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Coexistence of genetically different *Varroa destructor* in *Apis mellifera* colonies

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Abstract

The aim of this study was to investigate the genetic diversity of *Varroa destructor* parasitizing *Apis mellifera* colonies and to test for possible host–parasite association at the mitochondrial DNA (mtDNA) level. Six *A. mellifera* haplotypes (including a novel C2aa) and five haplotypes of *V. destructor* were detected in 29 analyzed colonies from eight sampling sites in Serbia. We revealed the presence of the K and S1 haplotypes as well as KS1 and KP1 heteroplasmic mite individuals in all localities, while the P1 haplotype was only found in four sampling sites. Significant differences in *V. destructor* genetic diversity were found at both apiary and colony levels, with mite haplotypes coexisting in almost all tested colonies. In addition, a significant correlation between the number of analyzed mites per colony and the number of identified *V. destructor* haplotypes was observed. However, no significant host–parasite relationship was found, suggesting that mites bearing different haplotypes as well as those heteroplasmic individuals are well adapted to the host, *A. mellifera*, independently of the identified haplotype present in each colony. Our results will contribute to future population and biogeographic studies concerning *V. destructor* infesting *A. mellifera*, as well as to better understanding their host–parasite relationship.

Keywords *Varroa destructor* · *Apis mellifera* · Haplotype · Heteroplasmy · Host–parasite

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Introduction

The honey bee (*Apis mellifera* L.), is considered the most important pollinator of crop monocultures worldwide (De la Rúa et al. 2009). Because of its pollination success and honey production, *A. mellifera* has been spread and repeatedly introduced in non-endemic regions, including eastern Asia (Moritz et al. 2005; Pirk et al. 2017). In this context, beekeeping management brought *A. mellifera* into contact with indigenous Asian honey bee species and their parasitic mites (Anderson and Morgan 2007), including *Varroa destructor* Anderson and Trueman and microsporidian parasites (Higes et al. 2006).

Varroa destructor is an obligate ectoparasitic mite, feeding on the fat body tissue of honey bees (Ramsey et al. 2019) and vectoring multiple viruses (Genersch and Aubert 2010). After it host shifted from the Asian honey bee *Apis cerana* Fabricius, it has spread globally (Anderson and Trueman 2000; Rosenkranz et al. 2010), significantly contributing to colony losses of *A. mellifera* worldwide (vanEngelsdorp et al. 2008; Genersch et al. 2010). Initially, only two mitochondrial DNA (mtDNA) haplotypes of *V. destructor* (Korea and Japan, K and J haplotype, respectively) successfully colonized *A. mellifera* outside Asia (Anderson and Trueman 2000). Although a high mtDNA diversity of *V. destructor* was detected primarily on *A. cerana*, four haplotypes parasitizing *A. mellifera* throughout Asia were described using more sensitive mtDNA markers (Navajas et al. 2010). Recent studies have reported well-established infestations of *A. mellifera* by related Luzon 1 mite haplotype and sibling *Varroa jacobsoni* Oudemans species, revealing new host shift events taking place in Southeast Asia and Oceania, respectively (Beaurepaire et al. 2015; Roberts et al. 2015).

Varroa destructor was detected in Serbia for the first time in 1976 (Lolin 1977) and mites originating from this region (former Yugoslavia) were later all identified as bearing the K haplotype as defined by Anderson and Trueman (2000). However, novel Serbia 1 (S1) and Peshter 1 (P1) haplotypes of *V. destructor* from Serbia were described in 2013, differing from the original K haplotype in single nucleotide polymorphisms (SNPs) within cytochrome c oxidase 1 (*cox1*) and cytochrome b (*cytb*) gene sequences, respectively (Gajić et al. 2013). Lately, nucleotide heteroplasmy has been discovered in this parasitic species and a molecular assay for identification of defined haplotypes and heteroplasmic individuals has been established (Gajić et al. 2016).

Biogeographic studies based on molecular analyses of the host species *A. mellifera* in Balkan countries revealed that two subspecies, *A. m. carnica* and *A. m. macedonica*, are present in Serbia (Stevanovic et al. 2010; Nedić et al. 2014, respectively). In total seven haplotypes were detected, all belonging to the East-Mediterranean (C) evolutionary lineage of honey bee subspecies, among them two newly-described, C2o and C2p (Muñoz et al. 2012). These findings are in agreement with a status of Balkan Peninsula as a centre of insect biodiversity (Milankov et al. 2009) with a central position of Serbia in *A. mellifera* C-lineage distribution area (Meixner et al. 2013).

