Research Report

Analysis of Grain Aphid (*Sitobion avenae*) Populations – genetic composition and the frequency of pyrethroid resistance

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CONTENTS

1. SUMMARY

1.1. Aim

To determine the genetic variation in relation to the insecticide resistance properties of *Sitobion avenae* (grain aphid) in samples collected from suction traps in the UK.

The grain aphid, *Sitobion avenae*, the bird cherry–oat aphid, *Rhopalosiphum padi.* and the rose–grain aphid, *Metopolophium dirhodum,* are the main pest aphids on cereals in the UK. Depending on the species, they can cause direct damage by feeding on developing grain heads, or they can transmit destructive viruses such as barley yellow dwarf virus (BYDV). They also transmit poytviruses in potato crops, such as potato virus Y (PVY) and potato virus A (PVA). *S.avenae* has been shown to be a more efficient vector of PVY than either *R. padi* or *M. dirhodum*[1].

Pyrethroid spray failures were first noted in cereal crops in June 2011 for some populations of *S. avenae*. Samples of *S. avenae* were analysed for evidence of resistance and were shown to have a gene mutation that is associated with reduced sensitivity to pyrethroids. This was confirmed with bioassays showing a Resistance Factor of ~40. Additional testing of suction traps and field specimens found the resistance mechanism was reasonably widespread in the English *S. avenae* population.

In the UK, *S. avenae* can develop sexually as well as asexually. In autumn, males and females are produced which can mate and lay eggs to overwinter on grasses. This is quite distinct from the well understood insecticide-resistant aphid, the peach–potato aphid, *Myzus persicae*, which is thought to rarely lay eggs in the UK, existing instead as a series of distinct asexual clones. It is possible that pyrethroid resistant *S. avenae* consist of one or a few clones (as it can reproduce asexually all year round in the UK) however, it is also possible that the resistance gene has spread into different genotypes. Each aphid has two matching sets of genes and, in all of the samples tested to date, only one set of the genes carries the knockdown resistance (kdr) mutation which confers reduced sensitivity to pyrethroids (the aphids are referred to as heterozygous; kdr-SR (susceptible resistant)). This probably means that the aphids are less resistant than if both sets of genes carried the mutation (homozygous; kdr-RR (resistant resistant)). By monitoring the population for the kdr mutation and understanding the population genetic structure, it may be possible to model the likely trajectory of the evolving insecticide resistance in this species, as has been possible for *M. persicae*.

1.2. Methodology

A high throughput PCR-based TaqMan assay for detecting the kdr mutation in individual *S. avenae* had been developed prior to the start of the project (HGCA-funded summer bursary; Rothamsted Research, 2012). DNA extracted for kdr genotyping at Rothamsted was sent to James Hutton Institute for microsatellite

[1] REF values: are used to indicate how efficient an aphid species/clone is at transmitting a virus. In the case of PVY and PVA, *M. persicae* is the most efficient vector and has a REF value of 1. The virus transmission efficiency of other aphid species is compared to *M. persicae*. *S. avenae* has a REF value of 0.6; *R. padi* has a value of 0.4, *M. dirhodum* has a value of 0.3. The REF values are used in conjunction with aphid flights data to produce risk indices that are communicated via the aphid monitoring activity. (See Potato Council research project R428).

analysis. Occasional sequencing of kdr gene fragments was also carried out in order to identify any novel mutations.

1.3. Key findings

Overall, the frequency of *S. avenae* carrying kdr-SR collected in the English suction traps was similar in 2012 and 2013 suggesting that this form of resistance may have stabilised. The frequency of kdr-SR varied between English sites in 2013 from 0% (at Starcross) to a maximum of just over 50% (Kirton). Kirton consistently showed the highest kdr-SR frequency which may reflect a higher pyrethroid selection pressure in that area.

The kdr-SR frequency in Scottish *S. avenae* was overall lower than in the English population with 8% and 27% in the Edinburgh and Dundee traps, respectively, in 2013. No kdr-SR aphids were recorded from the Elgin and Ayr traps in 2013.

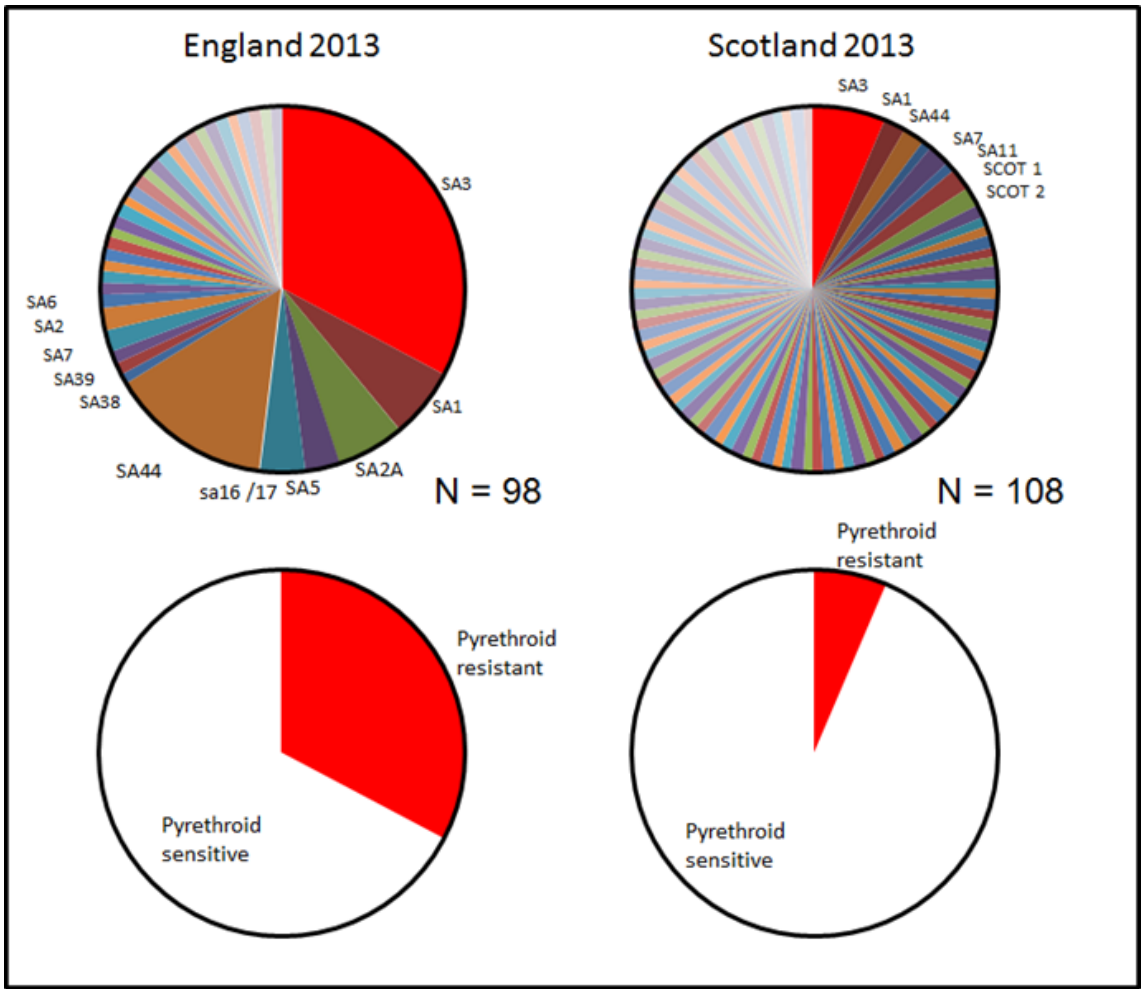
A subset of the *S. avenae* samples tested for the presence of the kdr mutation was also tested to understand their genetic make-up. If a genotype appears more than once during the analysis, this is considered as evidence of a clone (resulting from asexual reproduction). If a particular genotype appears to be unique then this suggests that the individual aphid has arisen from sexual reproduction.

