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Identification of novel Glutathione S-Transferases epsilon 2 mutation in *Anopheles maculipennis* s.s. (Diptera: Culicidae)

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ABSTRACT

Anopheles maculipennis complex comprises some important malaria vectors in Iran, Middle East, and Europ. The principal way to control of malaria remains on the use of chemical insecticides against its vectors because there is no vaccine for malaria prevention. Extensive use of organophosphate compounds has caused to emergence and distribution of insecticide resistance in *Anopheles* species in Asia. The current study aimed to the detection of three well-known amino acid substitutions (I114T, L119F, and F120L) in the Glutathione S-Transferases epsilon 2 (GSTe2) gene are associated with DDT and organophosphate insecticides resistance in an *Anopheles maculipennis* population collected from Iran. Adult samples of *An. maculipennis* were collected by hand and Total catch in Animal and Human Shelters from Azerbaijan-Gharbi and Zanjan provinces. Following morphological identification, DNA was extracted by YTA Genomic DNA Extraction Mini Kit for amplification of rDNA-ITS2 and GSTe2 fragments. ~500 bp fragment was amplified using F rDNA-ITS2 and GSTe2 primers. rDNA-ITS2 sequence analysis showed 100% similarity with *An. maculipennis*. GSTe2 nucleotide sequence similarity within species was 99–100%, while, it was 95–96 % when compared with *Anopheles sacharovi* GSTe2 sequences available in GenBank. Amino acid sequence comparisons showed a novel amino acid substitution in N148D position with 15.79% frequency. The current study reports new GSTe2 amino acid substitution in *An. maculipennis s.s.*, for the first time. The function of the mutation N148D and its association with resistance phenotype need to validate. However, the integration of these data into the malaria control program still remains a challenge.

1. Introduction

Estimation of worldwide malaria cases and death were 216 million and 445000 in 2016, respectively (WHO, 2017). About 2% of the cases were in the WHO Eastern Mediterranean region (EMRO) in 2016 (WHO, 2017). After the report of malaria from Iran in 1303, it still is reported from Iran (Jalali Muslim, 1955; WHO, 2017); however, it reduced to 0.01/1000 cases in 2017 (Vatandoost et al., 2019).

The members of *An. maculipennis* complex is considered as malaria vectors in Europ, Middle East and one of the eight Iranian malaria vectors (Sedaghat et al., 2003; Djadid et al., 2007; Sevgili and Simsek, 2012; Gholizadeh et al., 2013; Danabalan et al., 2014; Tabbabi et al., 2015). *Anopheles maculipennis* has been distributed in 20 provinces of Iran (Hanafi-Bojd et al., 2018). Among mosquitoes, *An. maculipennis* is the first complex species to be discovered (Falleroni, 1926; Van Thiel, 1927). Out of 24 members of this complex species, seven species including *Anopheles atroparvus, Anopheles labranchiae, An. maculipennis, Anopheles messeae, Anopheles melanoon, An. sacharovi* and *Anopheles persiensis* were reported from Iran (Sedaghat et al., 2003; Linton, 2004; Djadid et al., 2007; Harbach, 2017; Azari-Hamidian et al., 2019). Except for *An. maculipennis* and *An. sacharovi*, the remaining members of this complex could be identified using egg pattern, polytene chromosome, isoenzymes and rDNA-ITS2 molecular marker (Sedaghat et al., 2003; Nicolescu et al., 2004; Azari-Hamidian, 2007; Patsoula et al., 2007; Azari-Hamidian and Harbach, 2009).

Insecticide-based vector control tools have the main role in progress towards malaria elimination (WHO, 2013, 2017). Whereas, the frequent use of insecticides is increased resistance risks in mosquitoes and

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Table 1
The details of *Anopheles maculipennis* samples collected from Iran.

West Azarbaijan	Urmia	Nazloo, Jarchiloo, kooraneh	35
East Azarbaijan	Khodaafarin	Larijan	5
Zanjan	Zanjan	Gharabooteh	12
Gilan	Langrood, Astara, Masal, Talesh, Siahkal	–	8

consequently problems in control programs (Cuamba et al., 2010; Morgan et al., 2010; Casimiro et al., 2014; Clark et al., 2015) It could seasonal changes in susceptibility of *An. maculipennis* s.s to various insecticides in some areas in Iran and Turkey (Manouchehri et al., 1976; Floore, 2006; Akiner et al., 2013; Yousef Mogaddam et al., 2016).

