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Research

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Molecular Monitoring of Knockdown Resistance in Head Louse (Phthiraptera: Pediculidae) Populations in Iran

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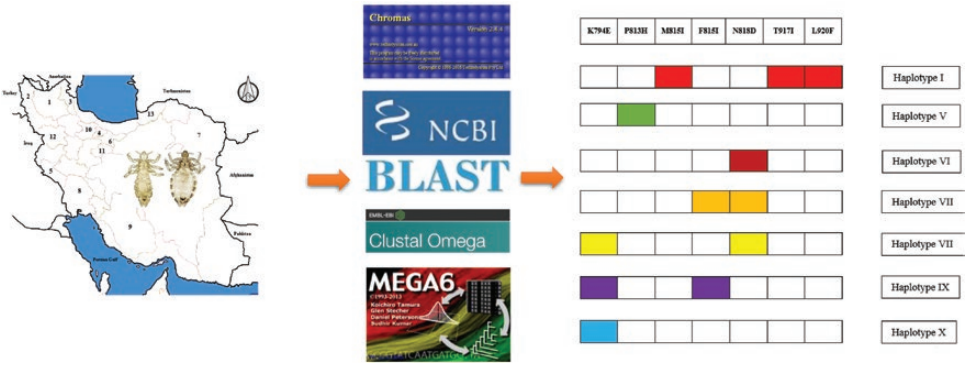
Abstract

Knockdown resistance (*kdr*) is a common mechanism of insecticide resistance in head lice to the conventionally used pyrethroid pediculosis and can be the result of various amino acid substitutions within the voltage-sensitive sodium channel (VSSC). In this study, 54 sequences from varied specimens were investigated to monitor well-known resistance mutations and probable new mutations. The *Pediculus humanus capitis* de Geer specimens were collected from 13 provinces in Iran. The specimens were stored in 70% ethanol until DNA extraction and PCR amplification of ~900-bp fragment of VSSC. The sequences were analyzed using different bioinformatics software for the detection of well-known *kdr* substitutions and additional mutations potentially associated with *kdr* resistance in head lice. There were six new and an old (haplotype I) *kdr* haplotypes within the Iranian head louse population. K794E, F815I, and N818D amino acid substitutions were reported for the first time. The P813H mutation was the most prevalent amino acid substitution in eight provinces. Among 53 sequences, 26 (49%) were homozygous susceptible, and 27 (51%) were heterozygotes. Thus, 51% of the head lice collected in Iran harbored only the P813H allele. The exact test for the Hardy–Weinberg (H–W) equilibrium showed that genotype frequencies differed significantly from the expectation in East-Azerbaijan and Tehran provinces. Moreover, these populations had an inbreeding coefficient (Fis) <0, indicating the excess of heterozygotes. This observation suggests that the populations of head lice from Iran are currently under active selective pressure. For the rest of the populations, H–W equilibrium and the expectations were significantly in harmony. The results of the current study highlight molecular techniques in the accurate detection of

resistance genotypes before their establishment within the head louse population. Accurate detection of resistant genotypes seems to be helpful in decision-making on lice control programs and resistance monitoring and management.

Graphical Abstract



Key words: Head louse, *vssc*, *kdr*, molecular resistance

Pediculosis, caused by the head or body lice, remains the most prevalent human ectoparasitic infestation (Gratz and Organization 1997, Kristensen 2005). The head lice (*Pediculus humanus capitis*) is a common public health problem in developed and developing countries, particularly in poor communities and in schoolchildren (Lee et al. 2000). No report is hitherto available on the disease transmission by the head lice, but its potential role as a vector in the transmission of infectious agents for typhus and trench fever needs more consideration (Robinson et al. 2003, Bonilla et al. 2009). *Acinetobacter baumannii* Bouvet and Grimont (Pseudomonadales: Moraxellaceae), a pathogen agent of ventilator-associated pneumonia, bloodstream infections, wound infections, and nosocomial meningitis, has been detected in the human head louse in Ethiopia (Kempf et al. 2012); however, its transmission by the head lice is obscure and requires further investigation.