So far, all of the *S. avenae* shown to have the kdr mutation (and therefore reduced sensitivity to pyrethroids) belong to a single genotype or clone. In addition to this clone, there were also at least seven other clones detected. These are all sensitive to pyrethroids (ie do not possess the kdr mutation). There were also many individual *S. avenae* that were a unique genotype.

England and Scotland differ in the proportion of the population that is clonal. In the areas of England sampled ~75% of the population was from one clone or another, whereas in Scotland, only ~10% was derived from a clone (see below). This is consistent with the hypothesis that the prevalence of individuals which have been derived from sexual reproduction increases at more northerly locations, where the conditions favour the sexual cycle.

Potato Council (PCL) is funding continuation of the survey of the frequency of the kdr-SR genotype and analysis of the genetic diversity of *S. avenae* populations in 2014. This will provide further information to determine if/how the *S.avenae* populations and frequency of the kdr mutation vary over time and across regions.

Genotypic composition of the Sitobion avenae UK population in 2013 and how this relates to pyrethroid resistance (SA3= kdr-SR, ie with reduced sensitivity to pyrethroids).



1.4. Practical recommendations

There are several sources of information on the occurrence of aphids during the growing season. AHDB provides funding towards the suction trap network, which provides information on the numbers of individual species including *S. avenae,* and towards monitoring aphids caught in yellow water traps.

www.rothamsted.ac.uk/insect-survey/bulletins
www.potato.org.uk/online-toolbox/aphid-monitoring
http://www.hgca.com/publications/2014/september/05/integrated-aphid-advisory-alerts.aspx

These tools can be used to indicate if/when numbers of *S. avenae* are present or increasing at susceptible crop stages. Consult a BASIS-qualified advisor regarding the aphid management options that may need to be applied.

2. INTRODUCTION

The grain aphid, *Sitobion avenae* (Fabricius) (Hemiptera: Aphididae) is a common pest of cultivated cereals throughout Europe and various other parts of the world (Kolbe and Linke, 1974). Its primary uncultivated hosts are various grasses and it is often associated with cocksfoot (*Dactylis glomerata*). In Britain it is a particularly important pest on barley, wheat and oats because it transmits at least four plant viruses including barley yellow dwarf virus (BYDV). However, it is also a vector of potato potyviruses and its relative efficiency in transmitting PVY was increased sixty-fold from 0.01 to 0.6 in 2013 (Potato Council project R428).

To control cereal aphids, including other species such as the bird cherry–oat aphid (*Rhopalosiphum padi*), foliar insecticide applications can be applied to cereal crops in the autumn (to prevent spread of BYDV) or in the spring and summer (to control direct feeding damage), with *S. avenae* often being the main target. Pyrethroids are a common choice because of their rapid aphid knockdown which aids in reducing virus transmission. In years when conditions favour aphids, there is, therefore, a risk of multiple applications of pyrethroids being used to control both *S. avenae* and *R. padi* during both the autumn and spring-summer period. On potato crops pyrethroids are also commonly used, particularly when there are no peach–potato aphids (*Myzus persicae*) present because this aphid species is now almost always resistant to pyrethroids in the UK (Foster and Dewar, 2013).

The widespread use of pyrethroids has, however, led to the selection of resistance in a range of insect pest species and is a serious problem for their continued, effective use. The most common resistance mechanism is termed knockdown resistance (kdr / super-kdr) and is caused by mutations in the voltage gated sodium channel gene that result in amino acid substitutions within the channel protein that reduce the sensitivity to pyrethroids (Davies *et al.,* 2007; Rinkevich *et al.,* 2013).

In England, during 2011, there were reports by growers and advisors of a reduced efficacy of pyrethroids sprayed against *S. avenae* on cereal crops in autumn. This has now been explained by the presence of the knockdown resistance (*kdr*) mutation (L1014F) in English samples of *S. avenae* (Foster *et al.*, 2014). Each aphid has two matching sets of genes and, in all of the samples tested to date, only one set of the genes carries the mutation (the aphids are referred to as heterozygous; kdr-SR (susceptible resistant)). This probably means that the aphids are less resistant than if both sets of genes carried the mutation (homozygous; kdr-RR). Adult aphids carrying the mutation kdr-SR display approximately 40-fold resistance to lambda-cyhalothrin, a representative pyrethroid used to control this pest (Foster *et al.,* 2014).

The recent identification of resistant aphids in England raised some obvious questions: how long has this mutation been around and how widespread is it? In this project we analysed UK samples of *S. avenae* for the presence of the kdr mutation using a PCR based allelic discrimination assay.

In a follow-on analysis, the same samples were tested with microsatellite primers to facilitate the analysis and understanding of the genetic diversity and structure of pyrethroid-resistant and -susceptible populations.

3. MATERIALS AND METHODS

Aphid samples

Sitobion avenae were collected from 12.2 m high suction traps across the UK. The location of the traps is provided in Figure 1. There were no grain aphids collected from the Wellesbourn site at the time the work was carried out. Use of the suction traps as the method of collection ensures that the aphid population is sampled randomly and, thereby, gives a good measure of population diversity for kdr and microsatellite genotype.

Figure 1. Location of UK suction traps.

DNA extraction and kdr genotyping

DNA was extracted from adult aphids using a modification of the sodium hydroxide method described by Malloch *et al*. (2006). Individual aphids were homogenised in the wells of a 96 well immunoplate with 20 µl of 0.25 M NaOH. The homogenates were heated at 99^{o}C for 3 minutes and neutralised with 10 µl of 0.25 M HCl, 5 µl of 0.5 M Tris HCl and 5 µl of 2 % Triton X-100. Samples were heated again at 99^{o}C for 3 minutes and the plates centrifuged at 4000 rpm for 5 minutes. Aliquots of the DNA supernatants were initially taken for kdr genotyping, with selected samples sent to James Hutton Institute for microsatellite analysis.

A PCR-based allelic discrimination assay (TaqMan) was used to detect the presence/absence of a mutation (kdr) in individual aphids and genotype them as susceptible (SS) or resistant (SR, RR) (Foster *et al*., in press). The technique uses

short fluorescent dye-labelled DNA probes that are selective for either the normal (susceptible) gene or the kdr (resistant) gene sequence. TaqMan PCR reactions were run on a Rotor-Gene 6000TM real-time PCR cycler using cycling conditions of 10 minutes at 95^{o}C, followed by 40 cycles of 95^{o}C for 10 seconds and 60^{o}C for 45 seconds. In total, 1,133 English aphids and 214 Scottish aphids were genotyped for the presence of kdr.

