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Molecular characterization of Babesia ovis in an enzootic focus in eastern Bosnia and Herzegovina

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Abstract

Purpose *Babesia ovis*, the agent of malignant ovine babesiosis, is transmitted by *Rhipicephalus bursa* within discrete enzootic foci. Whether the parasite varies genetically within a single focus and between transmission seasons is unknown. We examined 18S rRNA gene diversity within the enzootic focus of Rudo, eastern Bosnia and Herzegovina.

Methods Blood samples were collected from 41 clinically or subclinically infected sheep from 15 flocks in seven villages (approximately 36 km²) during four transmission seasons (2020, 2021, 2025 and 2026). A 549 bp fragment of the 18S rRNA gene was amplified by PCR and sequenced bidirectionally. Diversity indices, a TCS haplotype network and a UPGMA dendrogram were computed, and the association of haplotype with village and season was tested with chi-square and Fisher–Freeman–Halton exact tests.

Results Six haplotypes (H1–H6) with eight polymorphic sites were recovered. Genetic diversity was low (haplotype diversity 0.313 ± 0.093; nucleotide diversity 0.00098). H1 occurred in all villages and seasons and was identical to 23 of 48 reference sequences from eight countries. H4 matched the Turkish isolate MN611759. Each derived haplotype (H2–H6) was found in a single village and season, but neither association was statistically significant (exact test, P = 0.82 and P = 0.62, respectively).

Conclusion The 18S rRNA gene of *B. ovis* is not uniform within an enzootic focus. Rare haplotypes appeared sporadically, so repeated sampling across seasons is needed to capture local diversity, and higher-resolution markers are required to resolve the genetic structure of *B. ovis* within foci.

Introduction

Babesia ovis is the causative agent of malignant ovine babesiosis, a disease characterized by fever, marked depression, inappetence, anaemia, icterus and haemoglobinuria [1]. Its principal vector is the adult stage of *Rhipicephalus bursa*, with seasonal activity reported from May to August in the settings described in previous studies [2–4]. Ovine babesiosis characteristically remains confined to discrete, geographically circumscribed areas infested by *R. bursa*, termed enzootic foci, in which clinical cases appear roughly 15 days after adult ticks begin feeding on sheep [5]. The term “enzootic focus of ovine babesiosis” was introduced by Yeruham et al. [5]. In such areas, severe cases of malignant ovine babesiosis occur in sheep almost regularly from June to August, i.e. during the activity of adult vector ticks. Such foci are enzootically unstable areas, where host immunity and premunition are insufficient to reduce the occurrence, severity, and number of cases of malignant ovine babesiosis [5]. In the case of the enzootic focus in Rudo, outbreaks have caused substantial losses despite timely treatment with imidocarb dipropionate and preventive acaricide application [1, 4]. The processes underlying the establishment and persistence of these foci across successive transmission seasons remain poorly understood.

Over the past decades, the study of genetic diversity has become an important field of molecular parasitology. Levels of variation reveal differences that are not apparent from morphology or phenotype,

and they inform on parasite–host relationships, evolutionary forces, effective population size, differentiation between populations or lineages, and the degree of recombination [6–8]. In tick-borne apicomplexan parasites, these parameters also have implications for disease control: gene flow and selection can influence the spread of genotypes, while genetic diversity may affect the geographical applicability of diagnostic assays and candidate vaccines [9].

Despite its veterinary importance, the genetic diversity of *B. ovis* remains poorly understood. To our knowledge, only two studies have addressed it directly, and they differ in marker, scale and purpose. Spotin et al. [10] reviewed 81 publicly available 18S rRNA sequences and described 29 haplotypes worldwide, reporting higher haplotype diversity in Asian than in European isolates. Mira et al. [11] typed 107 Turkish isolates with eight mini- and microsatellite loci and found high genetic diversity (He = 0.799) together with substructuring between western and central/eastern subpopulations, which they attributed to restricted gene flow. Beyond these two surveys, no population-level information is available for *B. ovis*.

The dataset compiled by Spotin et al. [10] comprised sequences deposited in a public repository rather than sequences obtained through systematic population sampling. Therefore, the observed diversity may partly reflect differences in sampling effort and sequence submission practices. Mira et al. [11] worked with a purpose-built marker panel and a rigorous population-genetic framework, but their sampling unit was the province, and the study was cross-sectional. Notably, those authors observed that linkage equilibrium emerged only over shorter geographic distances, yet the available sample sizes did not allow this to be tested at the level of the individual flock or focus. Whether *B. ovis* varies within a single focus, and whether such variation persists across transmission seasons, therefore remains an open question.

The present study takes a different approach to sampling. First, sampling was repeated over four transmission seasons (2020, 2021, 2025 and 2026) within a single, clearly delimited enzootic focus in Rudo. Second, the focus corresponds to a defined *B. ovis* population in which no introduction of animals was recorded over the observation period. Third, and centrally, we examined whether detectable variation occurred among villages and flocks separated by only a few kilometres and whether haplotypes were shared across sampled transmission seasons. The 18S rRNA gene was retained deliberately: it is conserved and does not resolve fine evolutionary lineages, but it is the marker most widely applied to *B. ovis* worldwide and therefore the only one that allows the present focus to be placed in a global context [10].

