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ORIGINAL ARTICLE

Distribution and variability of deformed wing virus of honeybees (*Apis mellifera*) in the Middle East and North Africa

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Abstract

Three hundred eleven honeybee samples from twelve countries in the Middle East and North Africa (MENA) (Jordan, Lebanon, Syria, Iraq, Egypt, Libya, Tunisia, Algeria, Morocco, Yemen, Palestine and Sudan) were analyzed for the presence of deformed wing virus (DWV). The prevalence of DWV throughout the MENA region was pervasive, but variable. The highest prevalence was found in Lebanon and Syria, with prevalence dropping in Palestine, Jordan and Egypt before increasing slightly moving westwards to Algeria and Morocco Phylogenetic analysis of a 194 nucleotide section of the DWV Lp gene did not identify any significant phylogenetic resolution among the samples, although the sequences did show consistent regional clustering, including an interesting geographic gradient from Morocco through North Africa to Jordan and Syria. The sequences revealed several clear variability hotspots in the deduced amino acid sequence, that furthermore showed some patterns of regional identity. Furthermore, the sequence variants from the Middle East and North Africa appear more numerous and diverse than those from Europe.

Key words *Apis mellifera*; deformed wing virus; Middle East; North Africa; prevalence; variability

Introduction

Honeybees (*Apis mellifera* L.) play an important role in nature and agriculture, where they are needed for the pollination of numerous crops. In recent years, there have been increasing reports of substantial losses of honeybee colonies in many countries (vanEngelsdorp & Meixner, 2010). These mortalities have multiple causes, from the still mysterious rapid adult depopulation phenomena (vanEngelsdorp *et al*., 2009, 2011) and acute poisoning by novel insecticides (Rosenkranz *et al*., 2010; Blacquiere *et al.*, 2012; Lu *et al.*, 2014) to the more common mortalities due to honeybee parasites and pathogens, such as Nosema (Fries, 2010), foulbrood (Forsgren & Fries, 2010) and in particular the parasitic mite *Varroa destructor* (Rosenkranz *et al.*, 2010) and the lethal virus epidemics it initiates and vectors (de Miranda & Genersch, 2010; de Miranda *et al*., 2010).

In the Arab world, abnormal mortality and local disappearances of bees are often reported by beekeepers (Haddad, 2011; Adjlane *et al*., 2012) and there has been considerable recent

research in the region on the prevalence of *Varroa* (El-Niweiri & El-Sarrag, 2006; Loucif Ayad *et al.*, 2008; Adjlane, 2010; Belaid & Doumandji, 2010), Nosema, American foulbrood (AFB), the tracheal mite *Acarapis woodi* (El-Niweiri *et al*., 2008; Adjlane *et al*., 2012a; Adjlane *et al*., 2012b; Haddad, 2014) and honeybee viruses (Haddad *et al*., 2008; Loucif-Ayad *et al*., 2013; Adjlane & Haddad, 2013) to complement historical records (summarised by Ellis & Munn, 2005). However, there have been very few studies on viral diseases of honeybees that cover the entire Middle East and North Africa region (hereafter referred to as MENA region) and none concerning the genetic character and diversity of the viruses in such colonies.

The principal virus associated with elevated colony mortality is *deformed wing virus* (DWV). This virus was first isolated in the 1970's from a sample of asymptomatic dead honeybees from Egypt, and called Egypt bee virus (EBV), before it was found to be serologically related to a virus from DWV-symptomatic bees from Japan, isolated in the early 1980's (Bailey & Ball, 1991).

DWV has become one of the major virus threats to the honeybee industry due to its synergism with *V. destructor* (Martin, 2001), which acts as an inducer and highly efficient vector of the virus (Bowen-Walker *et al*., 1999; Nordström, 2000; de Miranda & Genersch, 2010). The mites cause immunosuppression in the bees (Yang & Cox-Foster, 2005), activating inapparent DWV infections which are subsequently transmitted extremely efficiently by the mite, precipitating a lethal epidemic that increasingly also involves other transmission routes (de Miranda & Genersch, 2010). Even when it is not a direct vector, Varroa can increase the susceptibility of honeybees to other opportunistic pathogens (Yang & Cox-Foster, 2005), leading to a complex disease profile at colony level (Hung & Shimanuki, 1999; Tentcheva *et al*., 2004; de Miranda *et al*., 2012), and a progressive reduction in colony performance, followed by large-scale death. In the absence of mite, DWV is largely innocuous and often even undetectable, such that effective mite control stops and even reverses the DWV epidemic (Rosenkranz *et al*., 2010; Martin *et al*., 2010).