Having in mind observed mitochondrial diversity of both *A. mellifera* and *V. destructor* in Serbia (Stevanovic et al. 2010; Muñoz et al. 2012; Gajić et al. 2013, 2016), we hypothesized that the distribution of existing *V. destructor* haplotypes and heteroplasmic mites depends on the host haplotype. Therefore, the aim of this study was to investigate the genetic diversity and distribution of *V. destructor* in genetically distinct *A. mellifera* colonies from Serbia and to examine possible association of the host and parasite based on mtDNA variation.

Materials and methods

Honey bee and mite samples

Honey bee workers and *V. destructor* females were collected in 29 *A. mellifera* colonies from eight localities throughout Serbia in 2012. Samples were taken from a single apiary at each locality (Fig. 1a). Colonies from five localities were stationary (Belgrade, Zlatibor, Niš, Suvi Do, Šaprance) whereas those from the remaining three localities (Palić, Lapovo, Boljevac) were migratory. All honey bee and mite samples were transported to

Fig. 1 Geographical distribution of collection sites for *Apis mellifera* and *Varroa destructor* samples (**a**) and hierarchical clustering of localities based on similarity of *Varroa destructor* mtDNA haplotypes (**b**)

the laboratory in 70% ethanol and stored at − 20 °C until DNA extraction. One honey bee worker per colony (n = 29) and 2–14 mites per colony (n = 245) were used for molecular analyses (Table 1).

DNA extraction

Total DNA was extracted from whole adult *V. destructor* females and from three right legs of one worker honey bee per colony (Evans et al. 2013). Individual mites were previously washed in distilled H_2O, dried on filter paper and crushed with sterile mini-pestles in 1.5-mL plastic tubes. DNA was then extracted using KAPA Express Extract Kit (KAPA Biosystems, South Africa), according to the manufacturer's protocol.

Molecular identification

Varroa destructor

Initial typing of all 245 *Varroa* mites was based on the presence/absence of SNPs and heteroplasmy using amplification refractory mutation system (ARMS) in partial *cox1* sequence and PCR-RFLP in *cytb* gene, according to the detailed protocols described elsewhere (Gajić et al. 2016). Final detection of one of the previously identified mtDNA haplotypes (K, S1, P1) or heteroplasmic *V. destructor* individuals (KS1, KP1) was done by coupling results obtained for individual *cox1* and *cytb* sequences.

Apis mellifera

For haplotype identification, the $tRNA^{leu}$-cox2 intergenic region of *A. mellifera* was amplified using KAPA *Taq* PCR Kit (KAPA Biosystems, South Africa). Reaction volume of 25 µL consisted of 14.4 µL nuclease free water, 2.5 µL 10× KAPA *Taq* buffer, 0.5 µL (10 mM) of dNTP mix, 1.25 µL (10 µM) of primers E2 and H2 (Garnery et al. 1991), 0.1 µL (5U/µL) of KAPA *Taq* DNA polymerase and 5 µL of DNA template. Amplification protocol comprised initial denaturation at 95 °C for 5 min, 36 cycles at 95 °C for 45 s, annealing at 47 °C for 60 s and extension at 72 °C for 90 s, followed by final extension at 72 °C for 10 min (Evans et al. 2013).

Sequencing

Mitochondrial amplicons of *cox1* and *cytb* fragments of two mites per colony (n = 58), also analyzed by ARMS and PCR-RFLP assays, were Sanger sequenced using the same primer sets as for PCR amplification to confirm accuracy of *Varroa* genotyping method. Amplicons of the $tRNA^{leu}$-cox2 intergenic region of one honey bee per colony (n = 29) were also Sanger sequenced with the primers used for PCR amplification. Bioinformatic analysis of resulting sequences was done using the software BioEdit v. 7.1.3 (Hall 1999).