Metabolic resistances are one of the main mechanisms of insecticide resistance to different insecticide classes through the multifunctional GSTs (Ranson and Hemingway, 2005). Glutathione S-transferases are members of a major family of intracellular enzymes caused detoxification of pesticides in insects and mammals (Hemingway and Ranson, 2000; Yang et al., 2001; Hemingway et al., 2002). GSTs from transferases superfamily are found in most aerobic eukaryotes and prokaryotes (Sheehan et al., 2001). Out of six classes of GSTs, Delta, Epsilon, Omega, Sigma, Theta and Zeta, the first two classes are arthropod specific with 12 and 8 members in Culicidae, respectively (Ranson et al., 2002; Ding et al., 2003; Tu and Akgül, 2005; Ketterman et al., 2011). Primary GSTs role is DDT metabolism to non-toxic combinations, and their secondary role is resistant to organophosphate insecticides (Hemingway et al., 1985; Ku et al., 1994; Huang et al., 1998). The GSTe2 is involved in resistance to organochlorides, organophosphates, and pyrethroids (Ranson et al., 2001; Ketterman et al., 2011; Riveron et al., 2014). Delta class of GST is classified as class I of insect GSTs which closely related to epsilin class based on sequence identity and phylogenetic analysis (Board et al., 1997; Sheehan et al., 2001; Ketterman et al., 2011). Delta GSTs in *Drosophila melanogaster* and *Musca domestica* are generally intronless, althogh some are interrupted by introns in 5´UTRs (Zhou and Syvanen, 1997; Lougarre et al., 1999; Sawicki et al., 2003).

Molecular analysis of GST genes in *Anopheles* mosquitoes can provide vital information to insecticide resistance monitoring and management. There is limited information on the molecular mechanisms of insecticide resistance of *An. maculipennis*. In the current study, the sequences of the *An. maculipennis* GSTe2 gene was studied and reported a novel mutation (N148D) in the GSTe2 gene in *An. maculipennis*. These findings are the first step in a survey of molecular insecticide resistance in *An. maculipennis* in Iran, and subsequently in the Eastern Mediterranean region.

2. Materials and methods

2.1. Study site and mosquito identification procedure

Adults of *An. maculipennis* were collected by hand and total catch in human and animal shelters from West Azarbaijan and Zanjan provinces of Iran during different collections from 2015 to 2018. More detail about sampling size and study area are presented in Table 1.

Mosquitoes were identified by using the key to Iranian Anophelines (Azari-Hamidian and Harbach, 2009) in Medical Entomology Lab. at School of Public Health (SPH), Urmia University of Medical Sciences (UMSU). Morphological identification confirmed by molecular techniques and sequencing of rDNA-ITS2 region using universal 5.8S and 28S universal primers (Djadid et al., 2007).

```
#MK421369    GGT GTC CTC TTT GCT CGG ATG CGG TTT GTG TTT GAG CGC ATT CTC TTC TTC GGA AAG TCG  [180]
#MK421370    ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...  [180]
#MK421371    ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...  [180]
#MK421372    ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...  [180]
#MK421373    ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...  [180]
#MK421374    ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...  [180]
#MK421375    ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...  [180]
#MK421376    ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...  [180]
#MK421377    ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...  [180]
#MK421378    ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...  [180]
#MK421379    ... ... ... ..C ... ... ... ... ... ..C ... ... ... ... ... ... ... ... ... ...  [180]
#MK421380    ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...  [180]
#MK421381    ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...  [180]
#MK421382    ... ... ... ..C ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...  [180]
#MK421383    ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...  [180]
#MK4213848   ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...  [180]
#MK4213849   ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...  [180]
#MK42138410  ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...  [180]
#MK42138411  ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...  [180]

#MK421369    CTG ACG GAC GAC TAT GTG GCC GGG CCG GTC ATG ACC GTT GCC GAT TTC AGT TGC ATT TCC  [300]
#MK421370    ... ... ... A.. ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...  [300]
#MK421371    ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...  [300]
#MK421372    ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...  [300]
#MK421373    ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...  [300]
#MK421374    ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...  [300]
#MK421375    ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...  [300]
#MK421376    ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...  [300]
#MK421377    ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...  [300]
#MK421378    ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...  [300]
#MK421379    ... ... ... A.. ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...  [300]
#MK421380    ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...  [300]
#MK421381    ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...  [300]
#MK421382    ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...  [300]
#MK421383    ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...  [300]
#MK4213848   ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...  [300]
#MK4213849   ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...  [300]
#MK42138410  ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...  [300]
#MK42138411  ... ... ... A.. ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...  [300]
```

Fig. 1. Multiple nucleotide sequence alignment of 19 GSTe2 open reading frames partial sequences (MK421369-MK421387) of *An. maculipennis* collected from Iran. Alignment was generated in MEGA6 program. A dot indicates identity with the relevant sequence. Transitions and transversion are highlighted in green and blue colores, respectively. Alighnments with 100% similarity was not shown.