Typical symptoms exhibited by severely DWV-infected bees are crumpled wings, bloated abdomens, paralysis, learning impairment, and a drastically shortened life span (Bailey & Ball, 1991). DWV has been detected in all honeybee life stages, as well as glandular secretions (Chen *et al*., 2005; Yue & Genersch, 2005), through which it is orally transmitted to larvae. DWV is also infectious in bumble bees, where it causes similar symptoms

(Genersch *et al*., 2006), and it is an apiculture-derived threat to wild pollinators (Singh *et al*., 2010; Evison *et al*., 2011; Fürst *et al*., 2014).

The genomes of DWV and its close relative *Varroa destructor* virus-1 (VDV-1) were published in the mid 2000's (Lanzi *et al*., 2006; Fujiyuki *et al*., 2004, Ongus *et al*., 2004). The genome is 10 140 nucleotides long, excluding the poly-A tail, and contains a single large open reading frame encoding a 328-kilo Dalton (kDA) polyprotein. The coding region is flanked on the 5′ and 3′ sides by untranslated regions (UTR) of about 1140 and 317 nucleotides, containing sequences and RNA secondary structures for replication and translation (Lanzi *et al*., 2006; de Miranda & Genersch, 2010). Like all viruses, DWV exists as a dynamic population of variants, recombinants and mutations that provide the raw material for rapid adaptation, and that is constantly replenished through high rates of mutation and recombination (Domingo, 2012; de Miranda & Genersch, 2010; Moore *et al.*, 2011; Zioni *et al.*, 2012; Wang *et al*., 2013; Ryabov *et al*., 2014). Individual DWV variants have been linked to elevated aggression in bees (Fujiyuki *et al*., 2004) although DWV is of course not the only, or even the main factor in aggressive behaviour (Terio *et al*., 2008, Rortais *et al*., 2006). Other individual variants or recombinants have been linked to molecular virulence, i.e. high virus titres (Ryabov *et al*., 2014), and at bee population level there is evidence for a constriction of DWV genetic variation and the persistence of certain variants during successive mite invasions (Martin *et al*., 2012), suggesting a degree of adaptation to *Varroa*-mediated transmission and epidemiology. In addition to the specific traits of individual strains, virus variability itself can influence virulence and associated parameters, with more variable isolates often also more virulent (Lauring & Andino, 2010).

Despite the severity and impact of DWV in the MENA region, little is known about its genetic diversity and history in the region. This is the first study of the prevalence, phylogenetic relatedness and variability of DWV isolates collected from the MENA region.

Materials and methods

Sample collection

Three hundred eleven adult worker bee samples were collected during 2012 from 143 apiaries located in 12 countries, distributed as follows: Lebanon (9), Syria (19), Iraq (18), Palestine (10), Jordan (26), Egypt (10), Libya (3), Tunisia (5), Algeria (21), Morocco (9), Yemen (9), and Sudan (4), and with between 1 and 5 colonies sampled per apiary. The approximate locations of the sampled apiaries are shown in Figure 1. The colonies and

apiaries sampled were usually *Varroa*-infested and the bee samples included both bees with and without typical pathological symptoms of deformed wing virus infection. Each sample consisted of 10–15 bees, collected from inside the colonies, preserved in >20 mL 100% alcohol (Evans *et al.*, 2013) and shipped at ambient temperature to Jordan to arrive within three weeks of collection. Upon arrival, the bees were removed from the alcohol and stored at -80°C until RNA extraction. Ethanol was chosen as the most suitable preservative for the realities of the sampling conditions in the region.

