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Impact of the Qinghai-Tibet Railway on Population Genetic Structure of the Toad-Headed Lizard, *Phrynocephalus vlangalii*

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Abstract Using data from nine microsatellite DNA loci and a population genetic approach, we evaluate the barrier effect of the Qinghai-Tibet Railway on toad-headed lizard, *Phrynocephalus vlangalii*. The study area is along a 20 km stretch of the railway on northern Qinghai-Tibet Plateau, and this section of the railway was constructed between 1958–1979. Both assignment tests and analysis of molecular variance (AMOVA) were used for data analysis. We found significant genetic differentiation between the populations from the study area and those from a further southeastern area, which are separated by a 20 km gap. This suggests the existence of population substructure at a fine-scale. However, we did not detect any difference between samples from the western and eastern sides of the railway within the study area, and concluded that the railway may not impose a significant barrier effect on these lizard populations at the present time. Available suitable habitat alongside the railway and bridge underpasses may have facilitated the gene exchange between the sides. The relatively short time since the completion of the railway may not allow the differentiation to accumulate to a detectable level. Since the Qinghai-Tibet Plateau maintains a unique and fragile ecosystem, long-term monitoring of such man-made landscape features is imperative for protecting this ecosystem.

Keywords Qinghai-Tibet Railway, barrier effect, population structure, *Phrynocephalus vlangalii*, microsatellite DNA, Bayesian assignment test

1. Introduction

Roads and railways are among the most conspicuous man-made landscape features and they impose major ecological impacts on the ecosystem (Forman and Alexander, 1998; Trombulak and Frissell, 2000). Various effects have been proposed and examined, including large numbers of fatalities (Gibbs and Shriver, 2005), altering individual movement patterns and abundance (Shine *et al*., 2004; Fahrig and Rytwinski, 2009), and more importantly the "barrier effect" that subdivides populations with significant demographic and genetic consequences (Epps *et al*., 2005; Riley *et al*., 2006).

Such impacts are particularly acute in a sensitive ecosystem, such as the Qinghai-Tibet Plateau. The recent construction of the Qinghai-Tibet Railway has created a storm of controversy surrounding its potential negative impacts on the local ecosystem (Yin *et al*., 2006; Xia *et al*., 2007). Measures, such as wildlife passages, have been put into place to reduce such impacts and some of them appeared to be functioning (Yang and Xia, 2008). The long-term effects, however, are largely unknown. Although the completion of the railway was in 2006, the first phase construction of the railway (Xining to Golmud section) started in 1958 and was completed in 1979 (Figure 1). This section of the railway has been in use for more than 30 years and provides a unique opportunity to examine its impact on the local ecosystem in a relatively large time span.

The Qinghai toad-headed lizard, *Phrynocephalus vlangalii,* is one of the dominant terrestrial species in

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the Qinghai-Tibet Plateau. Its preferred habitat is sand dunes with loose substrate and little vegetation. Both males and females were observed to excavate burrows. Using eleven microsatellite DNA loci, Wang *et al.* (2009) reported a surprisingly large amount of genetic diversity and high population differentiation within this species. Furthermore, the species demonstrated strong territoriality (Qi *et al.*, 2011). Such properties provide the potential to detect variations at a small spatial and temporal scale. Therefore, the species may serve as an excellent model system to examine the barrier effect of the Qinghai-Tibet Railway.

Recent developments in individual assignment methods have also greatly improved the ability to detect population structure at a fine scale (Manel *et al*., 2003; Crosby *et al.*, 2009). Using individual, rather than population, as the unit of examination, these methods do not require a pre-defined population boundary, which is particularly suitable for terrestrial species with low population density. Furthermore, these methods are very sensitive and are capable of detecting first generation migrants (Pritchard *et al.*, 2000; Piry *et al.*, 2004).

Figure 1 The Xining-Golmud section of the Qinghai-Tibet Railway. Arrow indicates sand dunes, which are suitable habitat for toad-headed lizards.

