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1 **Clonal mixing in the soldier-producing aphid *Pemphigus spyrothecae***
2 **(Hemiptera: Aphididae)**

3

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27 Clonal mixing in a soldier-producing aphid

28

1 **Abstract**

2 Illuminating the genetic relationships within soldier-producing aphid colonies is an
3 essential element of any attempt to explain the evolution of the altruistic soldier caste.
4 *Pemphigus spyrothecae* is a soldier-producing aphid that induces galls on the leaf
5 petioles of its host (trees of the genus *Populus*). At least a quarter of the aphids within
6 the clonally-produced gall population are morphologically and behaviourally distinct
7 first-instar soldiers that defend the gall population from predation. Using field
8 trapping and microsatellites, we investigated the degree of clonal mixing within
9 natural gall populations. Field trapping in the UK showed that all the migrants of *P.*
10 *spyrothecae* and of two other *Pemphigus* species were wingless first-instar soldiers.
11 The average degree of mixing estimated from trapping *P. spyrothecae* migrants was
12 0.68% (range = 0–15%). Microsatellite genotyping of 277 aphids from thirteen galls
13 collected in Italy revealed an average mixing level of 10.4% (range = 0–59%). Six
14 galls contained more than one clone (range = 2–5). Non-kin aphids were not restricted
15 to the soldier caste but were evenly distributed across instars. An additional gall, from
16 which 527 occupants were genotyped, contained 12 non-kin aphids distributed among
17 9 clones, showing that clonal diversity can be high even when mixing is very low.
18 These observations suggest that although soldiers migrate regularly and can moult and
19 reproduce within foreign galls, clonal mixing in this species is generally low and is
20 unlikely to provide a barrier to the evolution of investment by the aphid clones in an
21 altruistic soldier caste.

22

23 [250 words]

24

1 **Introduction**

2 Kin selection theory predicts that helping behaviour should be widespread among
3 clonal animals (Hamilton 1964, Hamilton 1987), yet helper castes have been found in
4 only a few groups: colonial invertebrates (Harvell 1994), polyembryonic parasitic
5 wasps (Cruz 1981) and aphids (Aoki 1977). One reason for this scarcity could be
6 mixing between individuals from different clones (clonal mixing), which will dilute
7 the benefit of cooperation by wasting it on unrelated clones. Therefore, measuring the
8 degree of clonal mixing is essential to understanding the evolution of social behaviour
9 in clonal animals (Hamilton 1987, Stern & Foster 1996).

10 Aphids are an excellent model for studying the factors that have influenced the
11 evolution of social behaviour because they have evolved altruistic castes at least 17
12 times (T. Fukatsu, pers. comm.), allowing associations between traits to be tested
13 using phylogenetic comparative methods (Harvey & Pagel 1991, Stern & Foster
14 1996). There are approximately 4,400 species of aphids in 25 subfamilies
15 (Remaudière & Remaudière 1997), but soldiers are found in only about 60 of the 475
16 species within the subfamilies Hormaphidinae and Pemphiginae, so an important
17 question is how to explain their limited taxonomic distribution (Stern & Foster 1997).
18 Aphids in these two subfamilies induce galls on their primary hosts, a trait that is
19 likely to be ancestral to both groups, suggesting that galls were probably an important
20 factor in the evolution of soldiers: a gall is a valuable, defensible resource, and a
21 potential barrier to intrusion by other clones that might dilute the benefits of sociality
22 (Foster & Northcott 1994).

23 For soldier-producing aphids, as for all social animals, the two primary issues
24 we need to address in order to understand their evolution are the ecology and the
25 genetic organization of the evolving colonies (Hamilton 1964). Due to their clonal

1 reproduction, genetic relatedness within aphid colonies is simpler than in any other
2 social organism: an aphid is either of the same clone as its neighbours or it is not.
3 Therefore, we need to determine the degree of clonal mixing within aphid colonies in
4 order to know to what extent the benefit of investing in soldiers is diluted by alien
5 clones. However, there are few data on the extent of clonal mixing in soldier-
6 producing species. The only detailed study found a high degree of mixing in seven
7 *Pemphigus obesinymphae* galls, where on average 41% of individuals were unrelated
8 to the foundress, and behaved and developed selfishly (Abbot *et al.* 2001).