Microsatellite genotyping

A sub-sample of 270 individual kdr-SS and kdr-SR *S. avenae* were selected and analysed for microsatellite genotype (no kdr-RR aphids were found). These aphids were collected from traps in Scotland in 2012 and 2013, and from traps in England in 2013 (Table 1). The small number of aphids collected at Silwood Park and Wye were not genotyped. In Scotland, in 2013, the aphids were sampled when peak numbers of *S. avenae* were flying, with samples being taken to give a good geographical representation of the population. For the English samples, aphid DNAs were selected randomly across the traps to give a roughly equal number of susceptible (SS) and resistant (SR) samples for genotype analysis.

Table 1. Collections of *Sitobion avenae* from suction traps used for microsatellite analysis

Location	Months of collection	No. of insects	No. of resistant insects
Dundee	June – September 2012	32	8
Dundee	July – August 2013	44	4
Edinburgh	July – July 2012	3	1
Edinburgh	July – August 2013	10	3
Elgin	July – August 2012	29	0
Elgin	July – August 2013	46	0
Ayr	July – August 2013	8	0
Kirton	May – July 2013	23	12
Rothamsted	May – July 2013	21	6
Broom's Barn	May – August 2013	15	6
Hereford	May – July 2013	13	3
Starcross	May – July 2013	5	0
Preston	May – July 2013	9	2
Writtle	May – July 2013	4	1
Newcastle	July 2013	2	0

Genotypes of individual *S. avenae* were examined at five microsatellite loci: Sm10, Sm12, Sm17, Sa4Σ and S16b. Sm10, Sm 12 and Sm17 were isolated from *Sitobion*

miscanthi and described by Wilson *et al.* (1997) and Sunnucks *et al.* (1996, 1997). Primer sequences are reported for the first time in Simon *et al.*, (1999). Primer Sa4Σ was cloned from *S. avenae* (Simon *et al.,* 1999) and primer S16b was isolated from *S. miscanthi* by Wilson and Sunnucks and its sequence was published in Wilson *et al.* (2004).

Table 2. Primer sequences

Primer	Sequence	Repeat	Size range published	Size range observed	Reference
Sm10f	TCT GCT GCA TTA CTG TTG GC	(CA)23	152-240	149-197	SIMON ET AL 1999
Sm10r	TCG TCT ACT TCG CCG TCA	(CA)23	152-240	149-197	SIMON ET AL 1999
Sm12 f	CAC CAT CGC GTT TCA TCT TA	(CA)33	127-177	112 (133)-154(175)	Llewellyn et al 2003
Sm12r	ACT CCC AAC CTC TGA TGA GC	(CA)33	127-177	112 (133)-154(175)	Llewellyn et al 2003
S16bf	ATA AAA CAA AGA GCA ATT CC	(CA) 14	166-206	158-281	Wilson et al 2004
S16br	GTA AAA GTA AAG GTT CCA CG	(CA)14	166-206	158-281	Wilson et al 2004
Sm17f	TGG ACA TTT CAT CGT TCG C	(TC)14AC(TC)3	174-185	88-97	Simon et al., 1999
Sm17r	ATG CGT TCG AGT TTA CCT GC	(TC)14AC(TC)3	174-185	88-97	Simon et al., 1999
SA4ΣF	GTG ACG TAT AAC GCG ATG CG	(AC)5TT(AC)16	162-176	155-213	Simon et al 1999
SA4ΣR	GAC GTC GAT ATT AGC CTA GCC	(AC)5TT(AC)16	162-176	155-213	Simon et al 1999

The above loci were selected for use as they had the greatest number of alleles reported in the literature (Table 3).

Table 3. The number of alleles reported for the loci used

primer	repeat	size range	no. of alleles
Sm 10	(CA)23	152-240	16
Sm12	(CA)33	127-177	19
Sm17	(TC14 AC (TC)3	174- 185	8
Sa4S	(AC)5 TT (AC)16	162-176	12
S16b	(CA)14	166-206	12

The size ranges are in base pairs. The Sm12F primer sequence we used is the redesigned version in Llewellyn *et al*. (2003). PCR products are 21bp smaller than those produced with the original primer set.

PCR was carried out in 8µl volumes using Illustra^{TM} Ready to Go PCR beads (GE Healthcare). When the bead is reconstituted, the concentration of each dNTP is 200µM in 10 mM Tris-HCl, 50 mM KCl and 1.5 mM $MgCl_2$. Each bead contains 2.5U of Taq DNA polymerase. PCR was carried out on a Biometra T Personal thermal cycler using the Touchdown programme described in Sloane *et al.* (2001).

4. RESULTS

An allelic discrimination (TaqMan) PCR diagnostic test which detects the presence of the kdr mutation (L1014F) has been developed for *S. avenae* (see Materials and Methods). The TaqMan assay is a PCR method that uses oligonucleotide probes that are dual labelled with a fluorescent reporter dye and a quencher molecule. Amplification of the probe-specific product causes cleavage of the probe, generating an increase in reporter fluorescence as the reporter dye is released from the quencher. By using different reporter dyes (VIC and FAM), cleavage of the allele-specific probes can be detected in a single PCR reaction. Comparison of control DNA from *S. avenae* of known genotypes allows discrimination of the wild-type and resistant (kdr) alleles. The assay uses two probes and an increase in fluorescence indicates whether the individual is a homozygous wild type individual (kdr-SS), a heterozygous mutant type (kdr-SR) or a homozygous mutant type (kdr-RR). To help assign the genotypes, software is used to plot fluorescence values for the two dyes on bidirectional scatter plots.

Kdr-SR frequency

The numbers and frequency of kdr-RS and sensitive (kdr-SS) *S. avenae* collected in the English suction traps in 2012 and 2013 are shown in Figs. 2–5 and in the Scottish suction traps in 2012 and 2013 in Figs. 6–9 (resistance data for Dundee only in 2012). The frequency of kdr-SR varied between English sites from 0% (at Starcross in 2013) to a maximum of just over 50% in others. Kirton consistently showed the highest kdr-SR frequency which may reflect a higher pyrethroid selection pressure in that area. The kdr-SR frequency in Scottish *S. avenae* was, overall, lower than in the English population with 14% in the Dundee trap in 2012 (the only trap catch tested) and 8% and 27% in the Edinburgh and Dundee traps, respectively, and no kdr-SR aphids being found in the Elgin and Ayr traps in 2013.

Figure 2. Number of kdr-SR and kdr-SS *Sitobion avenae* in English suction traps in 2012

