The Spread of Herbicide Resistance in *Lactuca serriola* at a Landscape Scale

Yi Qing Lu

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School of Agriculture and Wine

Faculty of Sciences

University of Adelaide

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Abstract

Several populations of *Lactuca serriola* have appeared with resistance to ALS-inhibiting herbicides within a small area of the northern Yorke Peninsula of South Australia. Resistance appeared quickly at a number of sites following the first initial report of resistance near Snowtown in 1994. One explanation for the widespread and rapid appearance of herbicide-resistant *L. serriola* in the area around Snowtown could be movement of resistant seed from the original field where resistance evolved. An alternative explanation is that resistance has evolved multiple times in the area. The aim of the study is to determine which of these two factors is more important for the spread of herbicide resistance in this area.

In this research, seeds were collected from various sites along roadsides and adjacent fields in 1999 and again in 2004. In 1999, seeds were collected in a 60 by 60 km area centred on Bute, South Australia. Seed were germinated and the seedlings tested for resistance to the ALS-inhibiting herbicide chlorsulfuron. As *L. serriola* is a self-pollinated plant, resistance in the progeny will indicate resistance in the parent plant. Of the samples tested for resistance, 75.0% of field sites and 58.8% of roadside sites contained resistant individuals. In the second collection, seedlings were collected from a smaller number of sites within the area. Seedlings were treated with chlorsulfuron and assessed for resistance. In this collection all field sites and 81.8% of roadside sites contained resistant individuals.

DNA was extracted from the leaves of plants and inter simple sequence repeat analysis (ISSR) was used to generate fingerprints for individuals in each population. A single

individual from 25 populations from the 1999 collection and two control samples was genotyped. A total of four different primers were used and 179 bands scored. Of these bands 49.7% were polymorphic across the samples tested. UPGMA analysis indicated the samples could be organised into 20 different clades. Only a small number of samples had identity. One group of three susceptible samples collected from 66 km apart had identical banding patterns to each other. A separate group of three resistant samples from 43 km apart had identical bands and a third group of four resistant samples from 48 km apart had identical bands. Among the resistant individuals in this collection, 13 different genotypes were identified.

From the 2004 samples, up to eight individuals from each population were genotyped. Within sites, many individuals shared the same genotype. However, some sites contained multiple genotypes. The same resistant genotype was identified in four different collecting points, two fields and two adjacent roadsides, within a 5 km radius. Within the samples tested, nine different resistant genotypes were identified.

This research has provided evidence for multiple independent evolutionary events resulting in the selection of chlorsulfuron resistance in *L. serriola* in South Australia. It is also likely that *L. serriola* can move from site to site, with the same genotype present across 66 km. Lastly, there are indications from the research that populations of *L. serriola* become locally extinct and sites are recolonised at a rapid frequency. This has probably contributed to the rapid spread of herbicide resistance in roadside populations of *L. serriola* in South Australia.

Declaration

This work contains no material which has been accepted for the award of any other degree or diploma in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text.

I give consent to this copy of my thesis, when deposited in the University Library, being available for loan and photocopying.

Yi Qing Lu

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Chapter 1 Introduction

Herbicides have revolutionised weed control since they first came into widespread use after 1946 and are currently the most reliable and economic method of weed control (Heap 1997). Herbicides have played a part in increasing food production and made cultivation easier, saving much labour. However, the efficacy of weed control by herbicides is threatened by the evolution of herbicide resistant weeds. There are currently about 181 weed species with herbicide resistant populations in the world, among which 46 are found in Australia and the number is increasing (Heap 2005).

Acetolactate synthase (ALS)-inhibiting herbicides inhibit the first enzyme in the biosynthetic pathway for producing branched chain amino acids (Preston and Mallory-Smith 2001). There are five different groups of ALS-inhibiting herbicides and all have the same target enzyme. Weeds resistant to one ALS-inhibiting herbicide may also be resistant to the other ALS-inhibiting herbicides, even though they have not been exposed to them (Tranel and Wright 2002). Resistance to ALS-inhibiting herbicides was first discovered in 1978 (Mallory-Smith *et al.* 1990a), and about 92 weed species now have populations with resistance to these herbicides (Heap 2005). Normally, mutations causing resistance occur in one of five domains within ALS gene (Boutaslis *et al.* 1999). Major mutations are at the Ala_{122}, Pro_{197}, Ala_{205}, Trp_{574} and Ser_{653} loci (Tranel and Wright 2002).

Lactuca serriola (prickly lettuce) is a common weed growing in crop fields, orchards, vineyards, riverbanks, along roadsides and railways, and is a problem weed in cereal and legume crops in some regions (Amor 1986, Alex 1992, Uva *et al.* 1997). It competes for water, sunlight and nutrients with crops, the flower buds are difficult to remove from harvested grains and the milky latex and stems clog harvest machinery and increase moisture of the harvested grain (Amor 1986, Schoennagel and Waller 1999). This species grows well in Mediterranean climates (Ferakova 1976, Mejias 1994) and has a large seed production (Arthur 1894). *L. serriola* is self-compatible and has wind borne seed (Weaver and Downs 2003, Mejias 1994). The first ALS-inhibiting herbicide resistant population of *L. serriola* was discovered in Idaho in 1987, after five years of being treated with the herbicide. This was the first report of resistance to ALS-inhibiting herbicides in the world (Mallory-Smith *et al.* 1990a). Since then resistance to ALS-inhibiting herbicides has also appeared at a number of sites in South Australia (Preston *et al.* 2005a).

The two main factors affecting weed resistance evolution are selection and genetic variability of populations (Diggle and Neve 2001). Herbicides apply a strong selection pressure for the evolution of resistance. Other important factors influencing resistance evolution are population dynamics, the pattern of inheritance of resistance and gene flow. Knowing the relationship and importance of these factors may help to manage herbicide resistance. Issues, such as comparing the contribution of individual selection events and gene flow to resistance spread may be important for practical management. *L. serriola* is self pollinated (Mejias 1994), and highly autogamous (Ferakova 1977), so gene flow is due to seed movement. Seeds are attached to pappus and spread by wind (Weaver and Downs

2003). These characters make *L. serriola* a model weed for the study of the importance of independent selection events and gene flow in the evolution of herbicide resistance. *L. serriola* can causes economic loss, particularly in the upper York Peninsula of South Australia, and the spread of herbicide resistance in this species is increasing.

Given that *L. serriola* seed can be widely dispersed by wind, it is possible that resistance in South Australia has spread from a single location. In order to determine whether resistance has spread from a single site or evolved independently at many locations, it is necessary to be able to determine the genetic relationship between different resistant individuals. Molecular markers are an effective method of determining the relationships within and between populations. Such markers can be used to determine whether the different ALS-herbicide resistant populations of *L. serriola* evolved independently.

In the following review, factors endowing herbicide resistance, biochemical and molecular aspects of resistance to ALS-inhibiting herbicide, the biology of *L. serriola* and the application of different molecular markers in plant research are discussed in detail.

Chapter 2 Literature Review

2.1 Herbicides and resistance

Weed control is important for agriculture, as weeds reduce crop yields, harvesting efficiency and contaminate produce (Powles *et al.* 1997). Herbicides are the most reliable and economic method of weed control and they have revolutionised weed control since they were first used in 1946. Since then weed control has relied on herbicides because they are cheap and reliable (Heap, 1997). Herbicides have also played a large part in food production being sufficient to support the increasing world population (Heap, 1997). Cultivation is made easier and farmers are saved much labour by using herbicides (Heap, 1997). However, herbicide modes of action are quite limited and few novel modes of action have been introduced recently (Powles *et al.* 1997).

One undesirable outcome of the widespread use of herbicides has been the evolution of herbicide resistance. Herbicide resistance has been increasing rapidly throughout the world over the past two decades (Powles *et al.* 1997). Herbicide resistance has evolved to almost every herbicide mode of action (Jasieniuk *et al.* 1996). The area infested with resistant weeds is also increasing rapidly (Jasieniuk *et al.* 1996). Herbicide resistance makes weed control more difficult by requiring the use of more expensive, more time consuming or less effective weed controls.

Herbicide resistance, is the ability for a weed to survive exposure to a dose of herbicide that would be expected to kill the wild type (Rubin 1991, O'Keeffe *et al.* 1993, Lee *et al.* 2000, Jutsum and Graham 2005). Herbicide tolerance is different to herbicide resistance and is defined as an ability inherent in a species to survive herbicide application. That is that species was never adequately controlled by the herbicide. Resistance to herbicides in weeds occurs because of the intensive selection pressure applied by herbicides.

In most cases after a herbicide is applied, over 90% weeds are killed. Although the number of weeds that survive is low, these survivors reproduce and set seed. If the same herbicide or herbicides of the same mode of action are used continuously, the proportion of herbicide resistant individuals grows larger and larger and eventually a herbicide failure occurs (Maxwell and Mortimer 1994).

2.1.1 History and current status of resistance

The first herbicide resistant population reported was *Senecio vulgaris* L. resistant to triazine herbicides (Ryan 1970). In the 1970s, many other weed species evolved resistance to herbicides. One case of herbicide resistance occurred each year from 1970 to 1977. However, from 1978 onwards, the number of herbicide resistance cases increased to 9 per year (Heap, 1997), with 33 new herbicide resistant weeds appearing from 1978 to 1983. Now there are 302 resistant biotypes of 181 species (108 dicots and 73 monocots) in over 270,000 fields in the world, and 46 resistant biotypes in Australia (Heap 2005).

In Australia, about A$30-80 ha^{-1} was spent on herbicides in cropping regions (Pratley *et al.* 1996). By 2000, this figure had risen to $583 million. About $4 billion was spent on weed management per year by farmers and a further $116.4 million is spent each year by governments to manage weeds in Australia (Sinden *et al.* 2004). Herbicide resistance adds extra cost to weed management and farmers are urged to invest more money to delay the onset of herbicide resistance (Weersink et al. 2004).

2.1.2 Biochemical mechanisms of resistance

Preston and Mallory-Smith (2001) described the major biochemical mechanisms of herbicide resistance in weeds as detailed in the following section. Herbicides affect plant metabolism and growth by binding to or interacting with specific proteins. It is possible for resistance to the same herbicide to be endowed by different biochemical mechanisms in different or the same plants. The main types of mechanisms that have been identified are target-site modifications, increased detoxification of the herbicide and changes in herbicide translocation.

2.1.2.1 Target -site-based resistance

Target-site-based resistance occurs when a modification of the herbicide binding protein decreases herbicide binding. The herbicide binds poorly to the target site, and the target enzyme is not inhibited. Another type of target site resistance is where the herbicide-binding proteins are over-produced (Preston and Mallory-Smith 2001).

Many examples of resistance to photosystem II-inhibiting herbicides are based on a

modification of the target site (Gronwald 1994). Triazine resistance occurs because of a modification of an amino acid residue in the Q_B-binding niche on the D1 protein (Mets and Thiel 1989, Fuerst and Norman 1991, Trebst 1991). This reduces the affinity of herbicides at the site so the herbicide can not compete for the exchangeable plastoquinone Q_B (Gronwald 1994). The mutation of serine to glycine, reduces the affinity of atrazine at the Q_B-binding site, reduces the rate of electron transfer between Q_A and Q_B, alters chloroplast ultrastructure and results in increased sensitivity to phenol-type herbicides (Bowes *et al.* 1980, Burke *et al.* 1982, Vaughn and Duke 1984, Gronwald 1994). Triazine-resistant weeds also show a modified galactolipid composition and an increase in unsaturated fatty acids (Pillai and St. John 1981, Lehoczki *et al.* 1985).

Another example of target site-based resistance is resistance to acetyl-coenzyme A carboxylase (ACCase)-inhibiting herbicides. ACCase, the key enzyme in fatty acid biosynthesis in eukaryotes and prokaryotes (Harwood 1988), is located in both the chloroplast, the primary site of plant fatty acid biosynthesis, and in the cytosol (Sasaki *et al.* 1995, Konishi *et al.* 1996). Choloroplastic ACCase is important in plant metabolism (Ohlrogge and Jaworski 1997). Aryloxyphenoxypropionate (APP) and cyclohexanedione (CHD) herbicides block fatty acid biosynthesis in the chloroplast of susceptible plants (Burton *et al.* 1989). Resistance to these herbicides is mostly due to alteration of the chloroplastic, eukaryotic-type ACCase (Evenson *et al.* 1997, Shukla *et al.* 1997, Menéndez and De Prado 1999).Target site-based resistance also often occurs with acetolactate synthase (ALS)-inhibiting herbicides. This will be discussed in more detail in the next section.

2.1.2.2 Herbicide detoxification-based resistance

The reason most crops survive when exposed to selective herbicides is that they metabolise the herbicide more rapidly than weeds. A similar mechanism can also occur in some resistant weed species that detoxify herbicides more rapidly than their susceptible counterparts. The study of these mechanisms is at the biochemical level and no specific mutations with genes endowing resistance have been found. Three enzyme systems have been identified as being involved in metabolism-based resistance. They are glutathione transferases, aryl acylamidases, and cytochrome P450 mono-oxygenases (Preston and Mallory-Smith 2001).

In some examples of triazine resistance, more rapid herbicide metabolism catalysed by glutathione transferases (GSTs) is responsible for resistance (Anderson and Gronwald 1991, Gray *et al.* 1996). In *Abutilon theophrasti*, the activity of two GST isoenzymes were increased in resistant plants (Anderson and Gronwald 1991). In *Alopecurus myosuroides*, the GST content was reported to be higher in resistant populations (Cummins *et al.* 1997). Likewise, in weeds resistant to propanil, aryl acylamidase activity has been shown to be increased (Leah *et al.* 1994). This enzyme detoxifies propanil. However, the major enzymatic system in herbicide detoxification in resistant weeds are the cytochrome P450 monooxygenases (Preston and Mallory-Smith 2001). These enzymes are responsible for metabolism of a wide range of herbicides and resistance as a result of increased cytochrome P450 mono-oxygenase activity have been found in many species, most notably *Lolium rigidum* and *A. myosuroides* (Preston 2004).

2.1.2.3 Other biochemical mechanisms of resistance

Herbicide resistance can also occur through changes in herbicide translocation. The best known examples are resistance to glyphosate and paraquat. Glyphosate resistance in *Lolium rigidum* (Lorraine-Colwill *et al.* 2002, Wakelin *et al.* 2004) and *Conyza canadensis* (Feng *et al.* 2004, Koger and Reddy 2005) is the result of decreased translocation from the leaves to the meristematic tissue and roots. Likewise, paraquat resistance in *Arctotheca calendula* (Soar *et al.* 2003) and *Hordeum leporinum* (Preston *et al.* 2005b) is the result of reduced translocation out of the treated leaves.

It is possible that other mechanisms may also contribute to resistance. For example, reduced absorption of diclofop-methyl has been proposed as a mechanism of resistance in *Lolium rigidum* (De Prado *et al.* 2005). However, these mechanisms have not yet been investigated in detail.

2.1.3 Genetics of herbicide resistance

2.1.3.1 Evolution of herbicide resistance

The evolution of herbicide resistance varies between species and herbicides (Powles *et al.* 1997). Selection pressure and the genetic variability of the population largely contribute to the evolution of herbicide resistance (Diggle and Neve 2001). Factors that contribute to the rapid evolution of herbicide resistance include: the intensity of the selection pressure; the frequency of resistance alleles in populations; the extent of the dominance of the resistant allele; gene flow within and between populations; population dynamics; and the nature and

extent of herbicide use (Diggle and Neve 2001). Herbicides are a very strong selection pressure for resistance evolution as they kill 90% to 99% of susceptible individuals. Therefore, each time a herbicide is used, susceptible individuals are killed and resistant individuals survive. In this way the frequency of resistance alleles in weed populations increases with selection and continuous use of herbicide with the same mode of action will lead to herbicide resistance (Kudsk and Streibig 2003). The intensity of selection pressure by herbicides is important in determining the rate of herbicide resistance evolution (Gressel and Segel 1978, Gressel 1982, Gressel and Segel 1990b, a, Maxwell *et al.* 1990). Three factors affect the amount of selection pressure imposed by herbicides. These are herbicide efficiency, use frequency and effect duration (Maxwell and Mortimer 1994). Herbicide efficiency is defined as either the mortality of the target weed or the reduction of seed production of the survivors. Reduction in seed return is a better measure of selection pressure, because seed production reflects the relationship of resistant and susceptible plants in the next generation (Gressel and Segel 1978). Highly effective herbicides leave few susceptible individuals to the next generation (Powles *et al.* 1997). This tends to drive the population rapidly to resistance (Figure 2.1). The effect duration is the period of phototoxicity imposed by the herbicide. Herbicides with high persistence in the environment will control weeds that emerge for a longer period and hence apply selection to more of the population. Use frequency relates to the number of times a herbicide is used. Selection for resistance to any one herbicide is restricted to the period when the herbicide is applied. Therefore, where herbicides are used in rotation, the appearance of resistance will be delayed (Jasieniuk *et al.* 1996). If it takes eight years for resistance to evolve with continuous herbicide application, it will take 24 years for resistance to occur when the same herbicide is used once in every three years (Jasieniuk *et al.* 1996).

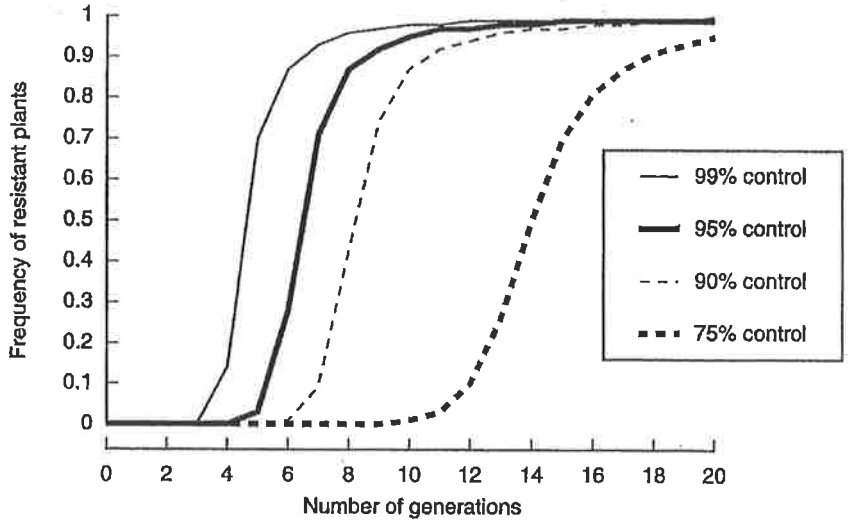

Figure 2.1 The effect of selection pressure of herbicide on the evolution of herbicide resistance in *L. rigidum* (Diggle and Neve 2001). The initial gene frequency is 1×10^{-8}, and herbicide is applied once every generation. The resistance trait is dominant and the population size is constant.

2.1.3.2 Genetic basis of herbicide resistance

Inheritance of resistance

In most cases, herbicide resistance is caused by a single nuclear-encoded gene (Darmency 1993, Maxwell and Mortimer 1994, Jasieniuk *et al.* 1996). Most examples of herbicide resistance are also dominant or semi-dominant in character (Jasieniuk, 1996). The exception is triazine resistance, which is controlled by a gene located on the chloroplast genome and is maternally inherited (Hirschberg and McIntosh 1983).

A new resistance mutation mostly arises in a heterozygous state, and the dominance of the mutation enables the individual with the mutation to survive herbicide treatment (Jasieniuk *et al.* 1996). For outcrossing species, the level of dominance is important as almost all of the fully recessive resistance genes will be present as heterozygotes and susceptible (Powles *et al.* 1997). For selfing species, the level of dominance is not so important, as the gene endowing resistance often becomes fixed as homozygotes (Powles *et al.* 1997). Dominant or partially dominant rare alleles also spread much faster than recessive alleles where selection is imposed. This is because the heterozygotes can be selected by herbicide use (James 1965, Charlesworth 1992).

Most herbicides target a single enzyme in a metabolic pathway (Macnair 1991). Under the strong selection pressure imposed by herbicide use, adaptation occurs when a few individuals in the population carrying a resistance allele survive (Macnair 1991). For polygenic inheritance of resistance, a number of favourable alleles are required to produce the resistant phenotype. This requires recombination among individuals for many generations. Therefore, this type of resistance is more likely to occur under weak selection with sublethal herbicide applications and to occur slowly (Jasieniuk *et al.* 1996).

Initial frequency of resistance alleles

The initial number of resistant individuals in a population will also influence the speed of resistance evolution (Powles *et al.* 1997). The average gene mutation rate was estimated as 10^{-9} (Haughn and Somerville 1987); however, the mutation rate of different genes may be different (Powles *et al.* 1997). The initial frequency of resistance in populations is also influenced by the relative fitness of the resistance mutation (Gressel 1982, Gressel and

Segel 1990b, Maxwell and Mortimer 1994, Jasieniuk *et al.* 1996). Fitness of herbicide resistance can be defined as the relative survival, growth, and reproduction of the resistant genotype compared to susceptible genotype when no herbicide is applied (Maxwell and Mortimer 1994). The frequency of mutations with little or no fitness penalty and controlled by a dominant allele will be 100 to 400 times the mutation rate (Jasieniuk *et al.* 1996). The initial frequencies of resistant individuals found in untreated populations have been surprisingly high (Powles *et al.* 1997). For example, the frequency of resistance in triazine resistant *Chenopodium album* was found to be 3×10^{-3} (Darmency and Gasquez 1990), and 1×10^{-2} in diclofop-methyl-resistant *Lolium rigidum* (Matthews and Powles, unpublished cited by Powles *et al.* 1997). Therefore, more resistant individuals might exist in a population than initially expected (Powles *et al.* 1997).

Mating system

For self-fertilized species, the rare heterozygous resistant individuals produce progeny which are a quarter homozygous resistant (Diggle and Neve, 2001). Many of world's major weeds, including several that have evolved herbicide resistance, are highly self-fertilizing (Brown and Marshall 1981, Brown and Burdon 1987). This greatly increases the chance of selecting recessive mutations (Charlesworth 1992).

In contrast, in outcrossing species with random mating systems almost no resistant plants will appear if the resistance mutation is recessive. Therefore, herbicide resistance is more likely to be endowed by dominant gene in outcrossing species while in selfing species, the probabilities of occurrence of a recessive or a dominant mutation are similar (Jasieniuk *et al.* 1996).

Gene flow

Gene flow can occur by both pollen dispersal and seed movement (Maxwell and Mortimer, 1993; Jasieniuk *et al.*, 1996). Pollen or seed from herbicide resistant plants in a field may spread to another area and provide an initial source of resistance alleles (Jasieniuk *et al.* 1996). The frequency of gene flow is believed to be higher than the frequency of mutation and therefore, may result in more rapid evolution of resistance than in situations without gene flow (Jasieniuk *et al.* 1996). There are not many studies examining the relative importance of mutation and gene flow as the source of resistance genes (Jasieniuk *et al.* 1996). A few studies suggested that resistant populations occurred independently where they were geographically separated, while the spread of resistant populations within a region was due to gene flow (Warwick and Marriage 1982, Gasquez *et al.* 1985). In early studies of herbicide resistance gene flow was largely ignored (Jasieniuk *et al.* 1996), because the rate of gene flow was believed to be too low to make a significant impact (Haldane 1924, Ehrlich and Raven 1969, Levin and Kerster 1974, Levin 1979, 1984). Later studies showed that migration rates were between 0.5 to 5.5 individuals per generation for allele distributions among populations in some species (Slatkin 1985a, b, 1987).

Since many weed species are highly self-fertilizing and pollen flow is minimal, seed dispersal will be the most important factor in gene flow (Jasieniuk *et al.* 1996). Grain harvesting equipment is one example of an activity that spreads herbicide resistant weeds (Jasieniuk *et al.* 1996). There are a variety of seed dispersal mechanisms, which can influence the rate of spread of resistance (Matthews 1994). For example, seeds of some species like *Kochia scoparia*, where the plant itself is wind dispersed, can spread over a

considerable distance and cause unexpected control failures (Matthews 1994). The spread of seed can be minimized by removal of weed seeds from harvesting operations and collection of weed seed (Matthews 1994).

Seed dissemination depends on forces such as wind, water, animals, machinery, crop seed, hay and straw. Some seeds can be carried on the surface of water runoff in streams, rivers, irrigation and drainage canals. Seeds like curly dock (*Rumex crispus*) have evolved special structures to help them float in water. These seeds may also remain viable for some time. As many as 55% of field bindweed seeds still germinated after 54 months kept in water (Anderson 1968). Animals and people carry seeds on their feet, clinging to their fur or clothes or in their manure. A study of Till *et al.* (1986) suggested that seeds could be spread by game birds, wildlife and domestic livestock. Barbs, hooks, spines and twisted awns are especially adapted to cling to animals and people. Weeds of some species can survive passage through the gut of animals or birds and be spread in that way (Anderson 1968). Tildesley (1937) showed that crupina (*Crupina* spp.) seeds could be spread by cattle, deer, horses and pheasants. Harvesting equipment such as combines, cultivation equipment and tractors also help to spread seeds. One study showed that after a harvester was cleaned in the usual manner, there were still 8% of the original seeds left. Another study showed as many as 790,000 seeds were released from a piece of farm equipment when it was moved 8 km from one field to another (Zimdahl 1993). It is possible for herbicide resistance to be spread between sites through all of these mechanisms.

