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Identification of Novel Voltage-Gated Sodium Channel Mutations in Human Head and Body Lice (Phthiraptera: Pediculidae)

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Abstract

In recent years, the increase of head louse infestation in Iran (7.4%) and especially in West-Azerbaijan Province (248%) has raised the hypothesis of insecticide resistance development. There are different mechanisms of resistance to various groups of insecticides, and knockdown resistance (*kdr*) is a prominent mechanism of resistance to pyrethroids, an insecticide group which is used conventionally for pediculosis control. For detection of *kdr*-type well-known amino acid substitutions (M815I–T917I–L920F) and additional sodium channel mutations potentially associated with *kdr* resistance in head and body lice, louse populations were collected from West-Azerbaijan and Zanjan Provinces of Iran. Six novel mutations were found to be located in the IIS1-2 extracellular loop (H813P) and IIS5 (I927F, L928A, R929V, L930M, and L932M) of the α-subunit. Genotyping results showed that all specimens (100%) have at least one of these or the well-known mutations. Therefore, the presence of *kdr*-related and novel mutations in the sodium channel is likely to be the reason for the frequent use of pyrethroid insecticides due to treatment failure against lice. Further studies are now required to evaluate the prevalence of the *kdr*-like mutant allele for monitoring of insecticide resistance and the management of head and body lice in other provinces of the country.

Key words: head louse, body louse, sequence analysis, novel voltage-gated sodium channel mutation, knockdown resistance

Head and body lice (*Pediculus humanus capitis* de Geer and *Pediculus humanus humanus* L. Linnaeus) are the cause of pediculosis that is the most prevalent human ectoparasitic infestation and creates a serious problem, particularly for school children worldwide (Gratz 1997, Kristensen 2005). Lice are morphologically indistinguishable and can be identified only based on their location on the human body (Ferris 1953, Boutellis et al. 2014). Light et al. (2008) assessed the taxonomic status of head and body lice using molecular data and found that body lice represent a morphotype of head lice (Light et al. 2008).

In the recent decade, pediculosis seems to be increasing in many parts of the world, but its infestation intensity varies in different countries (Gao et al. 2003, Burgess 2004, Soultana et al. 2009, Toloza et al. 2009, Oh et al. 2010, Manrique-Saide et al. 2011, Speare et al. 2014, Bartosik et al. 2015). Pyrethroids are the major commercially available pediculicides, and their extensive use has led to rapid development of resistance in lice (Clark et al. 2015). Widespread reports of treatment failure from Argentina, Australia, England, and lately Denmark, suggest that pyrethroid and carbamate pediculicide resistance is increasing (Picollo et al. 1998, Downs et al. 2002, Hunter and Barker 2003, Kristensen 2005).

Knockdown resistance (*kdr*), reduction in the sensitivity of the insect sodium channel to pyrethroids, was first described in the housefly, *Musca domestica* L. Linnaeus (Farnham 1977, Soderlund and Bloomquist 1990, Soderlund and Knipple 2003, Dong et al. 2014). The *para* voltage-gated sodium channel of insects is homologous with the α-subunit of the mammalian sodium channel (Catterall 2000, Davies et al. 2007, Rinkevich et al. 2013). The pore forming α-subunit of each channel consists of four homologous domains (I–IV), each containing six transmembrane helices (S1–S6) connecting with intracellular linkers (Catterall 2000, Davies et al. 2007, Dong et al. 2014).