Statistical analysis

The χ^2 test was used to compare: (1) the distribution of *V. destructor* haplotypes depending on locality, (2) the distribution of *V. destructor* haplotypes depending on beekeeping

Table 1 Distribution of honey bee haplotypes and *Varroa destructor* haplotypes and heteroplasmic individuals at observed localities

Locality	Coordinates	Beekeeping practice	No. of colonies/mites analyzed	No. of bee colonies with particular haplotype						No. of *V. destructor* mites with particular haplotype or heteroplasmy[a]				
				C1a	C2aa	C2c	C2d	C2e	C2i	K	S1	P1	KS1	KP1
Palić	46°06′32″N 19°46′02″E	Migratory	4/40	2			2			14 (35.0)	10 (25.0)	0 (0.0)	8 (20.0)	8 (20.0)
Belgrade	44°47′37″N 20°27′50″E	Stationary	5/50				2	2	1	23 (46.0)	13 (26.0)	2 (4.0)	11 (22.0)	1 (2.0)
Lapovo	44°11′10″N 21°04′59″E	Migratory	2/20				2			10 (50.0)	6 (30.0)	0 (0.0)	3 (15.0)	1 (5.0)
Boljevac	43°49′49″N 21°57′11″E	Migratory	3/23			1	1	1		2 (8.7)	7 (30.4)	0 (0.0)	6 (26.1)	8 (34.8)
Zlatibor	43°43′24″N 19°42′15″E	Stationary	4/33			1	2	1		3 (9.1)	17 (51.5)	7 (21.2)	5 (15.2)	1 (3)
Niš	43°18′33″N 21°54′31″E	Stationary	2/20					2		5 (25.0)	6 (30.0)	0 (0.0)	3 (15.0)	6 (30.0)
Suvi Do	43°02′43″N 20°07′15″E	Stationary	6/43		1		4	1		16 (37.2)	12 (27.9)	1 (2.3)	12 (27.9)	2 (4.7)
Šaprance	42°23′21″N 22°00′17″E	Stationary	3/16					3		5 (31.3)	7 (43.8)	1 (6.3)	1 (6.3)	2 (12.5)
Total	–		29/245	2	1	2	13	10	1	78 (31.8)	78 (31.8)	11 (4.5)	49 (20.0)	29 (11.8)

[a]Numbers in parentheses indicate the frequency (%) of mtDNA haplotypes of *V. destructor*

practice, and (3) the frequency of SNP and heteroplasmy depending on the analyzed sequence. General discriminant analysis (squared Mahalanobis' distance and its significance) determined the significance of differences in distribution of *V. destructor* haplotypes between the localities, between the hives, and between various bee haplotypes. The hierarchical model of the cluster analysis, based on the squared Mahalanobis' distances and the Ward's method of grouping, provided a dendrogram, pointing to groups of similar elements. Spearman's rank correlation coefficient quantified the dependence between the number of analyzed mites per colony and the number of identified *V. destructor* mtDNA haplotypes. Statistical analysis of experimental data was performed using software package STATISTICA v. 7.0 (Statsoft, Tulsa, OK, USA).

Results

Varroa destructor haplotypes

Independent analyses of cox1 and cytb sequences

In 48.2% (118/245) of mite samples, *cox1* sequence was identical to the reference sequence of the K haplotype (GenBank Accession No. GQ379056), showing no SNPs or heteroplasmy. *Cytb* sequence corresponding to the K haplotype (GenBank Accession No. GQ379094) was observed in 83.7% (205/245) of mites. However, SNPs or heteroplasmy was observed in 51.8 and 16.3% of mites in *cox1* and *cytb*, respectively. Moreover, the occurrence of SNPs and heteroplasmy was significantly dependent on the analyzed sequence ($\chi^2 = 67.190$, df = 1, $p < 0.001$). All sequencing results for 58 mites were in agreement with results obtained using ARMS and PCR-RFLP methods for *cox1* and *cytb* sequence, respectively.

Concatenated sequence analysis

Based on concatenated *cox1* and *cytb* sequence analysis, the majority of mite samples belonged to either K or S1 haplotypes (31.8% of each). *Cox1* heteroplasmy (KS1 individuals) was found in 20% of cases, *cytb* heteroplasmy (KP1 individuals) was observed in 11.8%, whereas 4.5% of mites were assigned to the P1 haplotype. There were no samples with simultaneously detected SNPs or heteroplasmy in both (*cox1* and *cytb*) analyzed sequences, neither individuals containing SNP in one sequence and heteroplasmy within the other (hypothetical S1-P1, S1-KP1, KS1-P1 and KS1-KP1 individuals) (Table 1).