In this study, we analyzed data from nine microsatellite DNA loci using assignment tests to examine the barrier effect of the Qinghai-Tibet Railway on the toad-headed lizard, *P. vlangalii*. Specifically, we addressed the question whether the railway formed a genetic barrier to the gene flow of this lizard. If the Railway significantly impeded gene flow, we would expect the populations from both sides of the railway to have a different genetic make-up. On the other hand, if the railway did not have significant impact, the individuals from both sides would make up one well-mixed population.

2. Materials and Methods

2.1 Sampling A total of 170 individuals of *P. vlangalii* were collected along a 20 km stretch of the Qinghai-Tibet Railway in July 2010, including 91 from the western side and 79 from the eastern side of the railway. The sampling area is located in the Qaidam Basin with elevations between 2700–2800 m above sea level. The individuals were collected from a total of 34 sites, with 1–11 individuals from each site. Our sampling strategy was to sample many sites but collect a few individuals from majority sites, which best suits the low population density nature of this lizard and the individual assignment analytic methods.

We collected an additional 56 individual lizards from 14 sites at an area located further southeast of our railway sampling area. There is a 20–25 km distribution gap between the railway sites and the southeastern sites; in the gap, the habitat is not suitable and lizards are rare. This additional collection is to confirm the natural occurrence of detectable population structure at a fine scale and to provide a comparison. All sampling sites and related information are presented in Figure 2 and Appendix I.

All individuals were collected and euthanized in the field. Liver tissues were collected and preserved in 95% ethanol. All voucher specimens are stored in the Herpetological Collection of the Chengdu Institute of

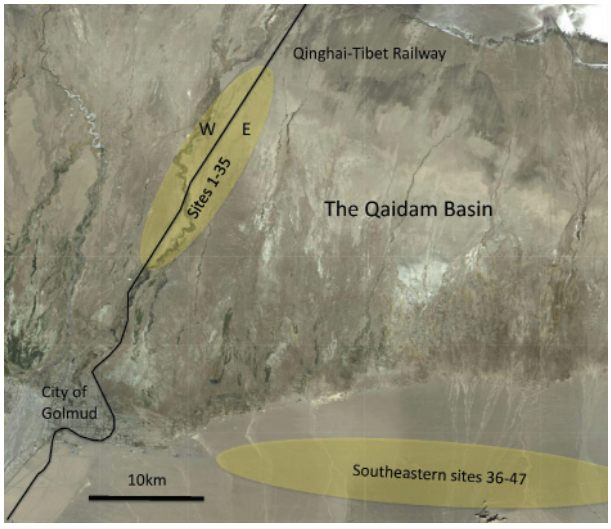

Figure 2 Sampling sites. Map is generated from Google maps using a satellite image. Circles show the sampling areas. Sites 1–35 are nearby the railway and from both sides; actual sites are too close together to be accurately marked on this map. Sites 36–47 are for the purpose of comparison. Some sites are off the map.

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2.2 Laboratory protocols Genomic DNA was extracted using TIANamp Genomic DNA kits (Tiangen Biotech) following the manufacturer's recommendation. Nine polymorphic microsatellite DNA loci (Phr27, Phr75, Phr79, Phr63, Phr160, Pvms12, Pvms18, Pvms35, Pvms38) were amplified by polymerase chain reaction (PCR) using primers developed by Urquhart *et al*. (2005) and Zhan and Fu (2009). The forward primers were labeled with one of FAM, TAMRA or HEX florescence dyes.

All PCR was conducted with individual pairs of primers in a 25 μL mix, including 20–50 ng of template DNA, 1U Taq DNA polymerase (TaKaRa), 2 mM MgCl2, 0.2 mM of each dNTP, and 10 pmol of each primer in 1X PCR buffer (TaKaRa). PCR cycling parameters were 5 min for initial denaturation at 95˚C, followed by 30 cycles at 94˚C denaturating for 30 sec, at 58˚C annealing for 30 sec, at 72˚C extension for 30 sec, and a final additional extension step at 72˚C for 5 min. PCR products with different fluorescent labels were then pooled and genotyped on an ABI 3730 genetic analyzer (Applied Biosystems). Electropherograms were visualized and analyzed by Gene Marker version 1.6 (SoftGenetics).