In the voltage-sensing part (S1–S6), the S5 and S6 segments are linked with hairpin loops. The *para* channel is the principal target site for pyrethroids, DDT, the synthetic analogue of N-alkylamides and dihydropyrazole derivatives (Davies et al. 2007, Dong 2007). The voltage-gated sodium channel gene contains 2104 and 2051 amino acids in *Pediculus humanus capitis* (GenBank AY191157) and *M. domestica* (GenBank MDU38814), respectively. A 53-amino-acid variation between the two species has caused different amino acid numeration in both species. Therefore, the domain IIS1-IIS5 is located between amino acids 782 and 940 in *Pediculus* and between 794 and 952 in the housefly *para* gene (Supp. Fig. 1 [online only]). In the past two decades, >50 sodium channel mutations or combinations of mutations have been reported that are responsible for or associated with *kdr* in various arthropods and disease vectors (Rinkevich et al. 2013, Dong et al. 2014). Co-occurrence of more than one *kdr* mutation in house fly (Lee et al. 1999b), bed bug (Yoon et al. 2008), cockroach (Tan et al. 2002), and *Anopheles gambiae* (Jones et al. 2012) has led to greater reductions of sodium channel sensitivity to pyrethroids, as compared to effects of a single mutation.

The early detection of resistance is crucial for choosing efficient management strategies and may delay the development of further resistance. Difficulty in collecting large numbers of live lice has complicated the early resistance detection using conventional bioassay-based monitoring methods (Clark 2010). As an alternative resistance monitoring tool, different molecular markers and techniques have been employed (Clark et al. 2001, Kim et al. 2004, Clark 2010). Thus, the establishment of a fast and accurate resistance monitoring system using molecular markers is essential to maximize the life span of available pediculicides. Otherwise, development and complete fixation of resistance to head and body lice will cause a serious problem in pediculosis control.

In Iran, permethrin 1%, lindane, malathion 5%, and benzyl benzoate 25%, which are found in shampoos, powders, lotion, and cream formulations, have been used for the treatment of pediculosis. Recommended treatments were changed to permethrin 1% shampoo, Dimeticone 4% lotion, and Lindane shampoo in 2016 (Control Disease Center [CDC], Iran). Permethrin had been the first line treatment of head louse during the past decade. The first nationwide survey on the prevalence of pediculosis in Iranian children and adolescents showed the pediculosis prevalence of 1.73% (Amirkhani et al. 2011). However, during 2009–2012, it increased by 23% in the country and by 248% in West-Azerbaijan Province. Availability of warm water for bathing and hair length have been reported as effective risk factors for increasing risk of head lice infestation in Urmia district in 2012 (Tappeh et al. 2012). Recently, the prevalence of head lice infestation has been estimated to be 7.4% among primary school children in Iran (Moosazadeh et al. 2015). Health workers believe that the increase of pediculosis in recent years has been due to treatment failure (A. Sadaghianifar and S. Firooziyan 2014, personal communication). To address this, the sequences of the *para*-orthologues head and body lice sodium channel gene fragments were studied. These sequences span the IIS1–IIS5 region, where most of the sodium channel gene mutations are located and associated with *kdr*. The present research is the first step in a nationwide survey of molecular insecticide resistance in head and body lice in Iran, and subsequently in the Eastern Mediterranean region.

Materials and Methods

Human head louse specimens were obtained from different geographical regions in Urmia (37° 33′19″ N and 45° 04′21″ E), Salmas (38° 19′75″ N and 44° 76′81″ E), and Zanjan (36° 66′44″ N and 48° 48′56″ E) and from families seeking treatment in health centers. Head lice treatment is conducted at health centers free of charge using permethrin shampoos. Body lice were collected from clothing of human volunteers in a rehabilitation camp in Urmia city. Regular changing of clothes, bathing, and application of dimeticone and ivermectin lotions were used for body lice treatment.

Prior to lice sampling, the objective of the study and the method of louse collection were explained to interested individuals, volunteers, and the parents or legal guardians of the children. Afterwards, written informed consents (in Persian) were obtained from these individuals. Owing to the use of protocols with human subjects, the details of information of infested humans, who willingly provided the collected samples for this study, are not presented. After lice collection, all patients received proper pediculicides. The protocol for collection of lice was reviewed and approved by the ethics committee in Urmia University of Medical Sciences (UMSU).