Fitness of resistance alleles

Fitness is one of the most important factors in the persistence of herbicide resistance

(Gressel and Segel 1990b, Maxwell *et al.* 1990) and it will also affect the rate of selection of resistance. Fitness is determined by survival and reproduction through the life cycle of the organism (Holt and Thill 1994). The factors generally considered to contribute to fitness include the average number of seeds, seed viability and plant fertility (Hartl 1980, Silvertown 1987). Herbicide resistance mutations are assumed to provide a fitness cost to the plant that carries them (Holt 1990), as many novel mutations affect the organism's ability to survive or reproduce (Lande 1983). However, some examples of herbicide resistance do not incur an easily measurable cost to fitness. Therefore, the fitness of resistant weeds should be evaluated for each case (Holt and Thill 1994), which will help to adjust management strategies (Roush *et al.* 1990, Maxwell 1992).

The most recognisable example of a fitness penalty with herbicide resistance is with triazine resistant weeds. It is well known that triazine-resistant weeds are less fit than susceptible ones (Gressel and Segel 1990b). Triazine resistant biotypes have a reduced photosynthetic rate, reduced quantum yield of photosynthesis, reduced biomass accumulation, lower fecundity, and lower competitiveness than susceptible biotypes (Holt 1990, Warwick 1991). In contrast, ACCase- and ALS-resistant weeds do not demonstrate significant differences in comparative growth and germination characteristics (Thompson *et al.* 1994, Gill *et al.* 1996, Weiderholt and Stoltenberg 1996a, b).

Seed bank

The seed bank is composed of dormant and non-dormant seeds in the soil. The seed bank conserves genes in a population. An important factor in seed bank dynamics is seed

longevity (Mexwell *et al.* 1990). Species with short-lived seed banks respond rapidly to herbicide selection (Powles *et al.* 1997). A longer lived seed bank will delay the evolution of resistance by acting as a buffer of susceptible individuals (Powles *et al.* 1997).

2.1.4 Management implications

Herbicides are the principal method used in weed control in recent decades, and they contribute greatly to world crop production (Powles *et al.* 1997). However, the successful use of herbicides is threatened by the evolution of herbicide resistant weeds (Powles *et al.* 1997). Herbicide resistance has increased rapidly throughout the world in the past two decades (Powles *et al.* 1997). It is possible to delay the onset of herbicide resistance by correct rotation of herbicides of different modes of action in an integrated weed management strategy (IWM) (Powles *et al.* 1997). However, as herbicides are the most efficient, easiest and most cost-effective way of managing weeds, resistance inevitably will lead to greater expense and difficulty in weed management.

One problem in the management of herbicide resistant weeds is if resistance enters a site from outside. Such weed movement may mean that despite the best efforts of the manager, resistance may still occur. Therefore, the effective control of weed seed dispersal can be important in slowing the spread of herbicide resistance (Matthews 1994). Harvesting and grain handling equipment, spraying equipment and other machinery and animals could help to spread resistant weeds, so strict hygiene standards should be enforced (Matthews 1994). For example, removal of weed seed from harvesting operation has been used as a means to manage herbicide-resistant *L. rigidum* in Australia (Matthews 1994). Weed seed should

also be removed from crop seed before crops are sown in the field (Matthews 1994). It is also possible that wind, water and other natural vectors can move herbicide resistant weed seed from site to site. It can be much more difficult to manage such weed seed movement as the farmer has limited control over the vectors.

2.2 Resistance to ALS- inhibiting herbicides

2.2.1 ALS-inhibiting herbicides

ALS-inhibiting herbicides are some of the most efficacious and widely used herbicides in the world (Tranel and Wright 2002). There are five commercially used herbicide chemical families that inhibit acetolactate synthase: sulfonylurea, imidazolinone, triazolopyrimidine, pyridinyloxybenzoate and sulfonylaminocarbonyltriazolinone (Singh and Shaner 1995). The first ALS-inhibiting herbicide was first marketed in 1982 and after that, many ALS-inhibiting herbicides have been marketed all over the world (Powles *et al.*, 1997). The current list of ALS-inhibiting herbicides used in Australia is given in Table 2.1.

ALS-inhibiting herbicides have several properties that have facilitated their adoption by farmers. They are effective at low rates (Bellinder *et al.* 1994), have a relatively broad spectrum of weeds they control and wide crop selectivity (Mazur and Falco 1989). They also have low toxicity to mammals (Mazur and Falco 1989).

Table 2.1 Group B herbicides used in Australia (Preston 2005c).

Herbicide	Trade names	Weeds controlled	Crops
Sulfonylureas			
Chlorsulfuron	Glean	Broadleaf weeds and some annual grasses	Wheat, barley, oats, triticale
Triasulfuron	Logran	Broadleaf weeds, annual ryegrass, paradoxa grass	Wheat
Metsulfuron-methyl	Ally	Broadleaf weeds	Wheat, barley, triticale
Bensulfuron-methyl	Londax	Arrowhead, dirty dora, and starfruit	Rice
Thifensulfuron-methyl	Harmony M (with metsulfuron-methyl)	Broadleaf weeds	Wheat, barley, triticale
Sulfometuron-methyl	Oust	Broadleaf weeds and grasses	None
Halosulfuron-methyl	Sempra	Nutgrass	Cane, maize, sorghum
Sulfosulfuron	Monza	Brome grass and some broadleaf weeds	Wheat
Rimsulfuron	Titus	Some broadleaf weeds	Tomato
Iodosulfuron-methyl-sodium	Hussar	Wild oats, annual phalaris and broadleaf weeds	Wheat
Ethametsulfuron-methyl	Bounty (with diflufenican)	Broadleaf weeds	Lupins
Trifloxysulfuron-sodium	Envoke	Broadleaf weeds and nutgrass	Cotton
Mesosulfuron-methyl	Atlantis	Brome grass, wild oats, annual ryegrass and annual phalaris	Wheat
Imidazolinones			
Imazapyr	Arsenal	Broadleaf weeds and grasses	None
Imazethapyr	Spinnaker	Some broadleaf weeds and annual grasses	Certain legume crops
Imazapic	Raptor	Some broadleaf weeds and annual grasses	Sugarcane
Imazamox	Flame	Some broadleaf weeds and annual grasses	Peas, peanuts, soybeans
Sulfonamides			
Flumetsulam	Broadstrike	Some cruciferous weeds	Field peas, wheat, medics
Metosulam	Eclipse	Some broadleaf weeds	Wheat, barley, oats
Pyrimidinylthiobenzoates			
Pyrithiobac-sodium	Staple	Broadleaf weeds and some grasses	Cotton

2.2.2 Mode of action of ALS-inhibiting herbicides and resistance mechanisms

ALS, also referred to as acetohydroxyacid synthase (AHAS), is the first enzyme in the biosynthetic pathway for the branched-chain amino acids isoleucine, valine, and leucine (Umbarger 1978). Sulfonylureas (Chaleff and Mauvais 1984, LaRossa and Schloss 1984, Ray 1984), imidazolinones (Shaner *et al.* 1984), triazolopyrimidines (Gerwick *et al.* 1990), pyridinyloxybenzoate (Takahashi *et al.* 1991) and sulfonylaminocarbonyltriazolinone (Hawkes 1989, Subramanian *et al.* 1990) directly inhibit ALS activity (Saari *et al.* 1994). The binding site of the herbicides has been proposed to be a residual quinone-binding site from pyruvate oxidase (Schloss *et al.* 1988). It is likely that the binding sites for different ALS-inhibiting herbicide chemistries are different, although overlapping (Preston and Mallory-Smith 2001). Inhibition of ALS by herbicides results in a build up of 2-ketobutyrate, changes in the amino acid pools, disruption to protein synthesis and disruption to photosynthate transport (Shaner 1991). At this stage, it is not clear which of these effects is responsible for plant death.

2.2.3 The evolution of resistance to ALS-inhibiting herbicides

Resistance to ALS-inhibiting herbicide was first discovered in 1987, only five years after the first ALS-inhibiting herbicide was commercialised (Mallory-Smith *et al.* 1990, Primiani *et al.* 1990). More weed species have evolved resistance to ALS-inhibiting herbicides than any other group of herbicides (Tranel and Wright 2002). By 2005, there were 90 species with biotypes resistant to ALS-inhibiting herbicides (Tranel and Wright 2005). The number of species with resistance to ALS-inhibiting herbicides has also increased more quickly

than for any other group of herbicides (Tranel and Wright 2002). Resistance in some species is so widespread that ALS-inhibiting herbicide can no longer be used to control these weeds (Tranel and Wright 2002). The widespread use of these herbicides over a large area, the high efficacy of the herbicides and their persistence in soil have all contributed to the widespread evolution of resistance to ALS-inhibiting herbicides (Tranel and Wright 2002). The distribution of ALS-inhibiting herbicide resistance in the world is shown in Figure 2.2.

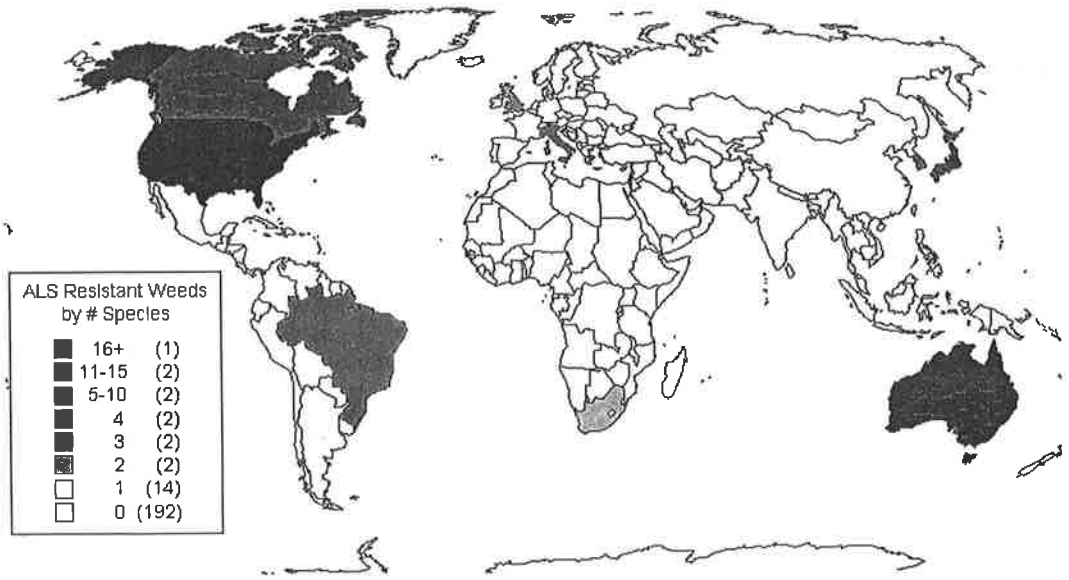

Figure 2.2 Distribution of ALS resistant species (Heap 2005).

Resistance to ALS-inhibiting herbicides is mostly the result of an insensitive target enzyme (Tranel and Wright 2002; Preston and Mallory-Smith 2001). However, there are examples of resistance as a result of more rapid detoxification of ALS-inhibiting herbicides (Christopher *et al.* 1991, Veldhuis *et al.* 2000). Where resistance to ALS-inhibiting herbicides is due to an altered target site, the resistance has been shown to be due to a

single dominant nuclear gene (Sebastian *et al.* 1989, Hart *et al.* 1993, Wright and Penner 1998, Foes *et al.* 1999) and follows Mendelian inheritance (Tranel and Wright 2002).

2.2.4 Molecular biology of resistance to ALS-inhibiting herbicides

The ALS gene does not contain introns (Tranel and Wright 2002). This has made the identification of mutations with the ALS gene simple. Substitutions at five conserved amino acids have been identified that result in target-site resistance to ALS-inhibiting herbicides (Tranel and Wright 2002). The sequences of these five conserved regions are shown in Table 2.2 with the amino acids where mutations occur underlined. While the amino acid sequence of Domain A is conserved for all amino acids in susceptible biotypes, Domain A is not protected from random mutations for neutral variations, not encoding amino acid modifications (Guttieri *et al.* 1992). The amino acid substitutions endowing resistance to ALS-inhibitors in identified weed species are shown in Table 2.3. Among all these mutations, changes at Pro 197 within Domain A are the most common (Saari *et al.* 1994), and any amino acid substitution at this site will result in an active enzyme and herbicide resistance (Guttieri *et al.* 1995). The eight different amino acid substitutions for Pro 197 indicate that herbicide-binding site is highly flexible on the ALS enzyme (Tranel and Wright 2002). For example, there were at least seven point mutations that have occured in ALS in *Kochia scoparia*, and each resulted in a herbicide resistant biotype (Foes *et al.* 1999).

Table 2.2 Peptide sequence of the five highly conservative domains in ALS gene. The substitution of the underlined residue confers resistance (Wiersma *et al.* 1989, Guttieri *et al.* 1992, Guttieri *et al.* 1995, Bernasconi *et al.* 1996, Woodworth *et al.* 1996).

Domain	Peptide sequence
A	AITGQV<u>P</u>RRMIGT
B	QW<u>E</u>D
C	VFAYPGG<u>A</u>SMEIHQALTRS
D	<u>A</u>FQETP
E	IP<u>S</u>GG

Table 2.3 ALS amino acid substitutions identified conferring herbicide resistance in weed populations (Tranel and Wright 2005).

Amino Acid Residue and Number[1]	Substitution conferring resistance	Weed Species	SU[2]	IMI[2]	PTB[2]	TP[2]	SCT[2]	Year first reported
Ala 122	Thr	*Xanthium strumarium*	S[3]	R[3]	S	ND	ND	1995
	Thr	*Amaranthus hybridus*	S	R	ND	ND	ND	1998
	Thr	*Solanum ptycanthum*	S	R	ND	ND	ND	2000
Pro 197	His	*Lactuca serriola*	R	R	S	r[3]	ND	1992
	Thr	*Kochia scoparia*	R	S	ND	R	ND	1992
	Arg	*Kochia scoparia*	R	ND	ND	ND	ND	1995
	Leu	*Kochia scoparia*	R	ND	ND	ND	ND	1995
	Gln	*Kochia scoparia*	R	ND	ND	ND	ND	1995
	Ser	*Kochia scoparia*	R	ND	ND	ND	ND	1995
	Ala	*Kochia scoparia*	R	ND	ND	ND	ND	1995
	Ala	*Brassica tournefortii*	R	S	ND	R	ND	1999
	Ile	*Sisymbrium orientale*	R	r	ND	R	ND	1999
	Leu	*Amaranthus retroflexus*	R	R	R	R	ND	2001
	Ala	*Raphanus raphanistrum*	R	S	ND	R	ND	2002
	Thr	*Raphanus raphanistrum*	R	S	ND	R	ND	2002
	Ala	*Lindernia dubia*	R	ND	ND	ND	ND	2002
	Ser	*Lindernia dubia var. major*	R	ND	ND	ND	ND	2002
	Gln	*Lindernia micrantha*	R	ND	ND	ND	ND	2002
	Ser	*Lindernia micrantha*	R	ND	ND	ND	ND	2002
	Gln	*Lindernia procumbens*	R	ND	ND	ND	ND	2002
	Ser	*Lindernia procumbens*	R	ND	ND	ND	ND	2002
	Ser	*Amaranthus blitoides*	R	S	r	r	ND	2003
	His	*Raphanus raphanistrum*	R	S	ND	R	ND	2003
	Ser	*Raphanus raphanistrum*	R	S	ND	R	ND	2003
	Thr	*Chrysanthemum coronarium*	R	r	R	r	R	2004
	Ser	*Chrysanthemum coronarium*	ND	ND	ND	ND	ND	2004
	His	*Papaver rhoeas*	R	S	ND	r	ND	2004

	Thr	*Papaver rhoeas*	R	S	ND	r	ND	2004
	Ser	*Papaver rhoeas*	R	S	ND	r	ND	2004
	Ser	*Bromus tectorum*	R	S	ND	ND	R	2004
Ala 205	Val	*Xanthium strumarium*	r	r	r	r	ND	1996
	Val	*Helianthus annuus*	r	R	ND	ND	ND	2003
Trp 574	Leu	*Xanthium strumarium*	R	R	R	R	ND	1995
	Leu	*Amaranthus rudis*	R	R	ND	R	ND	1996
	Leu	*Amaranthus hybridus*	R	R	ND	R	ND	1997
	Leu	*Kochia scoparia*	R	R	ND	ND	ND	1999
	Leu	*Sisymbrium orientale*	R	R	ND	R	ND	1999
	Leu	*Ambrosia artemisiifolia*	R	R	ND	R	ND	2001
	Leu	*Ambrosia trifida*	R	R	ND	R	ND	2002
	Leu	*Raphanus raphanistrum*	ND	ND	ND	ND	ND	2002
	Leu	*Amaranthus blitoides*	R	R	R	R	ND	2003
	Leu	*Camelina microcarpa*	R	R	ND	R	R	2004
	Leu	*Amaranthus retroflexus*	R	R	ND	ND	ND	2005
Ser 653	Thr	*Amaranthus powellii*	S	R	ND	ND	ND	2001
	Thr	*Amaranthus retroflexus*	S	R	ND	ND	ND	2001
	Asn	*Amaranthus rudis*	S	R	ND	S	ND	2001
	Thr	*Amaranthus rudis*	S	R	ND	S	ND	2001

(1) Amino acid number is standardized to the *Arabidopsis thaliana* sequence

(2) Herbicide family abbreviations:

SU = Sulfonylureas, IMI = Imidazolinones, PTB= Pyrimidinylthiobenzoates

TP= Triazolopyrimidines, SCT=Sulfonylaminocarbonyltriazolinone

(3) S =Susceptible biotype, r=Moderate resistance (< 10-fold relative to sensitive biotype), R=High Resistance (> 10-fold) ND = Not Determined

It should be noted that these classifications are generalizations.

Selected (i.e. laboratory selected) resistance-conferring ALS mutations are not in this table.

If a weed shows resistance to one ALS-inhibiting herbicide, it is also likely to be resistant to other ALS-inhibiting herbicides (Saari *et al.* 1994). For example, weeds resistant to one sulfonylurea herbicide are often cross resistant to all other sulfonylurea herbicides (Saari *et*

al. 1994). A population of *L. serriola* resistant to chlorsulfuron and metsulfuron-methyl was also resistance to eight other sulfonylurea herbicides as well as imazapyr and imazethapyr, but was not resistant to imazaquin (Mallory-Smith *et al.* 1990a). As a result of the identification of these patterns of resistance, resistance caused by altered ALS can be classified into three types, (1) SU and TP resistant, (2) IMI and PTB resistance, and (3) SU, IMI, TP and PTB resistance, the latter known as broad cross-resistance (Tranel and Wright 2002). Substitutions of Ala 122 and Ser 653 caused IMI but not SU resistance (Bernasconi *et al.* 1996). Substitutions of Pro 197 result in SU but not IMI resistance (Saari *et al.* 1994, Boutaslis *et al.* 1999). Substitutions of Trp 574 cause cross-resistance to SU and IMI herbicides (Tranel and Wright 2002). Fortunately, most weeds resistant to ALS-inhibiting herbicides did not show resistant to herbicide with other modes of action (Mallory-Smith *et al.* 1990a, Primiani *et al.* 1990).

2.2.5 Mutation rates

Mutation rates can play a role in determining resistance allele frequencies (Diggle and Neve 2001). The rate of mutation to chlorsulfuron and imazethapyr resistance in EMS-saturated *Arabidopsis thaliana* was found to be 3.2×10^{-5} (Jander *et al.* 2003). This level of mutation is likely to be several orders of magnitude higher than natural mutation rates.

The frequency of resistant individuals in natural populations with no gene flow will be a consequence of the mutation rate and the relative fitness of the resistant allele (Jasieniuk *et al.* 1996). This means that frequencies of resistant individuals could be quite a lot higher than the mutation rate. For example, Preston and Powles (2002) tested populations of *L.*

rigidum that had never been exposed to herbicides before and found the frequency of resistance to sulfometuron-methyl in these populations was between 4.6×10^{-5}-1.2×10^{-4} and resistance to imazapyr was between 1×10^{-5}-5.8×10^{-5}. This rate is more than 100 times the mutation frequency reported by Harms and DiMaio (1991) suggesting a low fitness penalty of resistant individuals. This high frequency in untreated populations allows rapid development of herbicide resistance once herbicides are used (Preston and Powles 2002).

The natural variability of ALS caused by spontaneous mutations among individual weeds can be selected by ALS-inhibiting herbicides (Tranel and Wright 2002). The high initial frequency of resistance to ALS-inhibiting herbicides in populations will result in rapid evolution of resistance to ALS-inhibiting herbicides (Tranel and Wright 2002). In addition to this high natural variation, there are a number of different amino acid substitutions within ALS that endow resistance. This is in contrast to some other herbicide modes of action where a much more limited number of substitutions are possible (Gronwald 1994, Bradshaw *et al.* 1997, Dill *et al.* 2000, Mengistu *et al.* 2000). This coupled with the low fitness penalty against resistance (Preston and Powles 2002), the intensive and widespread use of ALS inhibitors and the soil persistence of these products all mean resistance is likely to evolve regularly.

2.3 Biology of *Lactuca serriola*

L. serriola is diploid, $2n$=18 (Mulligan 1957), and belongs to the family of Asteraceae (Weaver and Downs 2003). *L. serriola* is a common weed growing along roadsides, railroads, in vacant lots, dumps, ditches, riverbanks, beaches, dockyards, quarries, gardens

and on farms (Schoennagel and Waller 1999). *L. serriola* is a winter or summer annual plant that dies after flowering. Seedlings germinate under conditions of sufficient moisture and temperature. The seedling develops into a rosette, which later develops one or more flowering stems from the centre of the rosette. The stem of the mature plant is erect and up to 1.5 m high. The leaf is oval and has a toothed-margined with a white central vein. The central vein bears a row of spines underside, which is one of the distinguishing features of the plant (Weaver and Downs 2003). *L. serriola* is sometimes referred to as the compass plant as its stem leaves are held vertical in a north-south plane. This is due to the growth response of the plant to sunlight (Werk and Ehleringer 1984). The phenotype of *L. serriola* can vary significantly within and between populations. Differences occur in leaf size and shape, the number of prickles and the degree of wax-covering of the epidermis (Fernal 1950, Werk and Ehleringer 1984).

L. serriola has numerous, small capitula flower heads which are about 8-12 mm in diameter. Flowers are in panicles at the end of the stem and branches (Weaver and Downs 2003). The seeds are spread by wind (Weaver and Downs 2003). The weight of the seed ranges from 0.45 to 0.8 mg, with an average of 0.6 mg (Prince *et al.* 1985, Werk and Ehleringer 1986a, Alcocer-Ruthling *et al.* 1992b, Gutterman 1992). Weaver and Downs (2003) reported the average seed weight of *L. serriola* in a soybean crop to be from 0.47 to 0.53 mg. Some of the factors that will affect the dispersal ability of a wind-dispersed seed are seed weight, the size of pappus, and velocity the seed can obtain. The seed is light, attached to a pappus of 4 to 5 mm long, on tall stems, which helps with wind dispersal (Frankto and Mulligan 1987, Alex 1992, Uva *et al.* 1997, Weaver and Downs 2003). Normally, seed released high on the plant could help the seed to gain velocity and the seed

will fly in the air for a longer time (Burrow 1986). Currently, there are limited data on the dispersal ability of *L. serriola*, and the dispersal ability could be various under different environmental conditions. Tackenberg (2003) reported nearly 25% of the total plants of *Senecio nemorensis* and *Cirsium arvense* dispersed over 100 m, with a wind speed ranging from 1.3 to 4.2 m s^{-1} and most from a release height of 1.5 m. There are some data on the mean settling velocity of some of the plant members in the Asteraceae family (Andersen 1992). The mean settling velocities of *Heterotheca grandiflora*, *Picris echiodies*, *Chrysopsis villosa* and *Aster exilis* are 1.016 m s^{-1}, 0.7018 m s^{-1}, 0.5135 m s^{-1} and 0.3731 m s^{-1} under conditions of no wind (Andersen 1992). However, the dispersal distance would be greatly influenced by wind speed for a wind borne species and could be suggested to range between 0.3 to 100 m with the occasional seed being carried even further when wind speeds are high or turbulant.