Materials and methods

Study area and definition of the enzootic focus

The study was carried out in an enzootic focus of ovine babesiosis in the municipality of Rudo, eastern Bosnia and Herzegovina. The focus was delimited on epizootiological grounds established in our previous investigations of this area [1, 4, 12]: recurrent clinical cases of malignant ovine babesiosis every

transmission season, and the predominance of *Rhipicephalus bursa* among ticks collected from sheep in those studies. In these previous studies, no tick species other than *R. bursa* was identified on the examined sheep. This finding refers only to ticks collected from sheep and does not exclude the presence of other species in the local tick fauna. In the present study, the presence of ticks on sheep was recorded at the time of blood sampling, but ticks were not collected for species identification (see Animals and sampling). The focus lies in the valley of the river Lim, in a hilly belt with holdings between 380 and 640 m a.s.l., surrounded by ridges rising to about 1400 m, where pastures adjoin the margins of coniferous pine forest (Fig. 1). The study area map was produced in Python 3.11 with the Matplotlib 3.10 library [13] from SRTM 1 Arc-Second Global elevation data (NASA/USGS, tile N43E019); relief is shown as a hillshade with an illumination azimuth of 315° and an altitude of 45°, and contours are drawn at 100 m intervals. Coordinates of the holdings were recorded in the field with a handheld GPS receiver and subsequently verified against satellite imagery in Google Earth.

The 15 holdings sampled lie between 43.595° and 43.664° N and between 19.321° and 19.456° E, and their convex hull covers approximately 36 km² within a rectangle of about 11 × 8 km. Fourteen of them are contained within some 13 km²; the fifteenth, in the village of Omarine, lies on the opposite side of the valley and is the only outlying holding. Distances between holdings ranged from 60 m to 11.0 km (mean 3.3 km, median 2.7 km), and the median distance from a holding to its nearest neighbour was 463 m, with holdings in the same village as little as 60 m apart. The sampled holdings are therefore located within a compact area of approximately 36 km²; however, the actual extent of sheep and tick movement between holdings was not measured. Sheep husbandry is smallholder-based: the 15 flocks held between 10 and 30 animals (median 20; 309 animals in total), and no introduction of sheep from outside the focus was recorded during the study period.

Animals and sampling

Blood samples were collected from clinically (n = 37) and subclinically (n = 4) infected sheep over four transmission seasons (2020, n = 10; 2021, n = 6; 2025, n = 17; 2026, n = 8). Clinical and subclinical infection were defined as in our previous studies [1, 4]. The main criterion for clinical suspicion of acute babesiosis was fever; chronic cases were recognized by a history of the acute disease treated with imidocarb dipropionate, together with cachexia, icterus and reduced appetite. Sheep with clinical suspicion and PCR-confirmed *B. ovis* infection were classified as clinically infected, whereas PCR-positive sheep without clinical signs at the time of examination were classified as subclinically infected. All sampling took place in June and July, coinciding with the activity of adult *R. bursa*. The presence of ticks on the sheep was recorded at the time of blood sampling; however, ticks were not collected, and their species composition and infestation intensity were therefore not determined in the present study. The 41 animals came from seven villages within the focus: Prebidoli (n = 22), Ljutava (n = 6), Resići (n = 6), Cikotsko polje (n = 2), Gojava (n = 2), Nikolići (n = 2) and Omarine (n = 1). The animals were between four months and two years old. All sheep in the study were of the Zackel type (local Pramenka sheep). The sex of the animals was not recorded by the attending veterinarians and could therefore not be

considered. Blood was collected from the jugular vein into EDTA tubes and stored at − 20°C until DNA extraction.

DNA extraction and PCR amplification

Genomic DNA was extracted manually from whole blood with the IndiSpin Pathogen Kit (Indical Bioscience, Leipzig, Germany) following the manufacturer's instructions. A 549 bp fragment of the 18S rRNA gene was amplified with the *B. ovis*-specific primers and cycling conditions described by Aktaş et al. [14], in a total reaction volume of 25 µl containing HiChrom PCR Master Mix (HiGenoMB, HiMedia Laboratories, Mumbai, India) on a Mastercycler Nexus thermal cycler (Eppendorf, Hamburg, Germany). Nuclease-free water and genomic DNA of *B. ovis* obtained from a naturally infected sheep in our previous study [1] (GenBank accession no. OM758309; this sequence is included in the present dataset) were used as negative and positive controls, respectively. PCR products were visualized by electrophoresis in a 1.5% agarose gel stained with Midori Green (Nippon Genetics Europe, Düren, Germany).

Sequencing and sequence editing

Amplicons were purified and subjected to bidirectional Sanger sequencing at Macrogen Europe (Amsterdam, the Netherlands), except for the two sequences obtained in our previous study (OM758309 and OM758310), which were sequenced at the Centre for Human Molecular Genetics, Faculty of Biology, University of Belgrade, Serbia [1]. Forward and reverse reads were inspected against their corresponding chromatograms, trimmed and assembled into consensus sequences using BioEdit 7.2 [15]. Only base calls unambiguously supported by both reads were retained. Samples yielding ambiguous bases or unresolved chromatogram regions were re-sequenced or discarded. The resulting sequences were deposited in GenBank under accession numbers OM758309, OM758310, QB011463–QB011484 and PZ748577–PZ748593.