Genomic DNA was extracted from the whole body of each louse stored in 70% ethanol using CinnaPure-DNA (CinnaGen, Tehran, Iran). Each louse was homogenized in 100 µl of prelysis buffer in liquid nitrogen. After the addition of 10 µl ribotinase, the mixture was incubated at 55 °C for 1–2 h. The rest of the DNA extraction process was followed based on the manufacturer's instructions.

A ~900-bp fragment in the α-subunit of the *para*-sodium channel gene was amplified with specific primers (HLF: ATTTTGCGT TTGGGACTGC and HLR: CCATCTGGGAAGTTCTTTATC) designed based on the GenBank AY191157 using Gene Runner (version 5.1.06, 1992-2016, Frank Buquicchio) and BLAST (http://www.ncbi.nlm.nih.gov/blast) software (accessed 17 May 2017).

PCR reactions (25 µl) were performed in thin-walled microcentrifuge tubes. The optimized reaction condition was 1 µl of genomic DNA, which was prepared as described above from a single louse, 1 µl each of the specific primers, 12.5 µl Master Mix (CinnaGen, Iran), and 7.5 µl ddH_2O. The amplification profile was set up with 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 90 s, and extension at 72 °C for 80 s, with 10 min extra extension time in the last cycle.

The PCR fragments were sequenced in both directions on an ABI 377 automatic sequencer with the primers used for amplification. The multiple sequences of lice were assembled and analyzed with the ClustalW (Thompson et al. 2002) and MEGA6 (Tamura et al. 2013).

Results

All school children and volunteers ($n = 11,115$) were examined for lice, and adult and immature stages of lice or nits were observed in 2,765 people. A total of 22 adult head lice and 14 adult body lice were collected. A ~900-bp fragment of the sodium channel gene was amplified and sequenced in 24 specimens randomly selected from head and body lice ($n = 12$ each). The complete reference sequence was deduced using direct sequencing, BLAST, and multiple sequence alignment and compared with the GenBank DQ062568 (Kristensen 2005) and AY191157 (Lee et al. 2003).

The amplified and sequenced fragment of the sodium channel gene was composed of three exons and two introns (Supp. Fig. 2 [online only]). The sizes of intron I in head and body lice were 86–87 bp and 86 bp, respectively (nucleotides 142–225; Supp. Fig. 2 [online only]). Multiple sequence alignments of intron I showed an insertion at position 176 and a missense mutation at position 145; transversion A/T in the head louse. Also, the intron I region in body

louse contained 2-bp mismatches at positions 145 and 167 (B3 and B10 specimens) as a transversion (T/A) and a transition (G/A), respectively (Supp. Fig. 2 [online only]). Sequence similarity within and between species was 97–100% in this noncoding region. Nucleotide and related amino acid sequences are available in GenBank, European Molecular Biology Laboratory (EMBL), and DNA Data Bank of Japan (DDBJ) databases [GenBank KX301981–KX302005].

The sequence sizes of the intron II region in head and body lice were 84–86 bp and 84 bp, respectively (nucleotides 408–492; Supp. Fig. 2 [online only]). Sequence similarity within 12 body louse specimens was 100%, whereas there were 2-bp deletion at positions 431 (A) and 432 (T) and a mismatched transition at position 433 (C/G), as compared with head louse sequences (Supp. Fig. 2 [online only]).

Exon I region in both species contained a nucleotide sequence of 141 bp. Of four mismatches (transition/transversion) at positions of 30, 33, 102, and 123 in exon I sequence of head louse, one mutation at position 102 (G/T) was not silent. This mutation led to the substitution of M815I in amino acid sequences (Figs. 1 and 2). Also, five mismatches were observed in exon I sequence of the body louse. In addition to the mutation at position 102 in head louse, there was an extra substitution at position 95 (A/C) in 69% of body louse sequences, which substituted amino acid P813H (Figs. 1 and 2). The prevalence of M815I was different in head and body lice (Table 1). The sequence size of exon II region was 178 bp with 100% similarity in nucleotide and amino acid levels in both species (Figs. 1 and 2).