Distribution of *Varroa destructor* mtDNA haplotypes

Distribution among localities

The K and S1 haplotypes as well as KS1 and KP1 heteroplasmic individuals were registered at all sampling sites. However, P1 haplotype was found only in four localities with stationary beekeeping practice (Belgrade, Zlatibor, Suvi Do and Šaprance) (Table 1). Moreover, the frequency of detected *V. destructor* mtDNA haplotypes was highly dependent on the practice performed by beekeepers that is migratory or stationary beekeeping ($\chi^2 = 14.258$, df = 4, $p = 0.007$).

At most sites, predominant haplotypes were K or S1, with KP1 individuals as the most frequent in Boljevac (Table 1). Genetic diversity of *V. destructor* populations was significantly different among localities observed simultaneously ($\chi^2 = 78.392$, df = 28, $p < 0.001$). In addition, significant differences were also detected among individual localities, showing different haplotype frequency of mites from Zlatibor compared to those from all other localities ($p < 0.001$) except from Šaprance.

Hierarchical clustering of localities according to the similarity of mite haplotypes using squared Mahalanobis' distance between localities and Ward's method of grouping, showed three clusters: (1) Boljevac, Niš, Palić; (2) Šaprance, Lapovo, Suvi Do, Belgrade; (3) Zlatibor (Fig. 1b).

Distribution among and within honey bee colonies

Most hives at the same apiary had similar distribution of mite haplotypes (Online Resource 1). However, based on the levels of squared Mahalanobis' distance, hives containing mites with P1 haplotype in Belgrade and Zlatibor were significantly different from their neighboring colonies ($p < 0.05$).

Number of identified mite mtDNA variants per hive ranged from 1 to 5, with 6.9% (2/29) colonies being infested by only S1 haplotype and with coexistence of mite haplotypes registered in 93.1% (27/29) colonies (Table 2). Spearman's rank correlation coefficient ($\rho = 0.711$, $p < 0.001$) showed strong dependence between the number of analyzed mites per colony and the number of identified *V. destructor* mtDNA haplotypes.

Table 2 Identified mtDNA haplotypes of *Varroa destructor* in colonies with different number of mites analyzed

No. of identified mtDNA haplotypes	*V. destructor* mtDNA haplotype	No. of colonies	No. of analyzed mites per colony
1	S1	1	2
	S1	1	3
2	K + S1	1	3
	K + S1	1	10
	K + KS1	1	2
	K + KS1	1	3
	S1 + KS1	1	3
3	K + P1 + S1	1	10
	K + S1 + KS1	1	8
	K + S1 + KS1	5	10
	K + S1 + KS1	1	12
	K + KP1 + S1	1	3
	KP1 + S1 + KS1	1	10
4	K + KP1 + S1 + KS1	8	10
	K + P1 + KP1 + KS1	1	10
	K + P1 + KP1 + S1	1	12
	P1 + KP1 + S1 + KS1	1	10
5	K + P1 + KP1 + S1 + KS1	1	14

Apis mellifera haplotypes

Based on the nucleotide polymorphism detected in the 571–573 bp long tRNA^{leu}-cox2 sequences, six *A. mellifera* haplotypes were identified, five of them previously described (C1a, C2d, C2e, C2i, C2c; GenBank Accession Nos. JQ977699–JQ977703). One sequence characterized by the presence of a new SNP was identified for the first time in this study and assigned to the novel C2aa haplotype (GenBank Accession No. MG788257) following the nomenclature of Rortais et al. (2011) and Chávez-Galarza et al. (2017). Number of detected honey bee haplotypes per locality ranged from one to three. The most frequent haplotype was C2d, followed by C2e, C1a and C2c, with the lowest frequency registered for C2i and C2aa haplotypes (Table 1).

Host–parasite association

Mites with K and S1 haplotypes were found in different proportion on honey bee colonies bearing all identified haplotypes, whereas mites with P1 haplotype were registered only in colonies with C2d and C2e haplotypes. KS1 and KP1 heteroplasmic individuals parasitized honey bees with C1a, C2d, C2e and C2c haplotypes, whereas mite KS1 individuals was also found in honey bees bearing with C2aa (Fig. 2). Significance levels of squared Mahalanobis' distances showed that genetic structure of *V. destructor* population was not dependent on the host haplotype ($p > 0.05$).