Reference sequences and quality criteria

Babesia ovis 18S rRNA sequences retrieved from GenBank were screened for coverage and sequence quality before inclusion. Each sequence was projected onto the 493 bp target region defined by the sequences generated in this study and retained only if it (i) covered at least 95% of this region, (ii) contained no ambiguous bases, and (iii) contained no internal insertions or duplicated segments relative to the target region. The final dataset comprised the 41 sequences from Rudo and 48 reference sequences from ten countries (Fig. 4). Sequences of *Babesia motasi* (AY533147) and *Babesia aktasi* (PV211451) were additionally included as outgroups in the dendrogram analysis.

Alignment and data analysis

Sequences were aligned with MAFFT v7 [16], and the alignment was restricted to a 493 bp target region. Because the alignment of local and reference sequences contained single-nucleotide insertions and deletions (indels), genetic distances were computed with pairwise deletion of gaps; complete deletion would have reduced the alignment to 453 positions and excluded a site that distinguishes one of the local haplotypes.

The number of haplotypes (h), the number of segregating sites (S), haplotype diversity (Hd) and its standard deviation, nucleotide diversity (π) and the mean number of pairwise differences (k) were calculated using DnaSP v6 [17]. These indices were also calculated separately for each village and transmission season. Genetic distances were computed as uncorrected p-distances, and a dendrogram was constructed by the unweighted pair group method with arithmetic mean (UPGMA) in MEGA 12 [18] with 1000 bootstrap replicates. UPGMA was chosen to display the matrix of p-distances graphically and to show the position of the local haplotypes relative to published sequences, not to reconstruct evolutionary history; the method assumes a constant rate of substitution, an assumption that cannot be verified for the present data, and the dendrogram should therefore be read as a summary of observed similarity rather than as a phylogeny.

The association of haplotype with village and with transmission season was assessed separately on the corresponding contingency tables using Pearson's chi-square test of independence in GraphPad Prism 10 (GraphPad Software, Boston, MA, USA). Because most expected cell frequencies were below five, P values were also obtained with the Fisher–Freeman–Halton exact test, estimated by Monte Carlo simulation with 200,000 replicates in Python 3.11. Relationships among haplotypes were inferred using TCS networks [19] constructed in PopART v1.7 [20], separately for the 41 local sequences and for the combined dataset of local and reference sequences. The network topology obtained in PopART was redrawn for publication using Python 3.11 and Matplotlib 3.10 [13], preserving the original connections among nodes and haplotype frequencies.

Results

Haplotypes within the focus

All 41 sequences obtained from the focus were identified as *B. ovis* and grouped into six haplotypes (H1–H6) across the 493 bp analysed region. Eight variable sites were identified at alignment positions 17, 138, 189, 205, 231, 232, 248 and 263, representing five transitions and three transversions. Six were singleton variable sites, and two were parsimony-informative. Each derived haplotype differed from H1 by one to three substitutions: H2 by T138G, H3 by T248C, H4 by A17G, H5 by A232G and G263C, and H6 by G189A, T205C and A231T. H1 was by far the most frequent (n = 34; 82.9%), followed by H2 and H3 (n = 2 each; 4.9%) and by H4, H5 and H6 (n = 1 each; 2.4%). The greatest pairwise sequence divergence within the focus occurred between H5 and H6, which differed at five nucleotide positions (uncorrected p-distance = 0.0101; 1.01%).

Diversity indices

Genetic diversity within the enzootic focus was low, with a haplotype diversity (Hd) of 0.313 ± 0.093, a nucleotide diversity (π) of 0.00098 and a mean number of pairwise nucleotide differences (k) of 0.483 (Table 1). For comparison, the 48 retained GenBank reference sequences yielded Hd = 0.731 ± 0.072 and π = 0.00475. Nucleotide diversity in the reference dataset was approximately 4.8 times that observed within the focus.

Table 1
Diversity indices of the *Babesia ovis* 18S rRNA sequences from the Rudo enzootic focus (n = 41), computed over the 493 bp analysed region.

Parameter	Value
Number of sequences (N)	41
Analysed length (bp)	493
Number of haplotypes (h)	6
Segregating sites (S)	8
Singleton variable sites	6
Parsimony-informative sites	2
Haplotype diversity (Hd ± SD)	0.313 ± 0.093
Mean pairwise differences (k)	0.483
Nucleotide diversity (π)	0.00098
Transitions / transversions	5 / 3
Ts/Tv ratio	1.67

Distribution across transmission seasons

All sequences obtained from 2020 (n = 10) and 2026 (n = 8) belonged to H1, resulting in Hd = 0 in both seasons. In 2021, six sequences represented two haplotypes, H1 and H4 (Hd = 0.333 ± 0.215). The highest observed haplotype diversity occurred in 2025, when five of the six haplotypes were detected among 17 sequences (Hd = 0.581 ± 0.131). H1 was recovered in all four sampled transmission seasons, whereas H2, H3, H5 and H6 were detected only in 2025, and H4 only in 2021 (Fig. 2). None of the rare haplotypes was recovered in more than one season.