There were six nucleotide mismatches as a transition and a transversion in the 162-bp nucleotide sequences of the exon III region in head louse sodium channel gene at positions 584, 592, 618–620, 622, and 628 (Supp. Fig. 2 [online only]). The mismatches caused five amino acid substitutions at positions T917I, L920F, L928A, R929V, and L930M in the head louse (Fig. 1), though the prevalence of these substitutions was the same, 58.33%, except for L928A (Table 1). Also, the pattern of amino acid substitution in the body louse included T917I, L920F, I927F, L928A, R929V, L930M, and L932M. The prevalence of T917F, V929R, and M930L substitutions in body louse was high (Table 1).

The genotyping results of *kdr* mutation sites in head and body lice showed that there are nine substitutions in the α-subunit of the *para*-sodium channel gene (Fig. 2). P813H, I927F, and L932M substitutions were specific for body louse, while the six remaining were common in both species. Four different haplotypes were identified in head louse based on amino acid sequence analysis. The most frequent was haplotype I (41.66%) with M815I, Tp17I, and L920F substitutions (Fig. 2), followed by haplotype II (25%; R929V and L930M). The frequency of both haplotypes III (L928A, R929V, and L930M) and haplotype IV (M815I, T917I, L920F, R929V, and L930M) was 16.67%. Haplotypes I and IV were collected from Urmia, whereas haplotypes II and III from Zanjan and Salmas, respectively. Interestingly, three well-known mutation sites (M815I, T917I, and L920F) were RR in both specimens of head and body lice; however, the remaining new mutation sites were RS (Table 1). The genotyping results were different in body lice. Amino acid sequence alignment of 12 body louse specimens indicated seven different haplotypes (Fig. 2).

Discussion

The first experiments in control of the louse were carried out in 1955 using 10% DDT dust and 1% gamma BHC (linden) dust in Iran (McLintock et al. 1958). One year later, the WHO susceptibility test with exposure to 0.25% BHC showed 61% mortality. The study by McLintock et al. (1958) recommended that BHC dust should not be used for the control of lice in Iran (McLintock et al. 1958); however, lindane is still used for louse control in the country (CDC, Iran) without re-assessing its resistance to linden. The current study, to the best of our knowledge, is the first report on the molecular insecticide resistance of head and body lice not only in Iran, but also in the Eastern Mediterranean Region. All the previous studies on pediculosis, except that of McLintock et al. (1958), were limited to the estimation of the infestation prevalence in the country (Poudat and Nasirian 2007, Motovali-Emami et al. 2008, Moradi et al. 2009, Amirkhani et al. 2011, Omidi et al. 2013, Dehghanzadeh et al. 2015). However, the prevalence of pediculosis in Iranian children and adolescents is low, but this infestation is on the rise in some provinces. Therefore, intensive public health measures as well as the monitoring and the management of insecticide resistance should be undertaken to control this communicable problem.

