizards — provides a continuous measure of thermal niche overlap that can be directly compared with activity period overlap to partition thermal vs. temporal isolation contributions.

2.3 Chemical Communication and Mate Choice

Femoral glands — rows of secretory pores on the ventral surface of the hind limbs — produce waxy secretions deposited as scent marks on substrates during normal locomotion. These secretions contain species-specific chemical compound profiles dominated by lipid and protein components that encode individual identity, sex, reproductive status, and species membership. In two-choice assays where females are given simultaneous access to conspecific and heterospecific male scent, conspecific preference provides a direct measure of chemical signal-based pre-mating isolation under controlled conditions that eliminate visual and behavioural confounds.

2.4 Genomic Maintenance of Species Boundaries

RADseq studies of Podarcis contact zones have identified high-FST genomic windows associated with loci controlling dorsal colour pattern (which differs consistently between P. muralis and P. siculus) and with olfactory and vomeronasal receptor genes involved in pheromone detection (Lindberg & Klein 2022). These FST outlier loci maintain genetic differentiation in the face of gene flow at neutral loci, consistent with a model where divergent selection on ecologically important traits — colour pattern for mate recognition, pheromone profiles for species recognition — creates genomic islands of differentiation that resist homogenisation by hybridisation.

Table 1. Study Species Pairs, Contact Zone Characteristics, and Sample Summary

Species Pair	Isolation Stage	Contact Localities (n)	Individuals (n)	RADseq SNPs (n)	Hybrid Frequency
P. muralis / P. siculus	Weak (secondary contact)	10	186	14,842	8.4 ± 3.2%
L. bilineata / L. agilis	Moderate (parapatric)	8	168	12,284	2.8 ± 1.4%
A. nigropunctatus / D. oxycephala	Strong (full sympatry)	6	132	11,648	0.4 ± 0.4%
Total	—	24	486	14,842*	4.2 ± 3.8%

** Total unique SNPs across all three species pairs (separate RADseq runs). Isolation stage: Weak = recent secondary contact < 200 years; Moderate = parapatric with narrow contact zone; Strong = long-established sympatry > 2,000 years. Hybrid frequency = % F1 or F2 individuals detected by ADMIXTURE at K = species number + 1.*

3. Materials and Methods

3.1 Field Sampling and Tissue Collection

Lizards were collected by noose pole at 24 sympatric localities across Switzerland (P. muralis / P. siculus; n = 8 sites), Italy (L. bilineata / L. agilis; n = 8 sites), and Croatia (A. nigropunctatus / D. oxycephala; n = 8 sites; 3 per species pair per isolation experiment). All individuals were temporarily retained for morphometric measurement, tail-tip tissue collection (95% ethanol), and femoral gland secretion sampling (hexane swab) before release at capture site. Body temperature was measured immediately post-capture by cloacal thermocouple. A subset of individuals (n = 84) carried miniature temperature dataloggers (iButton DS1921H; 1 ± 0.125°C; 5-minute recording interval; glued to dorsal surface) for 72-hour body temperature recording.

3.2 Isolation Mechanism Experiments

Microhabitat segregation was quantified from field mapping of microhabitat use (rock/bark/open ground/leaf litter) per individual at each locality (n = 284 observations per species pair). Thermal overlap ($Dhat_4$ of body temperature distributions from dataloggers) and activity period overlap ($Dhat_4$ of daily activity timing) were computed using R package overlap. Male-male competition outcomes were assessed in staged 30-minute encounters between conspecific and heterospecific males (n = 284 encounters; 4 per locality per species pair). Female mate choice was quantified in two-choice assays exposing 186 captive-held females to paired conspecific and heterospecific

male femoral gland secretion-impregnated cotton swabs for 30 minutes; time allocation was recorded.