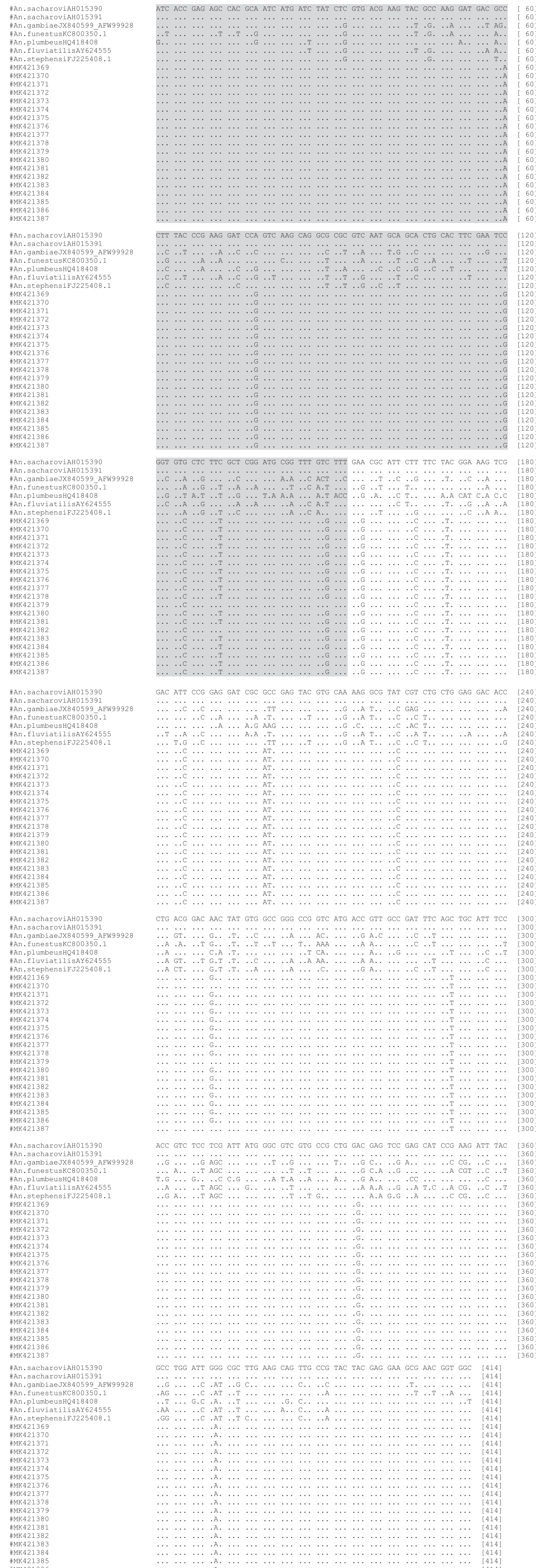

Fig. 2. Multiple nucleotide sequence alignment of 19 GSTe2 open reading frames partial sequences (MK421369-MK421387) of *An. maculipennis* collected from Iran (exons highlighted in grey and white colors), and available nucleotide sequences of *An. sacharovi*, *An. stephensi*, *An. fluviatilis*, *An. gambiae*, *An. funestus* and *An. plumbeus* in GenBank. Related accession numbers are included after the species name. Alignment was generated in MEGA6 program. A dash indicates deletion and a dot indicates identity with the relevant sequence.

Table 2
Similarity of *An. maculipennis* and available GSTe2 sequences of *Anopheles* species in GenBank.

Species	GenBank ID	Similarity %	Reference
An. gambiae	JX840599	78.74–79.23	(Mitchell et al., 2014)
An. funestus	KC800350.1	78.02–78.50	(Riveron et al., 2014) Direct submission
An. stephensi	FJ225408.1	80.43–80.68	(Djadid et al., 2006b)
An. fluviatilis	AY624555	77.29–77.78	(Djadid et al., 2006b)
An. plumbeus	HQ418408	77.54–77.78	(Ayres et al., 2011)

2.2. Genomic DNA extraction and PCR amplification

Genomic DNA was extracted from the whole body of each female mosquito using YTA Genomic DNA Extraction Mini Kit (Yekta Tajhiz Azma, Tehran, Iran) based on manufacturer's instructions and our recent experience (Firooziyan et al., 2018).