A number of interventions to control the head louse infestation are regular combing with an ordinary comb, using a plastic louse comb with very closely set fine teeth, and washing with shampoos containing commercial insecticides such as pyrethroids (i.e., phenothrin and permethrin) and organophosphates (i.e., malathion) (Yoon et al. 2014, Devore et al. 2015, Goddard 2016). Recently, benzyl alcohol, spinosad, and ivermectin have been used as effective treatments for pediculosis in the United States (Stough et al. 2009, Meinking et al. 2010, Pariser et al. 2012). In Iran, permethrin 1%, lindane, malathion 5%, and benzyl benzoate 25% were the insecticides utilized for the treatment of pediculosis (Control Disease Center [CDC] for Iran). Likewise, permethrin was the first-line treatment of head lice during the past decade in the country (Firooziyan et al. 2017). These insecticides are used in shampoos, powders, lotion, and cream formulations (Goddard 2016, Firooziyan et al. 2017). In 2016, the treatments were changed to 1% permethrin shampoo, 4% dimethicone lotion, and lindane shampoo (CDC for Iran). However, the intensive and continuous use of pyrethroids has led to the development of resistance and failure in control strategies in various countries, including Denmark, UK, France, Argentina, USA, Mexico, Russia, Chile, Madagascar, Honduras, and Iran (Kristensen 2005, Thomas et al. 2006, Durand et al. 2007, Toloza et al. 2014, Gellatly et al. 2016, Firooziyan et al. 2017, Ponce-Garcia et al. 2017, Eremeeva et al. 2019, Roca-Acevedo et al. 2019, Larkin et al. 2020). Resistance to lindane, gamma-BHC, has been reported in Iran since 1985 (McLintock et al. 1958).

Based on the evidence, the prevalence of head lice has been raising (Gao et al. 2003, Soultana et al. 2009, Toloza et al. 2009). During 2009–2012, the prevalence of pediculosis in Iranian children has increased from 1.73 to 23% (Amirkhani et al. 2011, Moosazadeh et al. 2015, Firooziyan et al. 2017). The infestation of pediculosis among Iranian primary schoolchildren has been reported to be 7.4% (Moosazadeh et al. 2015). Resistance to pyrethroids, major commercially available pediculicides, and the first-line treatment of pediculosis, is the main reason for increasing pediculosis infestations in the world (Clark et al. 2015). We have lately reported the presence of kdr-type well-known amino acid substitutions, M815I, T917I, L920F, and six novel mutations in the IIS1-2 extracellular loop (H813P) and IIS5 (I927F, L928A, R929V, L930M, and L932M) of the α-subunit, which are potentially associated with *kdr* resistance in the head and body louse populations from two settings in Iran (Firooziyan et al. 2017). The objective of the current work is to evaluate and monitor *kdr*-related mutations in the head lice collected from 19 geographical locations in 13 provinces of Iran.

Materials and Methods

Head Louse Samples

Human head lice were collected from health centers or schools in different geographical regions in Iran (Table 1). Sample collection was carried out by trained workers in accordance with sampling protocol for head lice collection approved by the ethics committee of the National Institute for Medical Research Development (NIMAD; the ethical code: IR.NIMAD.REC.1396.108). All the specimens were transferred to the Medical Entomology Laboratory, School of Public Health (SPH), Urmia University of Medical Sciences (UMSU), Urmia, Iran and preserved in 70% ethanol until further use.

Genomic DNA Extraction and PCR Amplification

DNA was extracted from each louse using YTA Genomic DNA Extraction Mini Kit provided by Yekta Tajhiz Azma, Tehran, Iran

Table 1. The details of head louse samples and distribution of different *kdr* haplotypes obtained from 13 provinces of Iran