Distribution among villages

H1 was the only haplotype found in all seven villages. Each of the remaining five haplotypes was confined to a single village: H2, H3 and H4 in Prebidoli, and H5 and H6 in Ljutava. Thus, none of the rare

haplotypes was detected in more than one village (Fig. 3). Multiple haplotypes were observed in Prebidoli (n = 22, four haplotypes, Hd = 0.403 ± 0.125) and Ljutava (n = 6, three haplotypes, Hd = 0.600 ± 0.215). Only H1 was detected in Resići (n = 6), Gojava, Nikolići, Cikotsko polje (n = 2 each), and Omarine (n = 1). Ljutava had the highest observed nucleotide diversity (π = 0.00338), although its six sequences originated from a single flock.

Association between haplotype and village or season

Neither association was statistically significant. For the village × haplotype table, $\chi^2 = 16.72$ (df = 30, asymptotic P = 0.976; exact P = 0.82); for the season × haplotype table, $\chi^2 = 15.62$ (df = 15, asymptotic P = 0.408; exact P = 0.62). Most expected cell frequencies were below five (98% and 88% of cells, respectively), and the number of sequences per village and season was unbalanced.

Relationship to published sequences

Within the combined dataset, H1 was the most widely distributed *B. ovis* 18S rRNA haplotype. It was identical to 23 of the 48 retained reference sequences across their overlapping regions. These sequences originated from eight countries: Türkiye (9), Iraq (5), Algeria (2), Iran (2), Spain (2), Portugal (1), Tunisia (1) and Bosnia and Herzegovina (a previously reported isolate from Gacko [1]; n = 1). Of these 23 sequences, 14 matched H1 across the entire 493 bp analysed region. In the haplotype network constructed from the combined dataset (not shown), H1 occupied a central position and was directly connected to 24 of the 27 remaining ingroup nodes, including 13 separated from H1 by a single mutational step, producing a pronounced star-like topology. H4 was identical to MN611759 from Türkiye, whereas H2, H3, H5 and H6 matched no published sequence and are reported here for the first time.

The UPGMA dendrogram, used to summarize uncorrected p-distances rather than to reconstruct evolutionary history, showed a pattern consistent with the haplotype network (Fig. 4). All *B. ovis* sequences formed a single cluster with 75% bootstrap support. Within this cluster, the six local haplotypes did not form a distinct subgroup. H1 grouped among the large set of identical sequences from Europe, North Africa and the Middle East. H4 clustered with the Turkish isolate MN611759, which had an identical sequence across the analysed region, and with OR395170, forming a small subgroup within the *B. ovis* cluster. Haplotypes H2, H3, H5 and H6 attached individually, without forming a geographically coherent clade.

Discussion

Variation in the 18S rRNA gene was detected within a single enzootic focus of approximately 36 km², with no recorded introduction of sheep from outside the focus during the study period (2020, 2021, 2025 and 2026). Six haplotypes were found among the 41 *B. ovis*-positive sheep. Five differed from the predominant haplotype (H1) by one to three nucleotide substitutions, and four of them have not been reported before. Although the overall level of genetic variation was low (Hd = 0.313; π = 0.00098), these findings demonstrate detectable local sequence variation in the 18S rRNA gene. To date, this relatively

conserved marker has been used primarily for the molecular detection and identification of *B. ovis* and for its differentiation from the other piroplasms of sheep [21, 22], rather than for assessing fine-scale genetic diversity.

The distribution of 18S rRNA variants within the focus was more informative than the overall level of genetic diversity. H1 was detected in all seven villages and during all four seasons, whereas each of the five derived haplotypes was restricted to a single village. H2–H4 were detected in Prebidoli, where most clinical cases recorded within the study area occurred, whereas H5 and H6 were confined to the village of Ljutava, which lies across the Lim from Prebidoli and where clinical disease was seen only in 2025 and was restricted to a single flock. No derived haplotype was shared between villages, despite the close proximity of sampled holdings, which were separated by a median distance of 2.7 km, with some neighbouring holdings located only 60 m apart.

Similar patterns of fine-scale population structure have been reported in other vector-borne protozoa, particularly when sufficiently polymorphic markers have been employed. In The Gambia, *Plasmodium falciparum* isolates collected from the same household were six times more likely to be genetically related than isolates from different villages, with much of the genetic differentiation accumulating between transmission seasons [23]. In Papua New Guinea, microsatellite markers distinguished *P. falciparum* populations primarily at the study-site level, whereas the more highly polymorphic *var* genes resolved population structure at the village level [24]. Comparable patterns have been observed among apicomplexan tick-borne parasites. *Theileria parva* sampled on a single farm in Uganda showed both high genetic diversity and detectable population substructure [25], whereas in *Theileria annulata* from Oman, evidence of local recombination was present at regional level but was no longer apparent when individual farms were analysed separately [26]. Our results tentatively suggest that *B. ovis* may fit the same pattern, in which individual villages and their associated grazing areas, rather than the enzootic focus as a whole, represent the epidemiologically relevant unit.

