# Invasive cattle ticks in East Africa: morphological and molecular confirmation of the presence of *Rhipicephalus microplus* in south-eastern Uganda

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# Abstract

**Background:** *Rhipicephalus microplus;* an invasive tick species of Asian origin and the main vector of *Babesia* species, is considered one of the most widespread ectoparasites of livestock. The tick has spread from its native habitats on translocated livestock to large parts of the tropical world, where it has replaced some of the local populations of *Rhipicephalus decoloratus* ticks. Although the tick was reported in Uganda 70 years ago, it has not been found in any subsequent surveys. This study was carried out to update the national tick species distribution on livestock in Uganda as a basis for tick and tick-borne disease control, with particular reference to *R.microplus*.

**Method**s: The study was carried out in Serere district, south-eastern Uganda, which is dominated by small scale livestock producers. All the ticks collected from 240 cattle were identified microscopically. Five *R. microplus* specimens were further processed for phylogenetic analysis and species confirmation.

**Results:** The predominant tick species found on cattle was *Rhipicephalus appendiculatus* (86.9 %; n=16,509). Other species found were *Amblyomma variegatum* (7.2 %; n=1,377), *Rhipicephalus evertsi* (2.3 %; n=434*)* and *R. microplus* (3.6 %; n= 687). Phylogenetic analysis of the 12S rRNA, 16S rRNA and ITS2 gene sequences of *R. microplus* confirmed the morphological identification.

**Conclusions** : It is concluded that *R. microplus* has replaced *Rhipicephalus decoloratus,* since the latter was not any longer found in this area. There is currently no livestock movement policy in force in Uganda, which could possibly limit the further spread of *R. microplus* ticks. Future surveys, but also retrospective surveys of museum specimens, will reveal the extent of distribution of *R. microplus* in Uganda and also for how long this tick has been present on livestock without being noticed.


# Background

The Asian blue tick, *Rhipicephalus microplus,(*(Canestrini, 1888)is one of the most important tick species infesting livestock in many parts of the world [1]. *R. microplus* has extended its distribution through the translocation of tick-infested cattle. In Africa, *R.microplus* has successfully competed and replaced the close related African blue tick, *Boophilus decoloratus* [2–4]. *R. microplus* is vector of *Babesia bovis* and *Babesia bigemina* causing extensive production losses [5–7].

*R. microplus* has been introduced from Asia on cattle exported to East and South Africa via Madagascar [3]. Similarly, *R. microplus* was introduced intoIvory Coast and Benin from Brazil 10 years ago [8]. Since then, it has spread to Burkina Faso, Mali, Togo and very recently into Nigeriaand Cameroon [3, 8–12]. *R. microplus* is now well established in the southern and eastern fringes of South Africa [2, 7]. There are a few isolated reports of *R. microplus* in East Africa, notably Tanzania and South Sudan [13, 14]. Some of these reports are over 30 years old [15, 16]; hence, this tick species could have spread to several parts of East Africa. Given that *R. microplus* is an invasive tick species, such isolated reports are likely to be due to a lack of regional or country-wide tick surveys and not to a patchy distribution pattern. Furthermore, the differential diagnosis of *R. microplus* and *R. decoloratus* in East Africa and *R. decoloratus, Rhipicephalus*

*annulatus* and *Rhipicephalus geigyi* in West Africa is difficult because of similarities in morphology and their small size [4].

There are no up-to-date Ugandan or East African tick surveys. As a result, despite records of *R. microplus* 70 years ago in the Uganda; Veterinary Department Annual reports of 1948 and 1950 [16], this has never been confirmed. This study was carried out in one of the high tick density districts of south-eastern Uganda to update national tick species data as a basis for a national tick and tick-borne disease control strategy.

# Methods

## Study area

The study was carried out in Serere district, south-eastern Uganda, in 2017. The district is made up of two rural counties (Kasilo and Serere), eight sub-counties (Bugondo, Kadungulu, Pingire, Labor, Atiira, Kateta, Chere and Serere/Olio) encompassing 254 cattle-owning villages. Tick collections were conducted in Kadungulu sub-county. Serere district was selected because it has a large number of small scale livestock producers (1–50 cattle per herd) whose potential to commercialise livestock production is primarily constrained by ticks and tick-borne diseases. Six of the 254 villages were randomly selected for this study (Fig. 1).