Province	Geographic coordinates	Collection year/m	No. of louse collection /analyzed/sequenced	Mutation	Position
East-Azerbaijan	38°05′55.0″N 46°16′25.8″E	2016–2017	17/5/5	K794E, P813H, N818D	IIS1, Linker IIS1–S2, IIS5
West-Azerbaijan	37°40′09.9″N 45°02′17.9″E	2015–2018	18/6/6	P813H, N818D	Linker IIS1–S2
Ardebil	38°14′55.7″N 48°17′54.4″E	2017	4/3/1	N818D	Linker IIS1–S2
Alborz	36°04′49.7″N 51°03′59.9″E	2018	30/5/5	-	-
Ilam	33°38′08.3″N 46°24′54.1″E	2019	46/5/4	N818D	Linker IIS1–S2
Tehran	35°41′31.7″N 51°23′02.9″E	2017	17/5/5	K794E, P813H, F815I	IIS1, Linker IIS1–S2, IIS5
Khorasan Razavi	36°25′37.1″N 59°38′33.1″E	2018	23/10/5	P813H, M815I, T917I, L920F	Linker IIS1–S2, IIS5
Khuzestan	31°32′26.3″N 49°13′15.2″E	2017	20/5/5	P813H	Linker IIS1–S2
Fars	29°44′34.3″N 52°29′54.9″E	2018	17/6/1	N/A	
Qazvin	36°15′43.6″N 49°59′07.6″E	2018	14/5/5	F815I, N818D	Linker IIS1–S2
Qom	34°39′10.5″N 50°47′27.3″E	2017	17/5/5	P813H	Linker IIS1–S2
Kurdistan	36°02′43.8″N 47°09′33.5″E	2018	11/5/4	P813H	Linker IIS1–S2
Golestan	37°26′56.9″N 56°05′07.5″E	2018	46/7/5	P813H	Linker IIS1–S2

(Firooziyan et al. 2018, Asadi Saatlou et al. 2019, Farmani et al. 2019). PCR reactions of the para-sodium channel gene were performed in a 25-µl volume of Master Mix (Yekta Tajhiz Azma). The *vgsc* fragment was amplified in a molecular laboratory using lice-specific primers (Firooziyan et al. 2017). The PCR amplification protocol was conducted in 3 µl of genomic DNA, 1 µl of each specific primer, 12.5 µl of Master Mix, and 7.5 µl of ddH_2O. The PCR reactions were optimized in a hot start at 95°C for 5 min, followed by 30 cycles of denaturation at 95°C for 1 min, annealing at 58°C for 1.5 min, and extension at 72°C for 1.33 min, with 10 min extra extension time in the last cycle. All the 56 samples were applied for sequencing in both directions.

Bioinformatics and Statistical Analysis

Sequences were analyzed using bioinformatics software viz Basic Local Alignment Search Tool (BLAST; http://www.ncbi.nlm.nih.gov/blast/), Chromas version 2.31 (http://www.technelysium.com.au/chromas.html), and Clustal Omega (Sievers and Higgins 2018). The final sequences were aligned with *vgsc* representative haplotype sequences in the GenBank in Molecular Evolutionary Genetics Analysis version 7.0. (MEGA7) (Tamura et al. 2013). Genotype frequencies were calculated by dividing the number of lice of each genotype (RR, RS, and SS) by the total number of analyzed head lice. All the frequencies were scored based on the chromatogram results and the double-peak signal in RS genotypes (Brownell et al. 2020). For each population, genotype frequencies at the 813 loci were compared with Hardy–Weinberg (H–W) expectations using the program Genepop (v. 4.2), option 1 (H-W exact tests), and suboption 3 (probability test) (Weir and Cockerham 1984). Genepop was also employed to estimate Wright's inbreeding coefficient (F_{is}) using the method of Weir and Cockerham (1984) and for populations out of the H–W equilibrium. These values were used to test heterozygote deficiency and excess (Genepop option 1, sub-options 1 and 2, respectively) using the *U*-test as described by Raymond et al. (Raymond and Rousset 1995).

Results

A total of 300 head louse specimens were collected from 19 geographical locations in 13 provinces of Iran (Fig. 1 and Table 1). Among the 300 collected lice, 70 were applied for DNA extraction, randomly. Five head louse specimens from each province were sequenced and analyzed, except for Kurdistan, Tehran, Ilam, and Ardebil provinces. Interestingly, despite DNA extraction in different rounds with the same method, PCR amplification reactions were negative for specimens collected from Shiraz district, Fars province (Fig. 1 and Table 1).