Two explanations are possible for these results. First, sheep in the study area generally graze on relatively fixed pastures close to their holding, and inter-flock mixing is limited; consequently, animal movement and parasite exchange may remain restricted even between neighbouring villages.

Second, the occurrence of rare haplotypes may reflect spatial structuring of local *R. bursa* populations. *Rhipicephalus bursa* is a two-host tick whose spread depends on the movement of its hosts, and the pastures here lie in separate patches along the forest edge. More generally, gene flow in ticks is strongly influenced by host mobility but may be further constrained by species-specific tick ecology and behaviour [27]. However, because *R. bursa* populations were not sampled in the present study, their contribution to the observed *B. ovis* haplotype distribution cannot be directly assessed. This explanation should therefore be regarded as a biologically plausible hypothesis consistent with, but not demonstrated by, the present sequence data.

Prebidoli was the only village sampled in all four transmission seasons, and the derived haplotypes detected there differed among seasons: H4 was detected in 2021, H2 and H3 in 2025, whereas only H1

was detected in 2020 and 2026. None of the derived haplotypes was detected in more than one transmission season across the study area. This pattern suggests that rare variants were detected only occasionally, consistent with their circulation at low frequencies. Consequently, a single cross-sectional survey of this focus might have detected only the H1 haplotype and concluded that the local population is uniform. However, sampling across the study area was uneven. Of the sequences analysed, 22 were obtained from Prebidoli across four transmission seasons, six from a single flock in Ljutava during one season, and only one from Omarine.

Therefore, the apparent restriction of H5 and H6 to Ljutava cannot be separated from the effect of limited temporal and spatial sampling at that location. Similarly, the higher occurrence of derived haplotypes in 2025 cannot be fully separated from differences in the villages sampled that year. Given this unbalanced sampling design and the small numbers of observations in some village-season combinations, the apparent spatial and temporal variation in haplotype distribution should be interpreted cautiously. Consistent with these limitations, no statistically significant association was detected between haplotype distribution and either village or transmission season.

In the global dataset, H1 was the most widely distributed *B. ovis* 18S rRNA haplotype. It was identical to 23 of the 48 reference sequences, which originated from eight countries across Europe, North Africa and the Middle East, and was directly connected to 24 of the remaining 27 haplotypes in the network. The same pattern has been described in *T. annulata*, in which a single haplotype was shared among isolates from India, China, Türkiye and Iran, whereas the other haplotypes were represented by single sequences confined to individual countries, with no clear geographical clustering [28]. These observations suggest that the occurrence of a widely distributed 18S rRNA haplotype may be a common pattern among tick-borne piroplasms, potentially reflecting the relatively conserved nature of this marker [29]. Among the local haplotypes identified in the present study, only H4 matched a previously reported sequence, being identical to the Turkish *B. ovis* isolate MN611759.

The two published studies of *B. ovis* genetic diversity approached the question from different directions. Spotin et al. [10] identified 29 haplotypes worldwide based on publicly deposited sequences rather than on sampled populations. In contrast, Mira et al. [11], using mini- and microsatellite markers, demonstrated genetic differentiation among *B. ovis* populations from different Turkish provinces and found evidence of local genetic exchange primarily over short geographical distances, without being able to test this at the flock level. The present data suggest that variation may be structured at distances of a few kilometres, and show that it is detectable even with a marker as conserved as the 18S rRNA gene.

A focus of this size appears to support an active exchange among the parasite, vector and host despite the relatively small susceptible host population. Mixed infections with *Anaplasma ovis* are frequent in this area, with 63.3% of *B. ovis*-positive sheep co-infected [30], and *A. ovis* itself exhibits high genetic diversity locally [30]. The main limitation of the present study, however, lies in the marker used. The 18S rRNA gene is conserved, so the lack of detectable differences among villages should not be interpreted as evidence that their parasite populations are genetically homogeneous. Likewise, the six haplotypes

identified here likely underestimate the genetic diversity of the local parasite population. Moreover, direct sequencing of the dominant PCR product from clinical blood samples cannot reliably detect multiple *B. ovis* genotypes co-infecting the same animal. A more detailed reconstruction of the population structure will require higher-resolution genetic markers, including the satellite marker panel already available for *B. ovis* [11], and mitochondrial markers [31]. Among the latter, *cox1* provides substantially greater resolution than 18S rRNA for assessing both intra- and interspecific genetic variation [32], while both *cox1* and *cytb* have been applied to related *Babesia* species [33, 34]. Applying these markers to parasites from the same flocks, together with genotyping of the local *R. bursa* populations, would provide a more robust framework for investigating the fine-scale genetic structure, transmission dynamics, and evolutionary history of *B. ovis* within this enzootic focus.