## Cattle herd and individual animal selection

Farmers in Serere district predominantly keep short-horn East-African Zebu cattle in communal village herds. In such communal grazing systems, tick populations are considered at the village level and not at the level of individual farms. Given that any animal sampled from each of these villages would be infested with ticks, the number of villages (n = 6) and animals selected (n = 240) need not to be based on any rigorous statistical methods. A small herd sampling proportion (6/254) is therefore sufficient to give a good indication of the different tick species infesting cattle in south-eastern Uganda. However, cattle were included in this study if they had not been sprayed against ticks for the past two weeks, were young [1–2 years] and non-fractious. Young non-fractious animals were preferred for inclusion because they were easier to restrain and pose very low risk of injury to themselves or personnel. An average of 40 cattle was sampled from each of the six selected villages. All cattle sampling sites were geo-referenced prior to tick collection.

## Tick collection and identification

Selected cattle were physically restrained before half-body tick collections were carried out. Each of the collected ticks was morphologically identified to the genus level before they were preserved in 70% ethanol. The tick samples were then transferred to Makerere University for further species identification

using taxonomic keys [17]. Five representative *R. microplus* specimenswere selected for molecular species confirmation based on 12S ribosomal RNA (12S rRNA), 16S ribosomal RNA (16S rRNA) and the internal transcribed spacer 2 (ITS2) gene sequences [4, 18]. A taxonomically and molecularly confirmed *R. microplus* specimen was photographed under a stereo microscope (Olympus model SZX7, Japan)

## DNA extraction

Prior to tick DNA extraction, each tick was cleaned in five one min steps, each step involving centrifugation at 10,000 rpm in freshly prepared 1.5 ml of Phosphate Buffered Saline (PBS). Individual clean ticks were immersed under liquid nitrogen for 5 min and thereafter crushed with a sterile mortar and pestle to create a tick homogenate. DNA was then extracted from each tick homogenate using DNeasy kit (Qiagen, 19300 Germantown Road, Germantown, MD - USA) following the manufacturer's guide. The presence and quality of DNA were checked by resolving 5µl of the extracted DNA on a 1 % agarose gel and viewing them under an ultraviolet transilluminator (Wagtech International, Thatcham, UK). The remaining DNA was stored at –20°C until use in the downward amplification steps.

## DNA amplification

PCR amplification was performed on 12S rRNA, 16S rRNA and ITS2 genes using primers (Table 1) and thermocycling conditions as previously described [4, 18]. Each reaction was prepared into a final volume of 50µl containing; 1x-reaction buffer (670 mM Tris–$HC_{l,}$ pH 8.8, 166 µM $(NH_4)_2SO_4$, 4.5 % Triton X–100, 2 mg/mL gelatin) (Bioline, Humber Road, London, UK), 0.25mM of each dNTP, 0.25 mM each of forward and reverse primers, 1.56U *BioTaq* DNA polymerase (Bioline, Humber Road, London, UK), 1.25 mM $MgCl_2$, 32.2 µl of PCR grade water and finally 5 µl of the template DNA.

The 16S ribosomal RNA gene was amplified in a thermo-cycler (Personal Thermocycler, Biometra, Germany)with initial denaturation of 94$^{o}$C for 5 min followed by 30 cycles at 94$^{o}$C for 30 sec, 48$^{o}$C for 45 sec, 72$^{o}$C for 45 sec and a final extension at 72$^{o}$C for 7 min. Amplification of the ITS2 and 12S ribosomal RNA was performed using similar thermocycling conditions to those of 16S at annealing temperature of 55$^{o}$C and 52$^{o}$C respectively. PCR products were resolved on 2% agarose gels. The resultant PCR products were sized against a 1kb DNA molecular ladder (Bioline, Humber Road, London, UK). The expected PCR product sizes ranged between 300–1,200 bp. PCR products were purified using QIAquick PCR purification kit (Qiagen, 19300 Germantown Road, Germantown, MD- USA) and commercially Sanger sequenced (Inqaba Biotec, 525 Justice Mahomed St, Muckleneuk, Pretoria, 0002, South Africa).