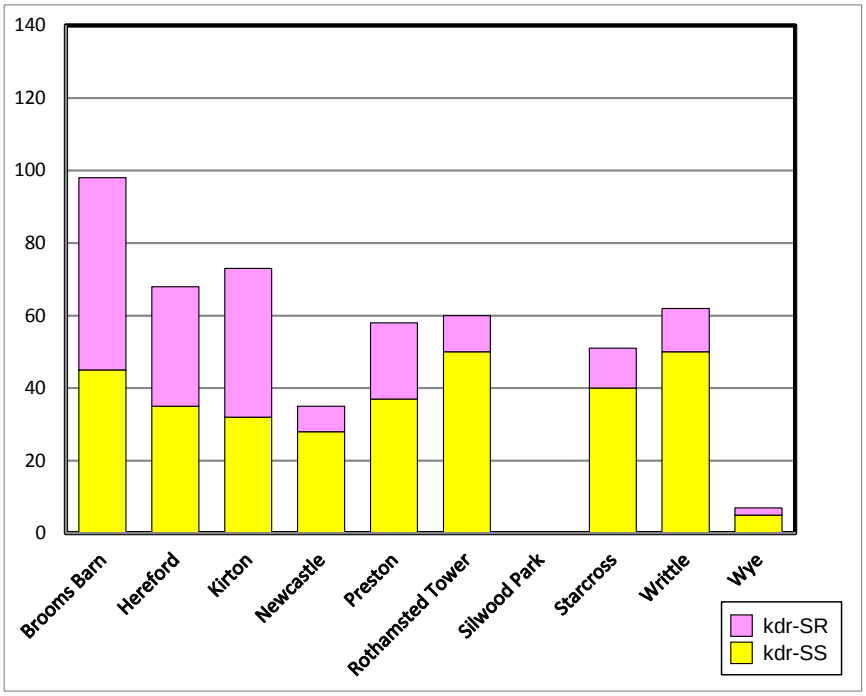


Figure 3. Number of kdr-SR and kdr-SS *Sitobion avenae* in English suction traps in 2013

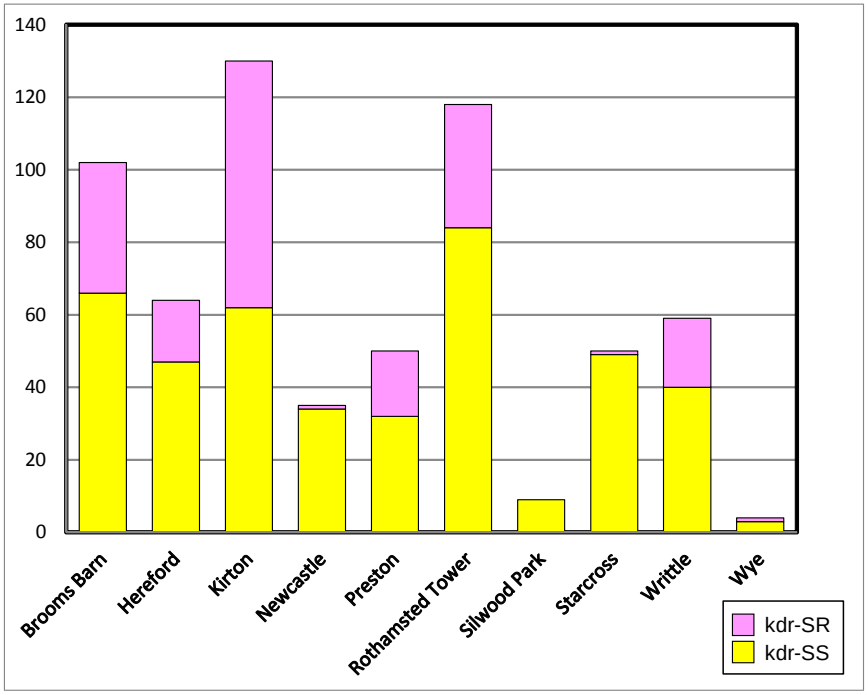


Figure 4. Frequency of kdr-SR and kdr-SS *Sitobion avenae* in English suction traps in 2012

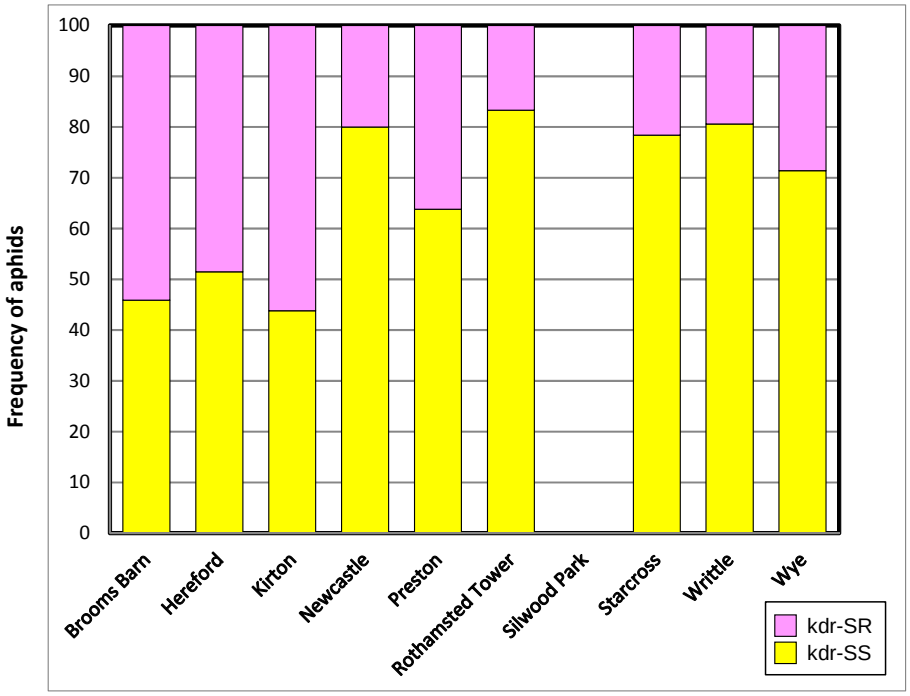


Figure 5. Frequency of kdr-SR and kdr-SS *Sitobion avenae* in English suction traps in 2013

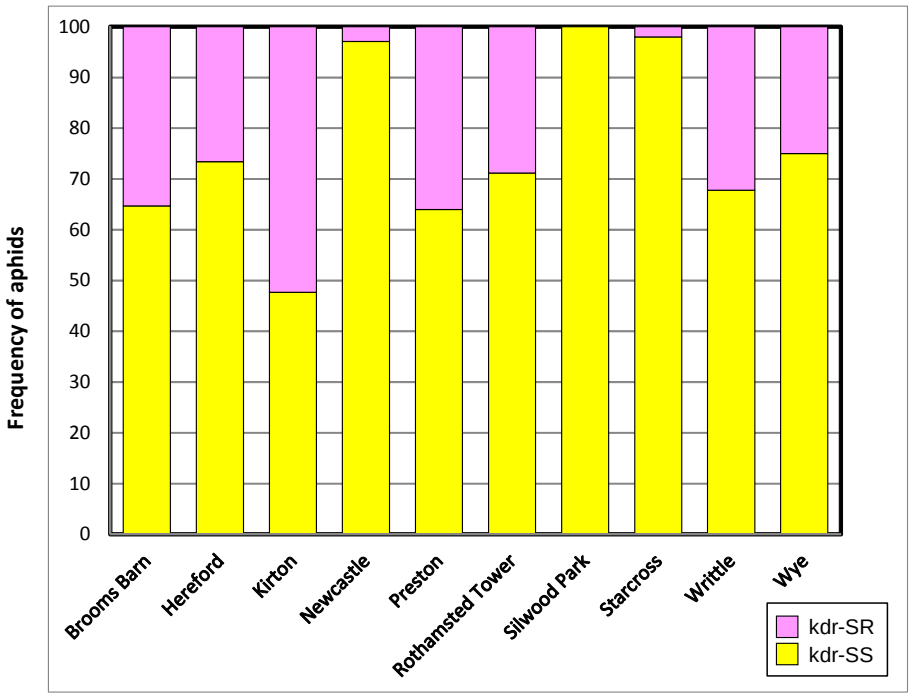


Figure 6. Number of kdr-SR and kdr-SS *Sitobion avenae* in Dundee suction trap in 2012