2.3.1 Distribution

L. serriola originated in Europe, western Asia and northern Africa (Munz 1959, Clapham *et al.* 1962, Ferakova 1976). Now *L. serriola* grows widely in North America, Australia, New Zealand, southern Africa and South America (Fernal 1950, Barkley 1986, Webb *et al.* 1988, Chapman 1991, Arnold and Wet 1993). The genus *Lactuca* originated in the Mediterranean Basin and Central Asia and *L. serriola* is the most common species in the genus (Ferakova 1976, Mejias 1994).

2.3.2 Growing conditions

L. serriola is drought-tolerant (Werk and Ehleringer 1985, 1986b) as the species originated

in a Mediterranean climate (Gallardo *et al.* 1996). In the northern hemisphere, it is normally distributed between latitudes 30 and 55 (Prince *et al.* 1978), where it grows mostly in sunny exposures (Weaver and Downs 2003). Climatic conditions do not prevent the plant from expanding to other regions (Carter and Prince 1985, Prince *et al.* 1985). When the plant is at the rosette stage, it tends to grow under moist, cool conditions but when it flowers and sets seed, it prefers dry, hot conditions (Werk and Ehleringer 1985, Jackson 1995). The Mediterranean climate could be one generally accordance with this weather pattern. The plant will have more branches after a long warm autumn (Prince *et al.* 1985).

L. serriola prefers dry soils, but it also tolerates a wide range of soil conditions (Weaver and Downs 2003). It appears on rich loams, clays, wet ditches, rocky outcrops, dry, gravely sand and forest clearings (Weaver and Downs 2003).

2.3.3 Agronomic importance

L. serriola occurs in cereals, vineyards, orchards, horticultural crops, pastures, turfgrass and nursery stock (Alex 1992, Uva *et al.* 1997). *L. serriola* can strongly compete for water with soybeans in dry seasons and only two plants per m^2 will reduce soy yields by 50% (Weaver and Downs 2003). Amor (1986) reported that the flowering time of *L. serriola* occurs during grain harvest in Australia. This means that flower buds are cut together with the grain and are difficult to screen out of the grain because they are similar in size. The result of this contamination leads to the value of the grain being discounted. Meanwhile, the thick stems of *L. serriola* clog the harvesting machinery. The leaves, stems and roots emit a milky sap when cut, which increases the moisture content of the harvested grain to

unacceptably high levels. Amor (1986) did not find a direct effect of the presence of *L. serriola* on yield in cereals or grain legumes in Australia. *L. serriola* can be harmful to livestock with cattle developing pulmonary emphysema after feeding on young fresh leaves (Kingsbury 1964).

2.3.4 Emergence, growth and development

The majority of *L. serriola* emerge in autumn followed by a small peak in spring (Prince and Marks 1982, Amor 1986). About 10 times more seedlings emerge in autumn than in spring or summer (Marks and Prince 1981). Amor (1986) reported 85% emergence occurred between April and June in Australia with a smaller peak in August and September. Under drought conditions in Australia, L. *serriola* only emerged between May and July. In Australia, stems start to elongate in July and rapid growth of stem and leaves occurs after October, with the plant reaching its final height in late December (Amor 1986). Marks and Prince (1981, 1982) in England reported peak emergence from October to December, with a smaller flush in April and May. Mortality of seedlings is highest after emergence and seedlings were most at risk with few mature plants dying. Earlier emerged plants also have higher fecundity (Marks and Prince 1981).

Growth in crops

L. serriola was reported as a frequent weed of wheat crops by Amor (1986), with an occurrence in 85% of crops. *L. serriola* that grew with little competition had 15 lateral branches arising from lower parts of the stem, while most of the *L. serriola* had only a

single stem when grown in competition with the crop (Amor 1986). Branching of the stem frequently occurred after the crop was harvested (Amor 1986). *L. serriola* reached a height of about 712 mm and a density of 1.1 plants m^{-2} when growing in wheat crops (Amor 1986). The density of *L. serriola* was 7.6 plants m^{-2} on unsown fallow and that density stayed constant throughout the year (Amor 1986).

2.3.5 Reproduction

L. serriola reproduces only by seeds (Weaver and Downs 2003). *L. serriola* is self-compatible and self-pollinated (Mejias 1994). Interspecific hybridization within the genus of *Lactuca* seldom occurs and the species are highly autogamous (Ferakova 1977). Pollen viability of *L. serriola* is 90% or more (Ferakova 1977). Seeds produced per capitulum varies between 12 to 22 (Arthur 1984, Prince et al. 1985, Alcocer-Ruthling et al. 1992b, Weaver and Downs 2003). Seeds produced per plant can be quite variable depending on the growing environment and the competitiveness of the crop. Alcocer-Ruthling et al. (1992) reported seed production of 4160 seeds for chlorsulfuron susceptible plants and 4870 seeds for chlorsulfuron-resistant plants. Amor (1986) reported *L. serriola* growing in crop stubble or wheat crop in Australia produced 48,000 to 90,000 seeds per plant. The number of capitula per plant was reported to be proportional to plant height in wheat stubble or soybeans (Weaver and Downs, 2003), and was reported between 500 and 5000 capitula per plant, giving 10,000 to 100,000 seeds per plant in open, recently cultivated areas without crop competition in England (Prince and Carter 1985).

The seed bank of *L. serriola* is short, lasting only one to three years (Marks and Prince 1981, 1982, Alcocer-Ruthling *et al.* 1992b). Seed viability is nearly 100% at harvest, and

declines with the length of seed burial (Weaver and Downs 2003). The half-life of buried seed is one and a half years in England (Marks and Prince 1982). Between 65 to 75% of seeds germinated in the first two years of burial, but none germinated after two years (Toole and Brown 1946). Marks and Prince (1982) reported seed viability declined to 75% in 80 weeks. Alcocer-Ruthling et al. (1992c) reported that seed viability declined from 100% to 0% with burial time from 17 to 30 months and buried seed survived longer than seed on the soil surface. The variability of surface seed declined to 0% after 12 to 18 months, while that of seed buried at 7.5 to 15 cm was 33 months (Alcocer-Ruthling *et al.* 1992b). There was no significant differences between the sulfonylurea susceptible and resistant biotypes in seed longevity (Alcocer-Ruthling *et al.* 1992b).

Seeds can germinate immediately after dispersal and have no primary dormancy (Marks and Prince 1982, Alcocer-Ruthling *et al.* 1992a). Both chlorsulfuron-susceptible and resistant *L. serriola* had 50% seeds germinating in two to four days (Alcocer-Ruthling *et al.* 1992b). Germination is responsive to temperature with almost no germination at a constant temperature below 8°C (Marks and Prince 1982). The ideal temperature for germination is between 12°C to 24°C, with higher temperatures of between 26°C to 35°C inducing secondary dormancy (Marks and Prince 1982, Gutterman 1992). However, herbicide resistant seeds were found to germinate twice as fast as susceptible ones (Alcocer-Ruthling *et al.* 1992).

2.3.6 Herbicide resistance in *L. serriola*

In 1987, sulfonylurea resistant *L. serriola* was first reported in Idaho in a continuous no-till winter wheat crop field (Mallory-Smith *et al.* 1990a) and became one of the dominant

weeds in the following five years. This was also the first time that resistance to an ALS-inhibiting herbicide had been discovered (Mallory-Smith *et al.* 1990a). Sulfonylurea herbicides were applied to this field every 6 to 14 months from 1982 to 1987 (Mallory-Smith *et al.* 1990a). However, when sulfonylurea herbicides were not used to control weeds on the farm from 1988 to 1990, the proportion of resistant *L. serriola* decreased by 25-86%, while the area with resistant *L. serriola* increased due to seed movement (Alcocer-Ruthling *et al.* 1992a).

In South Australia, *L. serriola* has evolved resistance to the ALS inhibiting herbicides chlorsulfuron and triasulfuron. This resistance was first recorded in 1994 following 4 applications of sulfonylurea herbicides (Preston *et al.* 2005). The resistant populations have cross-resistance to other ALS-inhibiting herbicides. It has already infested 101 to 500 sites and 101 to 500 acres and the number of sites and area infested are increasing (Table 2.4).

Table 2.4 The distribution of ALS-inhibiting herbicide resistant *Lactuca serriola* in the world (Preston *et al.* 2005d)

Place	Year first occurred	Numbers of sites	Acres infested
Australia (South Australia)	1994	101-500	101-500
USA (Idaho)	1987	11-50	10,001-100,000
USA (Washington)	1993	11-50	101-500
USA (Oregon)	1993	101-500	10,001-100,000

The rapid selection of sulfonylurea resistant *L. serriola* in South Australia is probably the

result of a relatively high frequency of initial resistance in untreated populations, the effective control of *L. serriola* at low rates of sulfonylurea herbicides, and the persistence of sulfonylureas in the soil due to the alkaline soils in the area (Preston *et al.* 2005a).

ALS-inhibiting herbicide resistance in *L. serriola*

The mechanism of ALS-inhibiting herbicide resistance in *L. serriola* from Idaho was studied by Eberlein *et al.* (1997) and determined to be the result of a resistant form of ALS. The mutation that contributes to resistance in *L. serriola* collected in Idaho is in Domain A of ALS with proline residue changed to histidine residue (Guttieri *et al.* 1992). Preston *et al.* (2005) investigated the mechanism of resistance to ALS-inhibiting herbicides in two populations from South Australia and determined the mechanism in these populations was also due to a modification of ALS. In this case a mutation of threonine for proline was found in both populations.

These amino acid substitutions in the ALS enzyme caused altered ALS activity in addition to resistance to herbicides (Eberlein *et al.* 1997, Preston *et al.* 2005a). The similarity of K_m (pyruvate) of ALS isolated from herbicide resistant and susceptible *L. serriola* from Idaho suggested the mutation did not impair pyruvate binding (Eberlein *et al.* 1997). ALS from resistant *L. serriola* had altered feedback inhibition compared to the enzyme from susceptible populations. Mutations of Pro 197 result in differences in branch chain amino acid feedback inhibition of ALS (Rathinasabapathi *et al.* 1990, Subramanian *et al.* 1991, Mourad *et al.* 1995, Eberlein *et al.* 1997). In the Idaho population (Eberlein *et al.* 1997), ALS activity in resistant *L. serriola* was 57% less than that of susceptible plants and ALS

from resistant plants was 12% to 30% less sensitive to the inhibition by valine and isoleucine. Leucine, valine and isoleucine concentrations in seed were 43%, 70% and 9% higher in resistant seed compared to susceptible seed. In the South Australian populations (Preston *et al.* 2005a), leucine and valine inhibited ALS activity by 47-57% and 63-68% respectively in susceptible *L. serriola*, compared with 19-24% and 47% respectively in resistant plants. K_m (pyruvate) for susceptible populations was about 10 mM, and 5 mM for resistant populations. In *L. serriola* from South Australia, the K_m (pyruvate) was reduced in the resistant populations compared to the susceptible populations, suggesting the mutant enzyme was more efficient (Preston *et al.* 2005a).

The different substitutions of Pro 197 in *L. serriola* resulted in different resistance across herbicides (Table 2.5) (Eberlein *et al.* 1997, Preston *et al.* 2005a). The two ALS-inhibiting herbicide resistant *L. serriola* populations collected 25 km apart in South Australia had the same mutation in ALS, which was a proline changed to threonine in Domain A (Preston *et al.* 2005a). However, different leaf morphology of the two populations suggested different origins of the mutations (Preston *et al.* 2005a).

Table 2.5 Comparison of ALS-inhibiting herbicide resistance in *L. serriola* from Idaho (Eberlein *et al.* 1997) and South Australia (Eberlein *et al.* 1997, Preston *et al.* 2005a).

Source	Lewiston , ID	Bute, SA	Kulpara, SA
Time of collection	1990	1996	1997
Mutation in ALS gene	Proline→Histidine in Domain A	Proline→Threonine in Domain A	Proline→Threonine in Domain A
Resistance status	High resistance to sulfonylureas, moderate resistance to imidazolinones and triazolopyrimidines	High resistance to chlorsulfuron, moderate resistance to imazethapyr and flumetsulam	High resistance to chlorsulfuron, moderate resistance to imazethapyr and flumetsulam
K_m (pyruvate) Resistant	7.3±1.5	5.4±0.1	4.9±0.4
K_m (pyruvate) Susceptible	7.1±1.5	10.0±0.6	9.7±1.5
Specific activity $nmol^{-1}$ mg^{-1} protein Resistant	2.8±0.6	8.0±0.3	9.7±0.2
Specific activity $nmol^{-1}$ mg^{-1} protein Susceptible	6.4±0.6	9.9±0.2	12.2±0.2

2.3.7 Summary

L. serriola is a common weed that will decrease harvesting efficiency when it occurs in fields (Amor 1986). The species is self-pollinated and the seed is wind-borne (Weaver and Downs 2003). Resistance to ALS-inhibiting herbicides first appeared in 1987 (Mallory-

Smith *et al.* 1990a), and the resistance is controlled by a single, dominant nuclear gene (Mallory-Smith *et al.* 1990b). Two substitutions at Pro 197 have been identified endowing resistance in different populations, and they resulted in multiple impacts on ALS enzyme activity, which may influence the frequency of the resistant allele in the environment (Eberlein *et al.* 1997, Preston *et al.* 2005a). The substitutions of Pro 197 were the same for two resistant populations collected in South Australia 25 km apart (Preston *et al.* 2005a).

As the seed of *L. serriola* is spread by wind, it is possible that resistance to herbicides could spread rapidly from one site to another. In order to understand the potential spread of herbicide-resistant *L. serriola*, it is important to be able to determine whether individual sites with resistance are related or not. To do so requires the use of tools that can distinguish the genetic relatedness of different populations with resistance. The next section will discuss the use of genetic markers as a tool for distinguishing between populations.

2.4 Molecular markers

2.4.1 Application of molecular markers to weed research

The development of powerful molecular markers has provided weed research with a larger array of genetic tools for studying population genetics, evolution and gene flow. A significant advantage of molecular markers is that they avoid environmental effects by measuring genotype instead of phenotype and enable better assessment of genetic relationships within and between populations (O'Hanlon *et al.* 2000). These techniques are cost-effective and can be less demanding than scoring phenotypes (O'Hanlon *et al.* 2000). Molecular markers are well established and currently widely used in weed research to

investigate genetic diversity, identify taxonomy, and determine the origin of introduced weeds particularly where multiple origins occur (Nissen *et al.* 1995, O'Hanlon *et al.* 2000). With the application of molecular markers, differentiation between taxa can be revealed. For example, a study of *Euphorbia esula* L. in North America using a combination of RAPDs and chloroplast RFLPs found the weed to have high inherent variability and/or multiple introductions into the area (Rowll *et al.* 1997). By using RAPD markers, Scott et al. (1998) identified two genotypes of *Chromolaena odorata* (L.) in North Queensland and identified the origins of both as Brazil.

Weed spatial dynamics are determined largely by seed movement with seed dispersal ranges determining colonization sites and extinction of local populations (Brown and Kodric-Brown 1977). Dispersal is also an important factor in evaluation of genetically modified organisms (Ellstrand and Hoffman 1990) and viability of fragmented populations (Ellstrand 1992, Ellstrand and Elam 1993). Long distance dispersal is important to determine colonization possibilities into new sites and influences on genetic variation (Ouborg *et al.* 1999). However, quantifying dispersal was difficult prior to the availability of molecular markers (Ouborg *et al.* 1999). Many researchers have measured the actual distances of dispersal by artificially analogising dispersal propagules (Nilsson *et al.* 1991), recapturing marked and released propagules (Johansson and Nilsson 1993), trapping seeds from the source (Huiskes *et al.* 1995, Ruckelshaus 1996, Thiede and Augspurger 1996), and using diffusion models (Greene and Johnson 1996, Cain *et al.* 1998). However, these studies tend to underestimate long distance dispersal (Cain *et al.* 1998). Molecular markers can be used to study the consequences of dispersal and, hence, become powerful tools to determine dispersal distances (Silvertown 1991). The most commonly used molecular

markers are AFLPs (amplified fragment length polymorphisms), RFLPs (restriction fragment length polymorphisms), RAPDs (random amplified polymorphic DNA), SSRs (simple sequence repeats) and ISSRs (inter simple sequence repeats) (Ouborg *et al.* 1999).

Different molecular markers have different characteristics and it is important to understand these characteristics before choosing a marker to use (Ouborg *et al.* 1999). Most variation expressed by molecular markers is selectively neutral, with the exception of SSRs and some other markers. Different molecular markers display different amounts of variability. There are codominant markers like RFLPs, which can be used to distinguish between homozygotes and heterozygotes, and dominant markers such as AFLPs and RAPDs, which cannot distinguish between homozygotes and heterozygotes. Using codominant markers, allele frequencies in populations can be more accurately estimated.

2.4.2 Amplified fragment length polymorphisms (AFLPs)

AFLP is a molecular marker technique that can generate a large number of polymorphisms. Therefore, this method can resolve extremely small genetic differences. For example, Maughan *et al.* (1996) used AFLPs to resolve small differences in the genome of soybean near-isogenic lines. AFLPs have also been used to uncover genetic variation in strains and closely related species of a wide range of taxa, including bacteria, fungi, plants and animals (Beismann *et al.* 1997, Heun *et al.* 1997, Russell *et al.* 1997, Triantaphyllidis *et al.* 1997). The application of AFLPs is also helpful in differing sets of clonally-descended individuals in plants and fungi (Beismann *et al.* 1997, Rosendahl and Taylor 1997, Majer *et al.* 1998, Muller *et al.* 1998). AFLPs have also been successfully applied in identifying novel

pathogens in hospital surveys (Koeleman *et al.* 1997), identifying microorganisms (Huys *et al.* 1996, Janssen *et al.* 1997) and nematodes (Semblat *et al.* 1998). AFLPs are efficient for inferring phylogenetic relationships between closely related species (Huys *et al.* 1996, Powell 1996a, Tohme *et al.* 1996, Beismann *et al.* 1997, Keim *et al.* 1997, Semblat *et al.* 1998), and are widely applied in the investigation of genetic variation within a species, identification of population structure and differentiation (Travis *et al.* 1996, Rosendahl and Taylor 1997, Semblat *et al.* 1998, Winfield *et al.* 1998). The method has also been used in evaluating gene flow and dispersal (Travis *et al.* 1996, Arens 1998, Majer *et al.* 1998, Semblat *et al.* 1998), outcrossing (Gaiotto *et al.* 1997), introgression (Tohme *et al.* 1996), and hybridization (Beismann *et al.* 1997, Arens 1998). AFLPs are also suitable to identify parentage (Mueller and Wolfenbarger 1999) and analyse relatedness and mating frequency because of the low level of artifacts (Jones 1997) and comigration of non-allelic fragments (Rosendahl and Taylor 1997, Rouppe Van Der Voort 1997). Koopman *et al.* (1998) used AFLPs to determine that *L. sativa* and *L. serriola* were conspecific.

Despite the benefits of AFLPs, there are some drawbacks. AFLPs are not efficient in identifying homologous alleles, and less useful for heterozygosity analyses (Mueller and Wolfenbarger 1999). The cost of the method is relatively high compared to other molecular markers (Robinson and Harris 1999). The number of polymorphisms is also affected by the restriction enzyme and primers used. For example, using the combination of *Pst* I/*Mse* I detected more polymorphisms than using *EcoR* I/*Mse* I (Ridout and Donini 1999). Chosen primers will largely influence the amount and quality of variation. For example, in research on hop cultivar diversity, primers consisting of adaptors and two selective nucleotides produced too many bands to score and separate on the polyacrylamide gels (Hartl and

Seefelder 1998). There is also a need to screen the primers prior to starting. In the work of Hartl and Seefelder (1998), 60 primer combinations were screened, but only eight combinations were reliable. Without preliminary screening of the primer combinations, randomly chosen primers may not give sufficient and reliable data (Robinson and Harris 1999). Additional screening of the primer combinations adds to the cost of the method (Robinson and Harris 1999).

2.4.3 Restriction fragment length polymorphisms (RFLPs)

With the application of RFLP method, changes in non-coding and coding sequences were analysed for the first time (Schotterer 2004). The first genetic maps and the successful associated studies were based on RFLP markers (Kerem *et al.* 1989). RFLPs were developed earlier than the other molecular markers and first used for human genome mapping (Botstein *et al.* 1980) and then plant genome mapping (Helentjaris *et al.* 1985, Weber and Helentjaris 1989). The method has been used in mitochondrial DNA and ribosomal DNA to study population genetics, conduct biogeographical surveys and phylogenetic studies (Avise 1994). For example, Kesseli (1991) used RFLPs to determien that *L. sativa* and *L. serriola* share 36% of alleles.

In RFLPs, restriction sites on DNA are recognised by one or more restriction endonucleases used, and the DNA cut wherever the recognised sequences are encountered (Loxdale and Lushai 1998). Common restriction endonucleases recognise restriction sites that are four to six bases in length (Loxdale and Lushai 1998). DNA changes such as indels (insertions or deletions), base substitutions and rearrangements involving the restriction sites causing

gain, loss and relocation of the restriction site will result in the change in the number and size of the fragment digested among individuals, populations and species (Loxdale and Lushai 1998, Liu and Cordes 2004). The cut DNA fragments can be visualized on ethidium bromide-stained agarose gels or polyacrylamide gels (Loxdale and Lushai 1998), so there is no need for radioactive isotopes (Binder 1997). However, radiolabelled probes developed from genomic libraries can be used to increase resolution (Bruford *et al.* 1991, Frischauf 1991, Avise 1994). Recent analyses have used PCR instead of the traditional southern blot method for detection (Liu and Cordes 2004). Relatively large deletions or insertions on the DNA sequence will result in different size of bands on gels (Liu and Cordes 2004).

RFLP is a codominant marker and all alleles can be observed in the analysis, which is its basic advantage (Liu and Cordes 2004). Fewer artificial factors will effect the scoring result as the size of fragments is often large (Liu and Cordes 2004). However, RFLP is time-consuming and labour intensive (Gupta *et al.* 1999) and not as efficient as the other molecular markers in revealing genetic variation, as the chances of change occurring in the DNA within the locus are low (Liu and Cordes 2004). The method also requires sequence information to develop probes and can be difficult to use in species without sufficient sequence information (Liu and Cordes 2004).

2.4.4 Randomly amplified polymorphic DNA (RAPDs)

RAPDs are DNA fragments generated via PCR, using randomly selected primers (Williams *et al.* 1990). The method requires small amounts of DNA (Virk *et al.* 2000) and no radioactive substances (Waugh and Powell 1992). It is inexpensive and can be easily

automated (Waugh and Powell 1992). RAPD fragments are specific makers and polymorphisms in numerous loci can be generated by the RAPD technique (Gostimsky *et al.* 2005).

RAPD is the most commonly used method to identify DNA polymorphisms in plants and has been used for detecting genome differences between closely rated organisms (Sivolap and Kalendar 1995, Oganisyan *et al.* 1996, Gostimsky *et al.* 2005). It has also been used for the identification of individuals within populations (Miller *et al.* 1996), paternity analysis, strain identification and phylogenetic analysis (Parker *et al.* 1998). For example, the method was used effectively for the identification of Sitka spruce (Van de Ven and McNicol 1995) and American elm (Kamalay and Carey 1996). Generally, sufficient loci can be obtained by RAPDs for genetic diversity estimation (Bai *et al.* 1997), taxonomic identification (Transue *et al.* 1994), characterizing population structure (Huff *et al.* 1993, Nesbitt *et al.* 1995), linkage mapping (Tulsieram *et al.* 1992), identifying hybrids (Baired *et al.* 1992), targeting chromosomes (Gourmet and Raybourn 1996), marker assisted selection and quantitative trait locus (QTL) mapping (Deragon and Landry 1992).

Randomly selected primers used in RAPDs do not require prior knowledge of the primer binding site sequences (Williams *et al.* 1990). Primers, usually 9-10 bp long with 60-80% GC composition, are used singly without combination with a second primer (Williams *et al.* 1990). Primers kits have been commercialised and more than 400 different 10-base primers can be ordered from different companies (Ritland and Ritland 2000). Annealing temperatures used in RAPDs are relatively low (Gostimsky *et al.* 2005). Regions amplified

by RAPDs are scattered across the whole genome, with quite a number of repetitive nucleotide sequences (Williams *et al.* 1990). Compared with RFLPs, RAPDs can detect higher levels of polymorphism (Williams *et al.* 1990).

Factors that cause the different banding patterns with RAPDs are presence or absence of priming sites, the level of priming completeness and the size of the fragment between priming sites (De Wolf *et al.* 2004). Point mutations, inversions, deletions and additions that occur in primer sites result in polymorphisms (De Wolf *et al.* 2004). PCR products are visualized by agarose or polyacrylamide gel electrophoresis (Williams *et al.* 1990). Several polymorphic loci can usually be generated by one primer within a population (Hadrys *et al.* 1992).