After the initial completion of genotyping all individuals, we randomly selected 20% of the samples and repeated the genotyping of these samples as a quality control measure. For three of the nine loci examined, a greater than 8% of inconsistency rate was detected, and therefore, all samples for these three loci were re-genotyped and inconsistent results were resolved. For the other six loci, the inconsistency rates were relatively low and the inconsistent results within these 20% repeated samples were resolved, but other samples were not re-examined.

2.3 Data analysis Genetic diversity was assessed with three indices. The number of alleles (A), the observed heterozygosity (H_O) and the expected heterozygosity (H_E) were calculated using Genepop web version 4.0 (Raymond and Rousset, 1995; Rousset, 2008). Departure from Hardy-Weinberg equilibrium (HWE) and linkage equilibrium (LE) was also examined using Genepop.

To assess the potential barrier effect of the railway, two sets of assignment tests were conducted. Using Structure version 2.3.1 (Pritchard *et al.*, 2009), a Bayesian model-based assignment test was employed to detect individual clustering and assign individuals to groups. The test uses multi-locus genotype data and a pre-defined number of clusters (K) to generate groupings that minimize the deviation from HWE and LE (Pritchard *et al.*, 2000). Individuals are assigned probabilistically to one or more clusters based on their genotypic data. Two sets of Structure analyses were conducted: 1) all samples were included; and 2) only the samples from the railway sites (sites 1–34) were included. The admixture model and independent allele frequencies model were used, and a total of 50 independent runs with 300 000 burn-in iterations and 100 000 post burn-in iterations were conducted at each value of K. The ten highest LnP(D) values from the 50 runs were averaged to estimate the best K. Since we were only interested in detecting potential differentiation between the two sides of the railway, we restricted the range of K from one to four. We assessed both LnP(D) values and the individual assignment probabilities to obtain the best value of K. In general, higher LnP(D) and large increase in the LnP(D) values between successive changes in K indicate a better fit to the data. Furthermore, the best K value should produce an assignment plot within which the majority of individuals are assigned to a proper cluster with high probabilities. Admixed individuals with less than 80% probabilities should be minimal.

A second set of assignment tests were conducted using GeneClass2 version 2.0.g (Piry *et al.*, 2004). This method estimates the likelihood that a genotype originates from a particular population given the allele frequencies of the population, and is considered more sensitive than the method implemented in Structure (Waser and Hadfield, 2011). Only the samples from the railway sites (sites 1–34) were subjected to this analysis and individuals were pre-defined as from the "western population" or "eastern population" based on where they were collected. Rannala and Mountain's (1997) resampling algorithm and Paetkau *et al.*'s (2004) simulation method (10 000 replicates) were used.

A locus by locus analysis of molecular variance (AMOVA) was also conducted to assess the barrier effect of the railway using Arleoquin version 3.5 (Excoffier and Lischer, 2011). Permutation tests (10 000 replicates) were carried out at four hierarchical levels: among groups, within groups among sites, among individuals within sites, and within individuals. Similar to the Structure analysis, two sets of analyses were conducted. First, all samples were included to test the natural occurrence of population genetic structure at a fine scale. Two groups were defined, one including the railway sites (1–34) and the other including the southeastern sites (35–47). Second, only the samples from the railway sites (1–34) were included to test whether the railway represents a

genetic barrier. Again, two groups were defined: samples from the western side of the railway were defined as one and those from the eastern side were defined as the other.

3. Results

The number of alleles (*A*), the observed heterozygosity (H_O) and the expected heterozygosity (H_E) are presented in Table 1. In concordance with previous studies, we found relatively high genetic diversity within this species. For example, the number of alleles on each locus ranges from 18 to 33.