The GSTe2 region of *An. maculipennis* was amplified using the E2F (5´-ATCACCGAGAGCCACGCAATCAT-3´) and E2R (5´-GCCACCGTTCGCTTCCTCGTAGT-3´) primers (Djadid et al., 2006a). Ribosomal DNA-ITS2 region was amplified using the 5.8S (5´-ATCACTCGGCTCGTGGATCG-3´) and 28S (5´- ATGCTTAAATT-TAGGGGGTAGTC-3´) primers. The PCR reactions were performed in a final volume of 25μl, containing a 12.5μl master mix (Yekta Tajhiz Azma, Tehran, Iran), 1μl of each primer, 8.5μl ddH_2O and 2μl of genomic DNA. Primary denaturation at 95 °C for 5 min followed by 35 cycles, 60-sec denaturation at 95 °C, 90-sec annealing at 56 °C and 75-sec extension at 72 °C with a 10 min final extension at 72 °C were PCR amplification profile of GSTe2 gene. These conditions for rDNA-ITS2 amplification was the same as GSTe2 except 53 °C annealing temperature and 30 cycles. PCR products were electrophoresed in %1.5 agarose gel stained with safe stain (Yekta Tajhiz Azma, Tehran, Iran) and visualized by UV transillumination.

2.3. Sequence analysis

The GSTe2 and rDNA-ITS2 PCR fragments in *An. maculipennis* were sequenced in both directions on an ABI 377 automatic sequencer with the E2F, E2R, 5.8S, and 28S primers. The sequences were double checked by Chroma software version 2.31 (http://www.technelysium.com.au/chromas.html) and analyzed using the Basic Local Alignment Tool (BLAST) (http://www.ncbi.nlm.nih.gov/blast). Clustal Omega online software (Sievers and Higgins, 2014) was used to sequence similarity comparison within and between sequences of different *Anopheles* species. The final sequences were aligned with representative sequences of various *Anopheles* GSTe2 and *An. maculipennis* rDNA-ITS2 sequences available in the GenBank in Molecular Evolutionary Genetics Analysis version 6.0. (MEGA6) (Tamura et al., 2011). Nucleotide sequences are available in the GenBank, European Molecular Biology Laboratory (EMBL), and DNA Data Bank of Japan (DDBJ) databases [GenBank ID: MK421369–MK421387].

3. Results

At the first phase of the current study, *An. maculipennis* specimens (n = 60) were identified based on morphological characters. A ~500 bp fragment of the rDNA-ITS2 region was amplified, and multiple sequence analysis showed 100% similarity with *An. macupennis* species. The sequences submitted to Genbank under GenBank ID: MK418775–MK4188782.

A ~500 bp fragment of GSTe2 was amplified in *An. maculipennis* specimens using E2F and E2R primers. The size variation was not noticeable in all amplified specimens collected from Azerbaijan-Gharbi (n = 13) and Zanjan (n = 6) provinces. The length of the sequenced fragment was varied from 491-498 bp. Basic Local Alignment Search Tool (BLAST) comparison with *An. gambiae* GSTe2 sequence (GenBank ID: JX840599) showed the existence of two exons and an intron between them. Both open reading frames consist of 414 bp in all *An. maculipennis* specimens. Multiple sequence alignment showed 99.28–100% similarity within species. The limited variation was due to two transitions (T/C, G/A) and a transversion (G/C) at 132, 250 and 150 nucleotides, respectively (Fig. 1).

The intron region in *An. maculipennis* contained 77–84 bp with 67.53–100% sequence similarity within specimens. There were 28 mismatches as deletion/insertion (n = 14) and transition/transversion (n = 14) (alignment was not shown).