From 1945 to date, various natural and synthetic insecticides with different modes of action have been available for the treatment of pediculosis (Durand et al. 2012). However, pyrethroids are the safest and most commonly used pediculicides (Lee et al. 2000). On the other hand, the large-scale and repeated application of pediculicides by infested persons or family members has led to the emergence and spread of resistance in several countries of the world (Chosidow et al. 1994, Rupes et al. 1995, Picollo et al. 1998, Downs et al. 1999, Hemingway et al. 1999, Pollack et al. 1999). Possible mechanisms of resistance to these insecticides include detoxification, esterification, oxidation, target site insensitivity, and *kdr* (Durand et al. 2012). M815I substitution has been reported as a *kdr* resistance associated mutation in louse (Lee et al. 2003) and other insect species (Williamson et al. 1996, Martinez-Torres et al. 1998, Lee et al. 1999a, Clark et al. 2013, Dong et al. 2014, Gholizadeh et al. 2014). In the current study, the sequence analysis of genomic DNA fragments in both louse species showed that in addition to M815I in 62.5% of the sequences (58.33% in head louse and 76.9% in body louse), there is a novel H813P mutation in 69.23% of body louse sequences. The high prevalence of both pyrethroid resistance mutations in head and body lice could be suggested as the cause of treatment failure and ineffectiveness of insecticides in pediculosis control. The association of other mutations (T917I and L920F) in the voltage-sensitive sodium channel gene with permethrin resistance has been indicated in head louse (Lee et al. 2000, 2003; Kristensen 2005). Kristensen (2005) also reported the existence of G931A substitution in the trans-membrane segment five of domain II on the sodium channel, which has not been identified in other insect species (Kristensen 2005). Sequence analysis in the current study showed that in addition to T917I and L920F in head louse and L920F in body louse, novel mutations, A928L, V929R, and M930L, are located in the trans-membrane segment IIS5 of voltage-sensitive sodium channel of both species. These mutations have not been detected in other insect species.

The frequency of the T929I-L932F *kdr*-like haplotypes in Danish head lice populations is linked to DDT cross-resistance and to the common use of permethrin for the control of head louse (Rosdahl 1975, Kristensen 2005). In the current study, the frequencies of A928L, V929R, and M930L mutations were 16–58% in head louse and 53–85% in body louse. The high frequency of these novel mutations in this relatively small sample from West-Azerbaijan and Zanjan Provinces, Iran, reveals that a larger survey is needed to confirm the pyrethroid resistance status of Iranian head and body lice populations.

In conclusion, sequence analysis of IIS1–S5 regions in the voltage-gated sodium channel gene of head and body lice collected