The *vgsc* fragment, containing three exons and two introns, of 54 head louse specimens, was sequenced and analyzed (Supp Fig. 1 [online only]). Multiple sequence alignments of exons showed 98.95–100% similarity within the sequences and 97.65–100% between previously reported haplotypes (Supp Fig. 1 [online only]). Seven out of nine mismatches within nucleotide sequences caused an amino acid substitution in positions 794, 813, 815, 818, 917, and 920 (Fig. 2). Two types of substitution, M815I, and F815I have also occurred in position 815 in samples collected from Khorasan-Razavi and Tehran provinces, respectively (Table 1). Nucleotide and related amino acid sequences are available in the GenBank, European Molecular Biology Laboratory (EMBL), and DNA Data Bank of Japan (DDBJ) databases (GenBank MW057864– MW057917).

Multiple sequence alignments in amino acid level showed 97.48%-100% similarity within and 96.15%-100% between four known haplotype sequences. Except for T917I and L920F in exon III, the remaining amino acid substitutions were located in the exon I region (Fig. 2). Virtually half of the sequences (48.15%) were comprised of at least one amino acid substitution, and 9.26% had more than one mutation at the same time (Table 1). Of note, M815I+T917I+L920F, previously reported as haplotype I, was observed in 1.8% of the samples collected from Torbat-Heidariyeh in Khorasan-Razavi province. P813H, the most prevalent substitution, was present in 33.33% of the samples. It was also the most prevalent haplotype distributed to eight provinces (Table 1). The next second prevalent substitution was N818D (7.4%) found in the Ilam, West-Azerbaijan, East Azerbaijan, and Ardebil provinces. F815I+N818D substitution was acquired from only samples collected from Qazvin province (3.7%). The prevalence of the three remaining haplotypes was 1.8% in Tehran and East-Azerbaijan provinces (Fig. 3). Amino acid substitutions were located in the linker sequence connecting IIS1 and IIS2 (78.95%) and in IIS5 (21.05%) where three known *kdr* mutations were located. It was notable that Alborz province was the only area that amino acid substitutions were not detected in its samples. The details of locations and provinces are presented in Table 1.

Fig. 1. Collection sites of head louse specimens in Iran. 1. East-Azerbaijan, 2. West-Azerbaijan, 3. Ardebil, 4. Alborz, 5. Ilam, 6. Tehran, 7. Khorasan-Razavi, 8. Khuzestan, 9. Fars, 10. Qazvin, 11. Qom, 12. Kurdistan, and 13. Golestan.

Based on amino acid sequences, the genotyping of *kdr* mutations in the head lice indicated that there are six new and an old (haplotype I) haplotype(s). K794E, F815I, and N818D were reported for the first time not only in lice but also in insect species. K794E was located in IIS1, and the remaining two mutations were placed in the linker IIS1-2 extracellular loop of the α-subunit. K794E mutation, a new amino acid substitution in the IIS1 α-subunit, was reported from East-Azerbaijan and Tehran provinces (Table 1, Fig. 1). New haplotypes were classified as V, VI, VII, VIII, IX, and X (Fig. 3).

P813H *kdr* substitution was the most prevalent haplotype detected in 27 head lice collected from eight provinces (Table 2). The occurrence of the nucleotide substitution of the CCC/CAC that codes for the P813H substitution resulted in the nucleotide number 2,440 in the *kdr* fragment (Supp Fig. 1 [online only]). Based on the chromatogram results, there were three head louse genotypes: a homozygous susceptible or wild-type allele (SS), a heterozygote (RS), and a homozygous-resistant mutant (RR). Resistance medium frequency varied from 10 to 50% in seven provinces (Table 2). Among obtained sequences, 26 (49%) were homozygous susceptible and 27 (51%) were heterozygotes. The *kdr*-resistant homozygote (RR) was not detected in the studied populations. Therefore, 51% of the head lice collected from Iran harbored only one mutant allele, P813H.

The exact test for the H-W equilibrium for five sites, including Tabriz, Bonab, and Bostan-Abad in East-Azerbaijan and Tehran and Damavand in Tehran provinces, showed that genotype frequencies differed significantly from the expectation. These populations had an inbreeding coefficient (F_{is})<0, suggesting an excess of heterozygotes (Table 2). For the rest of the populations, H-W equilibrium significantly harmonized with the expectations.