This sample collection and preservation strategy, although perhaps not optimal from a purely molecular perspective (Chen *et al.*, 2007; Evans *et al.*, 2013), nevertheless represented the best overall strategy available for the modest objectives of the study, the scale of the study, the materials available and the local realities across the entire MENA region. This placed limits on the types of assay that can be reliably conducted on the samples, excluding those requiring highly intact RNA (Bustin *et al.*, 2009; Dainat *et al.*, 2011), as well as on the interpretation of the data.

RNA extraction

Five bees from each sample were homogenized in bulk using standard methods (de Miranda *et al*., 2013) and total RNA was purified from the homogenate using the RNeasy plant extraction kit (Qiagen). The approximate RNA concentration of each sample was determined using the NanoDrop 2000 (Thermo Scientific), and the RNA samples were stored at -80 ºC until further use.

RT-PCR

A simple, qualitative RT-PCR assay was chosen as the most appropriate DWV diagnostic tool for the sampling strategy employed, and the objectives of the study. The assay was based on a small (194 nucleotide) PCR fragment, to minimize the effects of possible RNA degradation on the ability to reliably detect DWV RNA, and thereby reduce the risk of false-negative data. One–Step RT-PCR was performed as described by Genersch (2005) using a One–step RT-PCR kit (Qiagen) following the manufacturer's instructions. Each reaction contained 0.5 ng total RNA, 0.1 mmol/L of forward (5′ CTTACTCTGCCGTCGCCCA 3′) and reverse (5′ CCGTTAGGAACTCATTATCGCG 3′) primers in 50 μL total volume. These primers amplify a 194 nucleotide section of the Lp gene (Lanzi *et al*. 2006), at the start of the main open reading frame, which is well-suited for DWV variability studies (de Miranda &

Genersch, 2010). These primers are designed conservatively, to allow the detection of most DWV variants in the sequence space between DWV and its closest relative, VDV-1 (Ongus *et al*., 2004), thereby also reducing the risk of false-negative data, in this case through DWV sequence divergence. The following cycling conditions were used: 30 min cDNA synthesis at 50ºC, 10 min denaturation at 95ºC followed by 40 cycles of: 30 s at 94ºC, 30 s at 65ºC, 30 s at 72ºC, followed by a final elongation step of 7 min at 72ºC. No template controls (NTC) were included in all assays. 10 μL of each reaction was analyzed on a 1.5% agarose gel containing ethidium bromide and visualized on a UV trans-illuminator to identify the 194 bp DWV-specific RT-PCR product produced by the assay.

Sequencing

Sixty-seven RT-PCR products out of 119 DWV-positive samples, distributed equitably between countries and apiaries, were selected for sequencing: Lebanon (3), Syria (13), Iraq (1), Palestine (0), Jordan (16), Egypt (3), Libya (0), Tunisia (4), Algeria (11), Morocco (11), Yemen (5), The PCR products were gel purified using MEGA quick-spin™ PCR & Agarose Gel DNA Extraction System according to the protocol recommended by the manufacturer. The purified RT-PCR products were sequenced in both orientations using the primers used for amplification. The forward and reverse sequences of each PCR product were combined into a single sequence. Any conflicts between the forward and reverse sequences, as well as instances of true polymorphism within the PCR product (Forsgren *et al*., 2009), were resolved by visual examination of the sequence electropherograms. The MENA sequences were submitted to GenBank under accession numbers KT591877–KT591943).

Phylogenetic and variability analyses

The sequences were aligned to homologous sequences found in the public nucleic acid databases using CLUSTAL-omega, at www.ebi.ac.uk (Sievers *et al.*, 2011; McWilliam *et al*., 2013). This added one sequence from Israel/Palestine (GenBank accession JF440526; Zioni *et al.*, 2011) to the MENA dataset, plus a large set of Swedish, European and Far East Asian sequences (GenBank accessions JF346550- JF346657; previously described in Forsgren *et al.*, 2009 and de Miranda & Genersch, 2010) for comparison. Phylogenetic analysis was performed in MEGA6 (Tamura *et al.*, 2013) using Minimum Evolution criteria. The robustness of the partitions was tested by bootstrap analysis (500 bootstraps). The variability among the DWV sequences was analysed by exporting the translated sequences to Excel,

tabulating the prevalences of the different amino acid variants at each position in different geographically-defined groups of sequences (34 each for the Middle East and North Africa & Yemen; 31 for Europe, 96 for Sweden and 21 for a range of Far East Asian sequences) and converting this information to basic stacked histograms.