Only the samples from the railway sites were subjected to HWE and LE tests. Individuals were pooled as a single population. Six loci showed significant departure from HWE after Bonferroni correction (Table 1). Two (out of 36) pairs of loci were in significant linkage disequilibrium. These results were expected because of Wahlund effect. Such departure provides information for the assignment tests. Previous studies of the same loci for *P. vlangalii* did not detect any consistent linage disequilibrium between populations (Wang *et al.*, 2009), and therefore, we assumed these loci are not in physical linkage.

The Structure assignment tests revealed two clusters when all samples were included in the analysis; however, one cluster was detected when only the samples from the railway sites were included. Figure 3 presents a plot of LnP(D) against K values of 1 to 4, and Figure 4 presents the individual assignment probability bar graphs. When all samples were included, the LnP(D) showed a major increase when K changed from 1 to 2, and the increases of LnP(D) were minimal with the subsequent changes of K (Figure 3 A). In the bar graph, with K = 2, approximately half of the individuals received assignment

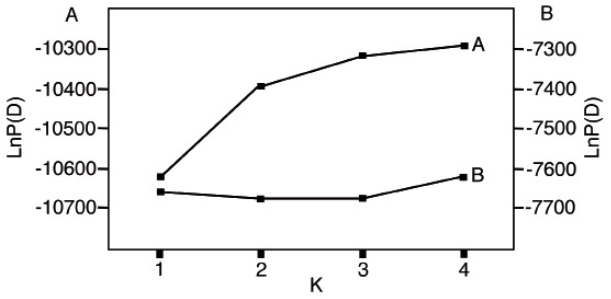

Figure 3 Average likelihood values for K = 1–4 from Structure (ver. 2.3.1). LnP(D) values are average of the 10 highest likelihood values of 50 independent runs. A: All samples are included; B: Only the samples from the railway sites are included.

probabilities greater than 0.90, and three quarters of the individuals had probabilities greater than 0.80 (Figure 4 A). Both measurements showed that K = 2 is the best fit for the data. It was also clear that the majority of individuals from the railway site made up one cluster and the majority from the southeastern sites formed the second cluster (Figure 4 A). When only the samples from the railway sites were included, the changes in K values did not produce a corresponding increase in LnP(D) values (Figure 3 B), suggesting that K = 1 is the best fit of data. In the bar graph, approximately 20% of individuals received assignment probabilities greater than 0.80, and no one received a probability of greater than 0.90 (Figure 4 B). Considering both the change in LnP(D) values and the individual assignment probabilities, there is clear genetic differentiation between the railway sites and the southeastern sites, but all samples from the railway sites appeared to belong to one population.

The results from the GeneClass2 analysis were consistent with these from the Structure analysis. Every individual from the railway sites received similar probabilities of being resident of both western and eastern

Table 1 Measurements of genetic diversity at nine microsatellite DNA loci.

Populations		Phr27	Phr75	Phr79	Phr63	Pvms12	Pvms18	Pvms35	Pvms38	Phr160
Railway	*A*	21	35	22	16	20	22	19	31	16
(west+east)	H_O	0.659	0.800	0.765	0.694	0.612	0.553	0.618	0.894	0.806
	H_E	0.907	0.915	0.918	0.877	0.701	0.888	0.855	0.893	0.827
	P_{HW}	**0.0000**	**0.0000**	**0.0002**	**0.0000**	0.0565	**0.0000**	**0.0000**	0.5834	0.2032
Southeastern	*A*	19	26	18	16	19	21	18	25	13
	H_O	0.491	0.527	0.673	0.782	0.655	0.582	0.800	0.927	0.691
	H_E	0.930	0.902	0.903	0.883	0.900	0.921	0.924	0.947	0.813
A by locus		22	37	23	19	23	26	24	33	18

A: Number of alleles; H_O: Observed heterozygosity; H_E: Expected heterozygosity; P_{HW}: *P* value of Hardy–Weinberg equilibrium test. Bold indicates significance after sequential Bonferroni correction at 0.01 level.