Discussion

The prevalence of pediculosis in the world and, consequently, in Iran has been growing during the last decade (Amirkhani et al. 2011, Tappeh et al. 2012, Moosazadeh et al. 2015). Our recent study on the molecular analysis of *kdr* sequence in West-Azerbaijan and Zanjan provinces has displayed the prevalence of new *vgsc* mutations in the head and body lice in the country (Firooziyan et al. 2017). In the current study, for the first time, we report *kdr* sequence analysis within head louse populations collected from 13 provinces of Iran.

M815I, T917I, and L920F are three well-known mutations in the *vgsc* α-subunit and recognized as *kdr* markers (Clark 2010, Gellatly et al. 2016, Firooziyan et al. 2017, Fox et al. 2020), the most common resistance mechanism to pyrethroid insecticides, as well as DDT (Picollo et al. 2000). These mutations have been reported in 15 countries in Asia, Africa, Europe, and America continents (Fox et al. 2020). Moreover, six novel *vgsc* mutations have been reported in the head and body lice collected from two northwestern provinces of Iran, West-Azerbaijan, and Zanjan

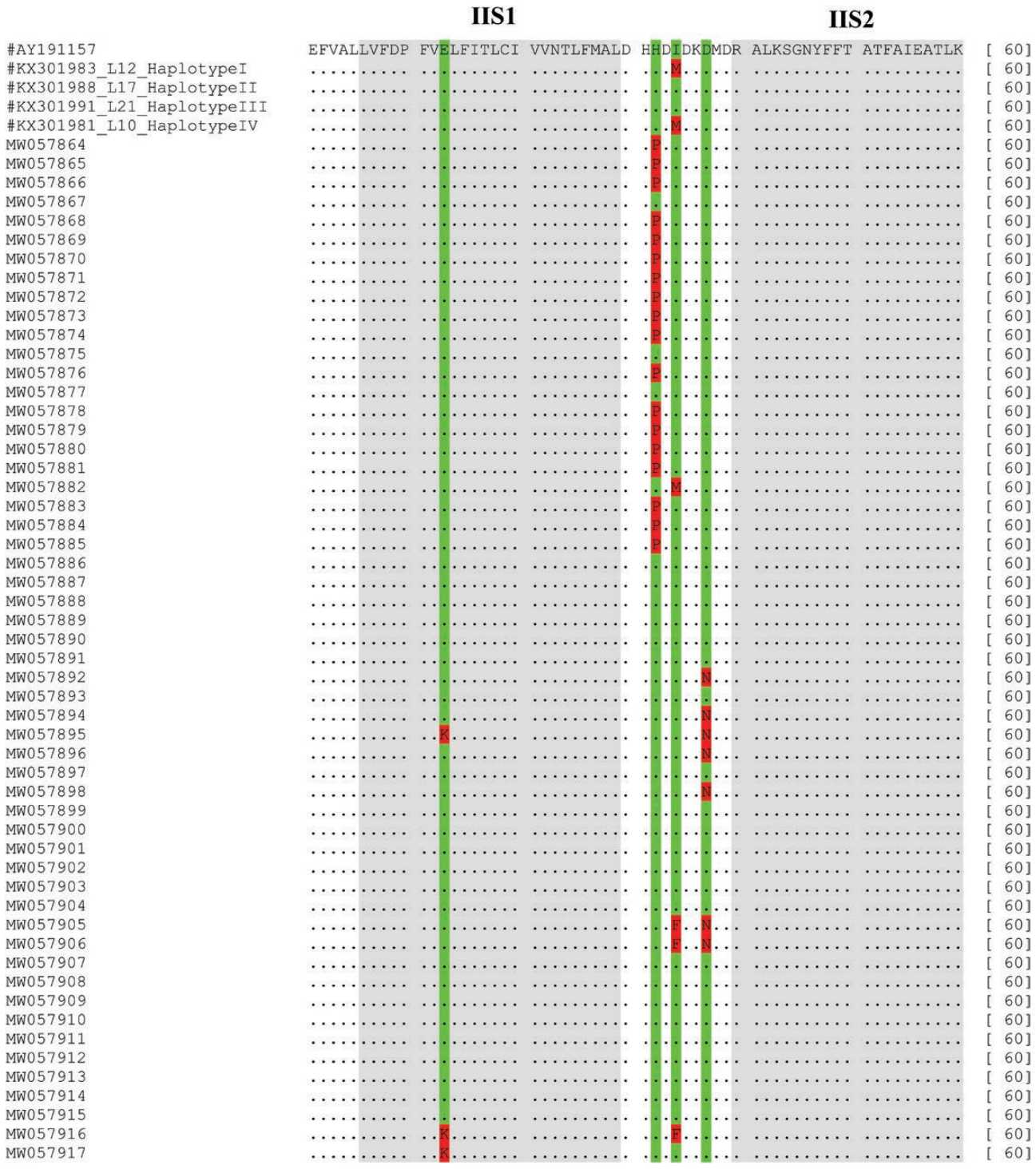


Fig. 2. The alignment of amino acid sequences of the partial sequence of the voltage-sensitive sodium channel α-subunit genes from the head lice collected from eight provinces in Iran. GenBank sequences, including KX301983, KX301988, KX301991, and KX301981, have been used as representative sequences for haplotypes I-IV, respectively. AY191157 was applied as a reference sequence from the GenBank. The segments S1–S5 of domains II is illustrated in gray and amino acid substitution in red color. MW057864-68 were collected from Qom, MW057869-71 and MW057891-93 from West-Azerbaijan, MW057872-74 from East-Azerbaijan, MW057875-79 from Golestan, MW057880 and MW057907-10 from Khuzestan, MW057881-82 and MW0579011-12 from Khorasan-Razavi, MW057883 and MW057913-15 from Kurdistan, MW057884-85 and MW057916-17 from Tehran, MW057886-90 from Alborz, MW057894 from Ardebil, MW057897-901 from Ilam, and MW057902-06 from Qazvin provinces.

(Firooziyan et al. 2017). In the current study, although samples were collected from 13 provinces, only two substitutions were obtained in positions 815, M815I, and F815I for the first time. The discrepancy between our past and present research work may be due to the variety and pressure of pediculicides used in the country.

In our former investigation, H813P mutation in the IIS1-2 extracellular loop region was observed in 69% of only body louse