Because the fragments amplified are short, some artifactual PCR products can be generated. Therefore, the control of DNA quality and PCR conditions are important to reproduce banding patterns (Carlson *et al.* 1991, Riedy *et al.* 1992, Scott *et al.* 1993). Replicates need to be included and only reproducible bands should be scored (Kump and Javornik 1996, Aagaard *et al.* 1998, Ge *et al.* 2003, Maltagliati *et al.* 2003). RAPDs are not codominant marker systems. The polymorphisms usually result in complete absence of DNA fragment and do not appear as change of positions of the banding patterns (Parker *et al.* 1998). Therefore, in heterozygotes, mutation on one of the DNA chains may only appear as lower band intensity, which is hard to detect (Parker *et al.* 1998). The nature of the scored genome change is unknown (Parker *et al.* 1998). There are possibilities that more GC-rich regions are screened because of the high GC composition in most RAPD primers and the

relatively low annealing temperatures in PCR (Harris 1999).

2.4.5 Simple sequence repeats (SSRs, Microsatellites)

Microsatellites or simple sequence loci are one of the variable number of tandem repeat (VNTR) loci (Nakamuara *et al.* 1987). Certain simple short sequence motifs such as poly (GT) and poly (GCG) ubiquitously occur in eukaryotic genomes in much higher frequency than other random sequences of similar length (Hamada *et al.* 1982, Tautz and Renz 1984). Microsatellites are one of the major sources of genetic variation (Tautz *et al.* 1986, Tautz 1989, Weber and May 1989). They comprise 10-50 copies of a short repeat motif of 1-10 base pairs (bp), usually 2-5 bp (Tautz 1989, Weber 1990). Microsatellites are distributed relatively evenly on all regions of the chromosomes (Liu and Cordes 2004), and are also present inside coding regions (Liu *et al.* 2001), including introns and exons (Liu and Cordes 2004).

The sequence, length and contiguity of the repeat motif affect the rate of mutation and levels of allelic variation (Cregan 2000). Microsatellite sequences arise as a result of DNA replication slippage (Levinson and Gutman 1987). Interruption of the core sequence results in arrays of repeats and generates polymorphisms between individuals (Cregan 2000). The polymorphisms of interrupted repeats are less variable than pure repeats (Richards and Sutherland 1994, Pepin *et al.* 1995, Petes *et al.* 1997). The distribution and prevalence of microsatellite motifs varies greatly across taxa (Stallings *et al.* 1991, Beckmann and Weber 1992, Lagercrantz *et al.* 1993, Booker *et al.* 1994, Jurke and Pethiyagoda 1995, Primmer *et al.* 1997).

The mutation rate of microsatellites is estimated at about 10^{-2}- 10^{-3} per locus per gamete, which means that on average a new microsatellite will arise in less than one thousand gametes (Weber and Wong 1993, Crawford and Cuthbertson 1996, Hartwell *et al.* 2004). This is much greater frequency than the mutation rate of a single nucleotide (10^{-9}) (Hartwell *et al.* 2004). The allelic distribution suggests a two-step, step-wise mutation model for microsatellites (Di Rienzo *et al.* 1994). The size and sequence of the repeat motifs and the sequences surrounding the motifs can vary within a species (reviewed by Scribner and Pearce 2000). Alleles of a certain size may not be identical by descent (Estoup *et al.* 1995, Garza and Freimer 1996, Angers and Bernatchez 1997). However, the rate of microsatellite mutation is low enough to be stable across several generations (Hartwell *et al.* 2004). Most microsatellite mutations result in insertions or deletions of one or two repeat units, which do not erase the information of ancestral states (Valdes *et al.* 1993, Weber and Wong 1993, Di Rienzo *et al.* 1994). Highly polymorphic and relatively stable microsatellites are used in linkage studies of human families, animals and plants (Hartwell *et al.* 2004). Microsatellites are a codominant maker and inherited in a Mendelian fashion (Liu and Cordes 2004).

Microsatellite markers are reproducible, multiallelic, relatively abundant, have a small locus size, and good genome coverage. Therefore, they are useful for a variety of applications in plant genetics and breeding (Powell 1996b). Microsatellites are efficient to link phenotypic and genotypic variation and are used in integrating genetic, physical and sequence-based physical maps in plant species (Gupta and Varshney 2000). Microsatellites are high-quality, provide strong bands and distinct allelic peaks in a number of studies (Cho 2000, Eujay 2001, Kota 2001, Thiel 2003, Nicot 2004, Saha 2004, Yu 2004). The

polymorphic microsatellite loci as genetic markers are being increasingly used in plant genome research because of the informativeness of the microsatellite markers and the potential of thousands of microsatellite sequences per genome (Cregan 1996). Genetic mapping of plant species such as maize (Senior and Heun 1993), rice (Wu and Tanksley 1993), *Arabidopsis* (Bell and Ecker 1994), soybean (Morgante *et al.* 1994), barley (Becker and Heun 1995) and tomato (Broun and Tanksley 1996) has used microsatellites to detect polymorphisms. This method provides a useful complement to the RFLP and RAPD markers.

Microsatellites are scored by PCR analysis. Primers are sequences that flank the repeats and therefore sequence information is required (Tautz 1989, Rassmann *et al.* 1991). PCR products are run on high resolution gels and variation in the number of repeats between alleles are detected and scored (Schlotterer 1998). For some species, the sequence of some microsatellites and their flanking sequences are available in databases (Schlotterer 1998).

Compared with RADP system, using microsatellite markers is time consuming for the steps required to identify the polymorphic loci (Cregan 1996). First the desired clones have to be identified by developing and screening a DNA library with a repetitive sequence oligonucleotide probe. Subcloning and identification of the appropriate subclone are required before sequencing if the library is composed of longer sequences. Another impediment to the widespread use of this method is the PCR products are often similar in length, which makes them difficult to detect. It has become popular to use ^{32}P to label PCR products, which offers rapid determination of the allelic constitution of three microsatellite loci from one PCR reaction. Cregan (2003) suggested that the elimination of secondary structure by denaturation might not be necessary to distinguish DNA fragments of

microsatellites, so a non-denaturing polyacrylamide gel separation followed by ethidium bromide or silver staining may be used.

2.4.6 Inter simple sequence repeats (ISSRs)

ISSRs have some advantages over the other four maker systems described above. Compared to ISSRs, the reproducibility of RAPDs is lower and the cost of AFLPs is higher. With SSRs, there is a requirement to know the flanking sequences for primer design (Meyer *et al.* 1993, Gupta *et al.* 1994, Wu *et al.* 1994, Zietkiewicz *et al.* 1994). ISSRs combine many of the benefits of AFLPs, SSRs and RAPDs (Reddy *et al.* 2002). ISSRs have been used in a number of different plant research areas, such as studies of genetic diversity, phylogenetic studies, gene tagging, genome mapping, and evolutionary biology of different plant species (Godwin *et al.* 1997). ISSRs are a dominant maker system and follow Mendelian inheritance (Gupta *et al.* 1994, Tsumura *et al.* 1996, Ratnaparkhe *et al.* 1998, Wang *et al.* 1998). However, ISSRs have been able to distinguish between homozygotes and heterozygotes in some cases (Wu *et al.* 1994, Akagi *et al.* 1996, Wang *et al.* 1998, Sankar and Moore 2001). Primers of ISSR PCR are not proprietary and the technique is highly reproducible (Reddy *et al.* 2002).

In ISSRs, the DNA segment between microsatellites is amplified (Reddy *et al.* 2002). The core sequence of the primers used in ISSRs is a microsatellite sequence of 16-25 bp of dinucleotide, trinucleotide, tetranucleotide or pentanucleotide repeats (Reddy *et al.* 2002). The sequence of the primer usually includes an anchor of 1-4 bp at the 3' or 5' end of the microsatellite sequence (Zietkiewicz *et al.* 1994). In some studies, unanchored primers have

been successfully used (Meyer *et al.* 1993, Gupta *et al.* 1994, Wu *et al.* 1994). Annealing temperatures depend on GC content of the primer and are usually between 45 to 65° C.

The primers used for ISSRs (16-25 bp) are longer than those used for RAPDs (10-mers), so annealing temperatures used in ISSRs (45-60° C) are higher than that in RAPDs. This results in higher stringency, with 92 to 95% of fragments being repeated in separate runs (Fang and Roose 1997, Moreno *et al.* 1998). ISSRs are also repeatable with varying amounts of template DNA. For example, in a 20 µl PCR reaction, DNA template of 10 ng, 25 ng and 50 ng yielded the same PCR products (Reddy *et al.* 2002).

Polymorphisms generated by ISSRs occur because of the high mutation rate of microsatellites. Primer binding sites can change when there are mutations at the flanking site of the microsatellite, which gives a presence or absence of a fragment (Reddy *et al.* 2002). Insertion and deletion of one or more nucleotides in DNA will also change the length of the amplified fragment in ISSRs. The differences between the numbers of nucleotides in different individuals will result in length polymorphisms (Reddy *et al.* 2002). Unanchored primers normally tend to slip within the repeat units in PCR and result in smears and the bands may not be clear (Reddy *et al.* 2002). Therefore, anchors are added at the 3' or 5' ends that make the primers only anneal in one direction, which reduces smearing (Reddy *et al.* 2002). Dinucleotide repeats with anchors were reported to reveal high numbers of polymorphism (Nagaoka and Ogihara 1997, Blair *et al.* 1999, Joshi *et al.* 2000) and are used more frequently than tri- and tetra-nucleotide repeats (Reddy *et al.* 2002).

Primers with (AG), (GA), (CT), (TC), (AC), and (CA) repeats usually generate higher numbers of polymorphisms than primers with other di-, tri-, or tetra- nucleotide repeats (Reddy *et al.* 2002). (AT) repeats are the most common di-nucleotides in plants, but primers consisting of (AT) repeats self-anneal and the PCR reaction will not work (Reddy *et al.* 2002). ISSR PCR primers based on (AG) and (GA), have been used to generate clear bands in rice (Blair *et al.* 1999, Joshi *et al.* 2000, Reddy *et al.* 2000, Sarla *et al.* 2000), trifoliate orange (Fang and Roose 1997), Douglas fir, sugi (Tsumura *et al.* 1996) and chickpea (Ratnaparkhe *et al.* 1998). Primers based on (AC) di-nucleotide repeats have been used to generate effective ISSR results in wheat (Nagaoka and Ogihara 1997, Kojima *et al.* 1998) and potato (McGregor *et al.* 2000). ISSR products are usually between 200 to 2000 bp and can be detected on polyacrylamide or agarose gels without the need for radiolabel (Tsumura *et al.* 1996, Kojima *et al.* 1998, Wolff and Morgan-Richards 1998, Arcade *et al.* 2000, Sankar and Moore 2001). Generally, more bands can be detected on polyacrylamide gels than on agarose gels (Moreno *et al.* 1998). High quality silver staining can be used to increase the ability to detect bands (Fang and Roose 1997).

ISSRs have been used to study genetic diversity within and between species in *Eleusine* (Salimath *et al.* 1995), gymnosperms (Tsumura *et al.* 1996), wheat (Nagaoka and Ogihara 1997), *Plantago* (Wolff and Morgan-Richards 1998), rice (Joshi *et al.* 2000), *Vigna* (Ajibade *et al.* 2000), sweet potato (Huang and Sun 2000), cocoa (Charters and Wilkinson 2000) and even fungi (Hantula *et al.* 1996). ISSRs have proved to be more reproducible and efficient than RFLPs and RAPDs in detecting diversity in trifoliate orange germplasm (Fang and Roose 1997). The method showed advantages in output quality and quantity data compared with RFLPs and RAPDs in detecting the diversity in the genus *Eleusine*

(Salimath *et al.* 1995). ISSRs also showed efficiency in characterizing genetic diversity at various level with a variety of species (Reddy *et al.* 2002). For example, 20 cultivars of *Brasssica napus* could be distinguished with the use of ISSRs (Charters *et al.* 1996). Only two ISSR primers were sufficient to distinguish 37 white lupins (Gilbert *et al.* 1999), three ISSR primers were sufficient to distinguish 16 genotypes of redcurrant (Lanham and Bernnan 1998) and only four primers were required to distinguish 34 cultivars of potatoes (Prevost and Wilkinson 1999).

ISSRs are simple, quick, efficient, highly reproducible and do not require the use of radioactivity. The main limitation of the method is that the molecular nature of the polymorphisms will not be known unless the DNA fragments are extracted from gel and sequenced (Reddy *et al.* 2002).

2.4.7 Summary

There is no perfect molecular marker system for all applications, and new markers are not necessarily better than the old ones (O'Hanlon *et al.* 2000). Choosing the best marker system depends on the aim of the study, technical considerations, availability of facilities and cost (Morell *et al.* 1995, Peakall 1997). Table 2.6 summarises the advantages of the five molecular markers described above.

Table 2.6 Comparison of the five molecular markers described above.

	AFLPs	RFLPs	RAPDs	SSRs	ISSRs
Co-dominant	No	Yes	No	Yes	No
Reproducibility	+++	++	++	+++	+++
Development time	Moderate	Long	Short	Long	Short
Cost	Moderate	High	Low	High	Low
Main tasks	Strain identification Genetic resource/ diversity analysis	Population structure	Species identification Hybrid identification	Strain identification Genetic resource/ diversity analysis	Strain identification Genetic diversity analysis
Usefulness for determining population relatedness	+++	++	++	+++	+++
Usefulness for identification of strains	+++	+	++	+++	+++
Ability to reveal variation	+++	+	++	+++	+++
Ease of use	Difficult initially	Labour intensive	Easy	Easy	Easy
DNA required (μg)	0.5-1.0	10	0.02	0.05-0.10	0.001-0.1
Level of polymorphisms (Hav[1])	0.32	0.41	0.31	0.60	Not in literature
Effective multiplex ratio[2]	19.2	0.25	1.56	1.0	Not in literature
Marker index (MI[3])	6.14	0.1	0.48	0.60	Not in literature

(Rafalski and Tingey 1993, Karp and J. 1995, Powell 1996a, Godwin *et al.* 1997, Parker *et al.* 1998, Mueller and Wolfenbarger 1999, Reddy *et al.* 2002, Liu and Cordes 2004)

Notes:

[1]Hav, average heterozygosity, which is the probability that two alleles taken at random can be distinguished.

[2]Effective multiple ratio, the number of polymorphic loci analysed per experiment in the germplasm tested.

[3]Marker index, the product of the average expected heterozygosity and the effective multiplex ratio.

2.5 Summary and aims of project

The widespread use of ALS-inhibiting herbicides has resulted in the evolution of resistance in many weed species. Of some interest in assessing the evolution of herbicide resistance in weeds is the relationship between mutation and gene flow in providing the resistance alleles to be selected in any population. This can be particularly important when significant amounts of gene flow occur between populations. ALS-inhibiting herbicide resistant *L. serriola* occurred in the Snowtown area of South Australia in 1994. Shortly thereafter, resistance was present in a number of sites across the Yorke Peninsula of South Australia. The main aim of this project was to determine the genetic relationships between resistant populations of *L. serriola* in order to determine whether gene flow or mutation was more important for the widespread distribution of resistance across the Yorke Peninsula.

In order to test this, seed collected from different populations across this area was tested for resistance to the ALS-inhibiting herbicide chlorsulfuron. ISSRs were used to genotype individuals and to determine their relatedness. This was repeated with plants from a second collection from the area.

Chapter 3 Materials and Methods

3.1 Plant material

In an earlier unpublished survey, seed from *Lactuca serriola* plants was collected across a region of approximately 60 km by 60 km centred on Bute, South Australia in 1999 (Figure 4.1). Seed were collected from individual plants growing in cereal fields and adjacent roadsides. Up to 30 plants within each population were individually sampled. A paper bag was placed over the flowering stalk and the stalk cut from the plant. These samples were transported back to the laboratory where they were dried and the seed removed from the heads and stored until required. A second collection of *L. serriola* seedlings was made in 2004 from fields and adjacent roadsides at several sites in the northern Yorke Peninsula, South Australia. Seedlings were transported to Adelaide and transplanted individually into pots. Seed of two susceptible populations were collected from waste ground in the Adelaide area where herbicides are not used.

Seeds were germinated by placing them on the surface of moist soil. Once seed had germinated, seedlings were thinned to five plants per pot. At the 4-leaf stage, leaf material was removed from the plants and frozen in liquid nitrogen. The leaves were stored at -80°C until required. After the leaves had been harvested, plants were treated with 15 g a.i. ha^{-1} chlorsulfuron to determine the resistance status of each plant. Survival was assessed 21

days after herbicide application. Survivors of the herbicide application were grown to maturity and seed collected. Individual plants were assigned a site number and an individual number for identification. DNA was extracted from these samples according to the CTAB method of Doyle and Dole (1987) for use in the investigation of genetic diversity of *L. serriola* for the region surrounding Bute, South Australia.

3.2 DNA extraction

CTAB method used for DNA extraction was modified from the method of Doyle and Doyle (1987). DNA was extracted from material by grinding 40 mg of leaves in 50 µl 2× CTAB buffer. To this 1×CTAB buffer was added to make a final volume of 500 µl, and incubated at 60° C for 2 h. Samples were cooled to room temperature, and 500 µl chloroform:isoamyl alcohol (24:1) was added to and mixed gently with the samples. The solution was centrifuged at 12000 g for 30 sec. The aqueous phase was removed and mixed with 0.5 vol. of 5 M NaCl and cold isopropanol added to reach a concentration of 40%. The samples were placed on ice for 2 h. The mixture was centrifuged at 12,000 g for 1 min and the supernatant poured off. The pellet was resuspended in 800 µl wash buffer (76% (v/v) ethanol and 10mM ammonium acetate, pH 8.0) for 20 min, and centrifuged at 12,000 g for 1 min. The supernatant was poured off and the pellet was left to dry by inverting the tube on a paper towel for 15 min. Resuspension buffer (10 mM ammonium acetate buffer, pH 8.0 with 0.25mM EDTA) was added to the dry pellet. RNAse (10 µg) was added and the DNA solution was incubated at 37° C for 2 h for RNA removal. The extracted DNA was stored at -80°C until required.

3.3 Extracted DNA testing and quantification

The extracted DNA was run on a 1% TAE agarose gel to check the purity and ensure the DNA template had not degraded. Samples of 1 µl were loaded with 1 µl loading buffer (10% glycerol, 2 mM EDTA, 0.1% xylene cyanol and 0.1% bromophenol blue). A high DNA mass ladder (Invitrogen Cat.# 10496-016) was run beside samples to quantify DNA. The gel was run at 70 v for 50 min and then stained in 1 mg/µl ethidium bromide for 12 min. The gel was gently washed by distilled water then visualised and photographed under 260 nm UV light. DNA mass was estimated by comparing the band intensity with the intensity of the DNA high mass ladder.

3.4 ISSR primer screen

Eight ISSR primers were screened by running PCR to test their effectiveness in genotyping. The PCR approach is detailed in section 6 of this chapter. These eight primers were 811 $(GA)_8C$, 818 $(CA)_8G$, 820 $(GT)_8C$, 880 HVH$(GT)_7$, 888 BDB$(CA)_7$, 889 DBD$(AC)_7$, 891 HVH$(TG)_7$ and 1423 HVH$(TGT)_5$ (GeneWorks). One DNA sample from the survey sites and the DNA samples from the two sites in Adelaide (Paradise and Waite) were used as PCR templates and run with each of these eight primers. A negative control PCR, which contained distilled water instead of DNA template, was included in each run.

PCR products were separated on a 1.5% TAE agorose gel at 70 V for 45 min and visualised as described before. Primers that gave clear banding patterns and with fragments amplified between 200-1500 bp were chosen for further testing. Chosen primers were used to amplify ten more samples and the products were run on polyacrylamide gels to determine the

presence of polymorphisms in the banding patterns. The primers that gave polymorphisms between populations were chosen for future use.

3.5 ISSR PCR program modification

A PCR program described by Charters *et al.* (1996) was used for reference as the basic PCR program to modify. Samples used for PCR program modification were five samples randomly chosen from the 1999 survey (samples 57-14, 68-15, 84-4 110-01 and 112-08), a positive control (Paradise) and a negative control (water). The PCR program modification was done four times with four primers (880, 888, 889, 891). Except for the modified conditions, all the other conditions were the same for each sample. PCR products were run on 2% TAE agarose gel at 70 V, for 45 min, and the PCR conditions that gave the clearest bands was chosen for use.

For the modification of the annealing temperature, PCR was performed on a master gradient authorised thermal cycler, which can provide a temperature gradient. Twelve annealing temperatures of 36° C, 36.2° C, 37.1° C, 39.1° C, 41.9° C, 43.0° C, 46.7° C, 49.4° C, 52.0° C, 54.2° C, 55.8° C, 56° C were set in the PCR program. The $MgCl_2$ concentration was determined based on a $MgCl_2$ concentration gradient of 1.5 mM, 2 mM, 2.5 mM, 3.0 mM. The most appropriate DNA concentration was determined by doing the PCR in which the undiluted extracted DNA samples and DNA samples diluted in distilled water in the following seven ratios, 1:1, 1:5, 1:10, 1:20, 1:50, 1:100, 1:200. The results of this experiment indicated the most appropriate DNA dilution for ISSR PCR was 1:100 while the annealing temperature was 52°C with a $MgCl_2$ concentration of 1.5 mM.

3.6 ISSR

3.6.1 PCR

PCR was performed at a total volume of 20 µl, with buffer A (1 mM Tris-HCl pH 9.0 at 25 °C, 5 mM KCl, and 0.01% Triton X-100), 1.5mM $MgCl_2$, 0.25 mM dNTP, 1 µM primer, 1 U T*aq* polymerase, and 5 ng DNA template. Distilled water was used as a negative control each time the PCR reaction was run. Primers used to test all samples were 880 $HVH(GT)_7$, 888 $BDB(CA)_7$, 889 $DBD(AC)_7$, and 891 $HVH(TG)_7$. The PCR program used was 30 cycles of 94° C, 15 sec; 52° C, 15 sec; 72° C, 30 sec and finished with 72° C, 2 min, followed by a step down to 25° C, 1 min before the PCR samples were held at 4° C. For long term storage conditions samples were placed in a -20° C freezer.

3.6.2 Electrophoresis on polyacrylamide gel

Electrophoresis was performed using a Multiphor Horizontal Flatbed System (Amersham Biosciences) at 10° C. The plate was cooled before the samples were loaded. Precast CleanGel 48 S DNA gels (Amersham Biosciences) were used for the separation of PCR products. The precast gel was rehydrated in gel buffer on a slowly rotating shaker for one hour before using. Kerosene (1 ml) was spread between the gel and cooling plate and air bubbles removed. Sample wells were dried by wiping off the buffer with CleanWipe tissues. Two electrode strips absorbed with electrode buffer were put on each side of the gel, with the electrodes resting on the outer edges of the strips. The samples (4 µl) were well mixed with 2 µl sample buffer (10 mM Tris, 1 mM EDTA, 1% xylene cyanol, 10 mg

bromophenol blue, pH 7.5) and the whole solution was loaded in a single well. In addition, 1 μl low DNA ladder (DMW-100L GeneWorks) with 3 μl distilled water and 2 μl sample buffer was loaded into the wells on each edge of the gel and in the centre of the gel as a marker. The gel was run at 200 V, 20 mA, 10 W for 20 min; 380 V, 30 mA, 20 W for 50 min and 450 V, 30 mA, 20 W for 30 min.

The gel was stained by using the DNA Plus One™ Silver Staining Kit (Amersham Biosciences). Reagents were prepared immediately before use. The gel was put in a fixing solution for 30 min, the staining solution for 30 min, and then washed with Milli-Q water for 1 min. The gel was developed for 6 min and placed in a preserving solution for 30 min. The gel was left in the air to dry overnight and then covered by plastic paper until scored.

3.6.3 ISSR replication check

ISSR PCR reactions were performed twice for all the 29 samples in population 1, 2, 3 and 4 collected in 2004. The duplicate PCR products were run on the polyacrylamide gel side by side.

3.7 Gel scoring and data analysis

The gels were scored on a light box. Bands running between 200-1500bp were scored. The presence of a band was recorded as 1, and the absence was recorded as 2, ambiguous bands were scored as 0. Scored data was analysed by using software package Tools for Population Genetic Analysis (version 1.3) (Miller 2000). Within the program, the following conditions were set: the data contained genotypes of individuals sampled from populations; the

organism type was diploid and the marker type was dominant; and Hardy-Weinberg equilibrium was assumed. Allele frequencies were estimated based on the Lynch and Milligan (1994) method and rounded to exactly match the observed sample.