However, distribution of *V. destructor* haplotypes and heteroplasmic individuals infesting honey bee bearing C2d haplotype in Boljevac and Zlatibor was significantly different from mite distribution in honey bees with C2d haplotype in the remaining localities ($p \leq 0.031$ and $p \leq 0.002$, respectively), as well as between Belgrade and Palić ($p = 0.029$). Moreover, distribution of *V. destructor* mtDNA variants parasitizing C2e honey bees in Zlatibor significantly differed from all other localities ($p \leq 0.005$).

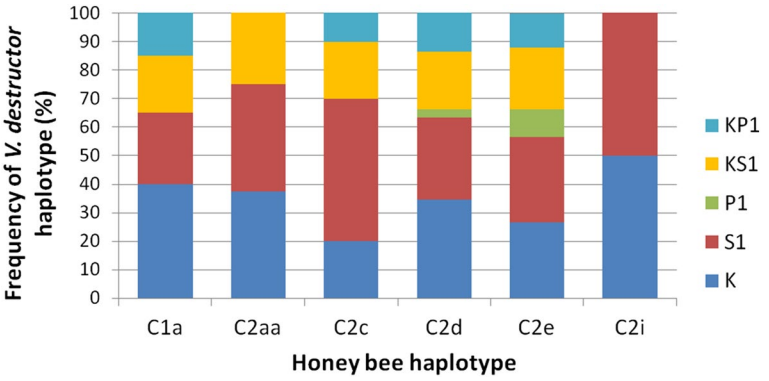

Fig. 2 Distribution of *Varroa destructor* haplotypes and heteroplasmic individuals versus *Apis mellifera* haplotypes

Discussion

In the current study, we revealed that different *V. destructor* haplotypes and heteroplasmic individuals are widely distributed and coexist in *A. mellifera* colonies throughout Serbia, with no strict host–parasite association at mtDNA level.

Based on independent sequence analysis, significantly more *V. destructor* samples from our study showed identity to the reference K haplotype in *cytb* compared to the *cox1* sequence (83.7 and 48.2%, respectively). However, Navajas et al. (2010) failed to detect any mutation in the *cytb* within the K haplogroup of *V. destructor* parasitizing *A. mellifera* in Asia. Additionally, in mites with SNPs or heteroplasmy detected in one sequence (*cox1* or *cytb*), the second analyzed sequence was always without observed SNP and heteroplasmy being therefore identical to the K haplotype. This SNP-heteroplasmy pattern put forward that point mutations in *cox1* and *cytb* occurred independently as well as in different mite populations of the original K haplotype.

Based on concatenated *cox1* and *cytb* sequence analysis, the K and S1 haplotypes were equally represented (each by 31.8%) in tested samples whereas the P1 haplotype was detected in only 4.5% of mites. All previous studies showed predominance of the K haplotype outside Asia (Anderson and Trueman 2000; Garrido et al. 2003; Solignac et al. 2005; Muñoz et al. 2008; Farjamfar et al. 2018) including the geographic region of former Yugoslavia (Anderson and Trueman 2000). Surprisingly low prevalence of the K haplotype (in less than one third of analyzed samples) observed in the current study can be explained by better adaptation or higher virulence of coexisting haplotypes and heteroplasmic mites. Moreover, temporal changes in *V. destructor* haplotype are not unusual, as it has been reported for the K haplotype displacing the J haplotype in Brazil (Garrido et al. 2003).

Heteroplasmic KS1 and KP1 individuals were found in 20.0 and 11.8% of samples, respectively, suggesting that heteroplasmy is common in *V. destructor* populations. In contrast to the high frequency of heteroplasmic individuals in all investigated localities in Serbia, heteroplasmy has not been reported in previous mtDNA-based studies concerning *Varroa* spp. regardless the number and length of analyzed mitochondrial fragments (Solignac et al. 2005; Navajas et al. 2010). In addition, site heteroplasmy in *V. destructor* has not been detected independently of whether DNA was extracted from individual mites (Zhou et al. 2004; Solignac et al. 2005; Muñoz et al. 2008; Navajas et al. 2010) or from a pooled mite sample (Maggi et al. 2012). Moreover, the absence of S1-P1, S1-KP1, KS1-P1 and KS1-KP1 *Varroa* haplotypes in this study emphasizes maternal inheritance of mtDNA in *V. destructor* and minimizes the role of paternal leakage as the cause of heteroplasmy in this species. Nevertheless, additional analyses are needed to elucidate the source and transmission mechanism of the observed heteroplasmy.