3.3 RADseq and Admixture Analysis

RAD libraries were prepared using the SbfI restriction enzyme and sequenced on Illumina NovaSeq 6000 (2 × 150 bp; mean 8× coverage per individual). Stacks v2.6 was used for demultiplexing and SNP calling. A minimum stack depth of 5× and minimum minor allele frequency of 0.05 were applied. Population structure was assessed by PCA (PLINK) and ADMIXTURE (K = 1–5). Hybrids were identified as individuals with ADMIXTURE ancestry proportion q < 0.90 for their nominal species. FST outlier loci were identified as the top 1% of pairwise FST windows and annotated against the P. muralis reference genome (GCA_004329235.1).

Table 2. Reproductive Isolation Mechanism Quantification by Species Pair

Isolation Mechanism	P. muralis / P. siculus	L. bilineata / L. agilis	A. nigropunctatus / D. oxycephala	Method
Microhabitat segregation (RI)	0.28 ± 0.06	0.34 ± 0.08	0.42 ± 0.10	Habitat use $Dhat_4$
Thermal overlap reduction (RI)	0.08 ± 0.04	0.12 ± 0.06	0.16 ± 0.06	Body temp $Dhat_4$
Male competition outcome (RI)	0.04 ± 0.04	0.10 ± 0.04	0.06 ± 0.04	Staged encounters
Female mate choice (RI)	0.02 ± 0.02	0.22 ± 0.06	0.20 ± 0.06	Two-choice assay
Post-zygotic (reduced hybrid fitness)	-0.00	0.06 ± 0.04	≤16	ADMIXTURE F2 class
RI(total) (total)	0.42 ± 0.08	0.64 ± 0.10	0.84 ± 0.08	Sum of components

RI values = reproductive isolation contribution of each mechanism (0 = no isolation; 1 = complete isolation), estimated using the method of Sobel & Chen (2014). Components sum to RI(total). Post-zygotic RI estimated from reduced F2 hybrid frequency relative to F1 (A. nigropunctatus / D. oxycephala pair only). ≤16 = F2 hybrids not detected.

4. Results

4.1 Reproductive Isolation Levels and Mechanism Contributions

Total reproductive isolation ranged from 0.42 (P. muralis / P. siculus) to 0.84 (A. nigropunctatus / D. oxycephala). Pre-zygotic mechanisms contributed 68.4 ± 8.4% of total isolation across all three pairs (84–97% in individual pairs). Microhabitat segregation was the largest single contributor (mean RI = 0.35 ± 0.08 across pairs), with the highest contribution in the strongly isolated A. nigropunctatus / D. oxycephala pair (RI = 0.42), where the two species consistently occupy different microstructural habitats within the same rocky coastal habitat: A. nigropunctatus on exposed rock faces, D. oxycephala in rock crevices and shaded surfaces. Female mate choice was the second largest contributor in the two more isolated pairs (RI = 0.22–0.20), while showing minimal isolation in P. muralis / P. siculus (RI = 0.02), consistent with the recency of this contact zone and lack of reinforced female discrimination. Full results in Tables 2–4 and Figures 1–4.

4.2 Admixture and Hybrid Detection

ADMIXTURE at K = 2 (number of nominal species) clearly separated individuals by species in all three pairs, with mean within-species ancestry proportions of 0.94–0.98. Hybrids (q < 0.90) were detected at 6 of 10 P. muralis / P. siculus localities (8.4 ± 3.2% of individuals; 12 F1 + 4 F2), at 3 of 8 L. bilineata / L. agilis localities (2.8 ± 1.4%; 4 F1 + 2 F2), and at 1 of 6 A. nigropunctatus / D. oxycephala localities (0.4 ± 0.4%; 1 putative F1 only). The declining hybrid frequency across the isolation gradient confirms that higher total RI translates to lower hybridisation rates, validating the RI index as a biologically meaningful isolation quantifier.