Conclusions

Within a single enzootic focus of about 36 km², sampled over four transmission seasons and with no recorded introduction of sheep, the 18S rRNA gene of *B. ovis* was not uniform. Six haplotypes were recovered among 41 animals, four of which were novel. Overall genetic diversity was low (Hd = 0.313; π = 0.00098). The predominant haplotype, H1, was present in all seven villages and across all transmission seasons and was identical to the most widely distributed haplotype among published sequences. H4 was identical to the previously reported Turkish isolate MN611759, while H2, H3, H5 and H6 matched no published sequence. Each of the five derived haplotypes was confined to a single village and none was recovered in more than one season, although neither association was statistically significant. These findings demonstrate that the relatively conserved 18S rRNA marker can reveal detectable intraspecific variation in *B. ovis* even at a fine geographical scale. Because the rare haplotypes were detected sporadically and were restricted to individual villages, repeated longitudinal sampling of the same villages and flocks will be necessary to determine whether these patterns represent persistent local variants or transient detections. Indeed, a single cross-sectional survey could have underestimated the haplotype diversity observed in this enzootic focus. The structure observed here represents only a baseline of intraspecific variation. Applying microsatellite or mitochondrial markers to the same flocks, together with genotyping of the local *R. bursa* population, could be the next step in studying this enzootic focus.

Statements and Declarations

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Competing interests The authors declare no competing interests.

Ethics approval The study was conducted in accordance with the Law on Animal Protection and Welfare of the Republic of Srpska (Official Gazette of the Republic of Srpska, no. 111/08). Formal ethical approval was not required, as all samples were obtained during routine clinical examinations carried out by local veterinarians.

Consent to participate Informed consent was obtained from the owners of all animals sampled.

Data availability The sequences generated in this study are available in GenBank under the accession numbers given in the Materials and methods section. All other data are available from the corresponding author on reasonable request.

Author contributions O.S. and G.V. conceived the study. O.S. and A.R. developed the methodology, curated the data and performed the formal genetic and statistical analyses; O.S. and A.R. also wrote the Python scripts used for data analysis and figure preparation. O.S., G.V. and I.S. carried out the investigation, including sample collection and laboratory work. O.S., G.V. and D.Kn. provided resources (animals, field access and materials). O.S., G.V., D.N. and D.Kn. administered the project. D.N., N.K. and M.A. supervised the study. D.Ka. and A.V. validated the results. O.S. prepared Figures 1–4. O.S. and A.R. wrote the main manuscript text, and M.C.U. and M.A. reviewed and edited the manuscript. All authors reviewed the manuscript and read and approved the final version.

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Figures

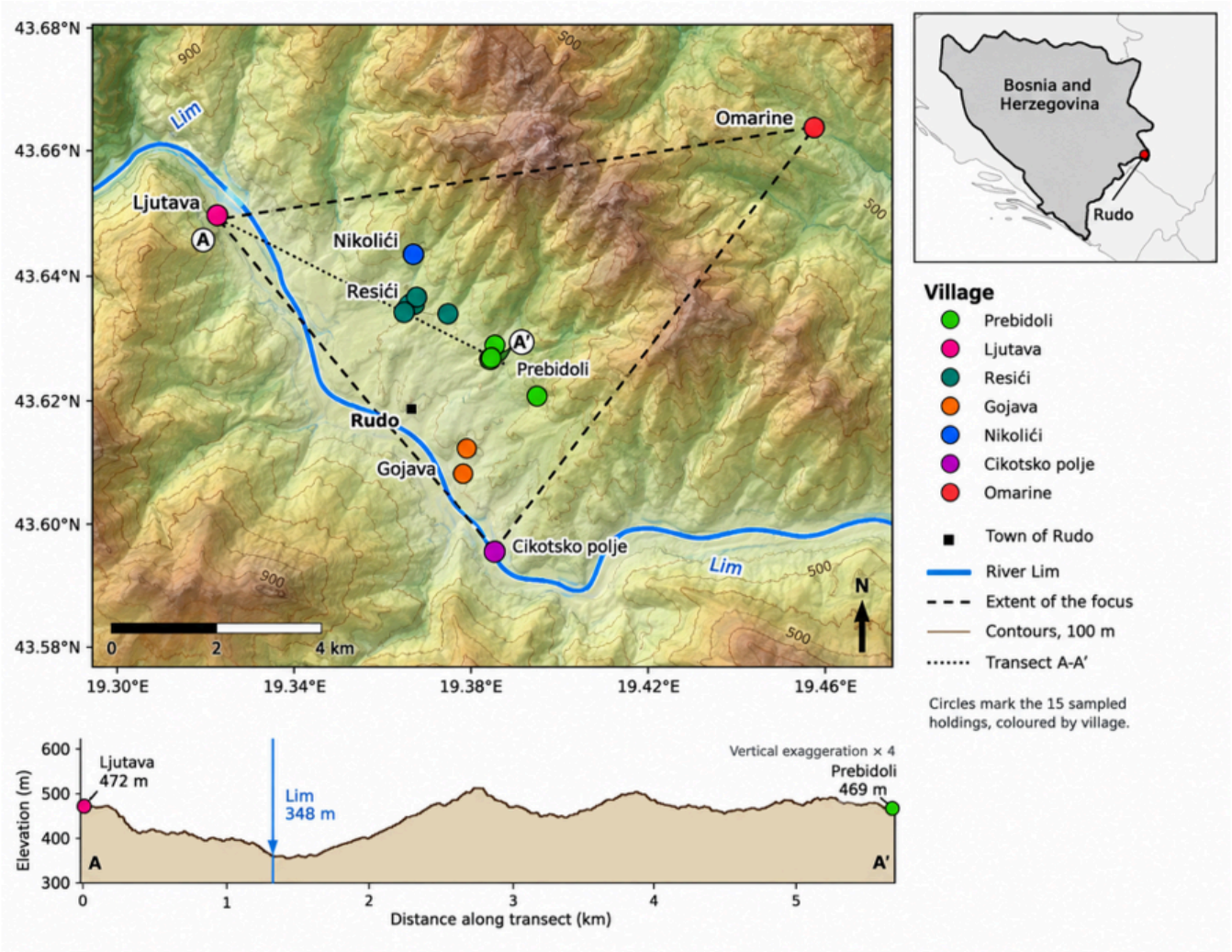
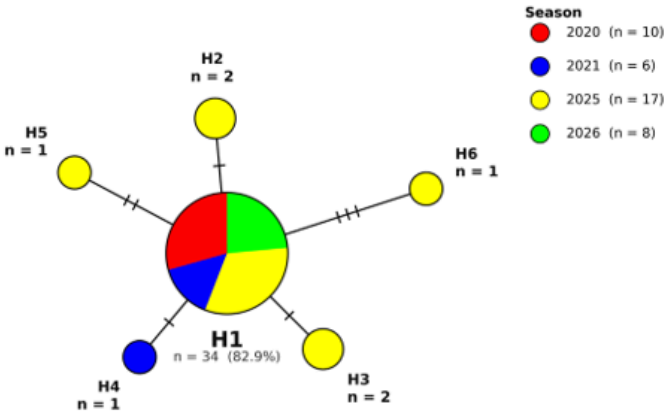