9 All soldier-producing aphids induce galls on the host-plants where the sexual
10 generation occurs (the primary host) (Stern & Foster 1996), and it might be thought
11 that being enclosed in a gall would eliminate clonal mixing. However, in all these
12 gall-living species, the gall must eventually open to allow waste products to be ejected
13 (Aoki 1980, Benton & Foster 1992), or to allow the winged aphids to migrate from the
14 gall. In addition, there might be a selective advantage to those clones that invest some
15 of their resources in individuals that habitually migrate to seek out and colonize other,
16 perhaps healthier, galls (Hamilton & May 1977), and there is good evidence in at least
17 one species (*Pachypappa marsupialis* Koch) that this does happen (Aoki 1979). There
18 is therefore undoubted potential for clonal mixing in these gall-living soldier-
19 producing species.

20 We looked at clonal mixing within galls of the genus *Pemphigus*. This is a
21 useful model genus, since soldiers are known from several species (Aoki & Kurosu
22 1986, Moran 1993, Rhoden 1997) and there is good evidence of high levels of
23 intergall migration in four species: *Pemphigus populitransversus* and *P. populicaulis*
24 (Setzer 1980) in which soldiers have not been reported, and *P. obesinymphae* (Abbot

1 *et al.* 2001) and *P. spyrothecae* (Llewellyn 1996), which have soldiers (Aoki &
2 Kurosu 1986, Moran 1993).

3 Most of our observations were made on *Pemphigus spyrothecae* Passerini,
4 which is widely distributed throughout Europe and western Siberia, and introduced
5 into Canada (Blackman & Eastop 1994). *P. spyrothecae* is cyclically parthenogenetic
6 on *Populus nigra*. Galls are formed on petioles by single sexually-produced
7 foundresses. All generations within the gall are clonal (Lampel 1969), so the sole
8 source of genetic variation within the gall (excluding mutation) is intergall migration.
9 Galls remain sealed from soon after initiation in spring until late July or early August,
10 when the colony makes an opening (the ostiole) just large enough to allow the exit of
11 honeydew and winged emigrants (Rhoden 1997; N Pike pers. comm.). Therefore, the
12 potential for clonal mixing appears to be restricted to the period following the
13 formation of the ostiole. *P. spyrothecae* has an unusual life-cycle, in that the aphid
14 colonies remain in the galls on the primary host and do not migrate in the summer to a
15 secondary herbaceous host as do most other species in the genus (Lampel 1969). The
16 colonies are therefore present in the galls for an extended period of time, which ought
17 to make intergall migration, and therefore clonal mixing, easier to detect.

18 Two morphological classes are born in the gall, soldiers (first-instar
19 virginoparae) and non-soldiers (first-instar sexuparae), both of which pass through
20 four moults to become adults (Foster 1990). It is the winged adult sexuparae that
21 migrate from the gall to the bark to the next stage of the life-cycle. *P. spyrothecae*
22 soldiers form a morphologically and behaviourally distinct altruistic soldier caste
23 (between one quarter and one half of the total gall population) that effectively defends
24 the colony against predators and removes waste products from the gall (Aoki &
25 Kurosu 1986, Foster 1990, Benton & Foster 1992, Foster & Rhoden 1998). All of the

1 foundress's offspring are soldiers (first-instar virginoparae), and all adult virginoparae
2 give birth to approximately equal numbers of soldiers and non-soldiers. Due to their
3 position in the gall pedigree, most of the second generation of soldiers (i.e. those that
4 are the granddaughters of the foundress), which make up the great majority of soldiers
5 in the gall, probably die within the gall without leaving offspring. Moreover, soldiers
6 remain as first instars for about three times as long as non-soldiers, increasing the
7 gall's defences but further reducing the probability that a second-generation soldier
8 will have time to reproduce (Rhoden 1997).

9 We made additional observations on two other species, *Pemphigus bursarius*
10 *L.* and *Pemphigus gairi* Stroyan, which also produce soldiers (PK Rhoden & WAF
11 unpublished data) but do migrate to a secondary host in the summer and therefore
12 have shorter-lived galls.

13 The aim of this study was to measure rigorously the degree of clonal mixing
14 within galls of *P. spyrothecae* using microsatellites and relate this to the amount of
15 intergall migration observed in field populations. We are interested in knowing not
16 only the absolute level of clonal mixing but also which morphs are involved in
17 intergall migration and how many distinct clones of alien aphids occur in a particular
18 gall. Only by measuring clonal mixing in this way can we assess the genetic context
19 within which soldiers have evolved in these social animals.