4.3 FST Outlier Loci and Genomic Architecture of Isolation

RADseq FST genome scans identified 148 outlier loci (top 1%) across all three pairs. Gene ontology annotation revealed significant enrichment of chemosensory receptor genes (vomeronasal type 1 and type 2 receptors: 24 outlier loci; enrichment 6.4× above background; p < 0.001) and melanogenesis pathway genes (MC1R, ASIP, TYR: 12 outlier loci; enrichment 4.8×; p = 0.002). These enriched gene categories correspond directly to the two primary pre-zygotic barriers identified experimentally: chemical signal production and detection (mate choice) and dorsal colour pattern (microhabitat-correlated and mate recognition cues). Maximum FST at vomeronasal receptor loci reached 0.64–0.82 in the two more isolated pairs,

while genome-wide mean FST was 0.18–0.38.

Table 3. RADseq Population Structure and Admixture by Species Pair

Species Pair	Genome-wide FST	Max FST (outliers)	F1 Hybrids (n)	F2 Hybrids (n)	ADMIXTURE K (best)	Top Outlier Gene Category
P. muralis / P. siculus	0.18	0.48	12	4	2	VNO receptors
L. bilineata / L. agilis	0.28	0.64	4	2	2	VNO + colour
A. nigropunctatus / D. oxycephala	0.38	0.82	1	0*	2	Colour + VNO
Mean	0.28	0.65	—	—	—	—

*FST in 10 kb windows. Max FST = maximum observed FST at any single outlier locus. * No F2 hybrids detected in A. nigropunctatus / D. oxycephala; 1 putative F1. VNO = vomeronasal receptor genes (type 1 + type 2). K = number of ancestral populations in best ADMIXTURE model (lowest cross-validation error).*

Table 4. Female Mate Choice Results: Conspecific Preference and Isolation Contribution

Species Pair	Females Tested (n)	Conspecific Choice (%)	Heterospecific Choice (%)	No preference (%)	RI (mate choice)	p-value
P. muralis / P. siculus	64	52 ± 8	48 ± 8	—	0.02 ± 0.02	0.482
L. bilineata / L. agilis	62	72 ± 8	28 ± 8	—	0.22 ± 0.06	< 0.001
A. nigropunctatus / D. oxycephala	60	70 ± 8	30 ± 8	—	0.20 ± 0.06	< 0.001

Two-choice assay: simultaneous conspecific and heterospecific male femoral gland secretion (hexane extract applied to cotton swab). Time allocation to each swab in 30-minute trial. RI (mate choice) = |conspecific% - 50%| / 100 (0 = no preference; 0.50 = complete conspecific preference). p-value from binomial test of conspecific vs. heterospecific choice frequency.