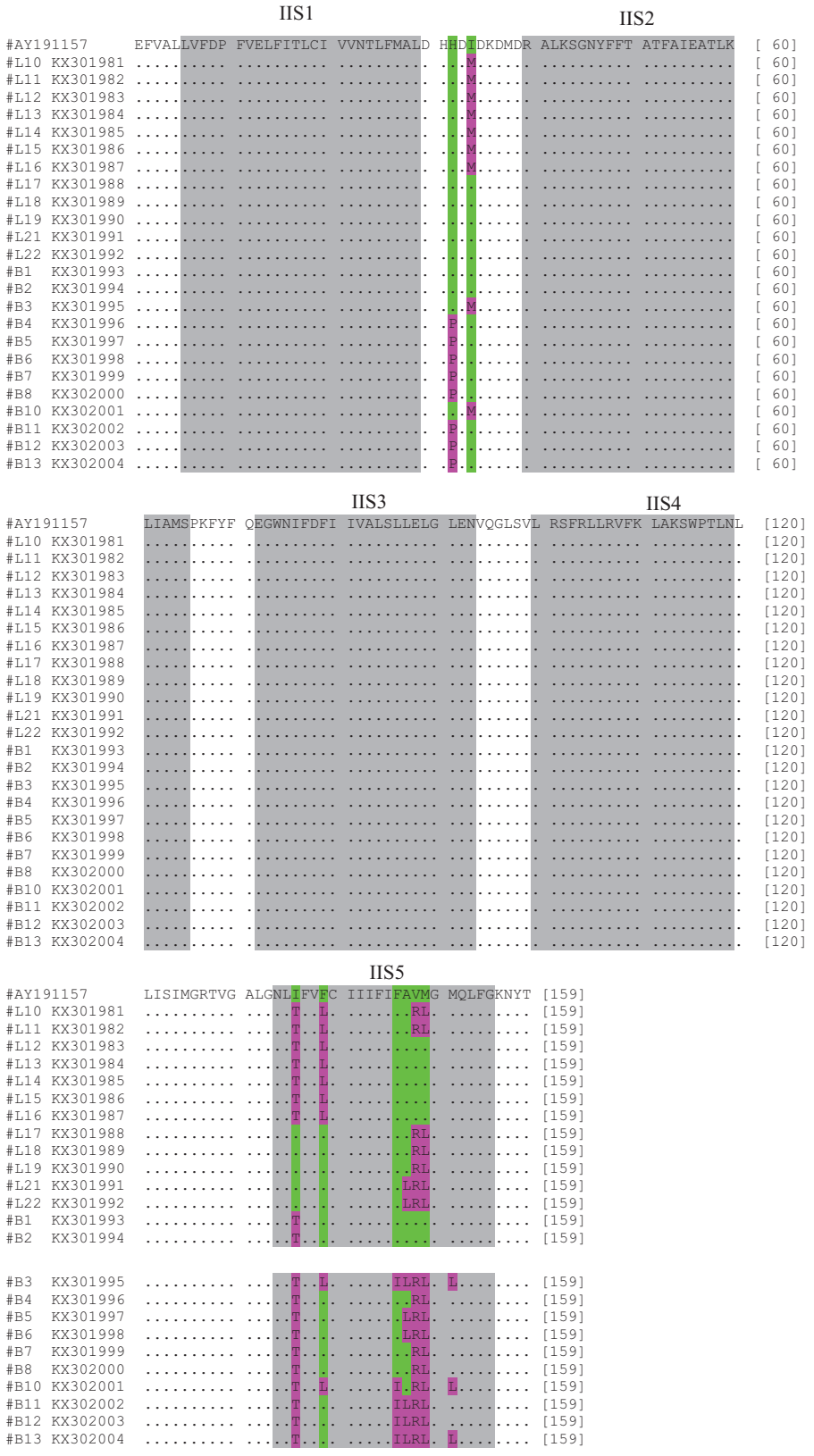

Fig. 1. The alignment of amino acid sequences of partial sequence of the voltage-sensitive sodium channel α-subunit genes from the head (L) and body (B) lice collected from Iran. The segments S1–S5 of domains II are illustrated in gray and amino acid substitution in purple color.

from Urmia, Salmas, and Zanjan revealed the presence of three well-known *kdr* type mutations that were responsible for nerve insensitivity and pyrethroid resistance at least when put into housefly sodium channel. Three mutations identified in the voltage-sensitive sodium channel α-subunit gene of permethrin-resistant human head lice abolish permethrin sensitivity of the house fly Vssc1 expressed