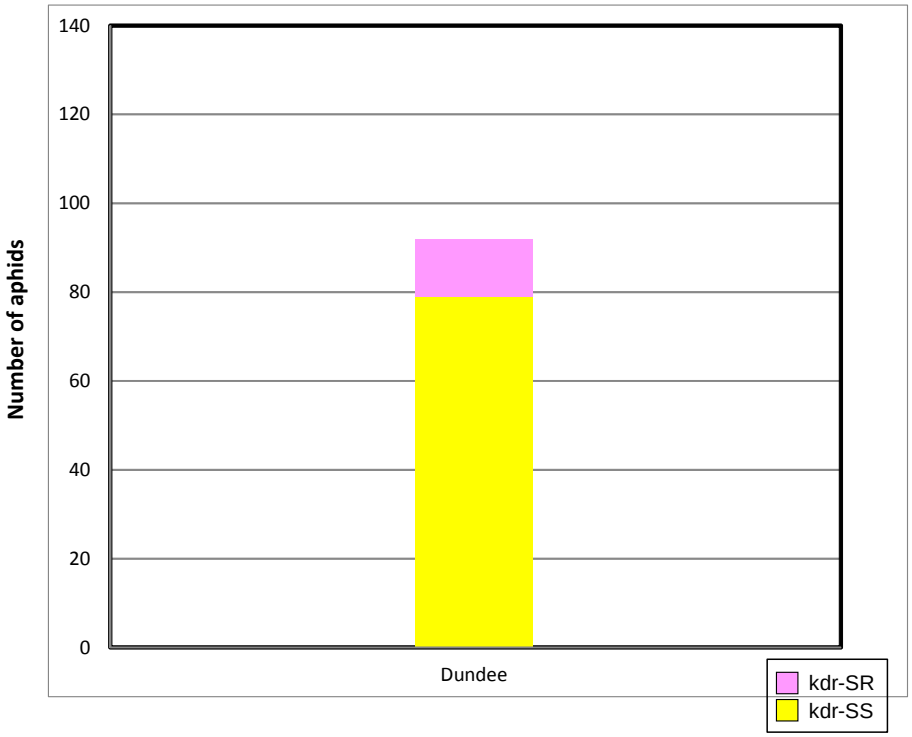


Figure 7. Frequency of kdr-SR and kdr-SS *Sitobion avenae* in Dundee suction trap in 2012

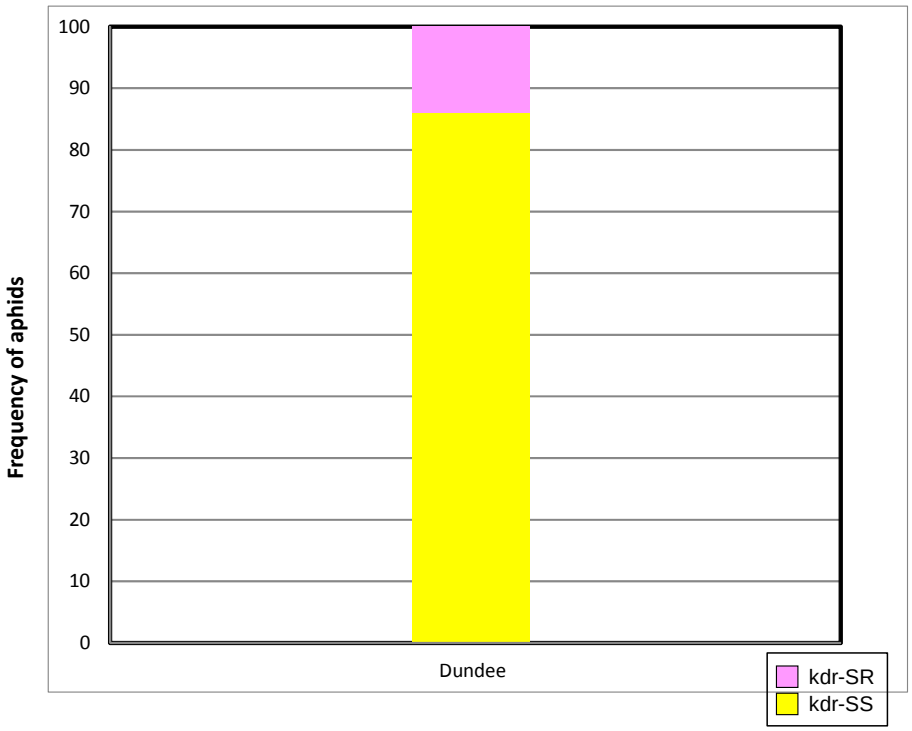


Figure 8. Number of kdr-SR and kdr-SS *Sitobion avenae* in Scottish suction traps in 2013

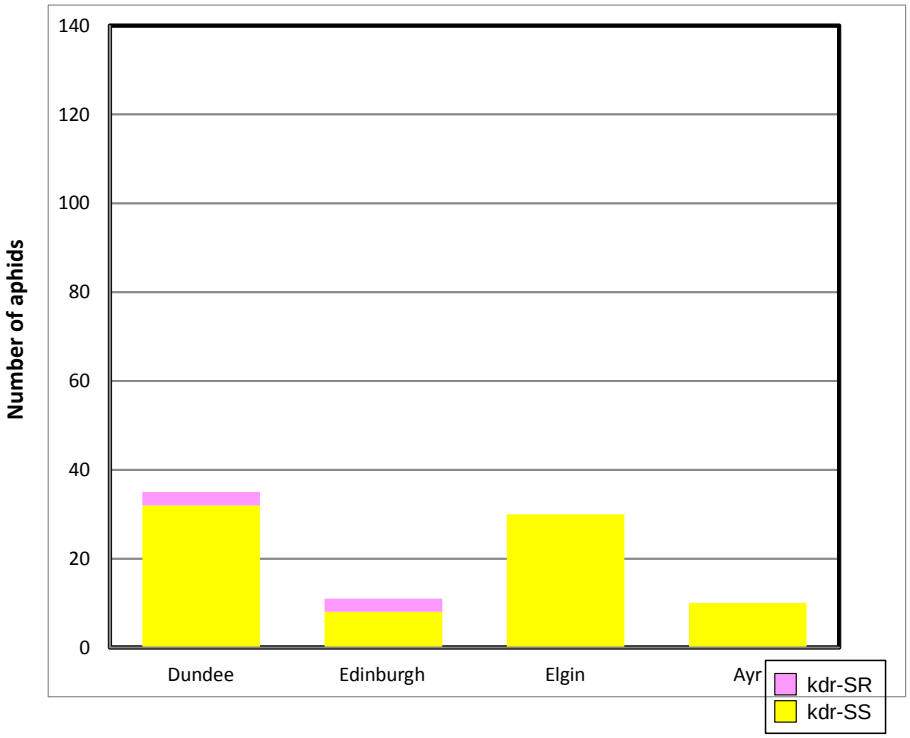
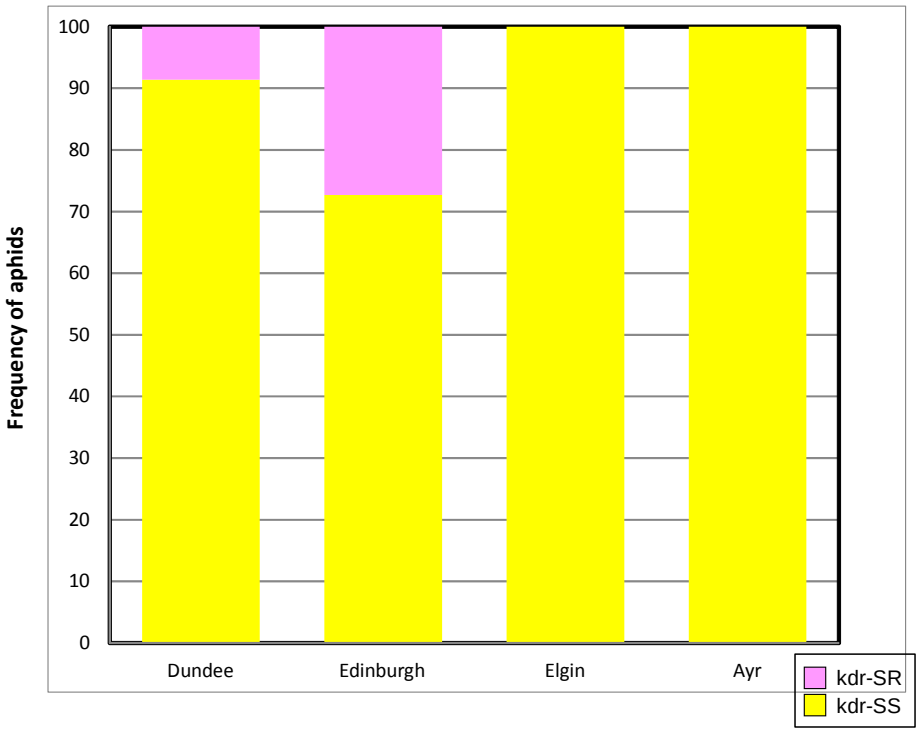