20

1 **Materials and Methods**

2 *Experiments on intergall movements*

3 We carried out experiments on galls of three species of *Pemphigus* on mature trees of
4 *Populus nigra* var. *italica* in the summer of 1995 in the following sites in and around
5 Cambridge, UK: *P. spyrothecae* at the University Library (52° 12' N, 0° 6' E; grid ref.
6 TL441584; these trees have since been felled) and the Cherry Hinton Cycle Path (52°
7 12' N, 0° 10' E; TL484573); *P. bursarius* near Abington (52° 6' N, 0° 14' E;
8 TL526472) and at Whaddon (52° 6' N, 0° 1.3' E; TL355465); and *P. gairi* at
9 Abington, Whaddon and the University Botanic Garden. The aphids in each study gall
10 were isolated from the rest of the tree by wrapping the petiole, or the branch next to
11 the petiole, with masking tape, which was then coated with sticky Boltac. Two sticky
12 traps were put in place for each gall, so that it was possible to distinguish emigrants
13 from immigrants. Galls and sticky traps were collected after one week. The galls were
14 collected over an extended period during which they were mature, i.e. with an opening
15 for the release of winged adult sexuparae and honeydew (Table 1). The aphids in each
16 gall and on each sticky trap were removed, counted and classified by eye to morph
17 (sexupara or virginopara) and instar (first, second, third, fourth or adult). We recorded
18 the shortest distance along branches to the nearest conspecific gall, the fresh weight of
19 each gall and of the whole leaf, and estimated gall volume from three perpendicular
20 dimensions, assuming the galls to be regular ellipsoids.

21

22 *Genetic analysis*

23 Fourteen galls were collected for genetic analysis between 4 and 17 September 1999
24 from ten Lombardy poplar trees (*Populus nigra* var. *italica*) at five sites selected for
25 high genetic variation: three trees at Gaggio near Modena in Italy (44° 37' N, 11° 0'

1 E); three at Ravarino near Modena (44° 43' N, 11° 5' E); two in central Modena (44°
2 40' N, 10° 54' E); one in Berlin (52° 31' N, 13° 24' E); and one in Uppsala (59° 52' N,
3 17° 38' E). We did not survey galls from the field sites used for the trapping program
4 because low microsatellite variation in the UK provided insufficient resolution for
5 distinguishing clones (PCDJ *et al.* unpublished data). Galls were stored in 80%
6 ethanol in individual vials at room temperature.

7 For the genetic analysis of the *P. spyrothecae* gall colonies we used two highly
8 variable microsatellite markers, 96PS20 and 98PS12 (Johnson *et al.* 2000).
9 Preliminary genotyping of one aphid from each of 97 galls collected from the 10 study
10 trees showed that genetic variation among clones within trees was high (locus
11 96PS20: mean expected heterozygosity calculated from allele frequencies (H_e) = 0.66,
12 range = 0.59–0.78; locus 98PS12: mean H_e = 0.69, range = 0.43–0.81) and genetic
13 structure was low, demonstrating that the two loci could provide sufficient resolution
14 to identify migrants with high probability (PCDJ *et al.* unpublished data). To
15 minimise the probability that an immigrant share the genotype of the natal clone, the
16 14 gall populations were selected for the rarity of their genotypes. Estimates of the
17 probability of detecting an immigrant clone, calculated by multiplying allele
18 frequencies, ranged from 98.0% to 99.98% across the fourteen galls (mean = 99.5%).

19 DNA extractions and PCRs were carried out according to Johnson *et al.*
20 (2000). As a gall can hold more than 1000 aphids, making exhaustive genotyping of
21 every colony unfeasible, an average of 23 aphids (sum = 300; range = 16–40) were
22 genotyped from each of thirteen galls, while 746 of the aphids within the remaining
23 gall were genotyped. In order to obtain reliable genotypes, 460 individuals were re-
24 genotyped at both loci, in addition to all that failed on the first attempt and any whose
25 genotype differed from the majority gall genotype. To reduce the risk of heterozygotes

1 being scored as homozygotes (Taberlet *et al.* 1996), a homozygous genotype that
2 differed from the majority in the gall was not considered reliable until confirmed by at
3 least two independent PCRs. Individuals that did not yield complete two-locus
4 genotypes were not included in the analysis. Before being genotyped, each aphid was
5 classified by eye to morph and instar. The most common genotype was assumed to be
6 the foundress genotype, because foundresses were not found in the galls, having
7 probably died earlier in the season.