The Lynch and Milligan (1994) method is described and explained briefly as follows. The sampling variance of a mean of parameter estimated is $\mathrm{Var}(\bar{z}) = \frac{1}{s(s-1)} \sum_{i=1}^{s} [z(i) - \bar{z}]^2$, in which s substitute sample size, z(i) substitute the individual estimated, and $\bar{z}$ is the mean of the estimation. Genetic diversity is calculated as two genes randomly drawn from population j, differ at the ith locus is expressed as $H_j(i)=2q_j(i)[1-q_j(i)]$. The mean gene diversity in the jth population over all L loci is $H_j = \frac{1}{L} \sum_{i=1}^{L} H_j(i)$. The sampling variance is given by $\mathrm{Var}(\hat{H}_W) = \mathrm{Var}_I(\hat{H}_W) + \mathrm{Var}_L(\hat{H}_W) + \mathrm{Var}_P(\hat{H}_W)$, in sampling of finite numbers of individuals, loci and populations with $\mathrm{Var}_I(H_W) = \frac{1}{n^2} \sum_{j=1}^{n} \mathrm{Var}_I(H_j)$ and $\mathrm{Var}_L(H_W) = \frac{1}{n^2} \sum_{j=1}^{n} \mathrm{Var}_L(H_j)$ (n substitute the sample size) (Lynch and Milligan 1994).

Unweighted Pair-Group Method Using Arithmetic Averages (UPGMA) cluster was generated based on Nei's (1978) unbiased distance, bootstrapping over loci 3000 times. A dendrogram of all the samples was printed out. Genetic distance was measured by the method of Nei's (1972, 1978).

The UPGMA is a simple method of tree construction, which employs a sequential clustering algorithm and local topological relationships are identified in order of similarity (Sneath and Snokal 1973). The phylogenetic tree is built in a stepwise manner. The two

individuals that are most similar to each other are first determined and are treated as a new single composite unit and then from among the new group of units, the pair with the highest similarity is identified and clustered. This continues until only two units are left (Sneath and Snokal 1973).

Bootstrapping is a statistical method of testing the reliability of the dataset (Felsenstein 1985). It is used to examine the frequencies of a particular cluster in a tree when loci are resampled with replacement. Bootstrapping is used to test the influence of stochastic effects in the distribution of characters. Pseudoreplicate datasets are generated by randomly sampling the original character matrix to create new matrices of the same size as the original. Bootstrap proportion is the frequency with which a given branch is found and recorded, which can be used as a measure of the reliability within limitations of individual branches in the optimal tree (Felsenstein 1985).

Chapter 4 Results

4.1 Herbicide resistance in the populations collected from the Yorke Peninsula

4.1.1 Populations collected in 1999

The aim was to collect paired populations from fields and the adjacent roadside. However, this was not always possible. Seeds of 85 populations were collected from 67 locations. Of the 85 populations, seed of 58 germinated, among which 24 populations were from fields and 34 were from roadsides. Resistance to chlorsulfuron was found in at least one individual in 38 *L. serriola* populations with 20 populations fully susceptible. The proportion of resistant populations was 65.5%. Of the 38 resistant populations, 20 were collected along roadsides and 18 were collected from fields. Resistance was present in 75.0% of field populations tested and 58.8% of roadside populations.

There was considerable variation in the number of resistant plants within populations. Some populations had high percentages of resistant individuals, while others had only one or two resistant plants. Resistance was also unevenly spread. Of the 13 paired populations where seed from both field and roadside populations germinated, seven had resistance in both populations, four had resistance in the field populations only and two did not have resistance in either population. The 58 populations that germinated are shown in Table 4.1 with their sources, the number of individuals in the population collected, and their response

to chlorsulfuron. As the genetic relationship between resistant populations was for the focus of this research and more resistant populations were present than susceptible ones, 18 resistant samples and seven susceptible samples were chosen for fingerprinting. Samples from both roadside and fields were chosen, pairing them according to geographical location where possible, and the proportions were generally in accordance with the number of samples germinating. In addition to this constraint, the 25 samples were chosen to cover the whole research area of 60 by 60 km as evenly as possible.

Table 4.1 The source, number of plants growing and their response to herbicide of the 58 germinated populations collected in 1999.

Site number	Population number	Source	Response to herbicide	Number of samples in the population	Used in ISSR*
1	1	Field	Susceptible	1	No
2	4	Field	Resistant	2	No
2	5	Roadside	Susceptible	10	No
3	6	Field	Resistant	10	Yes
4	9	Roadside	Susceptible	29	No
5	10	Field	Susceptible	10	No
6	12	Roadside	Susceptible	2	Yes
6	13	Field	Resistant	13	Yes
7	14	Field	Susceptible	11	No
7	15	Roadside	Susceptible	32	No
8	17	Roadside	Susceptible	10	Yes
9	18	Field	Resistant	11	No
11	20	Field	Susceptible	15	Yes
12	21	Roadside	Resistant	6	No
13	22	Field	Resistant	11	Yes
13	23	Roadside	Susceptible	13	No
14	24	Field	Resistant	10	Yes
14	25	Roadside	Resistant	32	Yes
15	26	Field	Resistant	14	Yes
16	29	Roadside	Resistant	5	No
23	40	Roadside	Susceptible	14	Yes
24	50	Roadside	Susceptible	14	No
26	53	Roadside	Resistant	2	No
28	57	Roadside	Resistant	21	Yes
29	58	Roadside	Resistant	2	No
29	59	Field	Resistant	1	Yes
31	62	Roadside	Susceptible	4	No
31	63	Field	Susceptible	1	No
32	64	Field	Resistant	4	No
32	65	Roadside	Resistant	2	No

34	67	Field	Resistant	1	No
35	68	Roadside	Susceptible	15	Yes
36	69	Roadside	Resistant	18	No
39	73	Roadside	Resistant	15	Yes
41	76	Roadside	Susceptible	3	Yes
43	80	Field	Resistant	1	No
43	81	Roadside	Resistant	17	Yes
44	83	Roadside	Resistant	16	Yes
45	84	Roadside	Resistant	4	Yes
46	86	Roadside	Resistant	5	Yes
47	88	Roadside	Resistant	6	Yes
49	92	Roadside	Resistant	10	No
51	96	Roadside	Resistant	4	No
52	98	Roadside	Susceptible	14	No
53	99	Field	Resistant	15	Yes
53	100	Roadside	Resistant	28	No
55	103	Field	Resistant	11	No
57	106	Field	Resistant	15	Yes
58	107	Roadside	Susceptible	16	No
58	108	Field	Resistant	10	No
59	110	Roadside	Susceptible	28	Yes
59	111	Field	Resistant	1	No
60	112	Field	Resistant	10	Yes
60	113	Roadside	Resistant	30	No
63	116	Field	Resistant	12	No
63	117	Roadside	Resistant	29	Yes
64	118	Field	Susceptible	30	No
66	120	Roadside	Resistant	31	No

* Molecular analysis using ISSRs undertaken a samples marked 'Yes'.

4.1.2 Populations collected in 2004

Eleven populations were collected from seven sites in 2004. At four sites, *L. serriola* was growing both in the field and along the adjacent roadside. At the other sites, *L. serriola* was only found growing along the roadside. Of the eleven populations collected, only two were susceptible, the others all contained resistant individuals. The proportion of resistant populations was 81.8%. All *L. serriola* populations collected from fields in 2004 contained resistant individuals, whereas 74.2% of the populations from roadsides contained resistant individuals.

The geographic location of the populations used in genotyping is demonstrated in Figure 4.1. The sources and their response to herbicide of each population and the number of plants collected from each population are shown in Table 4.2.

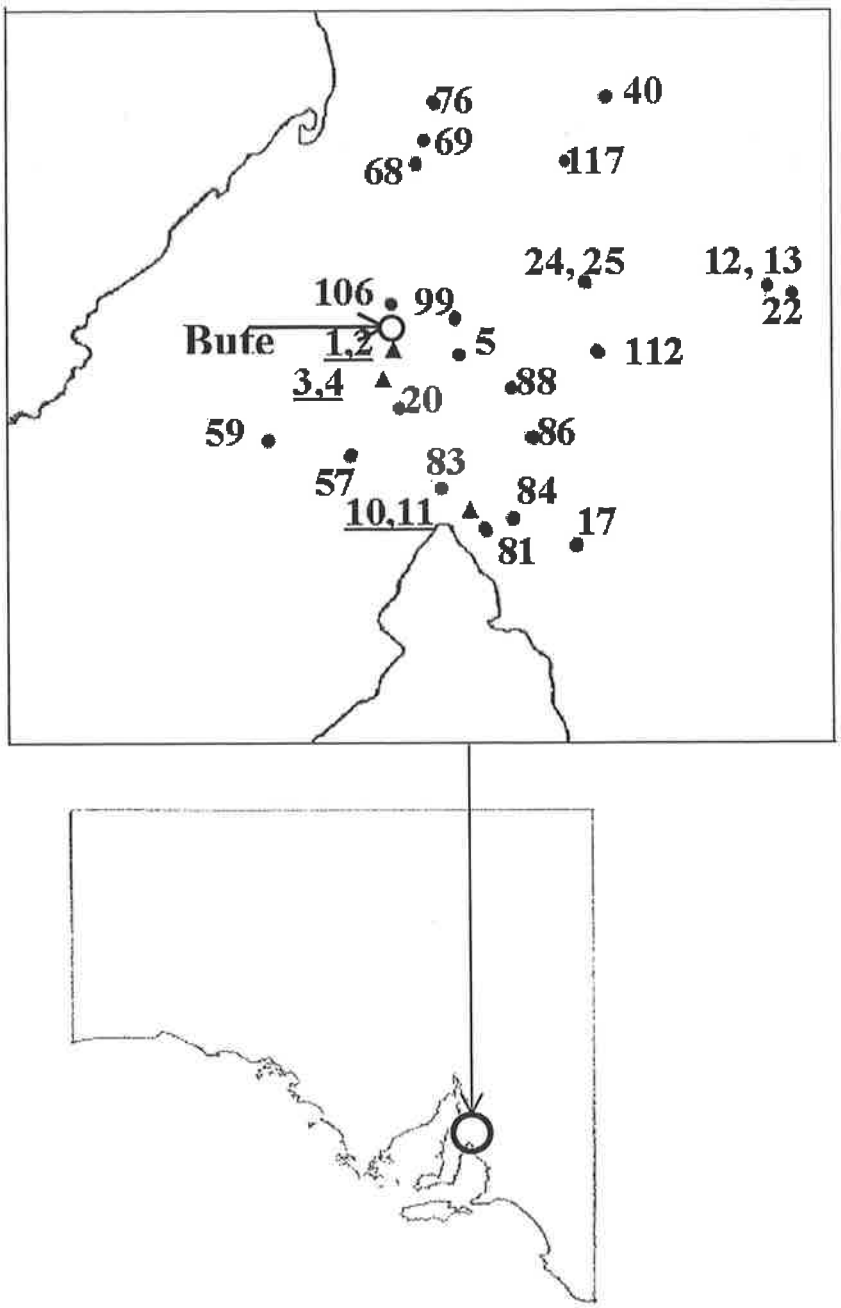

Figure 4.1 Geographic location of the populations used in genotyping by ISSR. Sites marked with • were collected in 1999, marked with ▲ and underlined were collected in 2004.

Table 4.2 The population source, the number of individuals growing in the population and their response to herbicide of the samples collected in 2004.

Site number	Population number	Source	Response to herbicide	Number of samples in the population	Used in ISSR*
1	1	Roadside	Resistant	18	Yes
1	2	Field	Resistant	8	Yes
2	3	Roadside	Resistant	16	Yes
2	4	Field	Resistant	11	Yes
3	5	Roadside	Resistant	9	No
4	6	Roadside	Resistant	18	No
5	7	Roadside	Susceptible	14	No
6	8	Roadside	Susceptible	11	No
6	9	Field	Resistant	13	No
7	10	Roadside	Resistant	11	Yes
7	11	Field	Resistant	5	Yes

*Molecular analysis using ISSRs undertaken a samples marked 'Yes'.

4.2 DNA quantity and quality

The extracted DNA for each sample on an agarose gel ran as a sharp and clear band with no obvious degradation. The intensities of the band for each sample were similar suggesting DNA could be readily extracted from each sample. Based on a standard from a high

molecular mass ladder, the concentration of DNA in the extracts was estimated to range between 20 and 100 ng μl^{-1}. An example of one of the photographs of some extracted DNA on 1% TAE agarose gel is shown in Figure 4.2.

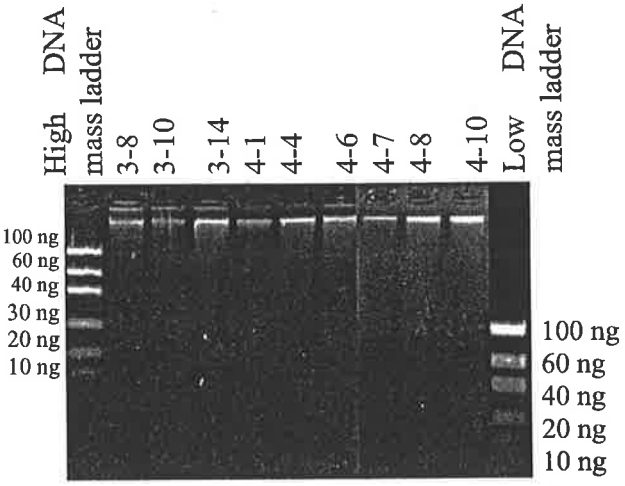

Figure 4.2 An example 1% agarose gel used for DNA quantification.

4.3 Primer screen

ISSR primers 811, 889, 888, 891 and 880 all amplified many DNA fragments between 200-1500 bp from DNA of *L. serriola*. For these primers there were 30 to 50 bands that could be readily visualised on polyacrylamide gels. Primer 811 PCR products did not show much polymorphism. For primer 818 and 820, most of the fragments amplified were larger in size, and poorly resolved on the gels, while with primer 1423, most of the fragments amplified were too small to be clearly resolved and scored. Therefore, the four remaining primers that amplified bands that were easy to resolve and score on the gel were used for all other ISSR experiments.

4.4 PCR program modification

The trend of therelationship between annealing temperature gradient and PCR products for the four primers was almost the same. With an annealing temperature gradient ranging from 36° C to 52° C, PCR products produced banding patterns which became clearer as the annealing temperature increased from 36° C to 52° C. Banding patterns were a smear at the lower annealing temperatures and the bands were not able to be scored. Banding patterns became unclear and fewer bands were amplified above 52° C. Therefore, the annealing temperature was set at 52° C in the PCR reactions (Figure 4.3).

Changing the $MgCl_2$ concentration demonstrated that a concentration of 1.5 mM was ideal for PCR. PCR was most effective at a DNA dilution ratio of 1:100 therefore, this dilution was used in experiments.

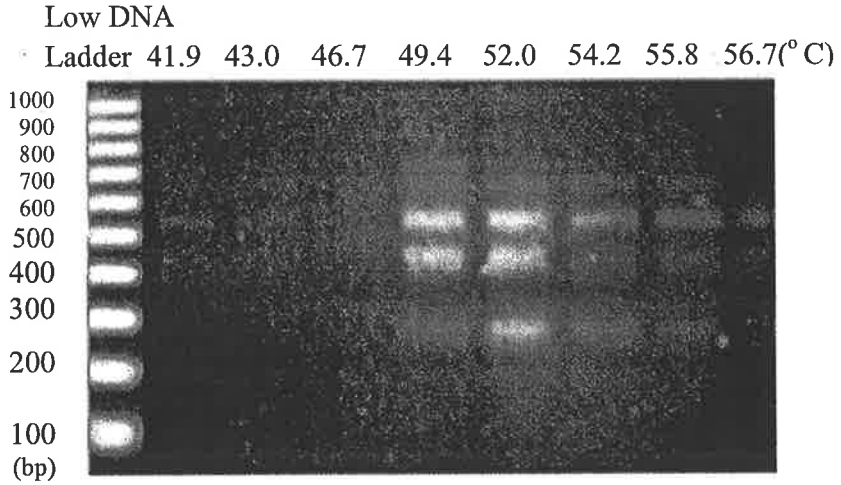

Figure 4.3 1% TAE agarose gel banding patterns produced at different annealing temperatures. The selective sample is 57-14, using primer 891.

4.5 ISSR banding patterns

ISSR analysis of 29 samples in Populations 1, 2, 3 and 4 collected in 2004 were performed in duplicate with all four primers. For each individual, banding patterns of the ISSR duplication were exactly the same. This proved that the method was repeatable.

4.5.1 Samples collected in 1999

For the 1999 survey, DNA from a single individual from each of 25 populations was used (Table 4.1), together with two susceptible populations from the Adelaide metropolitan area (Waite and Paradise).

4.5.1.1 Banding patterns and polymorphism on polyacrylamide gels

All 27 DNA samples amplified produced ISSR PCR products with the four primers used for screening. Across all four primers used, 179 bands, as 179 loci, on the polyacrylamide gel were scored for each sample. Of these, 89 loci were polymorphic, appeared as band present or band absent in different individuals, with the proportion of polymorphic loci varying between the primers (Table 4.3). Some examples of ISSR products on polyacrylamide gels are shown in Figures 4.4 to 4.9.

Table 4.3 The number of loci scored and the proportion of polymorphic loci for each primer for the 25 samples collected in 1999 and the two control samples collected from Waite and Paradise.

Primer	Bands scored	Number of polymorphic bands	Polymorphic bands %
880	46	9	19.6
888	44	21	47.7
889	34	19	55.9
891	55	40	72.7
Total data analysed	179	89	49.7

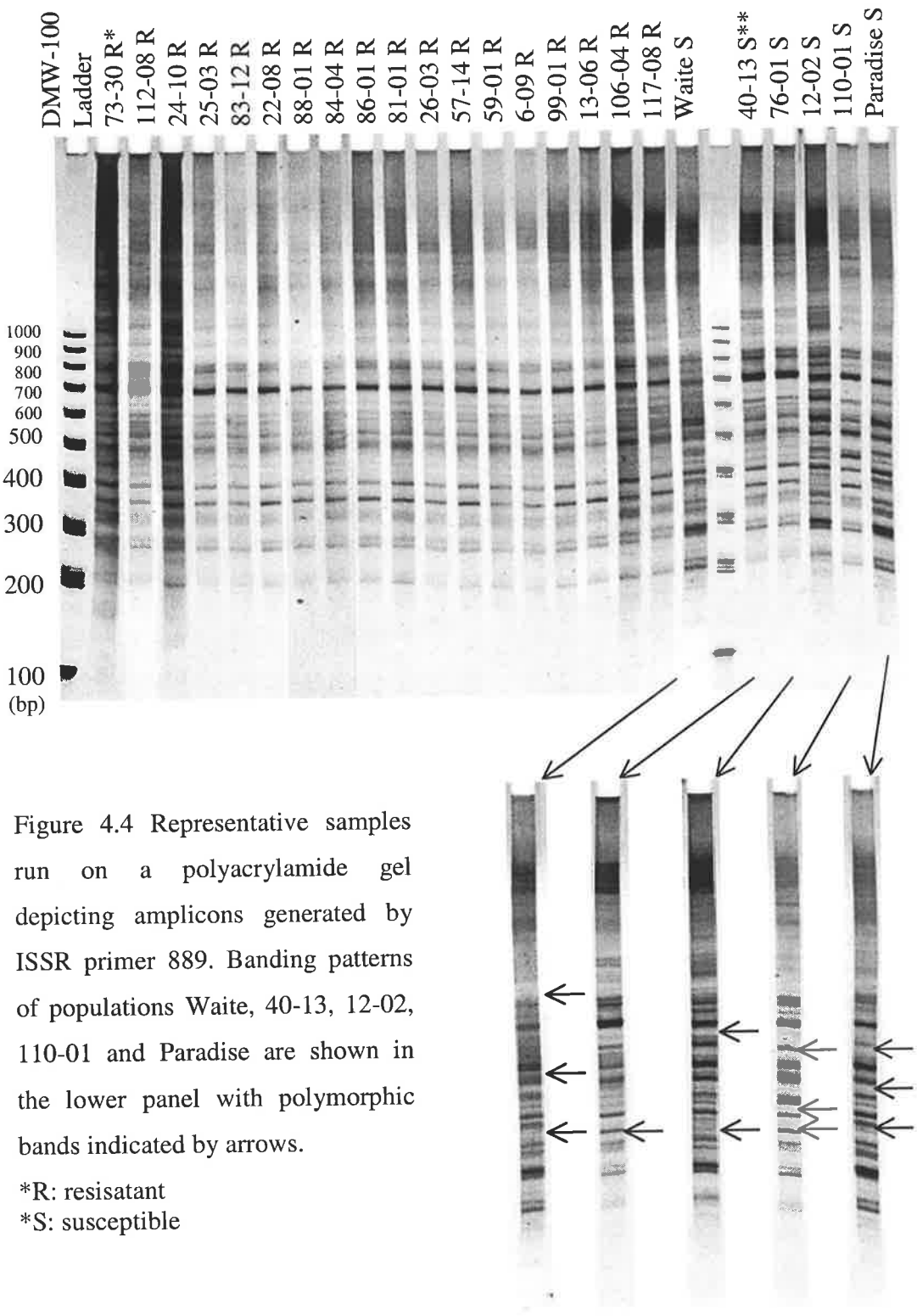

Figure 4.4 Representative samples run on a polyacrylamide gel depicting amplicons generated by ISSR primer 889. Banding patterns of populations Waite, 40-13, 12-02, 110-01 and Paradise are shown in the lower panel with polymorphic bands indicated by arrows.

*R: resisatant
*S: susceptible

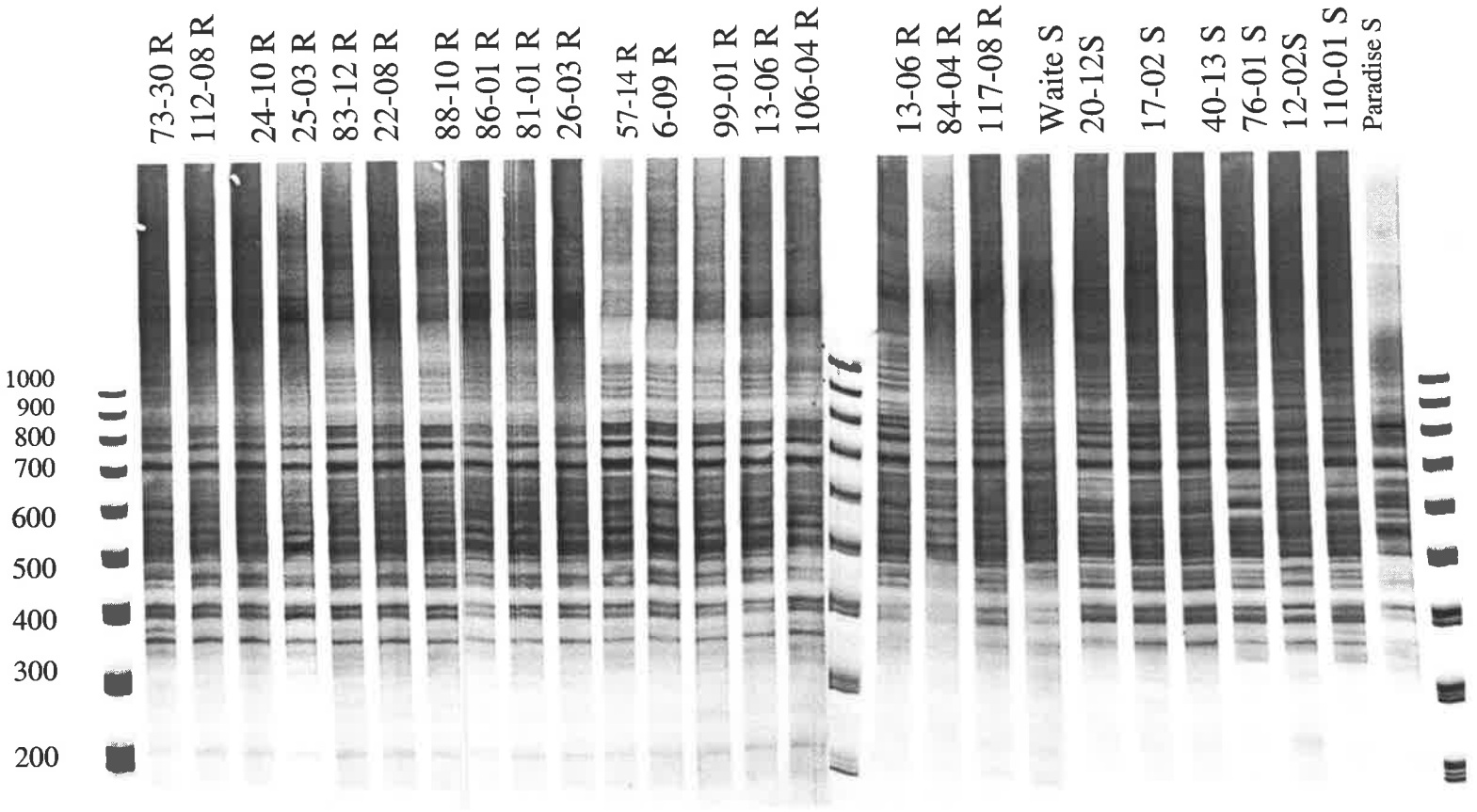

Figure 4.5 Polyacrylamide gel depicting amplicons generated by ISSR primer 888. Banding patterns of some representative samples are shown in Figure 4.6 (next page) to demonstrate polymorphic bands.