Figure 9. Frequency of kdr-SR and kdr-SS *Sitobion avenae* in Scottish suction traps in 2013



Genotypic composition of the English & Scottish *S. avenae* population

Microsatellite primers had been developed for *Sitobion* species in the 1990s. The technology of the time meant these were analysed using radioactive tracers and on large plate gel electrophoresis systems. The sizes were compared to a DNA sequencing ladder. However, there have been many advances in technology and the capillary fluorescent systems used now allow multiplexing and much greater accuracy of band sizing. There were complications associated with the development of the required new assays. These are described in Annex 1. However, from an initial screen of six primers a final analysis using two multiplex reactions was developed. It was unfortunate that the markers could not be analysed in a single multiplex as this would have been even more efficient.

Sitobion avenae clones

Once the microsatellite system was available, the first test was to examine the genetic composition of the kdr-SS and kdr-SR populations. Samples that represented both pyrethroid resistant and sensitive aphids were analysed. In the first run there appeared to be many genotypes in the kdr-SS population and a few in the kdr-SR population. The kdr-SR population was dominated by a single genotype. This fitted with a hypothesis that a single clone had mutated, but there were some exceptions. The exceptions were double checked by retesting the pyrethroid resistance using both TaqMan assays and gene sequencing. This changed the result after one retest. The pattern settled into all of the kdr-SR belonging to a single genotype or clone.

Apart from the common resistant clone, seven additional susceptible common clones were found in both England and Scotland (shown boxed in Table 4). The remaining aphids in the UK *S. avenae* population were of a unique genotype.

Table 4. Common *Sitobion avenae* genotypes

Genotype	Resistance	England 2013	Scotland 2013	Scotland 2012	UK 2013	UK 2012 / 2013
SA3	SR	32	7	9	39	48
SA1	SS	6	2	5	8	13
SA2A	SS	6	0	1	6	7
SA2	SS	2	0	0	2	2
SA5	SS	3	0	0	3	3
SA44	SS	14	2	0	16	16
SA16 17	SS	4	0	0	4	4
SA6	SS	2	0	0	2	2
SA7	SS	1	2	1	3	4
clone scot1	SS	0	2	0	2	2
SA11	SS	1	1	1	1	2
SA1D	SS	0	0	2	0	2
SA38	SS	1	1	1	2	3
SA39	SS	1	0	1	1	2
clone scot2	SS	0	2	0	2	2
other unique	SS	25	89	43	115	158
Total number		98	108	64	206	270

The distribution of clones between Scotland and England. The red boxes denote clones found in both countries, including the pyrethroid resistant clone SA3. In this sample, the remaining six clones were found in England or Scotland, but not both.

Table 5. Allele sizes of common clones

Clone	S16b	S16b	Sm12	Sm12	Sm10	Sm10	Sm17	Sm17	saΣ4	saΣ4	Location	Ref
SA3	173	211	115	146	161	163	92	96	162	163	resistant clone UK	This study
SA2A	173	211	115	126	160	163	92	96	163	172	UK	This study
SA2	173	211	115	126	149	163	92	93	162	172	ENGLAND	This study
SA7	173	217	115	115	160	163	92	96	163	172	UK	This study
SA11	173	215	115	126	161	163	92	93	162	163	UK	This study
SA 16 17	173	163	128	128	160	180	92	93	161	163	ENGLAND	This study
SA6	173	190	115	126	162	163	92	93	161	164	ENGLAND	This study
SA1	173	266	115	115	157	161	92	96	163	172	UK	This study
SA5	173	266	115	134	160	161	92	96	163	172	ENGLAND	This study
SA1D	211	217	115	115	161	163	92	93	163	172	ENGLAND	This study
SA38	211	241	115	136	160	161	92	96	161	163	UK	This study
SA39	211	279	115	115	160	161	92	93	161	163	UK	This study
SA44	266	279	115	128	160	161	92	96	161	164	UK	This study
Scot 2	158	215	132	134	161	163	92	93	157	164	SCOTLAND	This study
Scot 1	173	211	115	130	161	161	92	93	164	172	SCOTLAND	This study
geno2	150	206	165 (144)	165 (144)	164	166	178	179	163	169	FRANCE	Haack *et al.*, 2000
geno5	162	176	151 (130)	151 (130)	164	166	178	178	169	169	FRANCE	Haack *et al.*, 2000
clone 53	nt	nt	130	144	164	166	178	179	nt	nt	FRANCE and UK	Llewellyn *et al.*, 2003
clone114	nt	nt	138	138	152	166	178	178	nt	nt	FRANCE and UK	Llewellyn *et al.*, 2003
clone 58	nt	nt	118	118	164	168	183	183	nt	nt	UK	Llewellyn *et al.*, 2003
clone 30	nt	nt	118	132	164	164	178	183	nt	nt	UK	Llewellyn *et al.*, 2003
clone 20	nt	nt	118	118	160	164	178	183	nt	nt	UK	Llewellyn *et al.*, 2003
clone 21	nt	nt	118	118	160	164	183	183	nt	nt	UK	Llewellyn *et al.*, 2003

The sizes of the microsatellite markers in bp from the different clones in this study and earlier studies. Each locus has two columns for the size of each allele. There are five loci, producing ten columns. The other columns are the clone designation, the location and any citations.