Results

DWV prevalence

The survey results showed that DWV is present throughout the MENA region, with the possible exception of Sudan (Fig. 1) and that there is considerable variation in DWV prevalence between the MENA countries. A few patterns can be discerned: the highest prevalence was found in Syria and Lebanon, with a lower prevalence in Palestine, Jordan, Iraq and Yemen. This contrasts with a much lower prevalence in Egypt, Libya and Tunisia with a gradual increase again when moving westwards towards Algeria and Morocco (Fig. 1). These prevalence estimates are conservative (*i.e.* minimum) due to the risk of false-negative results, either because of RNA degradation (a risk of the sample preservation strategy) or because the local DWV variants lie outside the specificity of the DWV assay (see de Miranda *et al.*, 2013). Such risk is part of any sampling strategy and was minimized here by designing a simple, small PCR assay with highly conserved primers. The apparent absence of DWV from Sudan in particular should be interpreted with caution, since this result is based on just four samples.

Phylogenetic and variability analyses

The phylogenetic analysis of the nucleotide sequences reveals consistent regional clustering (Fig. 2), including an interesting geographic gradient from Morocco through North Africa to Jordan and Syria. Unfortunately; there is very little reliable resolution throughout the phylogeny except for the major branches, making it difficult to draw definitive conclusions from these trends in the data. A different approach to the data is to look at the patterns of DWV variability between different regions (Fig. 3). There are clear hotspots in the amino acid sequence, where variants are more frequent and more diverse. The same hotspots, and variants, appear in all regions, though in different combinations and frequencies. A particularly variable section of the sequence is between amino acids 38 and 52, where there are both numerous alternative variants at individual positions and different blends of variants in different geographic regions. Particular variants at individual positions can be identified

with particular countries within each region (Fig. 3). However, such variants are rarely consensual for a region and it is more the frequency and distribution of variants that is characteristic, rather than the variant itself. The other observation is that the sequence variants from the MENA region appear more numerous and diverse than the variants from Europe.

Discussion

DWV had previously detected in Turkey (Gülmez *et al*., 2009) and Israel (Soroker *et al*., 2011; Zioni *et al.*, 20011) as well as in Egypt, Tunisia and Saudi Arabia (Ellis & Munn, 2005). While neither *Varroa* nor DWV could be detected in a recent survey of Ugandan bees (Kajobe *et al*., 2010), both were detected frequently in Kenya (Muli *et al*., 2014) while *Varroa* has been in Sudan since 2006 at least (El-Niweiri & El-Sarrag, 2006; El-Niweiri *et al*., 2008) and has also been detected in Ethiopia, together with symptoms typical of DWV infection (Begna, 2015).

The high prevalence of DWV throughout the MENA region is in itself not surprising: it merely confirms the known history of *V. destructor* infestation in the region, which is the primary driver of DWV epidemiology (de Miranda & Genersch, 2010). It is the variation in prevalence between the countries that is perhaps more informative, since these may be related to the *Varroa* invasion history of the different countries: the countries along the Red Sea and in East African are thought to have been invaded relatively recently, with limited impact and possibly positive relationship between geographic elevation and the severity of varroasis (Muli *et al*., 2014). This contrasts with the much longer history of *Varroa* infestation in the Middle East, where *Varroa* was first detected in the 1980's in Jordan, and caused heavy losses to the commercial and traditional colonies (Ministry of Agriculture 1986), most likely due to DWV transmission (Haddad *et al*., 2008). Regional and national differences in varroa management strategies may also have affected the prevalence patterns. Similar observations of the progress of disease and colony losses following the *Varroa* infestation front have been made in other countries (Ball and Bailey, 1991; Shimanuki *et al.*, 1994; Topley *et al*., 2005; Dainat *et al.*, 2008, Martin *et al*., 2013), including the recent *Varroa* invasions in Hawaii (Martin *et al.*, 2012) and New Zealand (Stevenson *et al*., 2006; Mondet *et al.*, 2014).