## Gene sequence analysis

Each of the 12S rRNA, 16S rRNA and ITS2 tick gene sequences from this study were queried in a BLASTn search with default settings (NCBI BLASTn software version 2.6.10) [19] to reveal their identity. The query

sequence identity was assigned/matched based on the hits (tick species sequences returned) with the highest identity scores (≥80%) and most significant E-values (closest to 0.0). The identified query sequences from this study were annotated and submitted to GenBank under accession numbers: MK332390, MK332391, KY688455, KY688455, KY688459, KY688461 and KY688467.

Annotated gene sequences from this study were each analysed in a Multiple sequence alignment (MSA) with their corresponding reference gene sequences downloaded from GenBank using the ClustalW algorithm in MEGA version 10 [20]. The MSA files were used to infer nucleotide similarity between sequences from this study and their corresponding nucleotide reference sequences from the GenBank. Each of the data sequence sets were analysed in MSA using the ClustalW algorithm and trimmed in MEGA software version 10 [21, 22]. Phylogenetic analysis for each nucleotide sequence set was performed using the MEGA maximum likelihood method utilising the Tamura 3 parameter + Gamma distribution with 1000 bootstrap [21] as the best fit model to infer phylogenetic relatedness among the gene sets.

To evaluate the 12S rRNA, 16S rRNA and ITS2 gene sequence divergence of newly typed Ugandan *R. microplus* ticks and their corresponding reference sequences from the GenBank, pairwise genetic distances were calculated in MEGA software version 10 [21] using default settings for each gene.

# Results

# Tick collections

Adult ticks (n = 19,007) were collected upon completion of half body counts from 240 cattle. The majority (86.9 %; n = 16,509) of these ticks were *R. appendiculatus.* Other tick species identified were *Amblyomma variegatum (*7.2 %; n = 1,377), *R. microplus* (3.6 %; n = 687) and *Rhipicephalus evertsi (*2.3 %;n = 434*).* The mean adult tick density was 79 ticks per animal. On average, the numbers of adult *R. appendiculatus, A. variegatum, R. microplus* and *R. evertsi* per animal were 138, 11, 6 and 4, respectively.

A taxonomically and molecularly confirmed *R. microplus* specimen was photographed under a stereo microscope as shown in Fig. 2.Female *R. microplus* was characterized by hypostome teeth in a typical 4 + 4 column arrangement and internal margin palpal article 1 without protuberance and distinctly concave. Male*R. microplus* carried typical indistinct spurs on the ventral plates.

# Molecular confirmation of *R. microplus*

Five of the 687 ticks that were identified as *R. microplus* using standard taxonomic keys were further analysed and all confirmed *R. microplus* by assessing gene sequence variation of their 12S rRNA, 16S rRNA and ITS2 regions. The genetic diversity of *R. microplus* ticks recovered from Uganda and those from elsewhere ranged between 0.0 to 0.075 (Table 2). Phylogenetic analysis of the 12S rRNA (Fig. 3), 16S rRNA (Fig. 4) and ITS2 (Fig. 5) regions revealed polymorphic sub-grouping with *R.microplus* collected

from other parts of the world. The Ugandan *R. microplus* isolates were notably similar to those collected in Taiwan, Mozambique, Nigeria, USA and South Africa.

# Discussion

The high burden of adult ticks on cattle in Serere district, south-eastern Uganda confirms that ticks and associated diseases (anaplasmosis, babesiosis, theileriosis and heartwater) constitute a major constraint to livestock production in this region [23, 24]. It has been reported previously that *R. appendiculatus,* vector of *Theileria parva,* is the predominant tick species in Serere district [24–27]. Besides the discovery of *R. microplus* and the complete absence of *R. decoloratus,* the other tick species were the same as reported before in south-eastern Uganda [24–27]. However, the Ugandan tick population structure varies greatly between the different regions of the country, due to variation in microclimatic conditions [23, 24, 28]. For example, *Amblyomma lepidum, Hyalomma truncatum, Amblyomma gemma* and *Rhipicephalus pulchellus* thrive under the arid conditions of north-eastern Uganda [28, 29], and were therefore not found in this less arid study area.