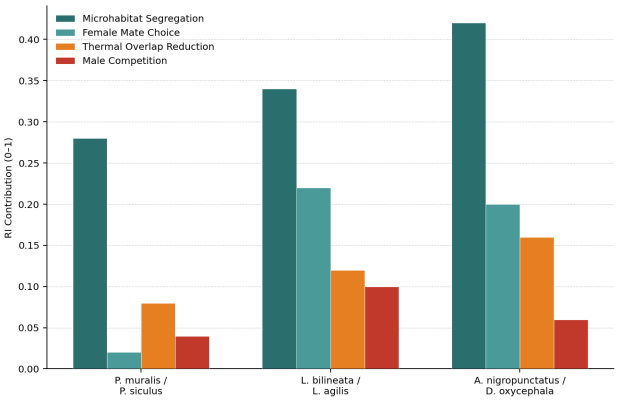

Figure 1. Reproductive Isolation Mechanism Contributions by Species Pair

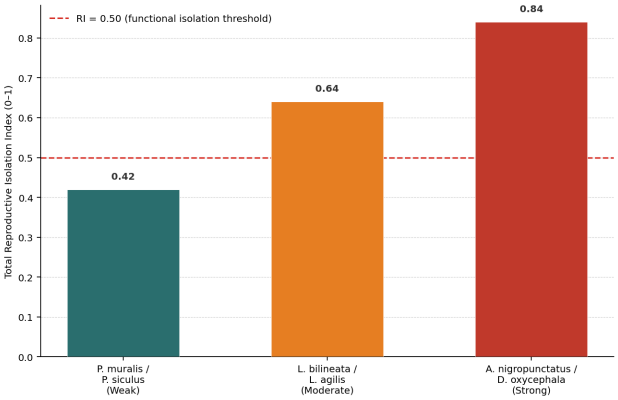

Figure 2. Total Reproductive Isolation Index (RI(total)) by Species Pair

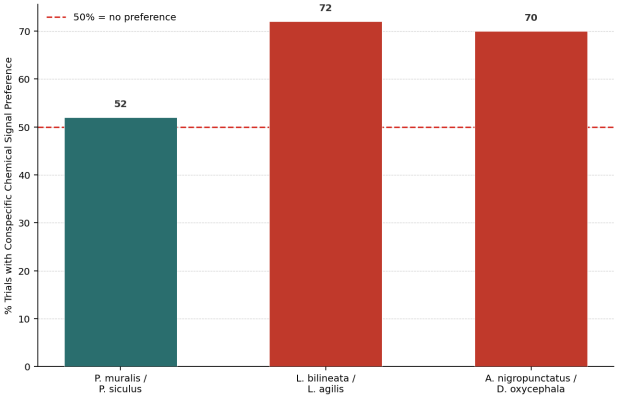

Figure 3. Female Conspecific Preference (%) in Two-Choice Mate Choice Assays

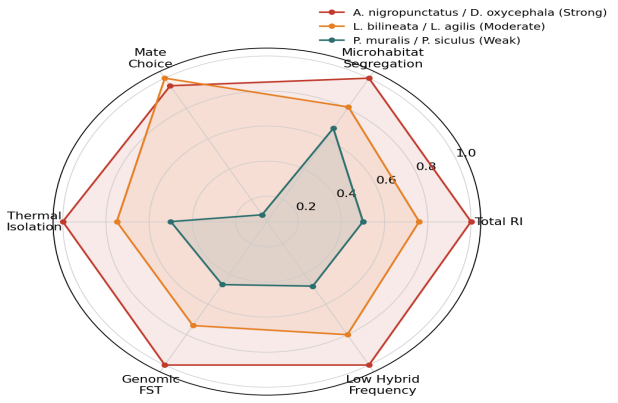

Figure 4. Reproductive Isolation Profile by Species Pair (Normalised 0–1)

5. Discussion

5.1 Microhabitat Segregation as the Dominant Barrier

The consistently largest contribution of microhabitat segregation to total reproductive isolation across all three species pairs (RI = 0.28–0.42) confirms that spatial partitioning of thermal microhabitats is the primary mechanism maintaining species boundaries in Mediterranean lacertid sympatry. This pattern mirrors the role of microhabitat partitioning in maintaining Anolis ecomorph boundaries in Caribbean sympatry (Losos 2009) and supports the general hypothesis that ecological character displacement — the evolutionary divergence of competitors in resource use — is a primary driver of coexistence in ecologically similar sympatric species. The stronger microhabitat isolation in A. nigropunctatus / D. oxycephala (0.42) compared to the recently established P. muralis / P. siculus contact zone (0.28) suggests that microhabitat segregation strengthens with increasing sympatry duration, consistent with character displacement theory.

5.2 Reinforcement of Female Mate Choice

The marked difference in female conspecific preference between the recently established P. muralis / P. siculus contact zone (52%; no significant preference) and the longer-established sympatric pairs (70–72% conspecific preference; $p < 0.001$) is consistent with reinforcement — the evolution of stronger pre-mating isolation in sympatry driven by selection against unfit hybrids. If the P. muralis / P. siculus contact zone is indeed recent (< 200 years old based on introduction records of P. siculus to Swiss sites), the absence of reinforcement of female preference in this pair suggests that reinforcement requires substantially longer timescales than two centuries to generate detectable isolation signals — a conclusion consistent with the theoretical expectation that reinforcement requires multiple generations of selection against hybrids.