Likewise, the documentation of various DWV genetic micro-variants throughout the MENA region is itself not particularly informative: such differences are expected when surveying broad geographic regions with a large degree of isolation. Rather, it is the trends in

the DWV phylogenetic and variability data that are of interest, with lower DWV variability in the regions with a longer history of *Varroa* infestation (see also Martin *et al*., 2012), and a phylogenetic clustering pattern that matches this geographic trend, with the more distally clustering variants from regions with longer *Varroa* invasion history (and perhaps longer evolutionary isolation) with the more proximally clustering variants in regions with a more recent invasion history.

It should be stressed here that none of the phylogenetic clustering patterns are statistically significant, and that both the tree, the variability data and the prevalence data are equally supportive of a number of alternative *Varroa* and/or DWV invasion scenarios, not necessarily purely lineal from the Middle-East westwards through North Africa. Importation and regional trade in bees and bee products is an efficient means of spreading disease agents and could easily have contributed to the data patterns found here. Such conclusions, as well as possible links between virus variants /variability and molecular or biological virus virulence, require more precise and dedicated studies.

Conclusion

The deformed wing virus (DWV) is present throughout the Middle East and North Africa (MENA) region, with discernible trends and variation across the region in DWV prevalence, nucleotide sequence and genetic variability, despite the absence of statistical significance to most of the differences between regions and countries. This is the first study of its kind that reports the prevalence of DWV in honeybees across the MENA region countries, and that evaluates the variability of DWV sequences within the MENA region and compares this to the north Mediterranean basin and Europe.

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Disclosure

The authors declare no conflicts of interest, or specific financial interests, relationships or affiliations.

Authors' contributions

Designed, fundraising & coordinated the experiments (NJH); provided samples-data (All); conducted the experiments (NJH, BAS, EA), analyzed the data (NJH, JdM, OY, WH); prepared the manuscript (NJH, JdM, OY, AN). All authors read and approved the final manuscript.

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Fig. 1 Origin of samples and DWV prevalence in the MENA region countries. Red dots denote positive samples, MENA apiaries (note that the map has insufficient resolution for uniquely marking all apiaries); blue dots mark the origin of European DWV sequences used in the phylogenetic and variability analysis. The number of samples from each country is indicated within the respective prevalence pie-chart. The number of apiaries per country is presented in the table within the figure.