Seasonal and geographical variation in clones

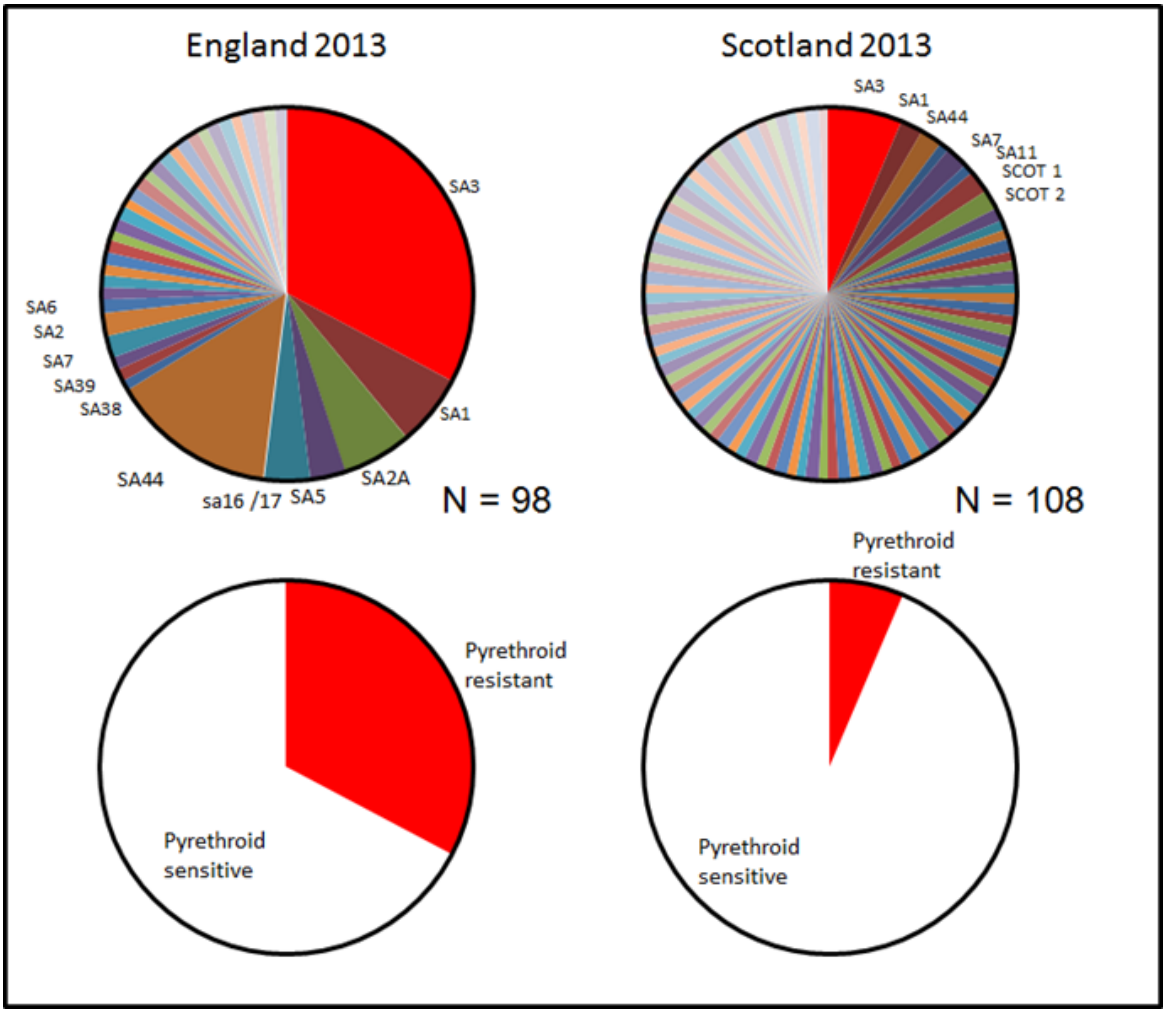
In both England and Scotland there were 158 genotypes of *S. avenae* from a total sample of 270 tested (Table 4). Wherever a genotype appears more than once, then this is considered as evidence of a clone. This contrasts dramatically with the peach–potato aphid, *Myzus persicae*, where, for the last four years, the entire population has

consisted of only two genotypes or clones. These observations confirm underlying differences in life history, with *S. avenae* being derived from sexual and asexual sources, whereas *M. persicae* is only derived from asexual sources in the UK.

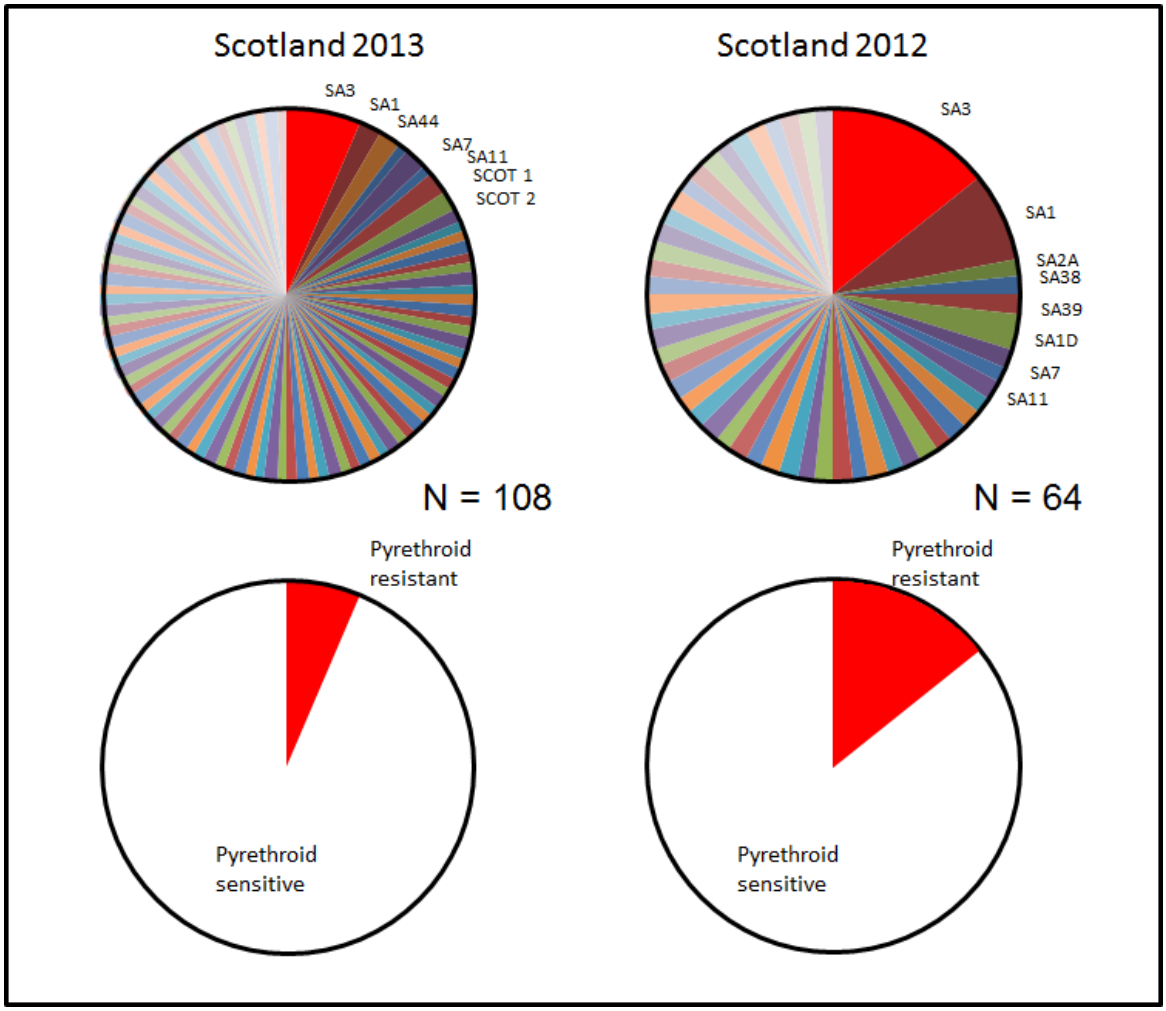
England and Scotland do differ in the proportion of the population that is clonal. In the areas of England sampled ~75% of the population was from one clone or another, whereas, in Scotland, only ~10% was derived from a clone (Figure 10). This is consistent with the hypothesis that the prevalence of individuals which have been derived from sexual reproduction increases at more northerly locations, where the conditions favour the sexual cycle.