Until to date, there are no GSTe2 sequences of *An. maculipennis* in the GenBank. The only available GSTe2 sequences from maculipennis species complex in the GenBank are GenBank IDs: AH015390 and AH015391 related to *Anopheles sacharovi*. Sequence comparison between both members on maculipennis complex showed 95.89–96.14% identity. There were 17 mismatches as the transition (n = 10) and transversion (n = 7) (Fig. 2). Sequence similarity between *An. maculipennis* and five other available species, *Anopheles gambiae*, *Anopheles stephensi*, *Anopheles*

```
#MK421369   DIPEDRIEYV QKAYRLLEDT LTDDYVAGPV MTVADFSCIS TVSSIMGVVP LGESEHPKIY  [120]
#MK421370   .......... .......... ...N...... .......... .......... ..........  [120]
#MK421371   .......... .......... .......... .......... .......... ..........  [120]
#MK421372   .......... .......... .......... .......... .......... ..........  [120]
#MK421373   .......... .......... .......... .......... .......... ..........  [120]
#MK421374   .......... .......... .......... .......... .......... ..........  [120]
#MK421375   .......... .......... .......... .......... .......... ..........  [120]
#MK421376   .......... .......... .......... .......... .......... ..........  [120]
#MK421377   .......... .......... .......... .......... .......... ..........  [120]
#MK421378   .......... .......... .......... .......... .......... ..........  [120]
#MK421379   .......... .......... ...N...... .......... .......... ..........  [120]
#MK421380   .......... .......... .......... .......... .......... ..........  [120]
#MK421381   .......... .......... .......... .......... .......... ..........  [120]
#MK421382   .......... .......... .......... .......... .......... ..........  [120]
#MK421383   .......... .......... .......... .......... .......... ..........  [120]
#MK421384   .......... .......... .......... .......... .......... ..........  [120]
#MK421385   .......... .......... .......... .......... .......... ..........  [120]
#MK421386   .......... .......... .......... .......... .......... ..........  [120]
#MK421387   .......... .......... ...N...... .......... .......... ..........  [120]
```