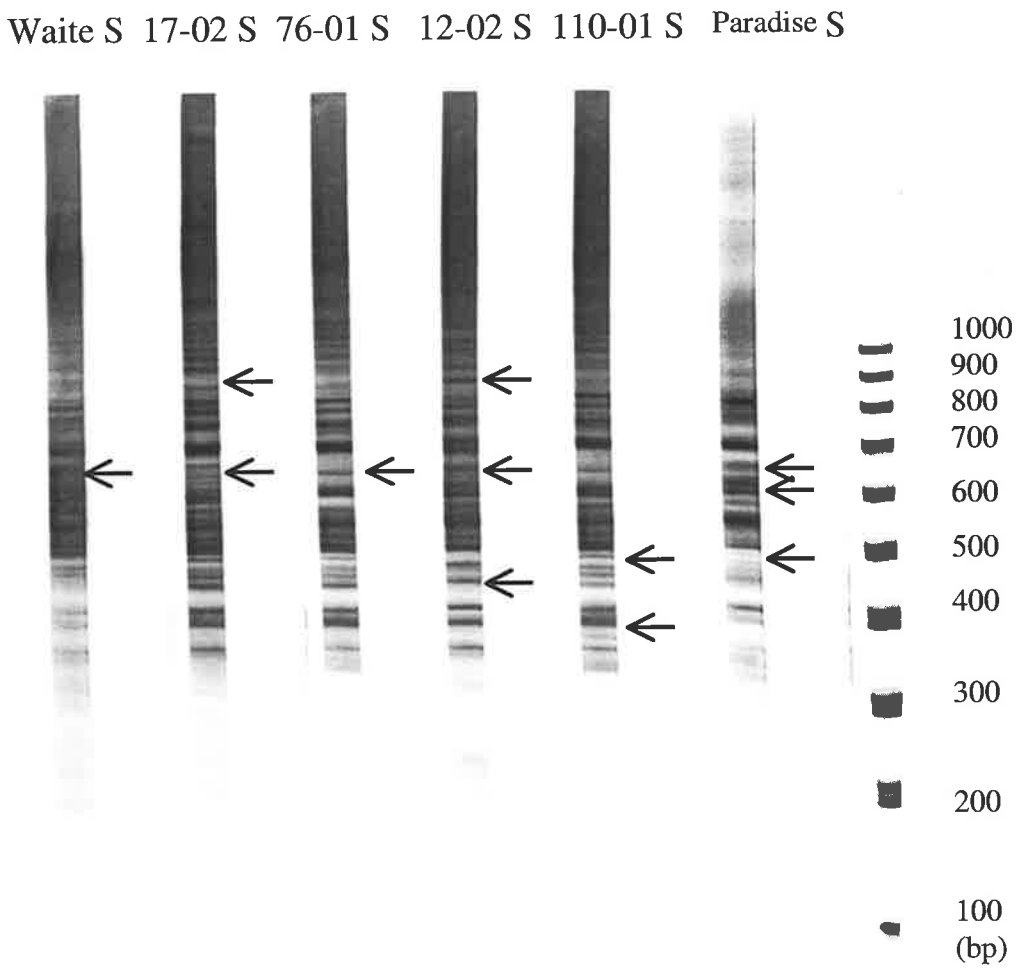

Figure 4.6 ISSR banding patterns generated by primer 888 of some of the samples- Waite, 17-02, 76-01, 12-02, 110-01 Paradise. Polymorphic bands are indicated by arrows.

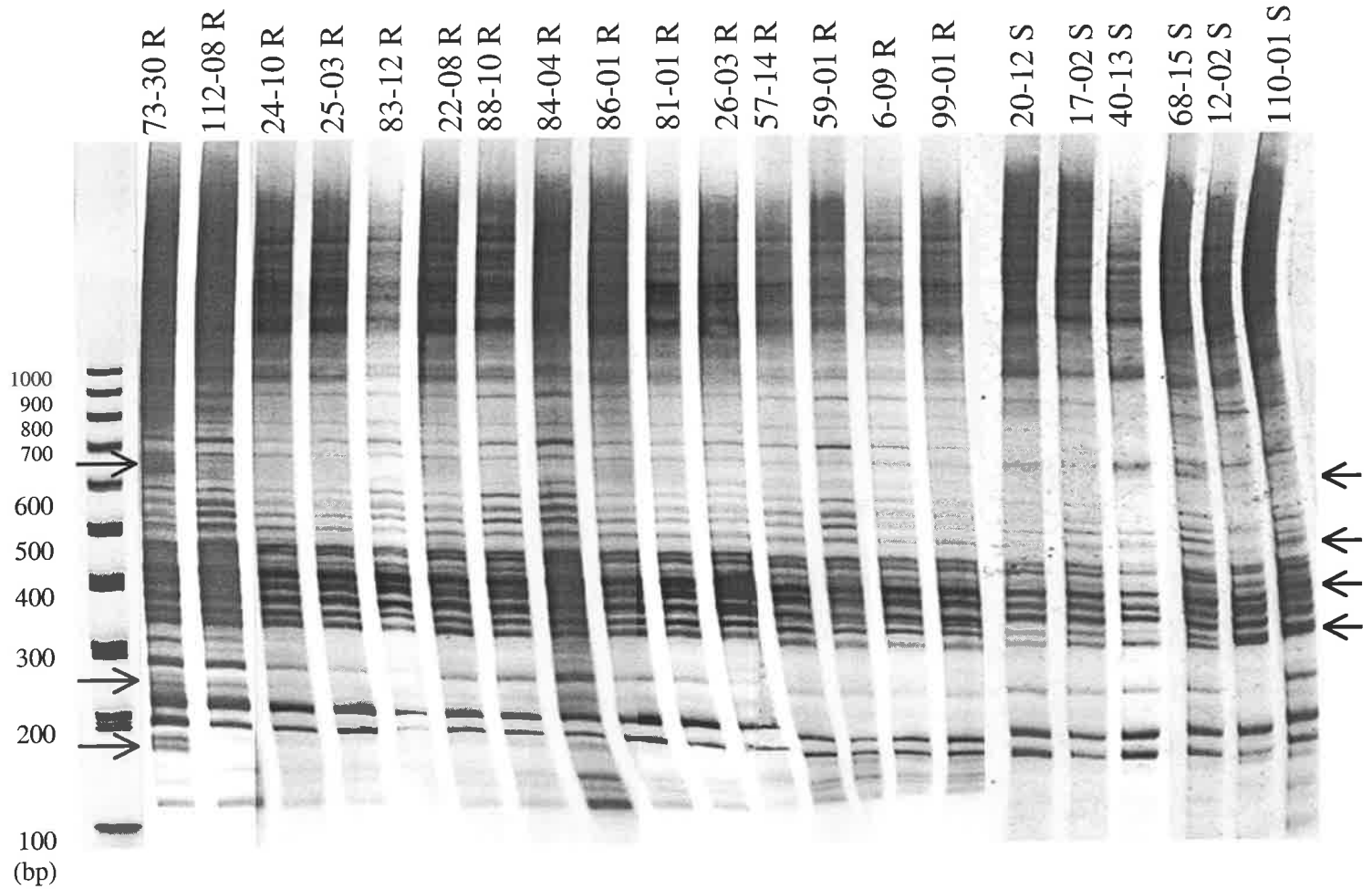

Figure 4.7 Some representative samples on a polyacrylamide gel depicting amplicons generated by ISSR primer 880. Some obvious polymorphisms are indicated by arrows aside the banding patterns of samples 73-30, 12-02 and 84-04.

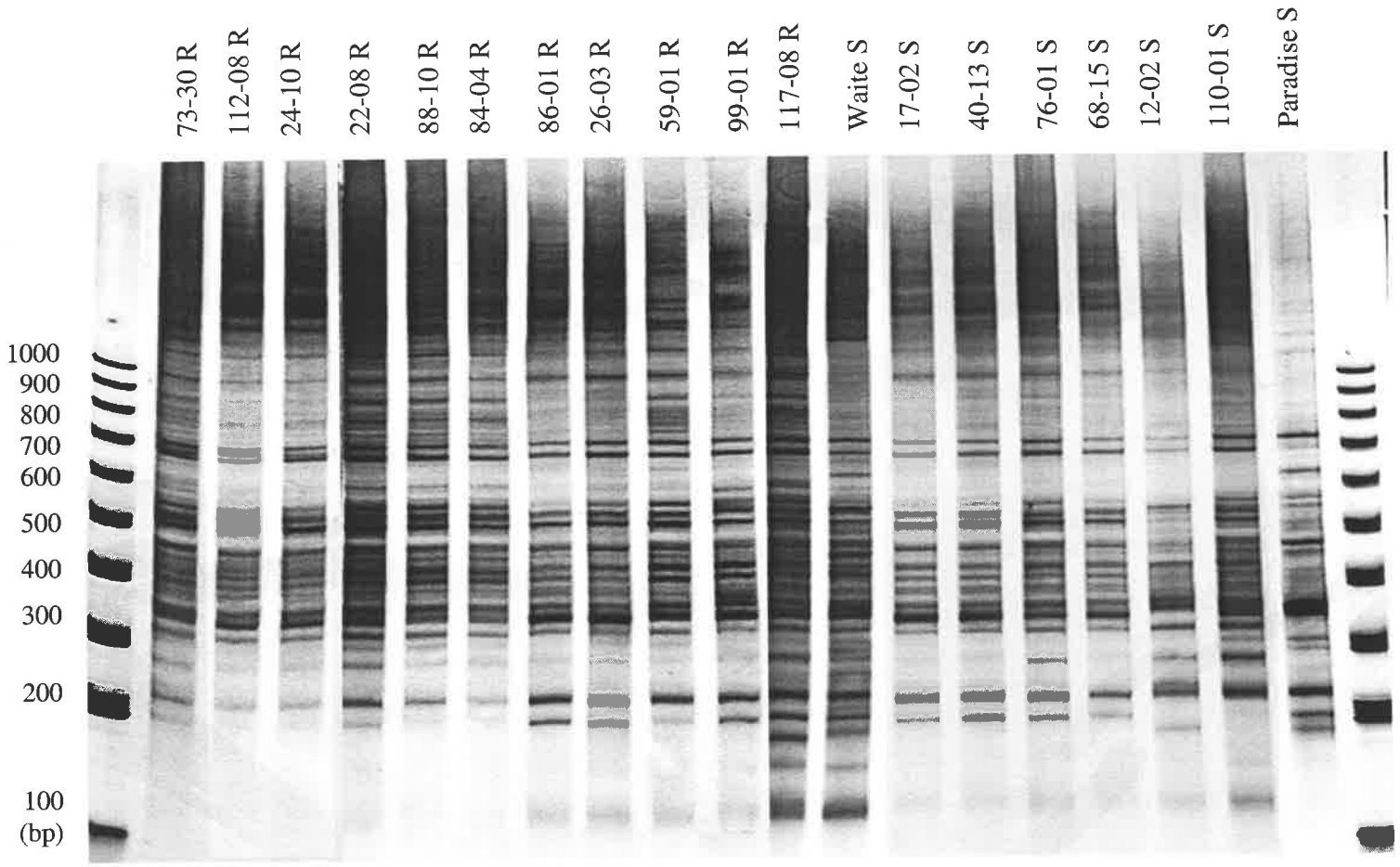

Figure 4.8 Some representative samples on a polyacrylamide gel depicting amplicons generated by ISSR primer 891. Banding patterns of selected samples are shown in Figure 11 (next page) to demonstrate

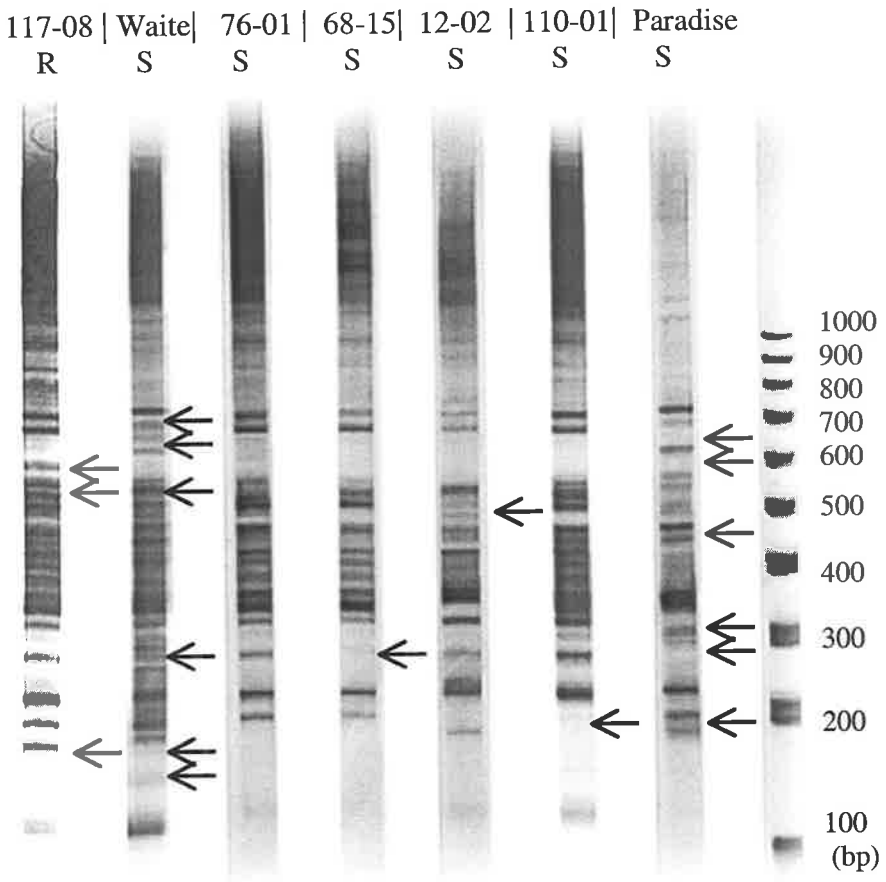

Figure 4.9 ISSR banding patterns of selected samples of 117-08, Waite, 76-01, 68-15, 12-02, 110-01 Paradise, generated by primer 891. Polymorphisms are indicated by arrows.

4.5.1.2 UPGMA clusters and genetic distance

The 27 samples formed 20 UPGMA clusters. From these samples, 17 genotypes were represented once and three more than once. Of the ten samples that grouped, one group of three individuals was susceptible and the other two groups of three and four individuals were resistant. The susceptible group contained three individuals, 17-02, 40-13 and 20-12, all of which originated from separate sites. Population 17 was 65.8 km from Population 40 and 28.9 km from Population 20, whereas Population 40 was 51.3 km from Population 20. One of the resistant groups of identical individuals contained samples 13-06, 106-04 and 99-01. Two of the samples were not close to each other. Population 13 was 42.87 km from Population 106, and 34.98 km from Population 99. However, Populations 106 and 99 were within 8.2 km of each other. The other resistant group had four identical individuals (84-04, 81-01, 22-08 and 83-12). Three of the samples were close to each other. Population 83 was 10.5 km from both Populations 81 and 84, and Populations 81 and 84 were only 4.0 km apart. Population 22 was over 43 km from all the other individuals in this group.

The two outlying samples Waite and Paradise were genetically the most different among the 27 samples studied. The genetic distances of Paradise and other samples were between 0.3156 and 0.4862 and the UPGMA node distance was 0.4008 (Nei 1978). The genetic distances between the Waite sample and other samples ranged between 0.2952 and 0.4017 and the UPGMA node distance was 0.3311. The genetic distance of these two control samples from each other was 0.4360.

Among the survey samples from the upper Yorke Peninsula, the largest genetic distance was 0.3432, which was between a susceptible individual (12-02) and a resistant individual (73-30). Susceptible sample 12-02 was also divergent from most of the other samples. The genetic distance of 12-02 from the other 24 survey samples was greater than 0.2405, over 0.3000 for nine samples and over 0.2500 for 14 samples. The UPGMA node distance of 12-02 was 0.2827. Sample 110-01, also a susceptible sample, was the second most genetically different sample within the survey. The genetic distance of 110-01 from the other samples was between 0.0864 (from 68-15) and 0.2691 (from 12-02), and all but one sample were over 0.1000. The UPGMA node distance was 0.1427. The resistant samples, while not identical, showed more similar banding patterns than the susceptible samples. The largest genetic distance among resistant samples was 0.1504, the genetic distance between 57-14 and 73-30. The UPGMA node distance of 57-14 was 0.0949, and of 73-30 was 0.0849. The UPGMA cluster (Nei 1978) dendrogram is shown in Figure 4.10 and the UPGMA node distances are shown in Table 4.4. The genetic distances (Nei 1972, 1978) of the samples are shown in Table 4.5.

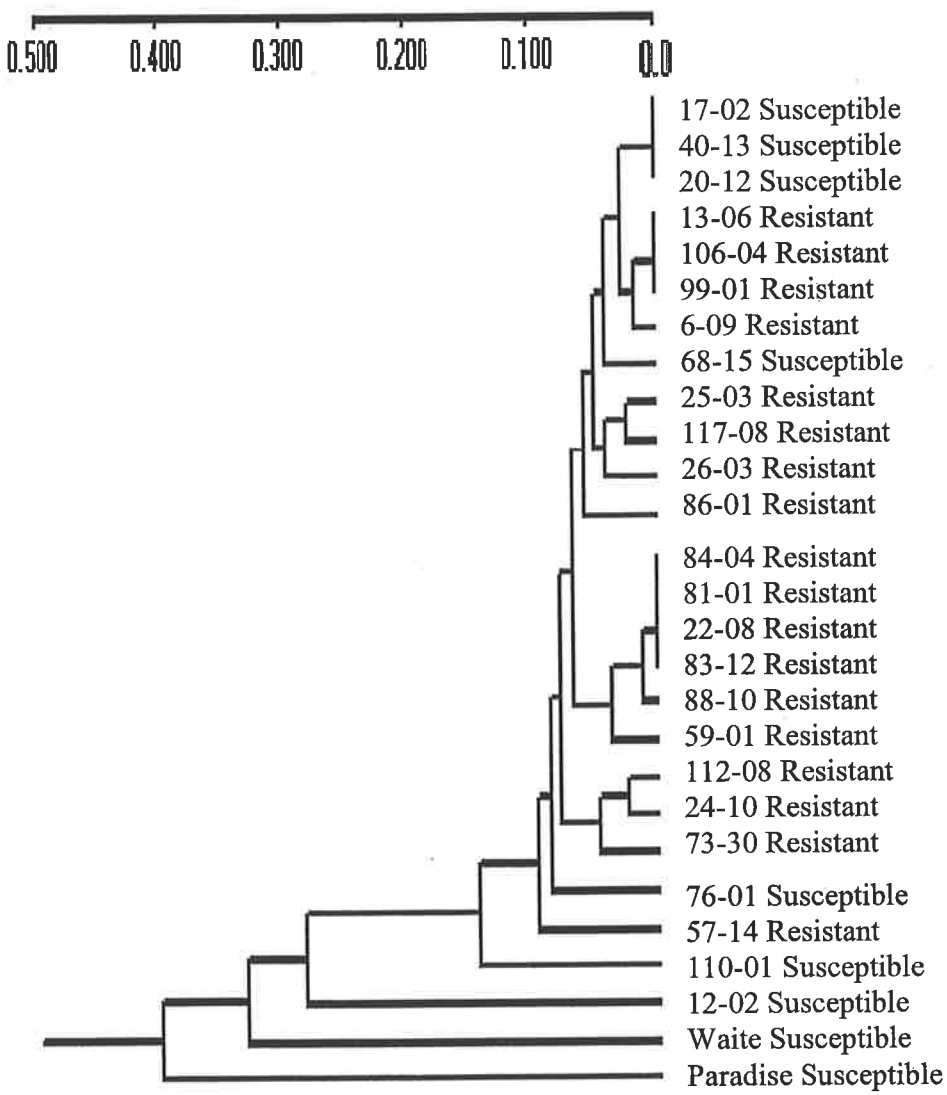

Figure 4.10 Dendrogram of the *L. serriola* samples collected in 1999 and the two control samples (UPGMA).

Table 4.4 UPGMA cluster node distance generated by TFPGA using Nei's (1978) unbiased distance.

Node Distance Includes Populations

1 0.0000 17-02 40-13
2 0.0000 20-12 17-02 40-13
3 0.0000 13-06 106-04
4 0.0000 99-01 13-06 106-04
5 0.0169 6-09 99-01 13-06 106-04
6 0.0272 6-09 99-01 13-06 106-04 20-12 17-02 40-13
7 0.0394 6-09 99-01 13-06 106-04 20-12 17-02 40-13 68-15
8 0.0226 25-03 117-08
9 0.0399 25-03 110-01-03 117-08
10 0.0488 25-03 110-01-03 6-09 99-01 13-06 106-04 117-08 20-12 17-02 40-13 68-15
11 0.0496 25-03 86-01 110-01-03 6-09 99-01 13-06 106-04 117-08 20-12 17-02 40-13 68-15
12 0.0000 88-01 81-01
13 0.0000 40-13 88-01 81-01
14 0.0000 83-12 40-13 88-01 81-01
15 0.0112 83-12 40-13 88-01 84-04 81-01
16 0.0364 83-12 40-13 88-01 84-04 81-01 59-01
17 0.0599 25-03 83-12 40-13 88-01 84-04 86-01 81-01 110-01 59-01 6-09 99-01 13-06 106-04 117-08 20-12 17-02 40-13 68-15
18 0.0226 112-08 24-10
19 0.0458 73-30 112-08 24-10
20 0.0715 73-30 112-08 24-10 25-03 83-12 40-13 88-01 84-04 86-01 81-01 110-01 59-01 6-09 99-01 13-06 106-04 117-08 20-12 17-02 40-13 68-15
21 0.0849 73-30 112-08 24-10 25-03 83-12 40-13 88-01 84-04 86-01 81-01 110-01 59-01 6-09 99-01 13-06 106-04 117-08 20-12 17-02 40-13 76-01 68-15
22 0.0949 73-30 112-08 24-10 25-03 83-12 40-13 88-01 84-04 86-01 81-01 110-01 57-14 59-01 6-09 99-01 13-06 106-04 117-08 20-12 17-02 40-13 76-01 68-15
23 0.1427 73-30 112-08 24-10 25-03 83-12 40-13 88-01 84-04 86-01 81-01 110-01 57-14 59-01 6-09 99-01 13-06 106-04 117-08 20-12 17-02 40-13 76-01 68-15 110-01
24 0.2827 73-30 112-08 24-10 25-03 83-12 40-13 88-01 84-04 86-01 81-01 110-01 57-14 59-01 6-09 99-01 13-06 106-04 117-08 20-12 17-02 40-13 76-01 68-15 12-02 110-01
25 0.3311 73-30 112-08 24-10 25-03 83-12 40-13 88-01 84-04 86-01 81-01 110-01 57-14 59-01 6-09 99-01 13-06 106-04 117-08 Waite 20-12 17-02 40-13 76-01 68-15 12-02 110-01

26 0.4008 73-30 112-08 24-10 25-03 83-12 40-13 88-01 84-04 86-01 81-01 110-01 57-14 59-01 6-09 99-01 13-06 106-04 117-08 Waite 20-12 17-02 40-13 76-01 68-15 12-02 110-01 Paradise

Table 4.5 Genetic distances of the samples generated by software TFPGA based on Nei (1972, 1978).