Figure 10. Genotypic composition of the *Sitobion avenae* UK population and how this relates to pyrethroid resistance. A: England vs Scotland, 2013. B: Scotland 2012 vs 2013

A.



B.



5. DISCUSSION

Overall, the frequency of *S. avenae* carrying kdr-SR collected in the English suction traps was similar in 2012 and 2013, suggesting that this form of resistance may have stabilised (at a maximum of just over 50% of the aphids collected at several sites) since its increase between 2009 and 2012. The frequency of kdr was overall lower in Scotland in the year it was tested (2013). This may reflect differences in climate, ecology or pyrethroid selection pressure affecting this species.

The population structure of *S. avenae* was genetically diverse, when compared to other aphids such as *M. persicae*, but clones of *S. avenae* were present. The simplest hypothesis is that common *S. avenae* clones or those occurring from year to year e.g. SA1, SA3 and SA44 are asexual and have lost the ability to produce sexual forms. This hypothesis is based on an assumption that the suction traps will not be as biased as samples taken directly from fields where unique individuals will have had an opportunity to reproduce asexually on the crop creating local seasonal clones. The candidate asexual clones include the resistant clone SA3 which survives from one year to the next. If this clone was capable of undergoing sexual reproduction to produce overwintering eggs then the kdr mutation would be found in new genetic backgrounds the following season. Clone SA3 has been recorded as early as 2009, so there have been four winters where sexual reproduction could have occurred, yet the kdr mutation has not been detected in any new genetic backgrounds. However, other factors could be at play, such as a requirement for a balancing physiology involving

other gene products which tolerate the kdr mutation. This would require the kdr mutation being maintained alongside other allelic combinations at different loci.

In addition to mating with different genotypes, the aphid life cycle also permits the SA3 clone to mate with itself, creating new recombinants some of which would also be homozygous-kdr (RR) aphids, carrying two copies of the mutated gene. These would be more resistant to pyrethroids gaining a greater selection advantage. However, this combination has not yet been found, despite many aphids from a range of locations in England being analysed. The absence of any kdr-RR *S. avenae* could also be due to the fact that the single (SR) clone that carries kdr, in the heterozygous form, can only reproduce asexually and is unable to produce sexual forms in the autumn, a hypothesis that can be tested. Alternatively, homozygote-resistant aphids may be being produced through sexual reproduction (which is thought to occur at a relatively low level in *S. avenae* in the UK) but these suffer some form of strong fitness cost in the absence of insecticides in a similar fashion to what is thought to occur with homozygote kdr *M. persicae* (Foster *et al*., 2011).

It seems most likely that the sodium channel gene in clone SA3 was in a suitable genetic background to tolerate the kdr mutation. Mutations will occur at a set frequency and if this was an abundant clone then the numbers alone would favour the clone, eventually mutating and insecticide selection increasing the frequency of this clone. It is fortunate that the 1997 and 1998 *S. avenae* population in the UK were analysed by Llewellyn *et al,*(2003, 2004) prior to the mutation event. However, it is not possible to directly compare the results of microsatellite analysis as the technology has diverged, but comparisons could be made by analysing stored suction trap samples dating from these study periods. It is clear from previous studies and the current study that clonal diversity of *S. avenae* varies, with more diversity in northern regions. There is evidence of the survival of asexual clones over extended periods of time in the absence of any insecticide selection. There is also evidence that genotypes can turnover, i.e. common clones can become less common and even disappear. The long term fate of the SA3 clone could be determined by natural turnover processes, such as winter hardiness. This may be more important to its long term survival than its selective advantage of containing the kdr mutation. Should the SA3 clone be found as a historically common clone, then this would suggest that it is robust and the mutation occurred in this background because it was a common clone. Conversely, if it had not been detected before 2009, then it is most likely a mutation occurred in a suitable genetic background in an ecologically less successful asexual clone and there is a reasonable chance that SA3 will slowly die out.

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7. APPENDICES

Annex 1: *Complicated history of recording* Sitobion avenae *microsatellite primer sequences*

References for primer sequences in many of the historical *S. avenae* microsatellite studies are incorrect. In most publications where *Sitobion* microsatellite primers are used, the authors refer to Sunnucks *et al*. (1996, 1997) and Wilson et al. (1997) for the primer sequences. However, the authors of these publications do not report the primer sequences in these manuscripts and suggest that the sequences will be made available on request. The first publication of primers Sm10, Sm11, Sm12 Sm17 and Sa4ε occurs in Simon *et al.* (1999). And the sequences of primer set S16b are recorded in Wilson *et al.* (2004). However, the sequences of SmS10 and SmS12 in Wilson et al. (2004) are not the same as those of Sm10 and Sm12 reported in Simon et al. (1999). These may be completely different primers designed to amplify a different region of DNA. The primer sequences reported in Simon et al. (1999), Sm10 and Sm12, are designed to amplify (CA)23 and (CA)33 .Those reported in reported in Wilson *et al.* (2004) named Sm S10 and Sm S12 are designed to amplify (AC)16 and (CA)8 repeat regions. In many of the *Sitobion* studies it is not clear which primer sets have been used.

In addition to the complications described above, primer Sm12f was revised and modified in Llewellyn *et al.* (2003) and the resulting PCR products are 21bp shorter than those amplified with the original primer. Furthermore, Simon *et al.* (1999) used a new reverse primer (Sm17r2 = ATG CGT TCG AGT TTA CCT GC) in conjunction with the original Sm17f reported to be described in Sunnucks *et al.* (1996, 1997) and Wilson *et al.* (1997). It therefore becomes impossible to determine which results can be directly compared. The primer sequences used in the current study are shown in Table 2.

The sizes of the products were different from the original allocations, although one primer (Sm17) produces microsatellite alleles (88-97bp) which are completely different to those reported in the literature (178bp-183bp) of Simon *et al.* (1999). We are not sure why, as we used the identical Sm17 primer pair reported in Simon *et al*. (1999). Inconsistencies with the reporting of primer sequences have made comparison of allele sizes between samples analysed for this study and those in previous publications very difficult. Comparison of the allele sizes of common *Sitobion* clones is shown in Table 5. If funding was available, it would be possible to obtain and analyse historical *S. avenae* samples from the same time period which would allow direct comparisons to be made.

8. ACKNOWLEDGEMENTS

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