8

1 **Results**

2 *Emigration from and immigration into the galls*

3 Very few aphids were trapped leaving or entering the galls in any of the three species.
4 In *P. spyrothecae*, where there was a higher rate of migration than in the other two
5 species, the average rate at which aphids moved into a gall was approximately once
6 every six weeks and only 17% of the galls were invaded by any aphids during the 10-
7 week period of observation (Table 1). All the migrant aphids in all three species were
8 first-instar soldiers, except winged adult sexuparae, which were assumed to be
9 migrating to the bark and therefore discounted. We recorded several features of the
10 galls to test whether there were any differences between galls from which aphids did
11 or did not emigrate: there were no significant differences in total gall population size,
12 fresh gall mass, gall volume or fresh leaf mass. Neither was there a significant effect
13 of date on the numbers of emigrants or immigrants in any of the species. However, *P.*
14 *bursarius* and *P. gairi* (but not *P. spyrothecae*) galls that were reached by one or more
15 immigrants were significantly closer ($P < 0.05$) to the nearest conspecific gall than
16 those that received no immigrants (Welch *t*-test (Sokal & Rohlf 1995)).

17 We calculated a direct estimate of percentage mixing in the galls by dividing
18 the number of immigrants in a particular week over the total population size and
19 multiplying by the total number of weeks of the period of observation, assuming that
20 immigrants survive and reproduce at the same rate as natives (Table 1). The average
21 mixing rates were low in the three species, but the level of mixing could on rare
22 occasions rise to quite high levels, particularly in *Pemphigus gairi*, which received
23 immigrants at a similar rate to the other species but had much smaller colonies. By
24 dividing the number of immigrants by the number of emigrants, we estimated the
25 migrant success rate (the probability of successfully reaching another gall). This was

1 surprisingly high, ranging from 1 in 4 in *P. spyrothecae* to almost 1 in 2 in *P.*
2 *bursarius*.

3

4 *Molecular data on clonal mixing*

5 Unambiguous two-locus genotypes were obtained from 277 (92%) of the 300 aphids
6 in the 13 galls that were sampled. The gall that was exhaustively genotyped yielded
7 527 complete genotypes (71%) from 746 aphids.

8 We found much higher levels of clonal mixing by genetic analysis than by
9 trapping (Table 2; Figures 1 & 2). The average frequency of aphids that did not share
10 the foundress genotype was 10.4% across the thirteen gall colony samples (range = 0–
11 59%). Six of the sampled galls contained aphids from more than one clone (range =
12 2–5 different clones). Every instar of both morphs was represented among the non-kin
13 aphids, except adult sexuparae, although only three of these were genotyped.

14 Low clonal mixing was detected in the gall that was exhaustively genotyped.
15 Twelve (2.3%) of the 527 occupants did not share the natal genotype, and these were
16 drawn from 9 clones. Nine of the 12 non-kin were soldiers, two were later-instar
17 virginoparae and one was a sexupara, representing a significant skew towards soldiers
18 ($\chi^2 = 16.6$, d.f. = 1, $p < 0.0001$).

19

1 **Discussion**

2 Our molecular data demonstrate rigorously the presence of multiple clones in the galls
3 of a soldier-producing species, *P. spyrothecae*, while our trapping data provide strong
4 evidence that intergall migration occurs in all three species studied and is undertaken
5 exclusively by soldiers. In addition, this is the first study to use species-specific
6 nuclear DNA markers to gauge clonal diversity within aphid galls.

7 If all migrants are soldiers, as indicated by our trapping data, then the later-
8 instar virginoparae and all the sexuparae found by genotyping must have moulted or
9 descended from immigrants. The inference that immigrant soldiers can grow and
10 reproduce and potentially produce winged emigrants in alien galls is important
11 because it suggests that migration could be an adaptive strategy of the clone, rather
12 than a chance event: in addition to defending the gall, soldiers might also have a role
13 as migrants to other galls. Migration might be either a bet-hedging strategy, the
14 clone's insurance against the possible extinction of the gall (Hamilton & May 1977),
15 or a sacrifice by the emigrant to free resources within a crowded gall, although our
16 data do not support a link between crowding and emigration. Soldiers appear well
17 adapted to evolving a role as migrants: they represent a small investment owing to
18 their small size; they are more sclerotized than non-soldier first instars and therefore
19 more resistant to desiccation outside the gall; and they are more mobile than the later
20 instars. Indeed, soldiers may have evolved from specialised migrants, rather than the
21 other way around (Stern & Foster 1996). However, the degree of specialization in
22 migration in *P. spyrothecae* does not begin to approach that observed by Aoki (1979)
23 in the dimorphic first instars of *Pachypappa marsupialis*.