	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	S	S	S	S	S	S	S	S
	73-30	112-08	24-01	25-03	83-12	22-08	88-01	84-04	86-01	81-01	26-03	57-14	59-01	6-09	99-01	13-06	106-04	117-08	20-12	17-02	40-13	76-01	68-15	12-02	110-01	Waite
112-08	0.0341																									
24-10	0.0575	0.0226																								
25-03	0.0936	0.0184	0.0694																							
83-12	0.0694	0.0575	0.0457	0.0457																						
22-08	0.0694	0.0575	0.0457	0.0457	0.0000																					
88-01	0.0694	0.0575	0.0457	0.0457	0.0000	0.0000																				
84-04	0.0814	0.0575	0.0457	0.0575	0.0112	0.0112	0.0112																			
86-01	0.0998	0.0574	0.0634	0.0516	0.0754	0.0754	0.0754	0.0754																		
81-01	0.0694	0.0575	0.0457	0.0457	0.0000	0.0000	0.0000	0.0112	0.0754																	
26-03	0.0998	0.0754	0.0634	0.0399	0.0634	0.0634	0.0634	0.0754	0.0457	0.0634																
57-14	0.1504	0.1248	0.1122	0.0998	0.0998	0.0998	0.0998	0.0998	0.0814	0.0998	0.0936															
59-01	0.1060	0.0936	0.0814	0.0575	0.0341	0.0341	0.0341	0.0457	0.0875	0.0341	0.0754	0.0998														
6-09	0.0998	0.0875	0.0754	0.0399	0.0516	0.0516	0.0516	0.0634	0.0575	0.0516	0.0575	0.0814	0.0339													
99-01	0.0814	0.0694	0.0575	0.0457	0.0457	0.0457	0.0457	0.0575	0.0516	0.0457	0.0516	0.0754	0.0341	0.0169												
13-06	0.0814	0.0694	0.0575	0.0457	0.0457	0.0457	0.0457	0.0575	0.0516	0.0457	0.0516	0.0754	0.0341	0.0169	0.0000											
106-04	0.0814	0.0694	0.0575	0.0457	0.0457	0.0457	0.0457	0.0575	0.0516	0.0457	0.0516	0.0754	0.0341	0.0169	0.0000	0.0000										
117-08	0.1185	0.0814	0.0694	0.0226	0.0694	0.0694	0.0694	0.0694	0.0516	0.0694	0.0399	0.0875	0.0694	0.0399	0.0457	0.0457	0.0457									
20-12	0.0814	0.0575	0.0457	0.0575	0.0575	0.0575	0.0575	0.0694	0.0339	0.0575	0.0283	0.0754	0.0575	0.0399	0.0226	0.0226	0.0226	0.0457								
17-02	0.0814	0.0575	0.0457	0.0575	0.0575	0.0575	0.0575	0.0694	0.0339	0.0575	0.0283	0.0754	0.0575	0.0399	0.0226	0.0226	0.0226	0.0457	0.0000							
40-13	0.0814	0.0575	0.0457	0.0575	0.0575	0.0575	0.0575	0.0694	0.0339	0.0575	0.0292	0.0754	0.0575	0.0399	0.0226	0.0226	0.0226	0.0457	0.0000	0.0000						
76-01	0.1219	0.1035	0.1035	0.0864	0.0945	0.0945	0.0945	0.1035	0.1219	0.0945	0.0855	0.1312	0.0945	0.0678	0.0591	0.0591	0.0591	0.0766	0.0591	0.0591	0.0591					
68-15	0.1247	0.0789	0.0641	0.2821	0.0864	0.0864	0.0864	0.0864	0.0641	0.0864	0.0641	0.0715	0.0864	0.0641	0.0423	0.0423	0.0423	0.0568	0.0280	0.0280	0.0280	0.0766				
12-02	0.3432	0.3122	0.2970	0.2821	0.3122	0.3122	0.3122	0.3122	0.2601	0.3122	0.2747	0.3046	0.2970	0.2457	0.2529	0.2529	0.2529	0.2529	0.2529	0.2529	0.2529	0.3267	0.2405			
110-01	0.2050	0.1645	0.1513	0.1513	0.1513	0.1513	0.1513	0.1513	0.1579	0.1513	0.1448	0.1846	0.1513	0.1319	0.1255	0.1255	0.1255	0.1255	0.1255	0.1255	0.1255	0.1127	0.0864	0.2691		
Waite	0.4017	0.3417	0.3686	0.3260	0.3471	0.3471	0.3471	0.3365	0.3365	0.3471	0.3365	0.3577	0.3156	0.2952	0.3054	0.3054	0.3054	0.2852	0.3260	0.3260	0.3260	0.2911	0.2776	0.3686	0.3502	
Paradise	0.4836	0.4244	0.4244	0.4017	0.4017	0.4017	0.4017	0.3905	0.3905	0.4017	0.3905	0.3905	0.3905	0.3905	0.4017	0.4017	0.4017	0.3795	0.3795	0.3795	0.3795	0.4362	0.4207	0.3156	0.4055	0.4360

4.5.2 Samples collected in 2004

A single individual was examined in each population from the samples collected in 1999. This provided evidence of between population variability, but not information on within population variation. The highly selfing nature of *L. serriola* would suggest limited variation within populations. However, as *L. serriola* seed is wind borne, there is a high probability of new immigration into populations. Therefore, in 2004 seedlings from a limited number of sites within the upper Yorke Peninsula were collected in order to examine within population variation as well as between population variation.

4.5.2.1 Banding patterns and polymorphism on polyacrylamide gel

The same four ISSR primers were used to amplify bands from DNA for these samples. From the polyacrylamide gels, 67 bands, as 67 loci, ranging from about 200 to 1500 bp for each of the 39 samples were scored, among which 15 loci were polymorphic. The proportion of polymorphic loci was 23.9%. The samples were much more homogeneous than the previous samples. This was to be expected, because they were collected from a much smaller number of sites over a smaller geographical area (Figure 4.1). This is reflected in the lower percentage of polymorphic bands scored. Some of the samples had different genotypes within the population, while others did not. Populations 2 and 3 contained a single genotype whereas Populations 10 and 11 each contained three genotypes. Additionally, some genotypes were shared between populations, whereas others were only present in a single population. The number of loci scored and polymorphic loci

generated by each primer are shown in Table 4.6. Some examples of ISSR banding patterns are shown in Figures 4.11 to 4.18.

Table 4.6 The number of loci scored and polymorphic loci of ISSR banding patterns for each of the four primers used for the 39 samples.

Primer	Bands scored	Number of polymorphic bands	Polymorphic bands %
880	19	5	26.3
888	11	4	36.4
889	13	3	23.8
891	24	4	16.7
Total data analysed	67	16	23.9

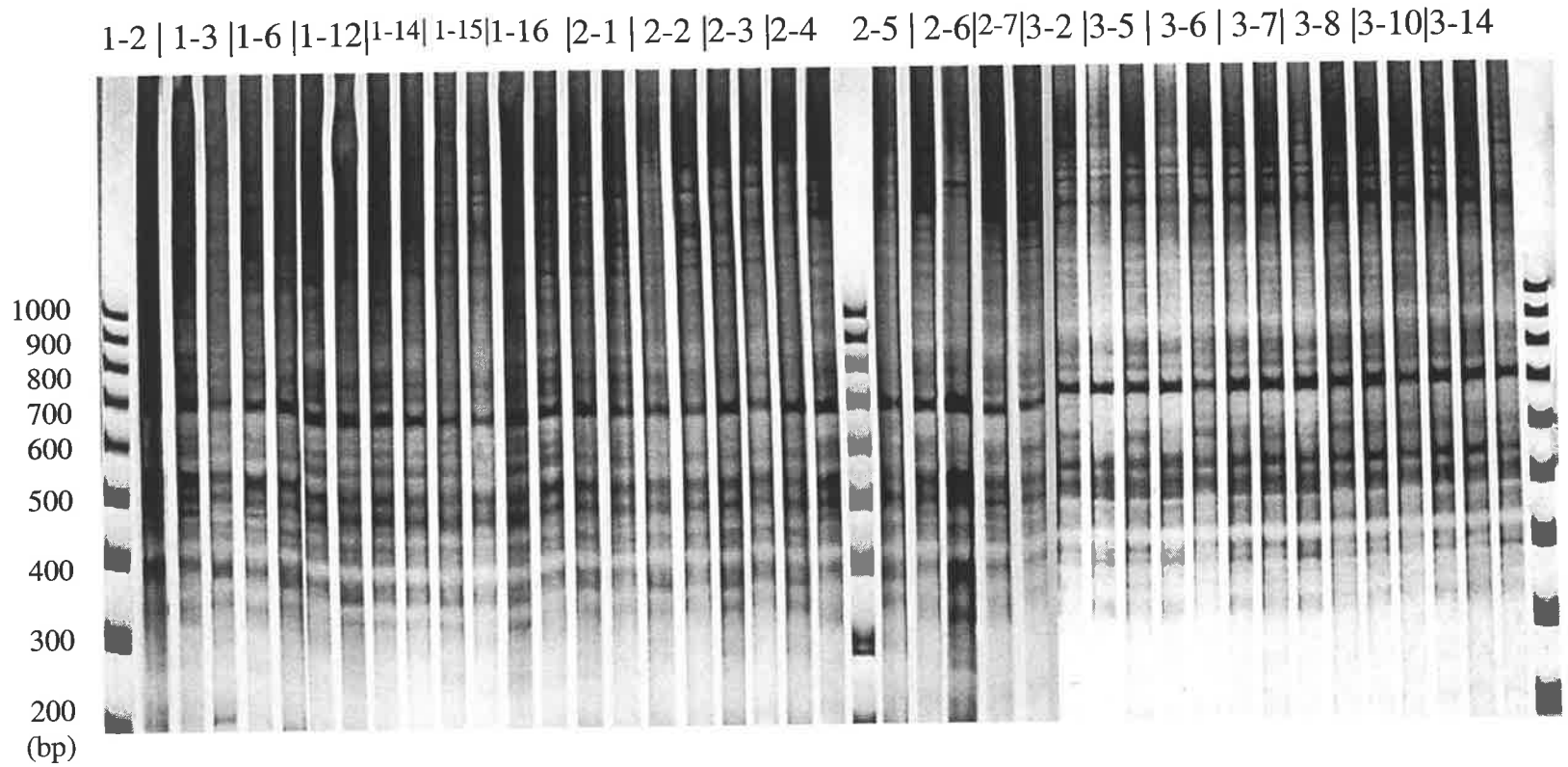

Figure 4.11 Polyacrylamide gel depicting duplicate amplicons generated by ISSR primer 888 of samples in Populations 1, 2 and 3. Banding patterns were identical in these populations.

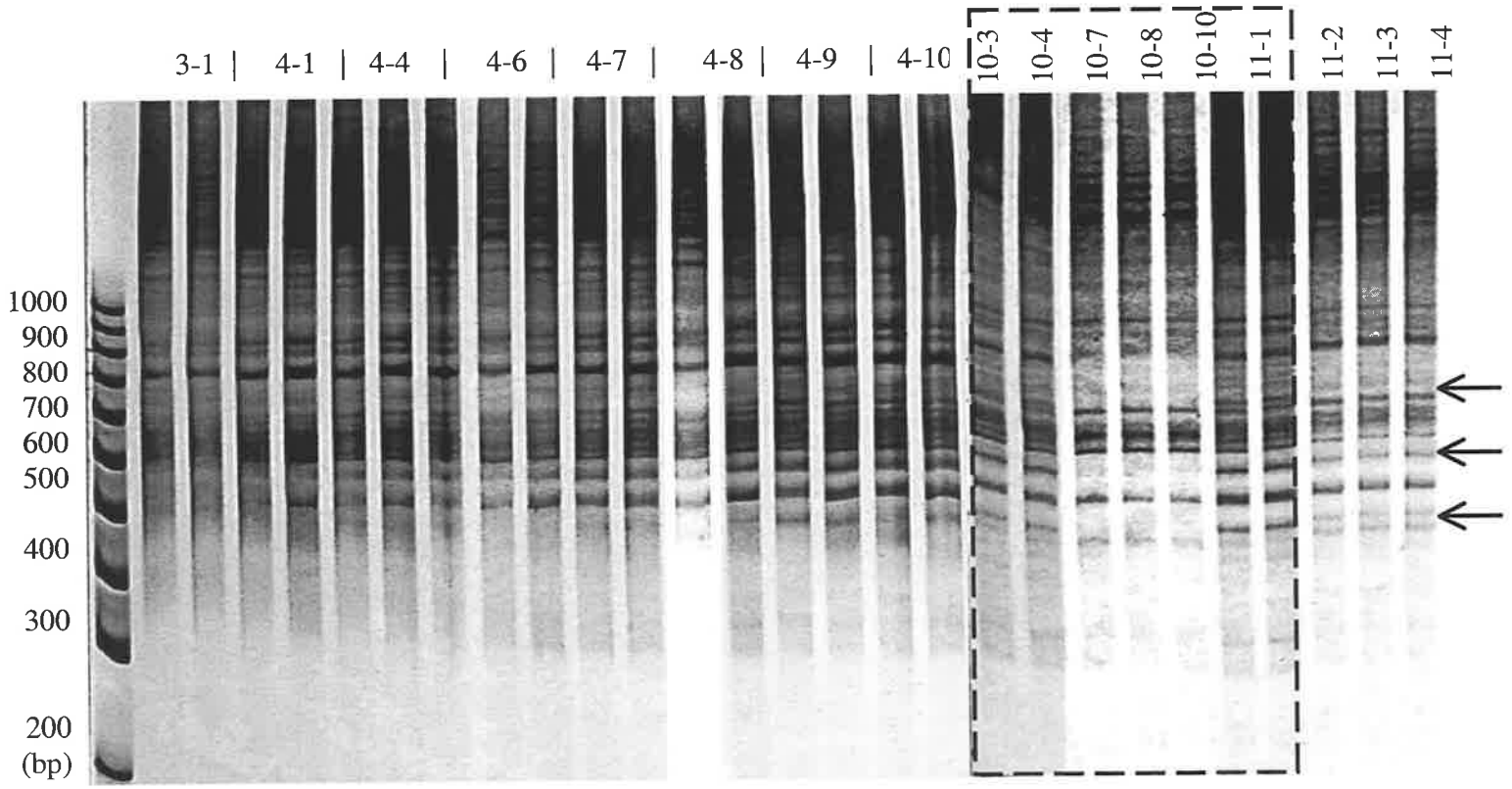

Figure 4.12 Polyacrylamide gel depicting duplicate amplicons of samples in Populations 3, 4 and amplicons in Populations 10 and 11 generated by ISSR primer 888 Polymorphic bands are shown within the black box and by the arrows along the side.

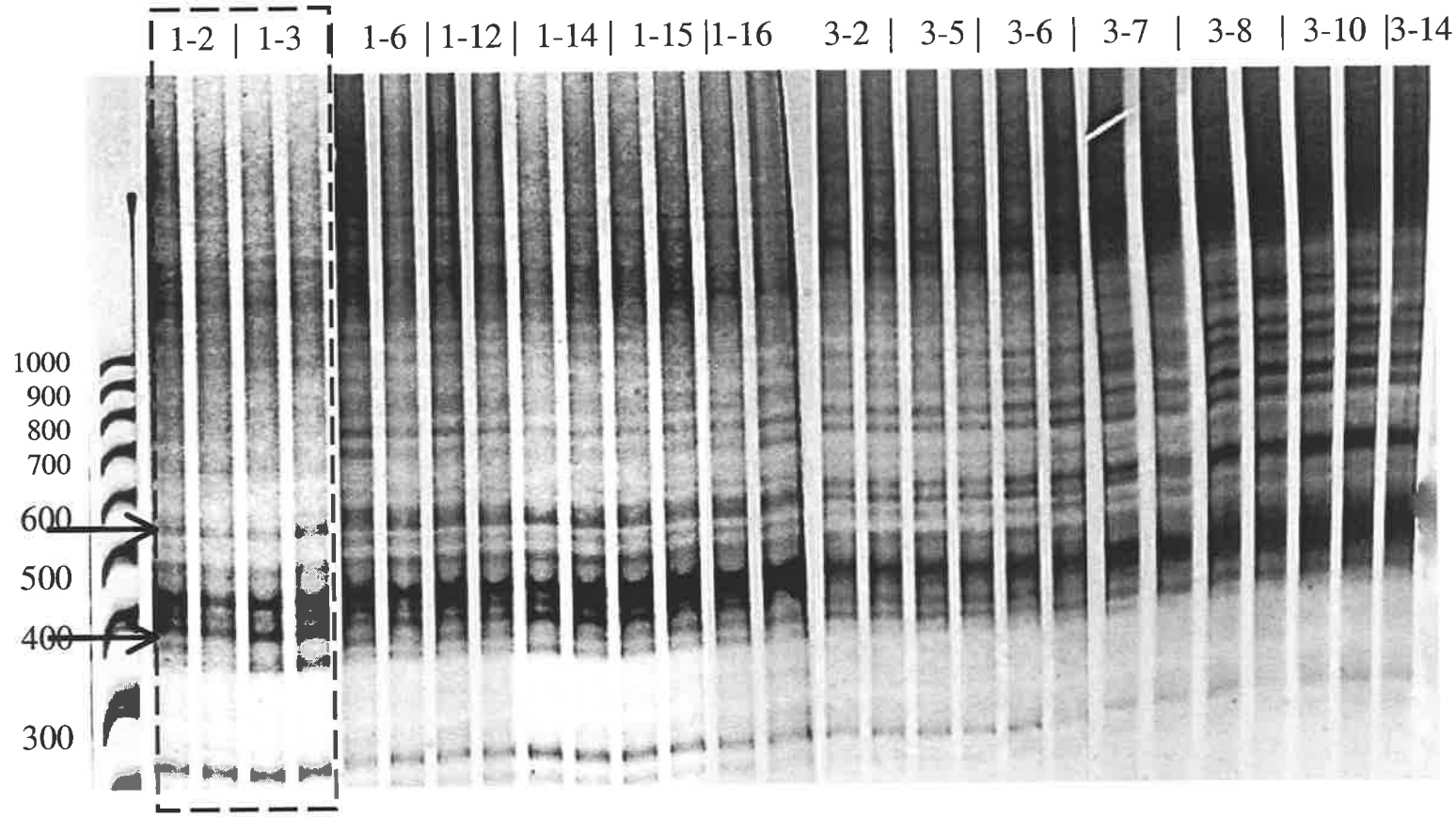

Figure 4.13 Polyacrylamide gel depicting duplicate amplicons generated by ISSR primer 880 of samples in Populations 1 and 3. Polymorphic bands are shown in lanes circled in black box and indicated by arrows along the side.

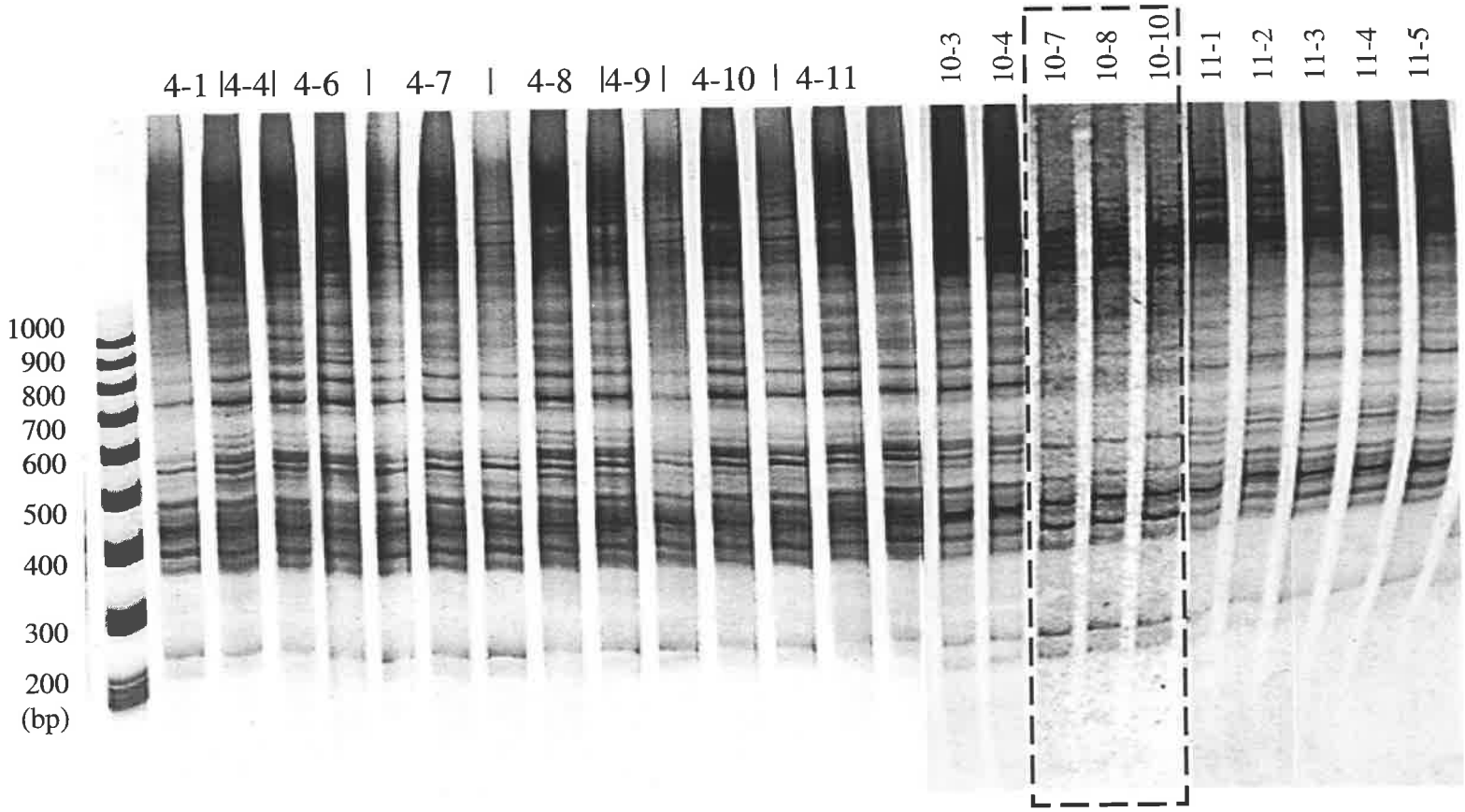

Figure 4.14 Polyacrylamide gel depicting duplicate amplicons of samples in Population 4 and amplicons in Populations 10 and 11 generated by ISSR primer 880. Polymorphic bands are shown within the black box and by the arrows along the side.

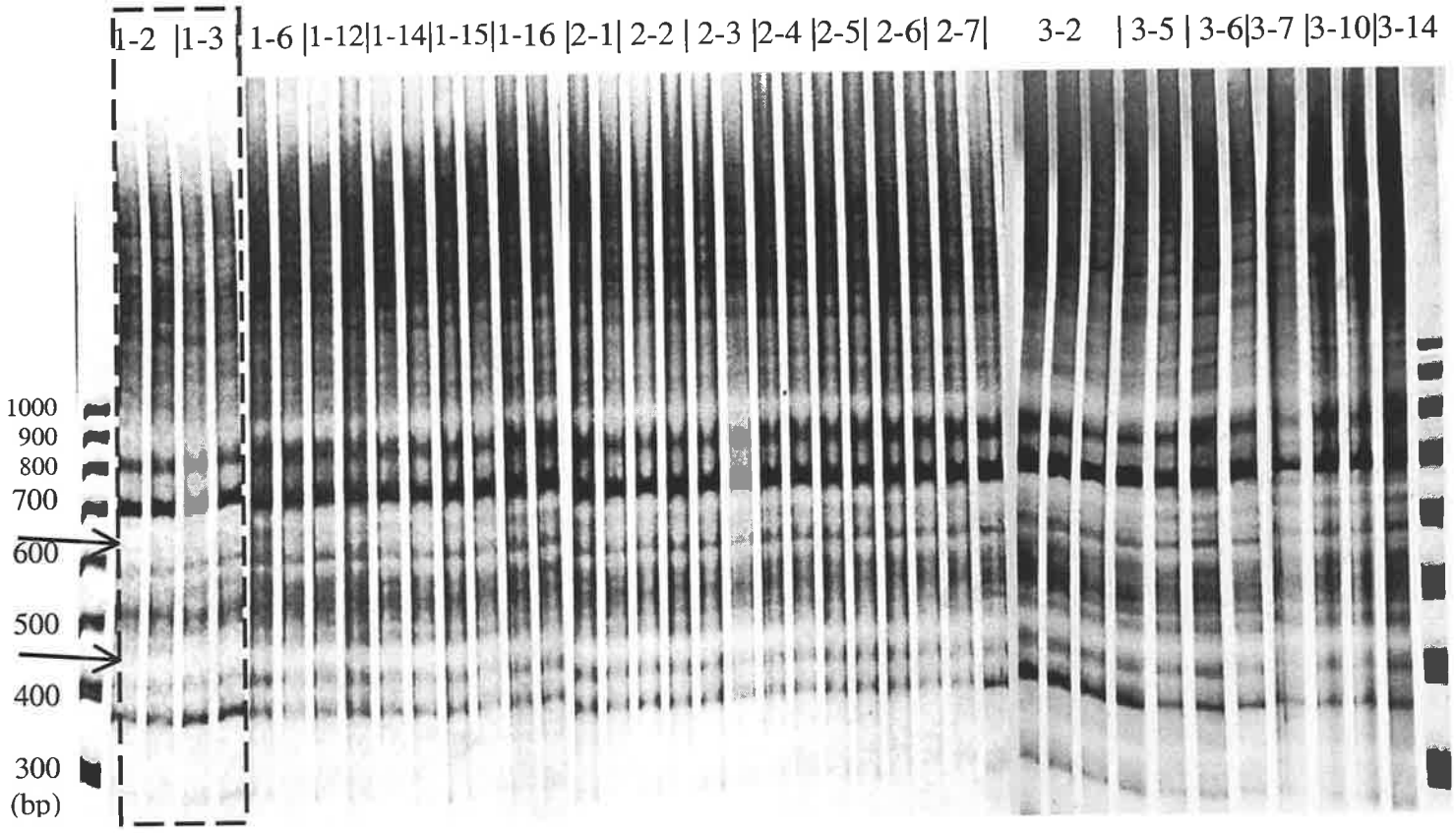

Figure 4.15 Polyacrylamide gel depicting duplicate amplicons generated by ISSR primer 889 of samples in Populations 1, 2 and 3. Polymorphic bands are shown within the black box and by the arrows along the side.