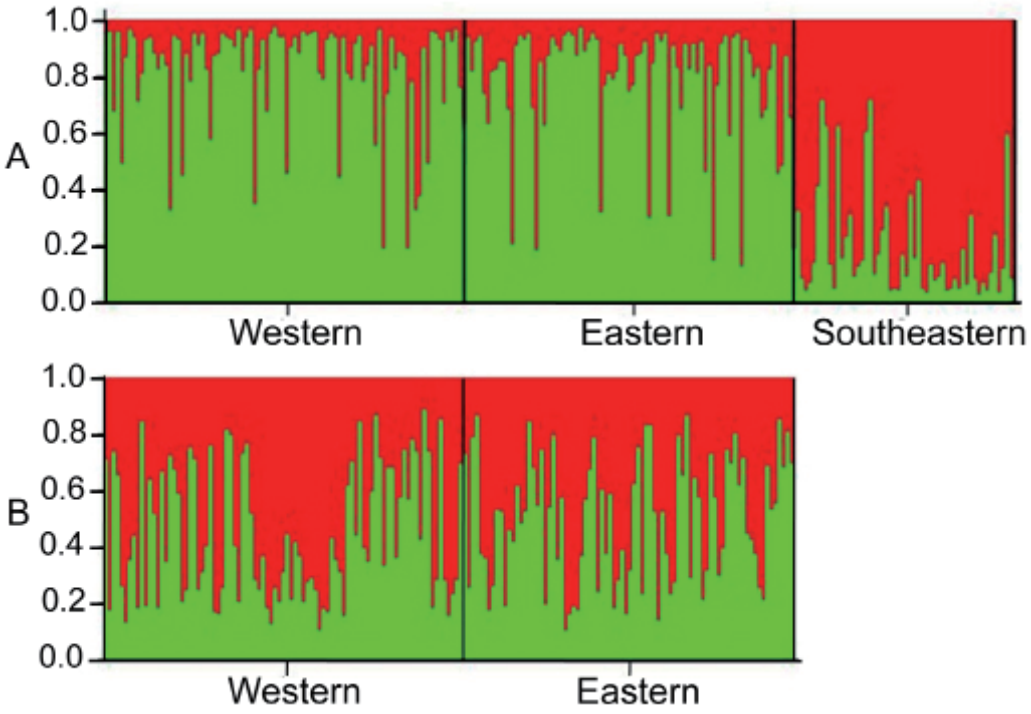

Figure 4 Individual assignment probability bar plots from Structure (ver. 2.3.1) for K = 2. Each vertical bar represents one individual. Individuals in both western and eastern sides of the railway are arranged in the order from north to south, and individuals from the southeastern sites are arranged in the order from west to east. Western = samples collected from the western side of the railway; Eastern = samples collected from the eastern side of the railway; Southeastern = samples collected from the southeastern sites. A: All samples are included; B: Only the samples from the railway sites are included.

populations; not a single individual received a particularly high probability of belonging to one side (probability difference > 0.5). This suggests that all samples from the railway sites make up one population. Twelve individuals were unlikely residents of either side the railway; their probabilities of being residents of any side were less than 0.01. These individuals are likely migrants or descendants of recent migrants from other areas.

AMOVA revealed a similar pattern (Table 2). When all samples were included, there was a small (3.27%) but significant difference between the railway site samples and the southeastern samples ($P < 0.001$; Table 2). When only the samples from the railway sites were included, there was no significant difference between the eastern sites and western sites ($P > 0.874$). Variation at all other hierarchical levels was significant.

4. Discussion

4.1 High genetic diversity This species has very high genetic diversity. Within the 20 km stretch of sampling area and 170 samples, the numbers of alleles per locus ranged from 16 to 35, with an average of 22.4 alleles per locus (Table 1). The diversity is much higher than that of many other lizard species in similar studies using microsatellite DNA loci. For example, the grand skink (*Oligosoma grande*) has an average number of alleles of 15.3 per locus (Berry *et al.*, 2003). Ernst *et al*. (2004) found 8–20 alleles per locus in the Florida scrub lizard (*Sceloporus woodi*), and Urquhart *et al*. (2005) found 7–25 alleles per locus for a different toad-headed lizard

Table 2 Results of the analysis of molecular variance (AMOVA).