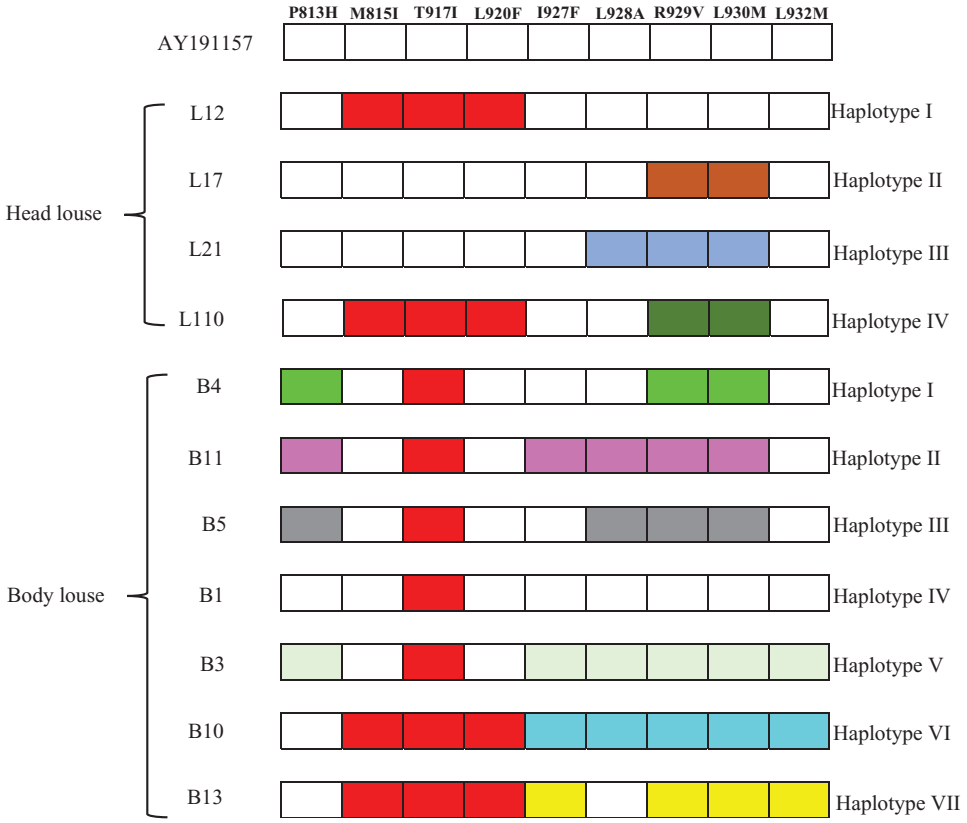

Fig. 2. Representative pyrethroid resistance haplotypes of head and body lice. L) head louse sequences (L12–L16 haplotype I, L17–L19 haplotype II, L21 and L22 haplotype III, L10, and L11 haplotype IV) and B) body louse sequences (B4, B7, and B9 haplotype I, B11 and B12 haplotype II, B5 and B6 haplotype III, B1 and B2 haplotype IV, B3, B10, and B13 haplotypes V, VI, and VII, respectively). Haplotypes I and IV of head louse were collected from Urmia, while haplotypes II and III were obtained from Zanjan and Salmas districts, respectively. Three well-known mutations associated with kdr resistance are presented in red color.

Table 1. Distribution of different *kdr* haplotypes obtained from 24 head and body lice samples collected from Iran. The nucleotide mutation and amino acid substitution in the *Pediculus* sodium channel gene and corresponding positions in housefly are also presented.

Amino acid substitution		Nucleotide mutation	Location	*kdr* Haplotypes	Head louse (%)	Body louse (%)	Origin of lice
Musca	*Pediculus*	*Pediculus*					
P813H	P813H	CAC/CCC	Linker IIS1–S2	RS	–	8 (69.23)	Urmia (37° 40′ N, 45° 0′ E)
M815I	M815I	ATG/ATT	Linker IIS1–S2	RR	7 (58.33)	4 (76.9)	Urmia
T929I	T917I	ACA/ATA, ACC	IIS5	RR	7 (58.33)	10 (100)	Urmia
L932F	L920F	CTT/TTT	IIS5	RR	7 (58.33)	2 (84.6)	Urmia
I939F	I927F	TTT/ATT	IIS5	RS	–	5 (46.15)	Urmia
L940A	L928A	GCG, GCC/TTG	IIS5	RS	2 (16.66)	6 (53.84)	Salmas (38° 11′ N, 44° 47′ E), Urmia
R941V	R929V	GTT/CGT	IIS5	RS	7 (58.33)	10 (84.6)	Zanjan (37° 8′ N, 47° 47′ E), Salmas, Urmia
L942M	L930M	ATG/TTG	IIS5	RS	7 (58.33)	10 (84.6)	Zanjan, Salmas, Urmia
L944M	L932M	ATG/CTG	IIS5	RS	–	3 (25)	Urmia
					$n = 12$	$n = 12$	

RS, heterozygous resistance; RR, homozygous resistance.

in *Xenopus* oocytes (SupYoon et al. 2008). Interestingly, the identified novel amino acid substitutions are clustered in the linker sequence connecting IIS1 and IIS2 or in IIS5 where three known *kdr* mutations are located (Fig. 3). However, it is not known whether the six new mutations are associated with permethrin resistance. However, computational modeling, electrophysiological and pharmacological analyses of *kdr* mutant sodium channels expressed in *Xenopus* oocytes will shed light on the association of novel mutations or combinations of them in lice pyrethroid resistance. Despite a small number of sequences, it is now clear that *kdr*-type mutations are widely distributed in head and body lice populations in Northern Iran. The presence of pyrethroid-resistant genotypes in lice populations indicated a critical need for reassessment of new approaches to management of lice infestation and development of new pediculicides. Molecular detection of insecticide resistance in head and body lice in all provinces of the country, together with