In “The Ixodid Ticks of Uganda”,by Matthysse and Colbo published in 1987 [16] the authors report a systematic survey of ticks on livestock conducted between 1965 and 1966, wherein not a single *R.microplus* tick was found. Interestingly, before this survey, *R.microplus* was reported from Uganda by S. G.Wilson, who conducted a limited survey on cattle along the borders of Karamoja district, closer to the border with Kenya [16]. It is unlikely that *R.microplus* would have been missed during the nation-wide extensive survey conducted by Matthysse and Colbo, now more than 50 years ago. Interestingly, our results clearly indicate that *R.microplus* has been overlooked for years, since it takes years to replace an indigenous population of *R.decoloratus* ticks.

*R. microplus* was introduced into West, South and East Africa on cattle imported from Asia and Latin America, where *R. microplus* is well established [2–4, 11]. Within the East-African region, *R. microplus* has been confirmed in Tanzania and more recently in South-Sudan [13, 14]. Given the invasiveness of this tick species, exacerbated by poor animal movement control and communal grazing practices within the East African region, it is likely that populations of *R. microplus* are now well established in Uganda.

Molecular phylogenetic analysis may be a useful tool to discern possible relationships between isolates collected from different geographical regions. In this study, the 12S rRNA and ITS2 regions of the tick isolates from Uganda were identical to those previously isolated from Taiwan, Mozambique, Nigeria, USA and South Africa. It is therefore plausible that the *R. microplus* ticks collected from cattle in south-eastern Uganda were introduced on livestock imported from the southern parts of Africa. In the past 10 - 15 years, there have been significant importations of dairy cattle into Uganda from South Africa to improve the Ugandan dairy herd through cross-breeding [30]. This requires more extensive genotyping of ticks collected from different geographical areas [25, 31].

Unprecedented levels ofacaricide resistant tick populations have recently been reported in Uganda [31]. The cause of this problem is due to farmer-related factors [acaricide overuse and misuse] potentiated by

lack of national acaricide and animal movement control policies [31–33]. Under such favourable conditions, *R. microplus* tick populations are known to rapidly become acaricide resistant as a result of target specific mutations and metabolic adaptations [34]. The introduction of *R. microplus* into Uganda is likely to exacerbate the already existing problem of ticks and tick-borne diseases in three ways. These include; (i) complete replacement of *R.decoloratus* by *R. microplus,* resulting ina national and probably regional upsurge of *R. microplus* populations*, (*ii) emergence of acaricide resistant *R. microplus* populations, and (iii) a proportional increase of bovine babesiosis given that *R. microplus* is an efficient vector of *B. bovis* [11, 25, 30]*.* Unless effective national acaricide and animal movement control policies are instituted, the Ugandan livestock sector will suffer severe losses due to the direct effects of *R. microplus* infestation and bovine babesiosis.

# Conclusions

It was expected to find *B. decoloratus* among other tick species on cattle during a survey conducted in south-eastern Uganda. Instead, we discovered that *R. microplus* has completely displaced *R.decoloratus,* an indigenous tick species previously known to this region. There is a need to determine the extent of spread of *R.microplus* throughout Uganda and to put in place effective control measures considering that *R. microplus* is capable of developing high levels of resistance towards the major classes of acaricides.

# Abbreviations

12S rRNA 12S ribosomal RNA

16S rRNA 16S ribosomal RNA

AAS African Academy of Science

BMGF Bill and Melinda Gates Foundation

DELTAS Developing Excellence in Leadership, Training and Science

MUII Makerere University-Uganda Virus Research Institute Centre of Excellence for Infection and Immunity Research and Training

MSA Multiple sequence alignment

NEPAD New Partnership for Africa's Development Planning and Coordinating Agency

DNA deoxyribonucleic acid

RNA ribonucleic acid

GPS Geographical positioning system

| | |
|---|---|
| ITS2 | Internal transcribed spacer 2 |
| PBS | Phosphate buffered saline |

# Declarations

# Acknowledgements

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# Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

# Authors' contributions

DM, JF,MM,TS, IH, NJ and FJ conceived and designed the study. DM, JB, CN, WA, SO, JN and FJ collected tick samples and performed taxonomic and molecular tick identification. DM, CN, SO, JN, FNM and RT undertook molecular confirmation of *R. (B.). microplus.* DM, FNM, RT, JF, MM, IH and FJ drafted and critically reviewed this manuscript. All authors read and approved the final manuscript.