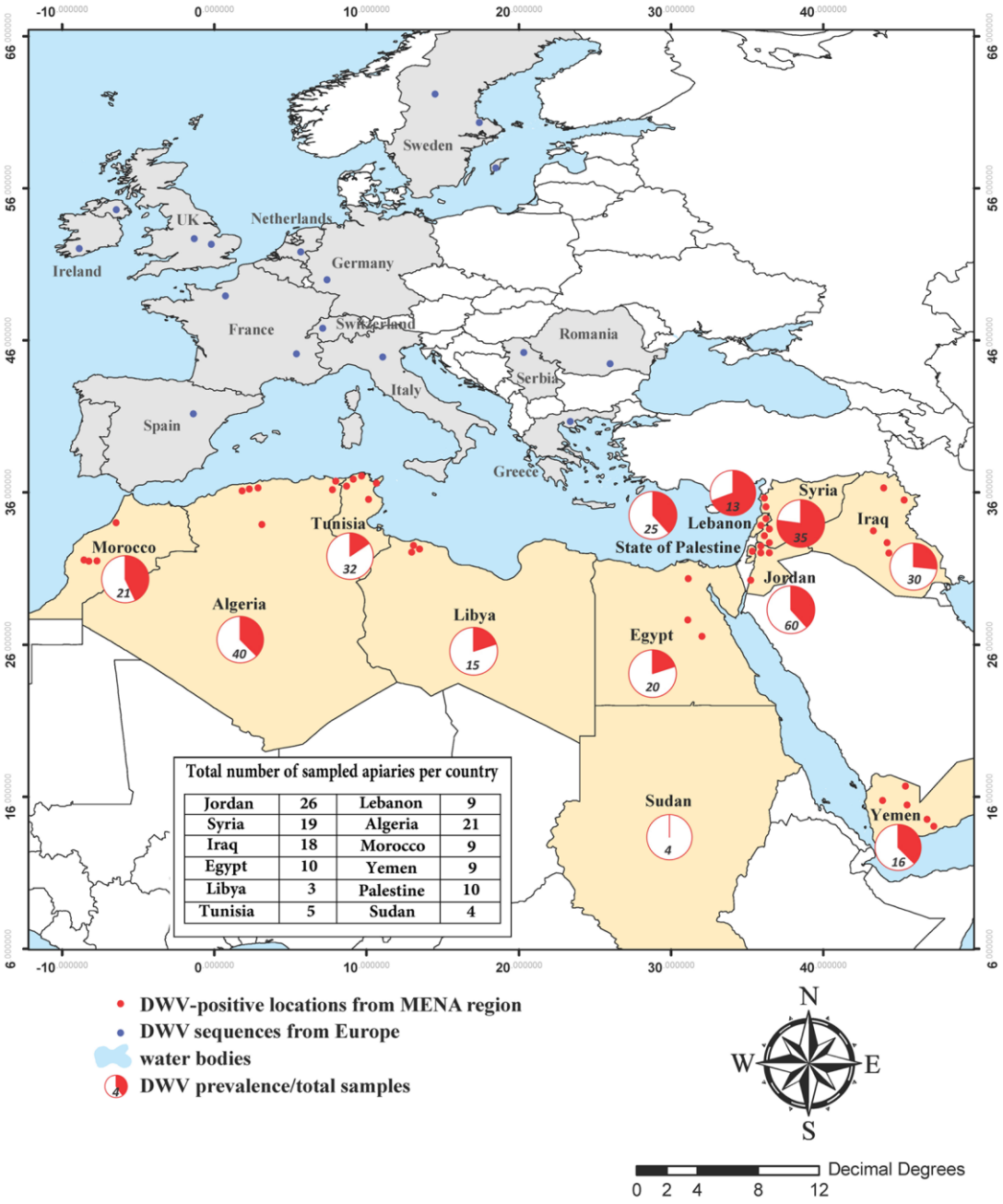

Fig. 2 Phylogram of the genetic relationships between the DWV isolates from the MENA region (current study), Europe and Asia, as determined from a 194 nucleotide fragment of the DWV Lp gene. The statistical strength of the nodes is shown as the percentage of correct partitions in a 500 replicate bootstrap analysis. Only bootstrap values > 65% are shown. Different geographic regions are identified by different coloured circles.

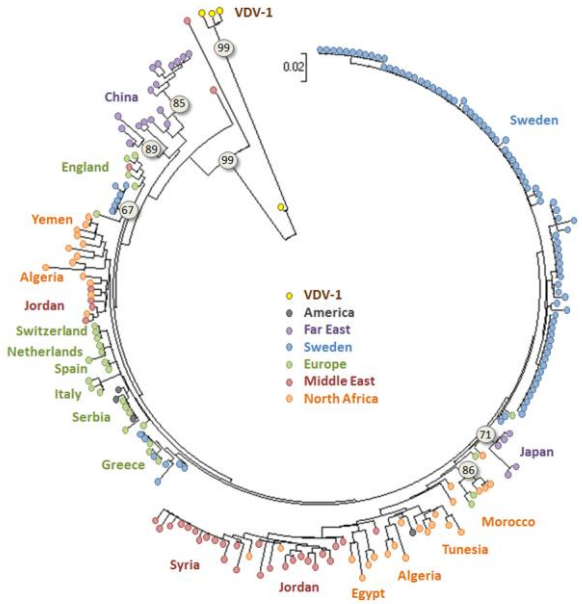

Fig. 3 Distribution and frequency of the DWV amino acid sequence variants within different geographic regions, based on the 194 nucleotide sequences of the DWV Lp-gene. The consensus amino acid sequence and the variant amino acids at each position are shown below the histogram. Those amino acid variants appearing only once in the entire dataset given in grey type, those appearing more than once are in black type. At each position the primary alternative amino acid variants are coloured blue in the histogram, followed by red, green and purple for the third, fourth and fifth variants.