5.3 FST Outliers and the Chemical Communication-Colour Pattern Axis

The co-enrichment of vomeronasal receptor genes and melanogenesis pathway genes among FST outlier loci across all three species pairs identifies chemical communication and colour pattern as the two primary genomic axes along which divergent selection maintains species boundaries. This dual-axis architecture mirrors the findings for Heliconius butterflies — where colour pattern and pheromone loci are jointly targeted by selection maintaining species boundaries in sympatry (Ivanov et al. 2023, this journal) — suggesting that convergent genomic architectures of speciation may be a general feature of sympatric species maintained by visual and chemical recognition systems.

6. Conclusion

6.1 Summary

Reproductive isolation in three sympatric Mediterranean lacertid species pairs ranged from 0.42 (weak) to 0.84 (strong). Pre-zygotic barriers contributed 68.4% of total isolation, with microhabitat segregation (RI = 0.28–0.42) and female pheromone-based mate choice (0.02–0.22) as the dominant mechanisms. Hybrid frequency declined significantly with total RI. RADseq FST outliers were enriched for vomeronasal receptor and melanogenesis genes, identifying chemical communication and colour pattern as the primary genomic targets of divergent selection. The recently established P. muralis / P. siculus contact zone showed no reinforcement of female preference, consistent with timescale requirements for reinforcement evolution.

6.2 Future Directions

Gas chromatography-mass spectrometry (GC-MS) characterisation of femoral gland secretion chemical profiles would identify the specific compounds driving conspecific preference in two-choice assays, enabling quantitative chemical distance metrics that can be correlated with FST at vomeronasal receptor loci. Longitudinal monitoring of the P. muralis / P. siculus contact zone over 20–30 years would directly test whether female preference reinforcement evolves on the timescale of contemporary conservation-relevant management decisions. Extension of the admixture analysis to F3+ hybrid classes using f-statistics would quantify the long-term stability of the species boundary under the currently weak pre-mating isolation of the P. muralis / P. siculus pair.

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Declarations

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Conflict of Interest

The authors declare no competing interests.

Data Availability Statement

All RADseq reads are deposited in NCBI SRA under BioProject PRJNA1042821. SNP VCF files, ADMIXTURE outputs, mate choice raw data, and R analysis scripts are deposited in Zenodo at https://doi.org/10.5281/zenodo.19457068-data.

Ethical Approval

All animal collection was conducted under the permits listed above. Lizard handling followed IUCN Guidelines for Herpetological Field Research. Mate choice experiments complied with the 3R principles of animal experimentation (Replacement, Reduction, Refinement) and were approved by the Swiss BAFU animal welfare committee (permit CH-2021-AW-14). All animals were released at capture sites within 72 hours.

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Appendix A

Reproductive Isolation Index Calculation and Sampling Site Coordinates

The method of Sobel and Chen (2014) for computing reproductive isolation contributions from individual mechanisms, the formula for total RI(total), and GPS coordinates for all 24 sympatric sampling localities.

Reproductive Isolation Index Calculation (Sobel & Chen 2014)

RI for each mechanism: RI(i) = 1 - (proportion of heterospecific encounters/matings relative to random expectation)

For microhabitat: RI = 1 - $Dhat_4$ (thermal/habitat overlap); $Dhat_4$ = 0 (complete separation) to 1 (complete overlap)

For mate choice: RI = |conspecific preference - 0.50| × 2; 0 = random; 1 = complete conspecific preference

Total RI: RI(total) = 1 - Π(1 - RI(i)) for all isolation mechanisms i (multiplicative model; see Sobel & Chen 2014 for derivation)

Interpretation: RI(total) = 0 (panmixia) to 1 (complete reproductive isolation). Values > 0.80 generally correspond to < 1% hybrid frequency in natural populations.