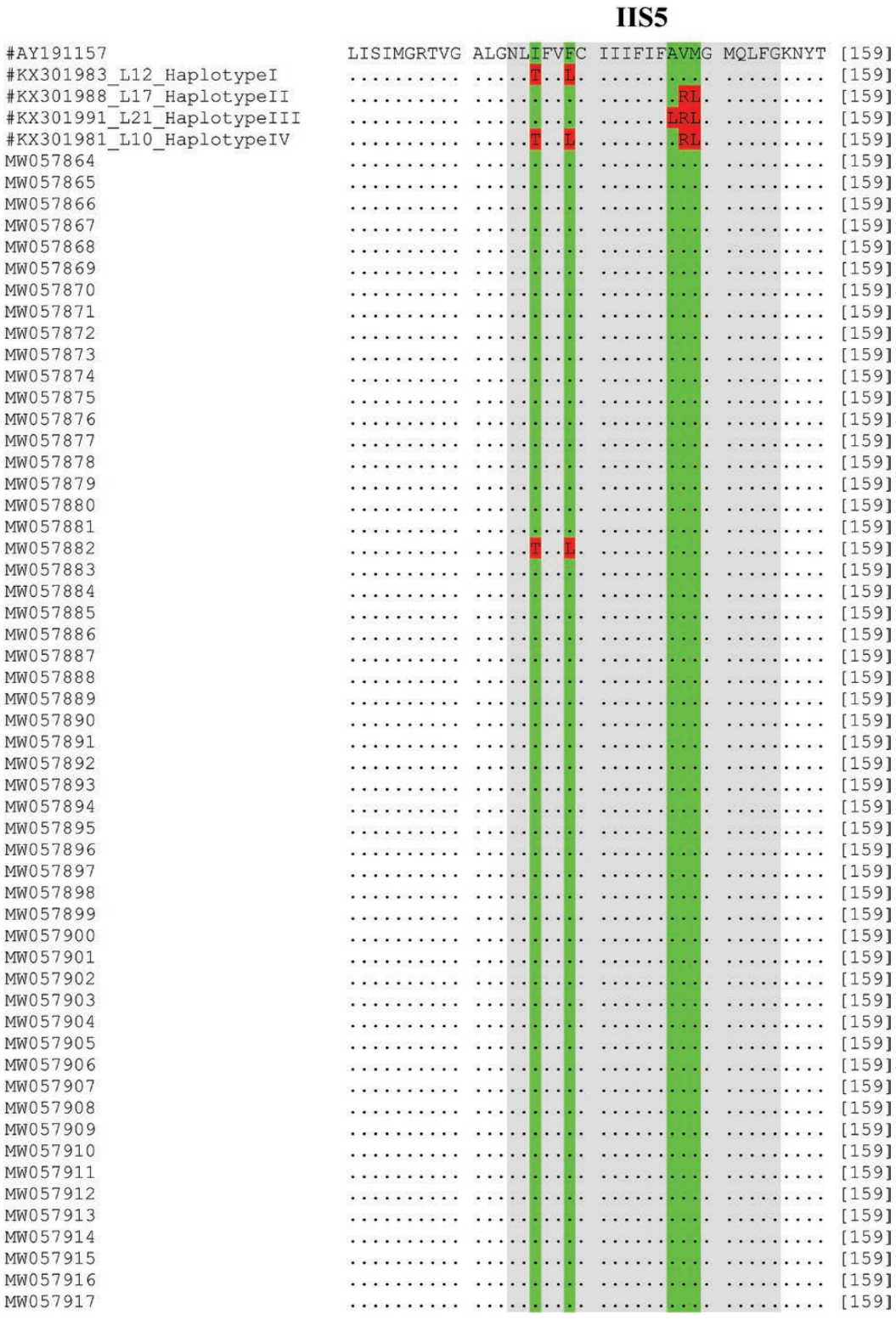


Fig. 2. Continued.

sequence samples (Firooziyan et al. 2017). In our present study, the mutation was distributed in 33.33% of head louse sequences collected from eight provinces of Iran (Table 1). Unlike our previous investigation in West-Azerbaijan province, in this study, we found a mutation, H813P, in specimens collected from the same province. These disparities in the results are possible owing to the increase in the number of samples or the spread of resistance in the aforesaid region and the country.

In addition to the most commonly *kdr* biomarkers recorded, six novel mutations located in the linker IIS1-2 extracellular loop and IIS5 have been reported (Firooziyan et al. 2017). Kristensen et al. (2005) have also identified the substitution of G931A in head lice

(Kristensen 2005). The mutations mentioned above have not yet been detected in other insect species. In our study, four new amino acid substitutions, K794E, P813H, F815I, and N818D, were identified in head