24 Of the six sampled galls in which we found immigrant genotypes, five
25 contained more than one immigrant from the same clone, as did the exhaustively

1 genotyped gall. We cannot know whether these occurred through repeated
2 immigration from the same clone, or reproduction by migrants within galls, or a
3 combination of both. However, the presence of sexuparae in all seven mixed galls
4 shows that migrants do reproduce, so it is likely that some duplicates are the result of
5 migrant reproduction.

6 Variable rates of migrant reproduction could account for the greater range of
7 mixing levels found by genotyping compared with those estimated by trapping
8 migrants (calculated under the assumption that migrants and natives reproduce at the
9 same rate), although differences between the sampling sites could also explain this
10 observation. Such variation in reproductive rate could be random, but in one closely
11 related species there is clear potential for migrants to out-compete the native clone: *P.*
12 *obesinymphae* migrants cheat in alien galls by withholding defensive behaviour and
13 accelerating their development (Abbot *et al.* 2001). Such cheating, if it adds
14 significantly to the cost to the native clone of immigration, should drive selection for
15 the development of defensive behaviours currently unknown in aphids, such as the
16 recognition and exclusion of non-kin.

17 Another observation that could be explained by variation in migrant
18 reproductive rate is predominance of soldiers among the 12 non-kin aphids in gall
19 3019, from which 527 aphids were genotyped, which contrasts with the relatively
20 even balance of morphs and instars found in the non-kin from the other galls, from
21 which 10–39 were genotyped. It is possible that all galls receive immigrants at an
22 approximately equivalent rate, but only in galls where one or more immigrant clone
23 has prospered do we find a level of mixing high enough to be detected by genotyping
24 a small sample. Therefore, we would expect to find a range of morphs and instars in

1 highly mixed galls, while the immigrants in galls with little mixing are likely to be
2 soldiers that have not reproduced, due to lack of time or competition.

3 The levels of mixing found here in *P. spyrothecae*, and inferred in *P. bursarius*
4 and *P. gairi* are low compared to those found in *P. obesinymphae* (mean = 41%) in
5 the only other detailed survey of clonal diversity in a soldier-producing aphid (Abbot
6 *et al.* 2001). These data, taken together with previous observations, provide no clear
7 trend of association between clonal mixing and soldier production. Setzer (1980)
8 recorded high levels (up to 25%) of clonal mixing in the North American species *P.*
9 *populitransversus* and *populicaulis*, and there is no evidence yet that these species
10 have soldiers (except for the one gall that was probably of *P. obesinymphae*). Lack of
11 clonal mixing was also recorded in the soldier-producing aphid *Ceratovacuna*
12 *nekoashi* by Fukatsu and Ishikawa (1994), on the basis of a very small sample size,
13 and in the non-soldier producing species *Melaphis rhois* by Hebert *et al.* (1991). As in
14 parallel arguments about genetic relatedness and the evolution of altruism in, for
15 example, the social Hymenoptera, we would not expect a simple relationship between
16 the degree of clonal mixing and soldier investment. Clonal mixing merely provides
17 the genetic context against which, based on ecological factors, soldier investment
18 might evolve. It would be interesting, therefore, to investigate clonal mixing in further
19 aphid species, especially those where levels might be predicted to be very high.

20

21 [3260 words]

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7

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1 **Author information box**

2 This work was conducted in the Department of Zoology of the University of
3 Cambridge, where John Whitfield and Paul Johnson were PhD students. William
4 Foster is senior lecturer in zoology and curator of insects. The research interests of his
5 group include the evolution of social behaviour in aphids and wasps, and the evolution
6 of forest canopy arthropods. Bill Amos is a lecturer in zoology. His laboratory focuses
7 on the use of molecular markers to reveal population structure and breeding behaviour
8 in a wide range of animals, and he has a particular interest in the evolution of
9 microsatellites.