Figure 1

The enzootic focus of ovine babesiosis in the municipality of Rudo, eastern Bosnia and Herzegovina. Circles mark the 15 sampled holdings, coloured by village; the dashed line is the convex hull of the sampled holdings. Relief and contours at 100 m intervals are derived from SRTM 1 Arc-Second Global elevation data (NASA/USGS), and the course of the Lim was traced from the valley floor of the same elevation model. The lower panel shows the topographic profile along transect A–A', from Ljutava across the Lim to Prebidoli, the only two villages in which haplotypes other than H1 were detected.



Haplotype distribution and diversity indices by season

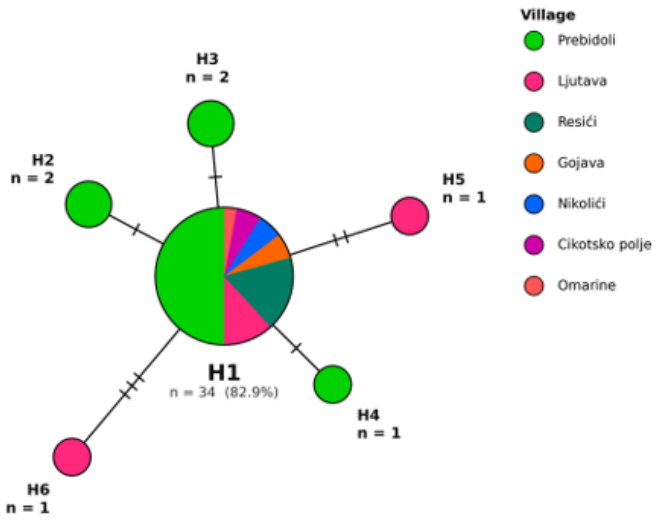
Season	H1	H2	H3	H4	H5	H6	n	h	S	Hd	k	π
2020	10	–	–	–	–	–	10	1	0	0.000	0.000	0.00000
2021	5	–	–	1	–	–	6	2	1	0.333	0.333	0.00068
2025	11	2	2	–	1	1	17	5	7	0.581	1.029	0.00209
2026	8	–	–	–	–	–	8	1	0	0.000	0.000	0.00000
Total	**34**	**2**	**2**	**1**	**1**	**1**	**41**	**6**	**8**	**0.313**	**0.483**	**0.00098**
% of total	82.9	4.9	4.9	2.4	2.4	2.4	100					

n = sequences; h = haplotypes; S = segregating sites; Hd = haplotype diversity; k = mean pairwise differences; π = nucleotide diversity. Overall Hd = 0.313 ± 0.093.

H1 was the only haplotype recovered in 2020 and 2026; all five derived haplotypes appeared in 2025 (four) and 2021 (H4).

Figure 2

TCS haplotype network of the 41 *B. ovis* 18S rRNA sequences from the Rudo focus, coloured by transmission season. Circle area is proportional to haplotype frequency and hatch marks on the branches denote single mutational steps. The accompanying table gives haplotype frequencies and diversity indices per season.