lice for the first time. These mutations were placed in the IIS1 and linker IIS1–S2 near the location of known *kdr*-related mutations (Table 1). However, their role and relation with insecticide resistance remain unclear and need additional investigation, with emphasis on standardized toxicological bioassays and on epidemiological studies to determine the frequency and magnitude of the exposition to current pediculicide treatments in Iran. Further studies by computational modeling and also by electrophysiological and pharmacological analyses of *kdr* mutant sodium channels expressed in *Xenopus* oocytes could unmask the role of H813P mutation in the nerve insensitive mechanism of *kdr* type-resistance (Yoon et al. 2008).

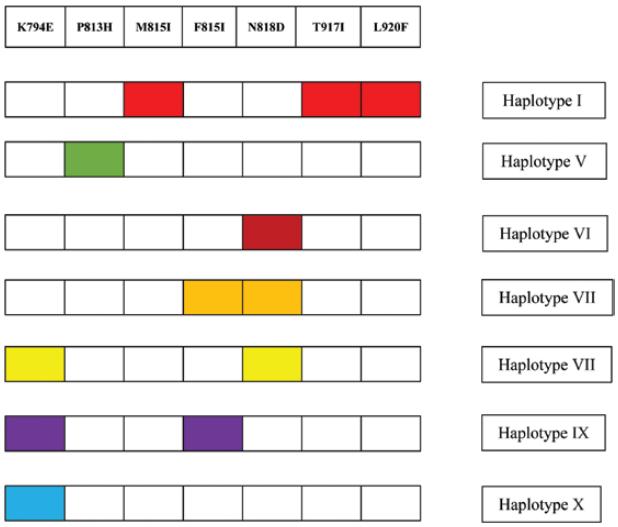


Fig. 3. Representative novel pyrethroid resistance haplotypes of the head louse. Three well-known mutations associated with *kdr* resistance are presented in red color (haplotype I). Haplotypes V-X are six new records in the head louse *kdr* sequences.