## Ethics approval and consent to participate

Written informed consent was obtained from cattle owners. Cattle restraint, tick collection, preservation, morphological and molecular analyses followed highest Veterinary care and Scientific standards as approved (SBLS/REC/16/136) by the Research and Ethics committee of Makerere University School of Biosecurity, Biotechnical and Laboratory Sciences and the Uganda National Council of Science and Technology (A 513).

## Consent for publication

Not applicable

## Competing interests

The authors declare that they have no competing interests

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# List Of Tables

Table 1: List of primer sets used in PCR amplification of 12S rRNA, 16S rRNA and ITS2 genes.

| Primer name | Primer sequence (5'-3') [4, 18] |
|---|---|
| 16S-F | TTAAATTGCTGTRGTATT |
| 16S-R1 | CCGGTCTGAACTCASAWC |
| ITS2-F | ACATTGCGGCCTTGGGTCTT |
| ITS2-R | TCGCCTGATCTGAGGTCGAC |
| T1B | AAACTAGGATTAGATACCCT |
| T2A | AATGAGAGCGACGGGCGATGT |

**Table 2:** Estimates of evolutionary divergence using Ugandan *R. (B.). microplus* 12S rRNA, 16S rRNA and ITS2 nucleotide sequence diversity compared to those obtained from the GenBank.

| ***R. (B.). microplus*** **sequences [This study]** | **GenBank Sequences** | **P distance** |
|---|---|---|
| **Nucleotide diversity based on 12S** | | |
| **KY688455 (*R. (B.). microplus* )** | DQ003008.1 *R. (B.). microplus* (Taiwan) | 0.006 |
| | EU921766.1 *R. (B.). microplus* (Mozambique) | 0.006 |
| **KY688459 (*R. (B.). microplus* )** | DQ003008.1 *R. (B.). microplus* (Taiwan) | 0.000 |
| | EU921766. *R. (B.). microplus* (Mozambique) | 0.000 |
| **Nucleotide diversity based on 16S** | | |
| **KY688461 (*R. (B.). microplus* )** | KY020993 *R. (B.). microplus* (Brazil) | 0.710 |
| | EU918182.1 *R. (B.). microplus* (South Africa) | 0.663 |
| | EU918187.1 *R. (B.). microplus* (Mozambique) | 0.071 |
| **Nucleotide diversity based on ITS2** | | |
| **KY688467 (*R. (B.). microplus* )** | U97715.1 *R. (B.). microplus* (South Africa) | 0.400 |
| | MF373428.1 *R. (B.). microplus* (Nigeria) | 0.003 |
| | MF373429.1 *R. (B.). microplus* (Nigeria) | 0.002 |
| | EU520392.1 *R. (B.). microplus* (USA) | 0.038 |