We found that coexistence of *V. destructor* haplotypes and heteroplasmic individuals within the same colony is widely present. This finding enables monitoring of seasonal dynamics of coexisting haplotypes and their cumulative effect on honey bee colony, but making at the same time the virulence estimation of particular mite haplotypes more challenging. Additionally, a high correlation observed between the number of the analyzed mites per colony and the number of identified mtDNA variants of *V. destructor* needs future studies in order to define the optimal number of mite samples for reliable molecular characterization.

A high mitochondrial diversity is present in *A. mellifera* colonies located in Serbia where eight haplotypes have been found, in contrast to neighboring countries where one (in Slovenia and Macedonia), two (in Albania) and four (in Croatia) haplotypes were detected

(Sušnik et al. 2004; Muñoz et al. 2009, 2012; Stevanovic et al. 2010). This high genetic diversity can be explained by the ecological history of the Balkan Peninsula that was one of the major Pleistocene ice age refugia for various species and consequently, the source for rapid post-glacial colonization of Europe (Hewitt et al. 1999).

A novel bee haplotype (C2aa) was identified in Syenichko-Peshterski region, where the micro-endemic, unique C2p haplotype was previously found (Muñoz et al. 2012). Importantly, C2aa, the only newly-found haplotype in this study, as well as C2p from previous study (Muñoz et al. 2012) originated from stationary apiaries situated at the altitude over 1000 m. The presence of those two haplotypes could be due to adaptation to specific ecological conditions as observed in the Carpathians (Coroian et al. 2014) if the beekeepers maintain their queens exclusively.

Our survey did not reveal any significant host–parasite haplotype correlation, despite mtDNA variation of *A. mellifera* and *V. destructor* in Serbia. These results are in line with those of Muñoz et al. (2008), who failed to detect significant relationship in the host–parasite haplotype distribution between *A. m. iberiensis* and *V. destructor* in an apiary in central Spain. However, we found that the frequency of observed *V. destructor* haplotypes was significantly dependent on the performed beekeeping practice. This finding indicates that migratory hives could facilitate dispersion of mites via horizontal transmission and lead to homogenization of *V. destructor* genetic structure, as it was reported at the apiary level (Beaurepaire et al. 2017; Dynes et al. 2017).

The lack of host specificity of Luzon 1 haplotype to both *A. mellifera* and *A. cerana* (Beaurepaire et al. 2015), together with the finding of two genetic haplotypes of the sister species *V. jacobsoni* reproducing on *A. mellifera* (Roberts et al. 2015) suggest the ongoing evolution and further host switching of *Varroa* spp. in Asia. However, coexisting haplotypes and heteroplasmic mites we detected in our study are apparently mutated descendants of the original K haplotype with the current host specificity to *A. mellifera* outside Asia, whose spreading potential should be carefully considered in future studies.

Conclusions

The coexistence of mtDNA haplotypes of *V. destructor* in *A. mellifera* colonies throughout Serbia emphasizes the need of analyzing more than one mite per colony for objective mite molecular typing and will make the virulence estimation of particular mite strain more challenging. In addition, high proportion of heteroplasmic mites detected in analyzed populations and specific reproductive behavior of *V. destructor* make this parasitic species a suitable model organism for any analysis concerning the cause, transmission and maintenance of heteroplasmy. Our findings will also contribute to future population and biogeographic studies concerning *V. destructor* infesting *A. mellifera*, as well as to better understanding their host–parasite relationship in other European countries.

Author contributions BG designed the study and wrote the manuscript; BG, IM and PDLR conducted molecular analyses and bioinformatics; NL performed statistical analysis; JS, ZK and ZS helped in interpretation of obtained results and critically revised the manuscript. All authors read and approved the final version of the manuscript.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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