Haplotype distribution and diversity indices by village

Village	H1	H2	H3	H4	H5	H6	n	h	S	Hd	k	π
Prebidoli	17	2	2	1	–	–	22	4	3	0.403	0.437	0.00089
Ljutava	4	–	–	–	1	1	6	3	5	0.600	1.667	0.00338
Resići	6	–	–	–	–	–	6	1	0	0.000	0.000	0.00000
Gojava	2	–	–	–	–	–	2	1	0	0.000	0.000	0.00000
Nikolići	2	–	–	–	–	–	2	1	0	0.000	0.000	0.00000
Cikotsko polje	2	–	–	–	–	–	2	1	0	0.000	0.000	0.00000
Omarine	1	–	–	–	–	–	1	1	0	0.000	0.000	0.00000
Total	**34**	**2**	**2**	**1**	**1**	**1**	**41**	**6**	**8**	**0.313**	**0.483**	**0.00098**
% of total	82.9	4.9	4.9	2.4	2.4	2.4	100					

n = sequences; h = haplotypes; S = segregating sites; Hd = haplotype diversity; k = mean pairwise differences; π = nucleotide diversity. Overall Hd = 0.313 ± 0.093.

H1 is the only haplotype shared among villages; H2–H4 occur solely in Prebidoli and H5–H6 solely in Ljutava.

Figure 3

TCS haplotype network of the same 41 sequences, coloured by village of origin. Only H1 is shared among villages; H2–H4 are restricted to Prebidoli and H5–H6 to Ljutava. The table reports the haplotype distribution and diversity indices for each village.

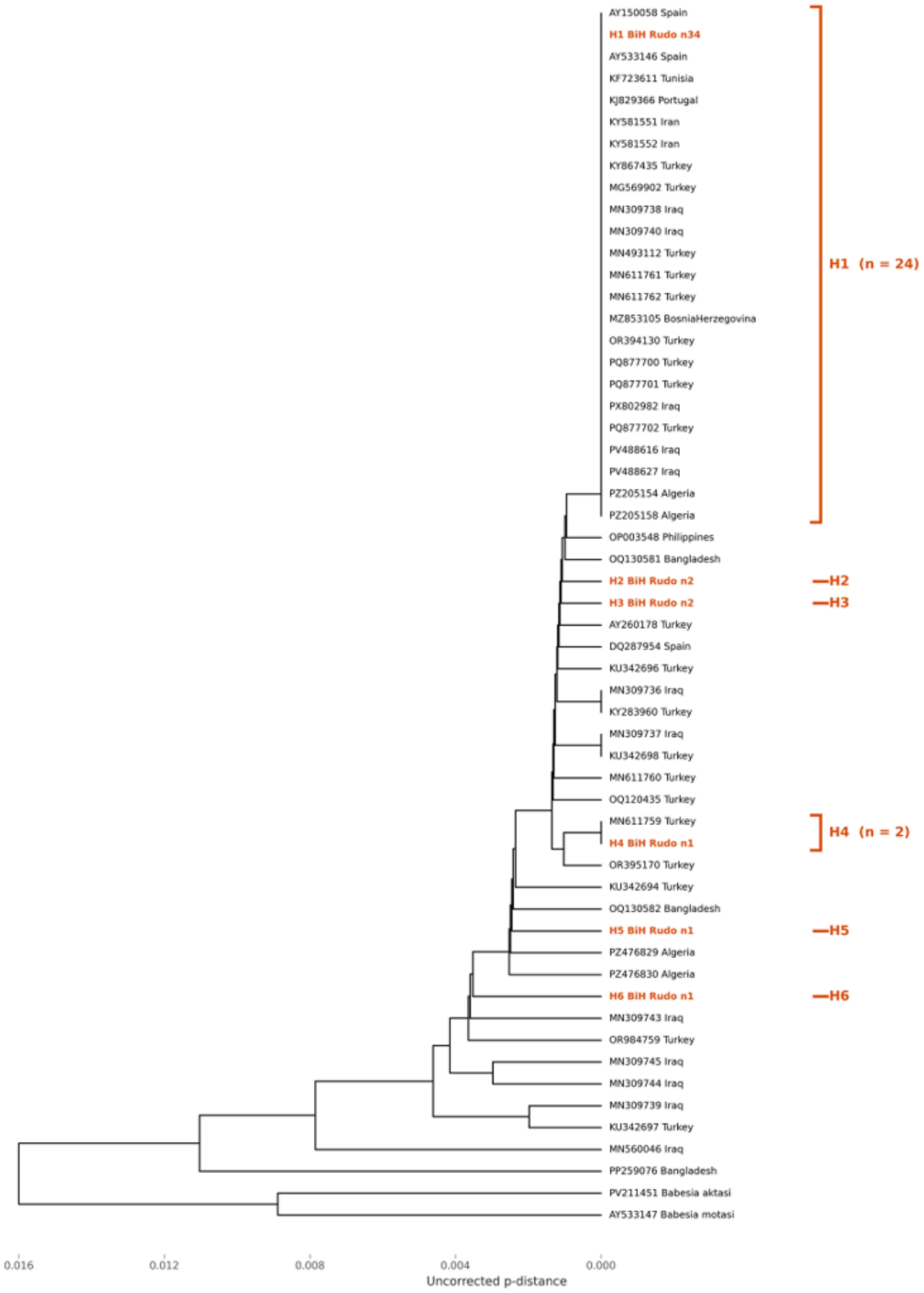


Figure 4

UPGMA dendrogram of the 56 sequences (41 from the Rudo focus reduced to six haplotypes, 48 GenBank references and two outgroups) based on uncorrected p-distances with pairwise deletion and 1000 bootstrap replicates. Brackets mark the positions of the local haplotypes H1–H6.