Source of variation	Sum of square	Variance components	Percentage variation
A: W+E *vs.* SE			
Among groups	28.600	0.13342	3.27337*
Among sites within groups	249.172	0.09868	2.42114*
Among individuals within sites	803.283	0.67201	16.48717*
Within individuals	716.500	3.17183	77.81832*
Total	1797.555	4.07594	
B: W *vs.* E			
Among groups	4.752	-0.00716	-0.18397
Among sites within groups	177.624	0.10550	2.70947*
Among individuals within sites	592.744	0.59535	15.29010*
Within individuals	544.000	3.20000	82.18440*
Total	1319.121	3.89368	

Grouping schemes are as following: A: All samples with two groups: 1) both sides of the railway sites, and 2) southeastern sites; B: Only the samples from the railway sites with two groups: 1) sites from western side of the railway, and 2) sites from eastern side of the railway. Average over 9 loci; number of permutations = 10 000; allowed level of missing data = 0.05. *: $P < 0.001$.

species, *P. przewalskii*. The revealed high diversity in this species is consistent with a previous study (Wang *et al.*, 2009). They recorded an average of 32.5 alleles per locus over a much large sampling area.

There is clear genetic structure among populations even within relatively short distance. The short gap (20–25 km) between the railway sites and the southeastern sites produced a detectable differentiation, by both the assignment tests and AMOVA. This observation also suggests that these tests are sensitive and able to pick up the signal from these genetic differences.

4.2 Does the railway function as genetic barrier to the lizard populations? Our data suggest that at the present time the Qinghai-Tibet Railway does not impose a detectable "barrier effect" on the toad-headed lizards. Individuals from both sides of the railway share similar genetic make-up and form one population. There is no detectable sub-structure or clustering within the population. There are several possible explanations.

The Qinghai-Tibet Railway may not significantly reduce the crossing of these lizards despite roads often obstructing reptile movement (Shine *et al*., 2004; Andrews and Gibbons, 2005). Railroads in particular have also been found to restrict gene flow in many other species (Bartoszek and Greenwald, 2009). Previous studies on large mammals found that the Qinghai-Tibet Railway indeed impeded crossing of these animals. For example, Yin *et al*. (2006) found that the Tibetan antelope (*Pantholops hodgsoni*), Tibetan gazelle (*Procapra picticaudata*) and Kiang (*Equus kiang*) were sensitive to roads, as well as other man-made structures. The raising embankment of the railway and human introduced objects, such as construction materials and the railway tracks, appeared to be the main cause of animal avoidance for large mammals (Xia *et al*., 2007). Such "alien" objects might not deter lizards as much as mammals. In addition, we noticed several bridges and culverts along the stretch of our sampling area, and lizards might use them as dispersal corridors, which has been demonstrated in many other species (Yanes *et al.*, 1995). Furthermore, on both sides of the railway, the construction activities produced many sand dunes that are suitable burrowing habitat. These dunes might attract lizards toward the railway and hence increase the exchanges between the two sides (Figure 1).

The temporal scale may be too small for any barrier effect to accumulate. It is possible that the railway does have a barrier effect but the accumulation of this effect is still below a detectable level at the present time. Nevertheless, microsatellite DNA data and assignment tests are very sensitive, and are able to detect population differences generated in a short period of time (Pritchard *et al.*, 2000; Piry *et al.*, 2004; Waser and Hadfield, 2011). For example, using microsatellite DNA data and assignment tests, Crosby *et al.* (2007) found significant differences between populations of woodfrog (*Rana sylvatica*) on both sides of Highway 401 in southern Ontario, which was completed 37 years prior to sampling time. In another study, Lesbarrères *et al*. (2006) found the populations (*Rana dalmatina*) separated by a highway, which has been open for only 21 years, had significantly more genetic structure (F_{ST} = 0.238) than the populations without a highway separation (F_{ST} = 0.022) in western France. Considering the high sensitivity of the Plateau ecosystem, it is imperative to continuously monitor any changes over long periods of time.