Fig. 3. The alignment of amino acid sequences of a partial sequence of the GSTe2 gene of *An. maculipennis* specimens collected from Iran. D148N mutation are highlighted in green colore. Alignment was generated in MEGA6 program. Alighnments with 100% similarity was not shown. A dot indicates identity with the relevant sequence.

```
#An.sacharoviAH015390         ITESHAIMIY LVTKYAKDDA LYPKDPVKQA RVNAALHFES GVLFARMRFV FERILFYGKS  [ 60]
#An.sacharoviAH015391.2       .......... .......... .......... .......... .......... ..........  [ 60]
#An.gambiaeAFW99928_JX840599  .......... .....G...S .......... ...S...... .........T ......F...  [ 60]
#An.funestusKC800350.1        .......... .....G...T .......Q.. .......... .........I ....F.....  [ 60]
#An.stephensiFJ225408.1       .......... .....G...S .......... .......... .........I .........T  [ 60]
#An.plumbeusHQ418408          V......... .......N.T .......... .......... ..I...L..I T.H.F.KHQP  [ 60]
#An.fluviatilisAY624555       .......... .....G..ET .......... .......... .........I ....F.F...  [ 60]
#AMU1                         .......... .......... .......... .......... .......... ......F...  [ 60]
#AMU3                         .......... .......... .......... .......... .......... ......F...  [ 60]
#AMU7                         .......... .......... .......... .......... .......... ......F...  [ 60]
#AMU8                         .......... .......... .......... .......... .......... ......F...  [ 60]
#AMU9                         .......... .......... .......... .......... .......... ......F...  [ 60]
#AMU23                        .......... .......... .......... .......... .......... ......F...  [ 60]
#AMU29                        .......... .......... .......... .......... .......... ......F...  [ 60]
#AMU30                        .......... .......... .......... .......... .......... ......F...  [ 60]
#AMU31                        .......... .......... .......... .......... .......... ......F...  [ 60]
#AMU32                        .......... .......... .......... .......... .......... ......F...  [ 60]
#AMU33                        .......... .......... .......... .......... .......... ......F...  [ 60]
#AMU34                        .......... .......... .......... .......... .......... ......F...  [ 60]
#AMU35                        .......... .......... .......... .......... .......... ......F...  [ 60]
#AMZ6                         .......... .......... .......... .......... .......... ......F...  [ 60]
#AMZ7                         .......... .......... .......... .......... .......... ......F...  [ 60]
#AMZ8                         .......... .......... .......... .......... .......... ......F...  [ 60]
#AMZ9                         .......... .......... .......... .......... .......... ......F...  [ 60]
#AMZ10                        .......... .......... .......... .......... .......... ......F...  [ 60]
#AMZ11                        .......... .......... .......... .......... .......... ......F...  [ 60]

#An.sacharoviAH015390         DIPEDRAEYV QKAYRLLEDT LTDNYVAGPV MTVADFSCIS TVSSIMGVVP LDESEHPKIY  [120]
#An.sacharoviAH015391.2       .......... .......... .......... .......... .......... ..........  [120]
#An.gambiaeAFW99928_JX840599  ......V... ..S.E..... .V.DF....T ..I....... .......... .EQ.K..R..  [120]
#An.funestusKC800350.1        ......V... ..S....... .K.DF...SK ..I....... .I........ .EQ....R..  [120]
#An.stephensiFJ225408.1       .L....V... ..S....... .L.DF....A ..I....... .I.......A ..KA...R..  [120]
#An.plumbeusHQ418408          ......K... .T..H..... ...QF....H ..I....... S.A.L..L.. MEK.A.....  [120]
#An.fluviatilisAY624555       ......V... ..S....... .V.DF....N ..I....... .....V.... .EK..Y.R..  [120]
#AMU1                         ......I... .......... ...D...... .......... .......... .G........  [120]
#AMU3                         ......I... .......... .......... .......... .......... .G........  [120]
#AMU7                         ......I... .......... ...D...... .......... .......... .G........  [120]
#AMU8                         ......I... .......... ...D...... .......... .......... .G........  [120]
#AMU9                         ......I... .......... ...D...... .......... .......... .G........  [120]
#AMU23                        ......I... .......... ...D...... .......... .......... .G........  [120]
#AMU29                        ......I... .......... ...D...... .......... .......... .G........  [120]
#AMU30                        ......I... .......... ...D...... .......... .......... .G........  [120]
#AMU31                        ......I... .......... ...D...... .......... .......... .G........  [120]
#AMU32                        ......I... .......... ...D...... .......... .......... .G........  [120]
#AMU33                        ......I... .......... .......... .......... .......... .G........  [120]