To date, various studies have assessed the great sensitivity and excellent specificity of the melting curve analysis genotyping method (Marcoux et al. 2010, Drali et al. 2012, Karakus et al. 2020). It is a single-step and rapid method that simultaneously detects the frequency and point mutation related to permethrin resistance without sequencing (Kim et al. 2004). This technique is suitable only for the detection of known mutations in large-scale epidemiological monitoring studies (Drali et al. 2012). Using melting curve analysis, Karakus et al. (2020) have newly reported resistance allele frequency (RAF) for three well-known mutations associated with resistance to pyrethroid insecticides in Turkey; however, gene sequencing and bioinformatics analysis may find new mutations in head louse populations (Firooziyan et al. 2017). As per the results of the current study, the evaluation of the frequency of six new mutations by using melting curve analysis is suggested.

Our previous and current reports on *kdr* mutation in head and body lice in Iran demonstrate the spread of insecticide resistance in the louse population in the region. Excess heterozygotes are likely due to the reason that the mutations have no link with resistance to insecticides being currently used and/or that *P. humanus capitis* in Iran is undergoing successive bottlenecks and passive genetic drift. To handle and inhibit the distribution of resistance alleles in the head louse populations, we recommend applying pyrethroid insecticides as a routine rotation policy, but with careful use, for effective treatment and long-lasting treatment in areas of resistance (Burgess et al. 1995). The *kdr* frequency map in Iran uncovers a critical need for a fundamental revision of national protocols for lice treatment and the development of new treatment strategies by categorizing pediculicides into three distinct treatment lines, the same as a malaria treatment protocol. Taken together, the establishment of molecular monitoring systems and continuous surveillance of insecticide resistance in head lice are necessary and would be helpful for the control and management of insecticide resistance.

Table 2. Distribution of *kdr*-like allele P813H in head louse populations from Iran

Province	No. of head lice analyzed (No. of infested subjects)	Genotype[a]			Resistant allele frequency (%)	H-W (χ2)[b]	Fis[d]
		S/S	S/R	R/R			
Qom	5(4)	1 (20)	4 (80)	0	40	2/22	–0.600
West-Azerbaijan	6(4)	2 (33,3)	4 (66,7)	0	33/3	1/5	–0.429
East-Azerbaijan	5(3)	0	5 (100)	0	50	5[c]	–1,000
Golestan	5(3)	2 (40)	3 (60)	0	30	0/91	–0.333
Khuzestan	5(1)	4 (80)	1 (20)	0	10	0/06	0.000
Khorasan-Razavi	4(2)	2 (50)	2 (50)	0	25	0/44	–0.200
Kurdistan	4(1)	3 (75)	1 (25)	0	12.5	0/08	0.000
Tehran	4(4)	0	4 (100)	0	50	4[c]	–1,000
Alborz	5 (1)	5 (100)	0	0	0	–	NA
Ardebil	1(1)	0	1 (100)	0	50	1	NA
Ilam	4(1)	4 (80)	1 (20)	0	10	0/06	0.000
Qazvin	5(2)	3 (60)	2 (40)	0	20	0/31	–0.143
Total	**53(27)**	**26 (49)**	**27 (51)**	**0**	**25.9**	**6.19[c]**	**–0.468**

[a]S and R are susceptible and resistant alleles. Between brackets are the percentages of each genotype proportion.
[b]Populations were tested for the Hardy–Weinberg equilibrium by a χ^2 test (χ2 = 3.84, df = 2, $P < 0.05$).
[c]Values that are statistically significant at $P < 0.05$. Significance level indicates the rejection of the null hypothesis $F_{is} = 0$ at $P < 0.05$.
[d]F_{is} values > 0 indicate heterozygote deficiency, whereas F_{is} values < 0 implies heterozygote excess.

Supplementary Data

Supplementary data are available at *Journal of Medical Entomology* online.

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