The small spatial scale may also contribute to our inability to detect any barrier effect of the railway. All of our sampling sites along the railway are within an approximately 20 km stretch. We, however, did detect differences between the railway sites and the southeastern sites, despite the small gap (20–25 km) between the two sampling areas. Other fine-scale studies of reptiles found significant population structure at a distance of less than 500 m (*Sphenodon punctatus*; Moore *et al*., 2008). Dileo *et al.* (2010) found significant population sub-structure of foxsnake (*Mintonius gloydi*) within a distance of 5 km. More research on the dispersal patterns of these lizards is needed to fully understanding the impact of the railway.

Sampling bias could have masked the existing population differentiation. Within the railway sampling area in this study, many suitable habitat patches are nearby the railway (Figure 1), and such patches become fewer farther away from the railway. Consequently, many samples were collected fairly close to the railway from both sides. The short distance between sites from different sides of the railway may make it difficult to detect any barrier effect unless the effect is very strong.

5. Conclusion

We did not detect any significant barrier effect of the Qinghai-Tibet Railway on a lizard species. This conclusion is contradictory to the findings from studies on large mammal species, such as antelopes and gazelles. The railway structure itself may not impede lizard movement in a significant way, and/or the existing bridge underpasses or culverts may facilitate crossing. On the

other hand, the railway may in fact have significant barrier effect, but temporal scale is too small for variations to accumulate. Therefore, continuing the monitoring is important.

The Qinghai-Tibet Plateau maintains a unique and fragile ecosystem, which is sensitive to disturbance. With the increased economic development in the region, we expect the traffic volume will be increased significantly, and more roads will likely be constructed in the near future. Carefully evaluating and monitoring the impact of such habitat alteration is crucial to protect this ecosystem.

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Appendix I Sample site and sample size (N) information.

Sites	N	Latitude (˚N)	Longitude (˚E)	Group assignment	Sites	N	Latitude (˚N)	Longitude (˚E)	Group assignment
1	3	36.65896	95.06916	Western	25	1	36.6209	95.03953	Eastern
2	4	36.64955	95.06145	Western	26	5	36.61631	95.03472	Eastern
3	4	36.64612	95.0586	Western	27	1	36.60283	95.02392	Eastern
4	3	36.64101	95.05428	Western	28	8	36.6011	95.02233	Eastern
5	1	36.6363	95.05052	Western	29	3	36.58434	95.01044	Eastern
6	2	36.63604	95.05025	Western	30	11	36.5827	95.0096	Eastern
7	10	36.62437	95.03752	Western	31	7	36.56934	95.00228	Eastern
8	5	36.61147	95.03008	Western	32	15	36.56903	95.0039	Eastern
9	2	36.60889	95.0282	Western	33	2	36.5404	94.98188	Eastern
10	8	36.59799	95.01914	Western	34	16	36.52213	94.96795	Eastern
11	6	36.58957	95.00943	Western	35	1	36.35925	95.047	Southeastern
12	11	36.5892	95.01217	Western	36	5	36.32434	95.26437	Southeastern
13	1	36.5859	95.01216	Western	37	3	36.33952	95.36497	Southeastern
14	6	36.56981	95.00185	Western	38	1	36.35709	95.45422	Southeastern
15	6	36.5582	94.9942	Western	39	1	36.37288	95.61159	Southeastern
16	1	36.55534	94.99097	Western	40	4	36.38054	95.74513	Southeastern
17	7	36.53059	94.972	Western	41	1	36.37277	95.79305	Southeastern
18	1	36.52745	94.9709	Western	42	11	36.38325	95.98775	Southeastern
19	8	36.52617	94.96852	Western	43	10	36.38324	96.13561	Southeastern
20	2	36.67026	95.07748	Eastern	44	14	36.37457	96.19838	Southeastern
21	1	36.66687	95.08364	Eastern	45	3	36.37812	96.2617	Southeastern
22	2	36.66637	95.0765	Eastern	46	1	36.38238	96.38237	Southeastern
23	2	36.65367	95.06547	Eastern	47	1	36.38136	96.40668	Southeastern
24	5	36.63562	95.05228	Eastern					