#AMU34                        ......I... .......... ...D...... .......... .......... .G........  [120]
#AMU35                        ......I... .......... ...D...... .......... .......... .G........  [120]
#AMZ6                         ......I... .......... ...D...... .......... .......... .G........  [120]
#AMZ7                         ......I... .......... ...D...... .......... .......... .G........  [120]
#AMZ8                         ......I... .......... ...D...... .......... .......... .G........  [120]
#AMZ9                         ......I... .......... ...D...... .......... .......... .G........  [120]
#AMZ10                        ......I... .......... ...D...... .......... .......... .G........  [120]
#AMZ11                        ......I... .......... .......... .......... .......... .G........  [120]

#An.sacharoviAH015390         AWIGRLKQLP YYEEANGG [138]
#An.sacharoviAH015391.2       .......... ........ [138]
#An.gambiaeAFW99928_JX840599  ...D...... ....V... [138]
#An.funestusKC800350.1        E..D...... ........ [138]
#An.stephensiFJ225408.1       G..D...... ........ [138]
#An.plumbeusHQ418408          ..VE...R.. ........ [138]
#An.fluviatilisAY624555       E..D...K.. ........ [138]
#AMU1                         ...E...... ........ [138]
#AMU3                         ...E...... ........ [138]
#AMU7                         ...E...... ........ [138]
#AMU8                         ...E...... ........ [138]
#AMU9                         ...E...... ........ [138]
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#AMU32                        ...E...... ........ [138]
#AMU33                        ...E...... ........ [138]
#AMU34                        ...E...... ........ [138]
#AMU35                        ...E...... ........ [138]
#AMZ6                         ...E...... ........ [138]
#AMZ7                         ...E...... ........ [138]
#AMZ8                         ...E...... ........ [138]
#AMZ9                         ...E...... ........ [138]
#AMZ10                        ...E...... ........ [138]
#AMZ11                        ...E...... ........ [138]
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Fig. 4. The alignment of amino acid sequences of a partial sequence of the GSTe2 gene of *An. maculipennis* specimens are collected from Iran and available sequences of *An. sacharovi*, *An. stephensi*, *An. fluviatilis*, *An. gambiae*, *An. funestus* and *An. plumbeus* in GenBank. Related accession numbers are included after the species name. Alignment was generated in MEGA6 program. A dash indicates deletion and a dot indicates identity with the relevant sequence. A dash indicates deletion and a dot indicates identity with relevant sequence.

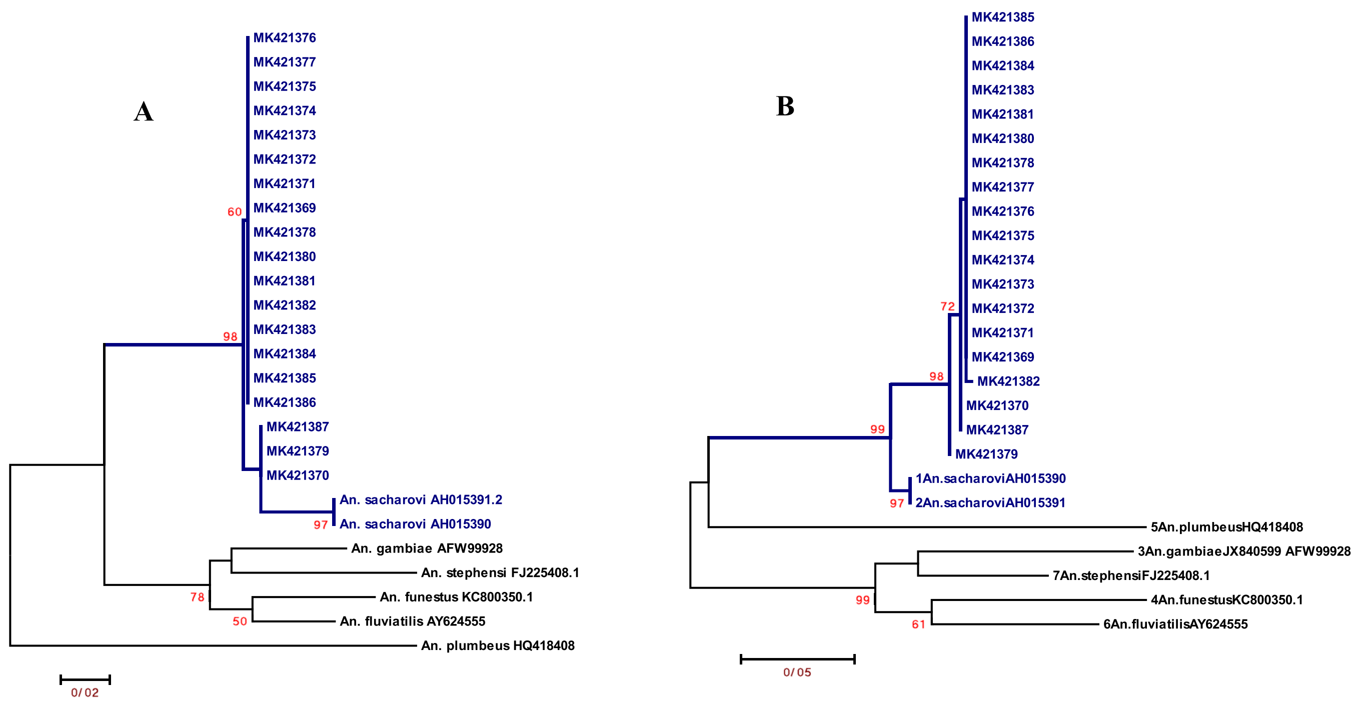

Fig. 5. Maximum likelihood tree based on GSTe2 amino acid (A) and nucleotide (B) sequences for 19 Iranian *An. maculipennis* specimens and available sequences of *An. sacharovi*, *An. stephensi*, *An. fluviatilis*, *An. gambiae*, *An. funestus* and *An. plumbeus* in GenBank. Bootstrap values (1000 replicates) >50 have been shown above the lines.

fluviatilis, *Anopheles plumbeus*, and *Anopheles funestus* are presented in Table 2.

Despite three nucleotide mismatches, the only amino acid substitution was Aspartic Acid (D) to Asparagine (N) in 148 positions in 15.8% of samples collected from Urmia (Nazloo and kooraneh) and Zanjan (Gharabooteh) (Fig. 3). Amino acid sequence comparisons between *An. maculipennis* and *An. sacharovi* showed 96.33–97.10% identity. There were five amino acid substitutions as Y121F, A131I, N148D, D176G, and G188E in all *An. maculipennis* sequences except for N148D which occurred in 84.2% (Fig. 4). The detail of amino acid sequence comparison between *Anopheles* species presented in Table 2 and Fig. 4.

Before phylogenetic analysis, multiple sequence alignment of GSTe2 nucleotide and amino acid sequences were carried out to explain the relationship among *An. maculipennis* and other main malaria vectors. Both constructed phylogenetic tree were similar in topology (Fig. 5). *Anopheles maculipennis* sequences were clustered in a clade together with *An. sacharovi*. Limited differences between AMU3 (GenBank ID: MK421369), AMZ11 (GenBank ID: MK421387), and AMU33 (GenBank ID: MK421379) with other sequences of *An. maculipennis* could be due to some nucleotide mutations and amino acid substitutions. There was a close relationship between *An. maculipennis* and *An. sacharovi*, two members of maculipennis complex, *An. stephensi* and *An. gambiae*, *An. fluviatilis* and *An. funestus* (Fig. 5).

4. Discussion and conclusion