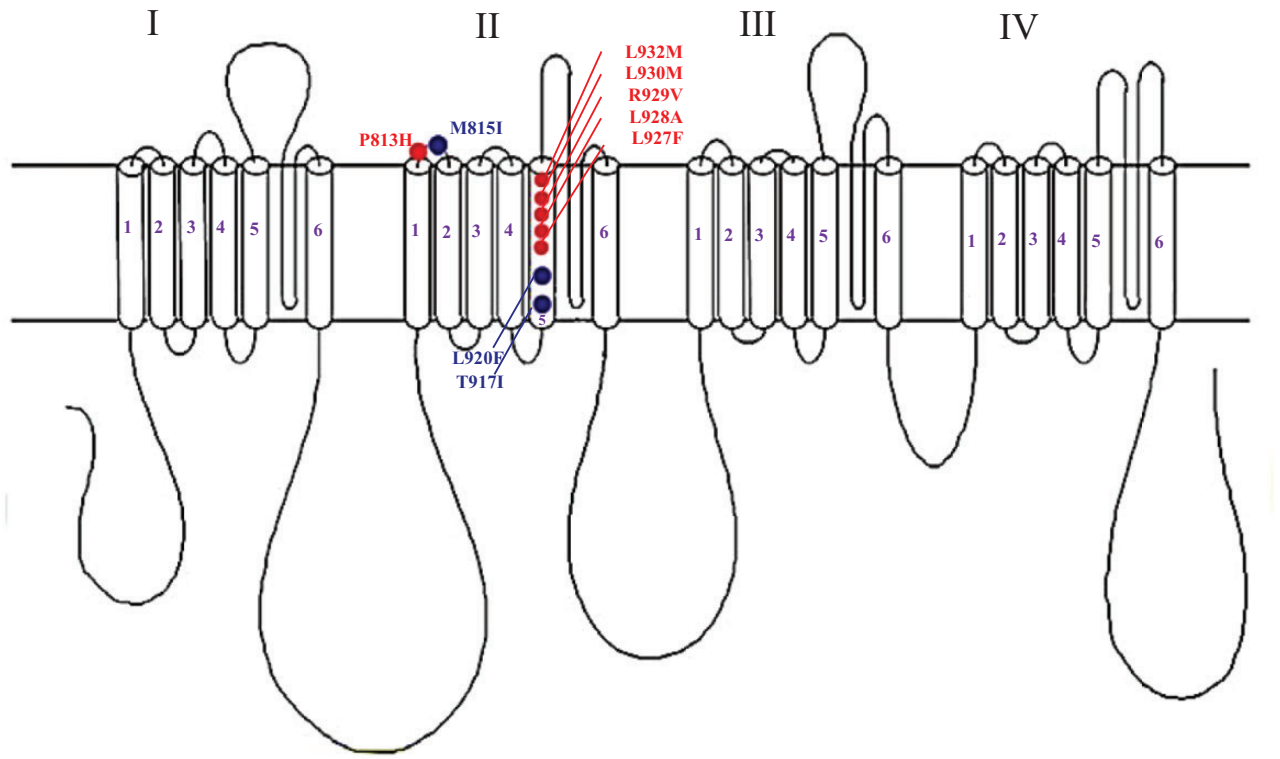

Fig. 3. Topology and schematic drawing of the sodium channel protein. Well-known mutations are presented in blue and novel mutation in red circles.

performing toxicological studies, will help to draw a *kdr* frequency map for decision making and policy making in the control of pediculosis.

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