10

11

12

1 **Figure legends**

2

3 **Figure 1** Microsatellite variation at locus 98PS12 revealing the presence of 5 clones
4 among 27 aphids from a *P. spyrothecae* gall (ref. 3072) (V = virginoparae; S =
5 sexuparae; 1–4 = instar number; A = adult).

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8 **Figure 2** Proportions of different clones among *P. spyrothecae* aphids from fourteen
9 galls. Sample sizes are given above the bars. Common shading patterns between bars
10 do not indicate that the same clones are present in different galls. The proportions of
11 the 9 non-kin clones in gall 3019 were too small to be distinguished here.

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Tables

Table 1 Observations on migrating behaviour in three *Pemphigus* species. Aphids caught in sticky traps on leaf petioles.

Species	No. of galls examined	Duration of experiment (weeks)	Mean total population in the galls (\pm SE)	Mean no. of emigrants per gall per week (range)	Mean no. of immigrants per gall per week (range)	% galls with at least one immigrant	Mean % mixing (range)	Migrant success rate
<i>spyrothecae</i>	59	7.8 – 19.10.1995 (10.3)	428 \pm 32	0.64 (0 – 10)	0.17 (0 – 2)	17	0.68 (0 – 15.4)	0.26
<i>bursarius</i>	60	26.5 – 25.7.1995 (7.6)	112 \pm 9.3	0.22 (0 – 3)	0.1 (0 – 2)	8.3	0.64 (0 – 17.7)	0.46
<i>gairi</i>	67	26.5 – 25.7.1995 (7.6)	38.3 \pm 3.5	0.37 (0 – 13)	0.15 (0 – 3)	10.4	2.58 (0 – 42.1)	0.4

Table 2 Clonal mixing among *P. spyrothecae* aphids from fourteen galls, revealed by two microsatellite loci.

Gall ref.	No. of migrants/no. of aphids genotyped			Total	Mixing level	No. of clones
	Soldiers	Other virginoparae	Sexuparae			
3003	0/0	0/2	0/8	0/10	0%	1
3044	0/10	0/3	0/14	0/27	0%	1
3067	0/10	0/3	0/13	0/26	0%	1
3078	0/10	0/1	0/15	0/26	0%	1
3081	0/3	0/1	0/8	0/12	0%	1
3085	0/4	0/2	0/11	0/17	0%	1
3308	0/6	0/2	0/7	0/15	0%	1
3019	9/138	2/62	1/327	12/527	2.3%	10
3291	0/3	0/1	1/6	1/10	10%	2
3063	2/12	1/5	1/22	4/39	10%	3
3017	0/10	1/3	2/14	3/27	11%	4
3032	1/5	0/3	3/15	4/23	17%	3
3030	0/2	0/3	5/13	5/18	28%	3
3072	6/10	2/3	8/14	16/27	59%	5

Figures

Figure 1

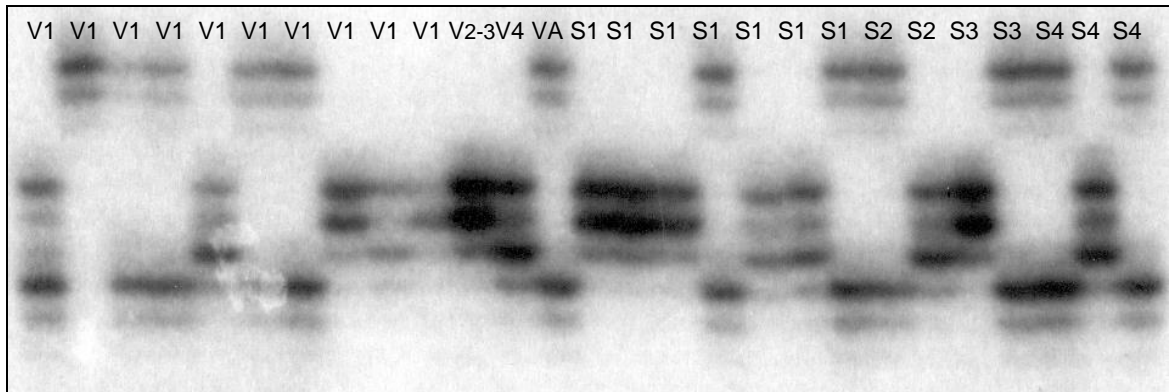


Figure 2