Anopheles maculipennis is distributed in 15 different provinces of Iran, including Azarbaijan-Gharbi province (Yousef Mogaddam et al., 2016). The first report on the insecticide resistance of this *Anopheles* species is reported in 1976 when DDT was used in cotton fields in northern Iran (Manouchehri et al., 1976). Recently, its resistance to propoxur, bendiocarb, and malathion are reported in northwestern Iran (Azerbaijan--Gharbi province) using WHO susceptibility tests (Chavshin et al., 2015). However, there is limited information on the molecular insecticide resistance status of this species in the country and in our best knowledge in the world. The current study, reports GSTe2 sequence analysis in *An. maculipennis* not only in Iran but also in the Eastern Mediterranean Region for the first time.

Two insecticide-based interventions, insecticide-treated nets (ITNs) and indoor residual spraying (IRS), remain core WHO recommended interventions to fight against malaria in the last few years (WHO, 2015). Due to the increase in pyrethroid insecticides resistance of malaria vectors in Africa (N'Guessan et al., 2007; Mnzava et al., 2015), and successful use of IRS in African countries (Mabaso et al., 2004), IRS with non-pyrethroids is in reintroducing as a primary vector control strategy. The biggest threats to control and malaria elimination strategies are the resistance of *Anopheles* vectors to insecticides (Quiñones et al., 2015). Despite RTS,S/AS01 (RTS,S), the first malaria vaccine to date, showed partial and age dependent protection against *Plasmodium falciparum* in phase III clinical trial (Rts, 2015; Draper et al., 2018). In mosquitoes, the primary role of GSTs is the metabolism of DDT to non-toxic compounds, however, they also have a secondary role in resistance to some organophosphate insecticides via metabolization of OP insecticides by conjugation of GSH-dependent route (Hemingway et al., 1991; Fournier et al., 1992). Population genetic studies have shown a correlation between GSTe2 and *kdr* mutations. Both *Gste2*-114T and *Vgsc*-1014F mutations were significantly associated with resistance of *An. gambiae* to DDT, and on the margin of the significance between *Gste2*-114T and *Vgsc*-1575Y mutation. Interaction among *Gste2*-114T, *Vgsc*-1014F, and *Vgsc*-1575Y increased survival probability of *An. gambiae* from 50% to 93% after one-hour exposure to DDT (Mitchell et al., 2014). *An. funestus*, a single substitution at position L119F of GSTe2 has led to a high level of metabolic resistance to DDT in Africa (Mulamba et al., 2014; Riveron et al., 2014). Multiple sequence analysis of GSTe2 in *An. stephensi*, *An. fluviatilis* and *An. culicifacies* collected from Iran showed no amino acid substitution, despite the resistance of *An. stephensi* to DDT 4% (Djadid et al., 2006b). Recently, the investigation of the presence of resistance alleles in an *An. arabiensis* population in Cabo Verde showed the presence of 37 haplotypes, 16 polymorphic sites and high genetic diversity in GSTE2 sequences (da Cruz et al., 2019). Well-known GSTe2 mutation (I114T, L119F, and F120L) were not detected in *An. maculepennis* species, therefore, they could be considered as susceptible alleles. However, a study on the expression level of GSTe2 marker using quantitative PCR assays is recommended to the precise evaluation of insecticide resistance status. In addition, the nucleotide and amino acid sequence analysis in *An. maculipennis* showed that there is a novel D148N mutation in 15.8% of *An. maculipennis* sequences.

In summary, our finding may be correlated with WHO susceptibility tests result in northwestern Iran which reports tolerance of *An. maculipennis* to dieldrin and resistance to malathion insecticides (Chavshin et al., 2015). Although, they do not use molecular data to species confirmation. The D148N mutation has not been detected in other insect species GST sequences. Various studies have only been reviewed the role of insect GSTs (not mutations) in insecticide resistance (Sheehan et al., 2001; Enayati et al., 2005). On the other hand, the conformational dynamics of the GSTE class on insecticide resistance in *An. gambiae* showed noticeable rearrangement for AgGSTE2-F120L and confer increased DDT resistance (Pontes et al., 2016). However, it could be postulated that conformational dynamics study will be helpful in the understanding of the potential impact of D148N mutation on the function of GSTe2.

Declarations

Author contribution statement

Zahar Asadi Saatlou, Saber Gholizadeh: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Mohammad Mehdi Sedaghat: Conceived and designed the experiments; Wrote the paper.

Behrooz Taghilou: Performed the experiments; Wrote the paper.

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Competing interest statement

The authors declare no conflict of interest.

Additional information

No additional information is available for this paper.

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