# Figures

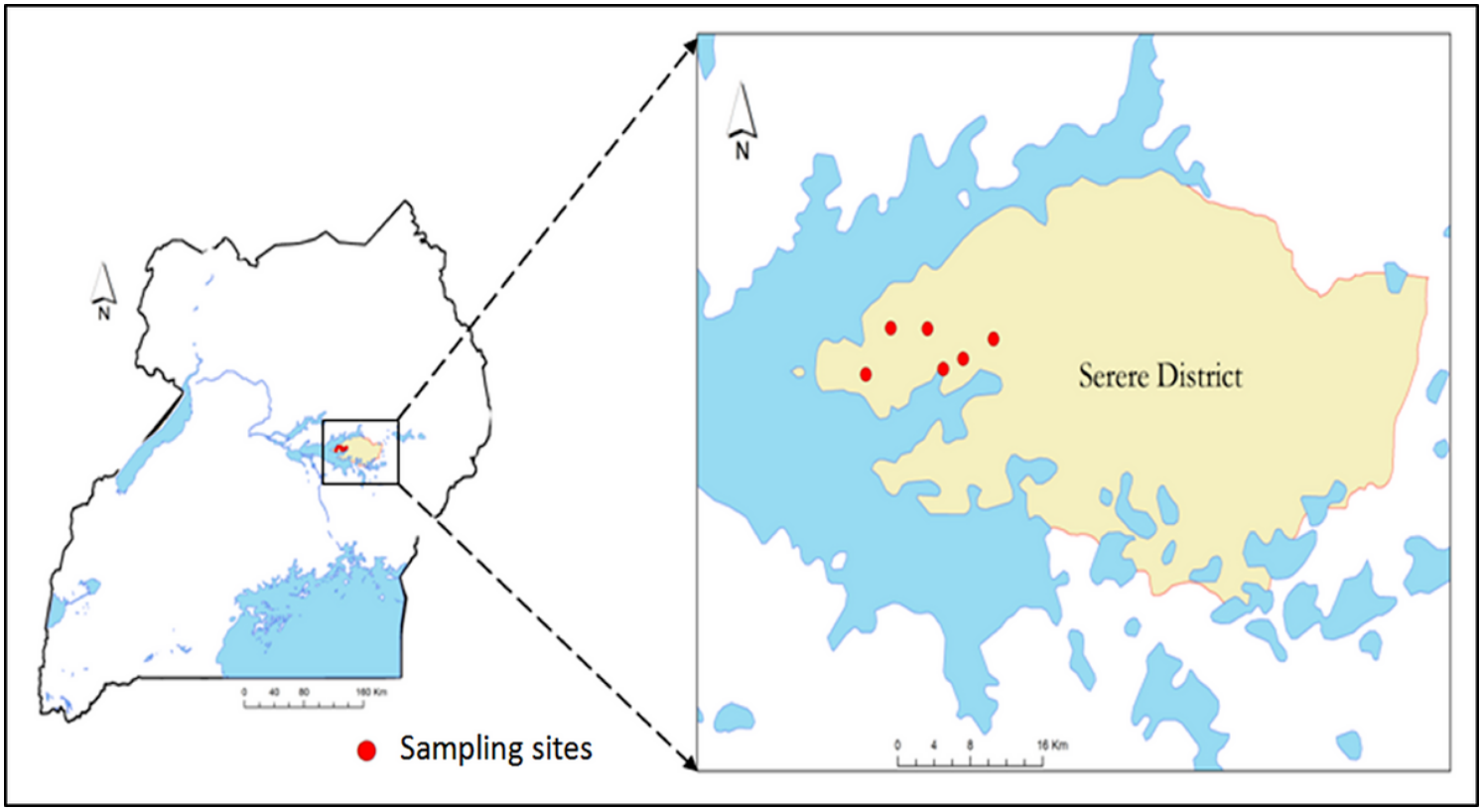


**Figure 1**

Study area: Serere district, south-eastern Uganda Red dots indicate the locations of the 6 villages from which 240 cattle were randomly selected and included in this study

**Figure 2**

Morphological identification of R. (B.). microplus Plate A| Ventral View of a female R. microplus tick ; 1: Hypostomal teeth in a typical 4 + 4 column arrangement, 2: Short Palp of internal margin article 1 [has no protuberance and is distinctly concave], 3: Cornua. Plate B| Ventral View of a male R. microplus tick; 4: Distinctly small adenal plates; 5: Ventral plate spurs (small accessory adanal plates); 6: Narrow caudal appendage, 7: Genital aperture with a broad U shape.

**Figure 3**

Phylogenetic analysis based on the tick 12S ribosomal RNA gene A Phylogenetic tree based on the 12S rDNA tick gene sequences of ticks. The tree was generated by the Maximum Likelihood method based on

the Tamura 3-parameter model. The analysis involved 26 nucleotide sequences.

**Figure 4**

Phylogenetic analysis based on the tick 16S ribosomal RNA gene A Phylogenetic tree based on the 16S2 rDNA tick gene sequences of ticks. The tree was generated by the Maximum Likelihood method based on the Tamura 3-parameter model. The analysis involved 25 nucleotide sequences.

**Figure 5**

Phylogenetic analysis based on the ITS2 ribosomal RNA gene region of ticks A Phylogenetic tree based on the ITS2 rDNA gene sequences of ticks. The tree was generated by the Maximum Likelihood method based on the Tamura 3-parameter model. The analysis involved 32 nucleotide sequences.

# Supplementary Files

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- Graphicabstract.jpg