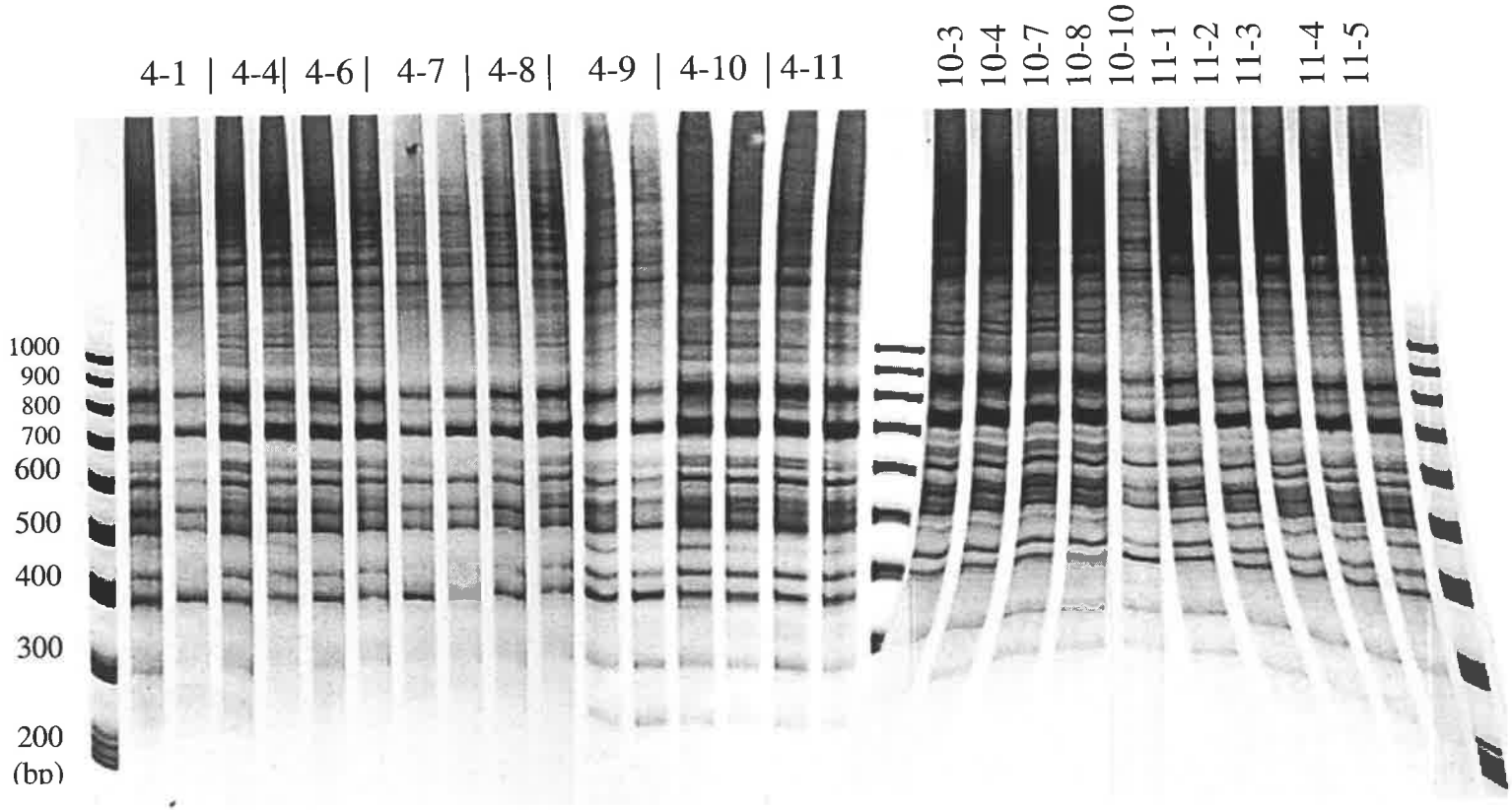

Figure 4.16 Polyacrylamide gel depicting duplicate amplicons of samples in Population 4 and amplicons in Populations 10 and 11 generated by ISSR primer 889.

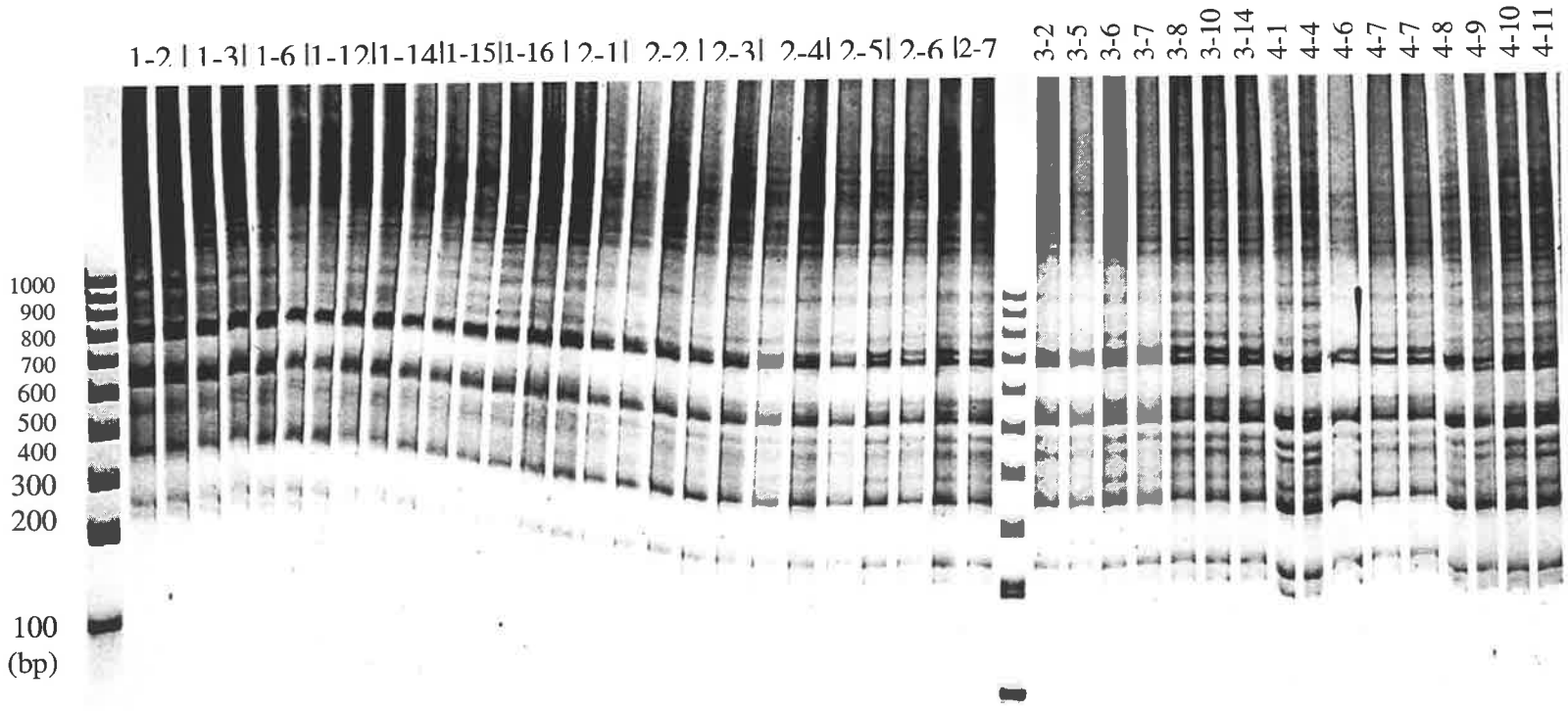

Figure 4.17 Polyacrylamide gel depicting duplicate amplicons of samples in Populations 1 and 2, and amplicons in Populations 3 and 4 generated by ISSR primer 891. Banding patterns with this primer in these populations were identical.

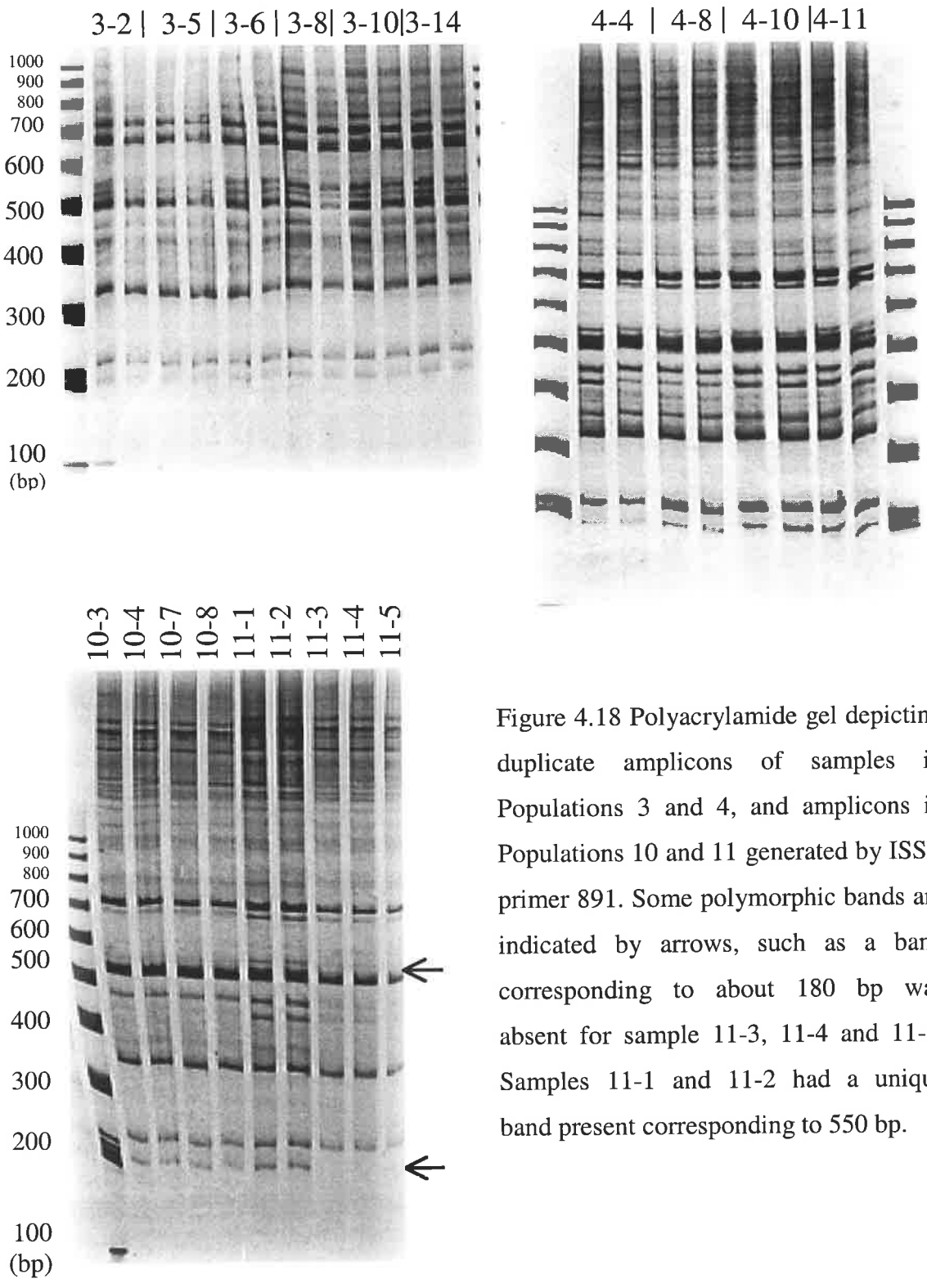

Figure 4.18 Polyacrylamide gel depicting duplicate amplicons of samples in Populations 3 and 4, and amplicons in Populations 10 and 11 generated by ISSR primer 891. Some polymorphic bands are indicated by arrows, such as a band corresponding to about 180 bp was absent for sample 11-3, 11-4 and 11-5. Samples 11-1 and 11-2 had a unique band present corresponding to 550 bp.

4.5.2.2 UPGMA clusters and genetic distance

The 39 samples examined formed nine clusters in the UPGMA dendrogram. There were 25 identical individuals. These were five of seven individuals in Population 1, all the seven individuals in each of Populations 2 and 3 and six of the eight individuals in Population 4. Populations 1 to 4 all came from a small area about 5 km in diameter. These consisted of paired roadside and field populations. The other two individuals in Population 4 (4-7 and 4-8) formed a separate cluster. The UPGMA node distance was 0.0148. Samples 1-2 and 1-3 also separated from all other members of these populations.

Individuals in Populations 10 and 11 separated into five genetic clusters. Individuals 11-1 and 11-2 were identical and 10-3 and 10-4 were identical. These two groups of individuals formed a single group with a relatively close relationship to the 25 identical individuals from other populations. Individuals 11-3, 11-4 and 11-5 were identical, and formed a separated cluster. Individuals 10-7, 10-8 and 10-10 formed two separated clusters, closely related to each other, but more diverse from the other individuals.

The genetic distance between individuals in Populations 1 to 4 were small, whereas Populations 10 and 11 were both more diverse and more different to Populations 1 to 4. Populations 10 and 11 were collected about 26 km from Populations 1 to 4. The UPGMA cluster (Nei 1978) dendrogram is shown in Figure 4.19. Genetic distances (Nei 1972, 1978) of the non-identical samples are shown in Table 4.7.

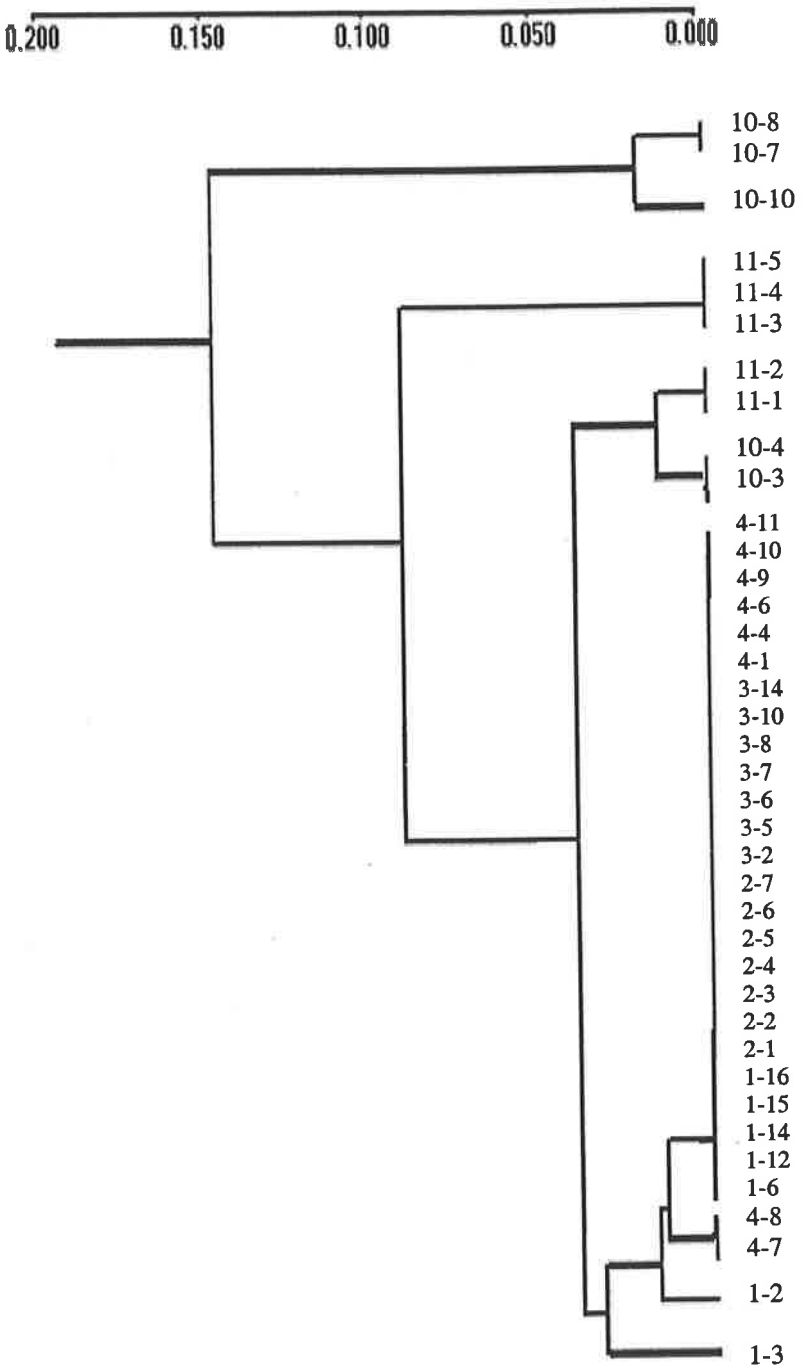

Figure 4.19 Dendrogram of the 39 samples collected in 2004 (UPGMA).

Table 4.7 Genetic distance of the non-identical samples generated by TFPGA using Nei's (1978) unbiased distance.

	1-2	1-3	II1234*	10-3, 10-4	10-7, 10-8	10-10	11-1, 11-2
1-3	0.0458						
IDP1234*	0.0299	0.0451					
10-3,10-4	0.0451	0.0606	0.0451				
10-7,10-8	0.1616	0.1793	0.1272	0.0938			
10-10	0.2283	0.2542	0.2029	0.1542	0.0206		
11-1,11-2	0.0606	0.0764	0.0299	0.0148	0.0938	0.1542	
11-3,11-4, 11-5	0.1252	0.0764	0.0924	0.0764	0.1358	0.1782	0.0616

*II1234=identical individuals in populations 1, 2, 3, and 4 : 1-6, 1-12, 1-14, 1-15, 1-16, 2-1, 2-2, 2-3, 2-4, 2-5, 2-6, 2-7, 3-2, 3-5, 3-6, 3-7, 3-8, 3-10, 3-14, 4-1, 4-4, 4-6, 4-7, 4-10 and 4-11

Chapter 5 Discussion

5.1 Seed movement and individual mutation

5.1.1 The ability for seed of *L. serriola* to spread in the Yorke Peninsula

Empirical studies on seed dispersal demonstrated that morphological attributes of both diaspores and maternal plants affect seed dispersal (Swaine and Beer 1977, Rabinowitz and Rapp 1981, Lee 1984, Augspurger and Franson 1987, Okubo and Levin, 1989). The height of seed release determines average dispersal distances in many species (Sheldon and Burrows 1973, Watkinson 1978, Morse and Schmitt 1985, Schmitt et al. 1985). It is determined by the combination of plant height and infructescence length (Thiede and Augspurger 1996), and it is one of the parameters in models predicting dispersal distances, which are also influenced by skewness and kurtosis (Thiede and Augspurger 1996). The average seed (achene) weight of *L. serriola* is 0.40 mg. The pappus structure of *L. serriola* is shown in Fig 21. The average height of *L. serriola* in the farming area is about 1.0-1.5 m, so the maximum seed release height was about 1.5 m. Therefore the terminal velocity in free fall should be attained rapidly and be sensitive to fluctuations in wind speed. This suggests that seed have the potential to disperse over wide areas, depending on local wind speeds. Seeds of *L. serriola* could also be whisked up into the air again or dragged along the ground after alighting at one place in the same way as a medium size seed would be (Burrow 1986).

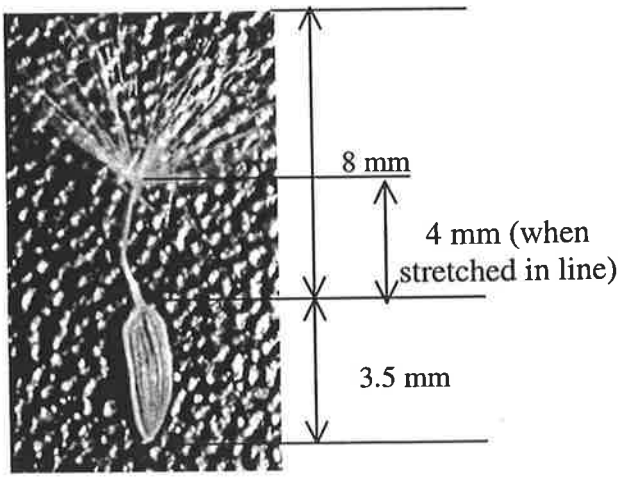

Figure 5.1 Structure of *L. serriola* seed pappus (Seed from the 2004 survey).

Environmental conditions are also important to seed dispersal (Thiede and Augspurger 1996). Seed dispersal distances were increased when wind speed is high (Sheldon and Burrows 1973, Augspurger and Franson 1987). The Yorke Peninsula has a Mediterranean climate with cool wet winters and hot dry summers. *L. serriola* germinates either in autumn or spring. Autumn-germinating plants stay as rosettes during the winter, elongating to flower during spring. Spring-germinating plants elongate a little later. Both cohorts flower in summer. During summer, weather conditions are conducive to the dispersal of *L. serriola* seed. The pappus becomes more efficient as a dispersal mechanism at low humidity, because the drag coefficient increases (Sheldon and Burrows 1973). According to the climate averages in the past 80 years, the summer average mean relative humidity was 35.5%, and the mean rain days were only 3.7 days during summer (Australian Government Bureau of Meteorology 2001). The mean wind speed at 3 pm was 17.2 km hr^{-1} (4.78m s^{-1}) in the 3 months during which seed dispersal occurs. The climatic conditions and the seed structures would favour the spread of herbicide resistant *L. serriola* widely across the Yorke Peninsula.

Seeds of many weed species are spread through contaminated farming equipment, and strict hygiene standards can be effective in minimising seed dspersal (Matthews 1994). Weed seeds can also be dispersed as contaminants of crop seed (Matthews 1994). In upper Yorke Peninsula, South Australia, crops are harvested in November or early December, when most of the *L. serriola* is just starting to flower. The tops of the stems are chopped off by the harvest equipment and clog the machine, but roots and stems still remain and continue to grow until late December and January when the plants flower and set seeds. Under these conditions, the probability of seed spread by harvesting equipment or through the contamination of crop seed for *L. serriola* is not high. In summary, while equipment hygiene and avoidance of contaminantion of crop seed are important, they will not stop the spread of herbicide resistant *L. serriola* as seed will still spread by wind. Dispersal distance is also affected by the surroundings. For example, obstacles formed by vegetation can reduce seed dispersal (Cremer 1965, Sheldon and Burrows 1973, McEvoy and Cox 1987, Telenius and Torstensson, 1989).

5.1.2 The spread of herbicide resistance in the Yorke Peninsula

5.1.2.1 Seed movement has contributed to the spread of herbicide resistance

The genotyping of individuals from the 1999 survey indicated a number of examples of individuals from different sites with the same genotype. The results of the study examining herbicide-resistant *L. serriola* suggest seed movement could play a significant role in the spread of resistance. Three identical susceptible individuals (17-

02, 20-12 and 40-13) suggest *L. serriola* genotypes can spread as far as 65.8 km, which is the longest distance in the survey. The second longest spread distance between identical individuals of 51.3 km also occurred with susceptible individuals. The three individuals, 17-02, 20-12 and 40-13 were not distributed along the same road, which confirms that wind is the most likely vector for seed dispersal. Long distance spread could occur over one or several generations.

The majority of the samples studied were from resistant plants. Two groups of identical resistant *L. serriola* suggested resistance had been spread over both short and long distances. Three identical resistant individuals, 81-01, 83-12 and 84-4, which were about 4 km to 11 km from each other demonstrated short distance spread. There was a fourth individual (22-08) identical to these three individuals about 43 km to 48 km from these three comparatively close individuals. This showed long distance spread of resistant *L. serriola* also occurred. The other group of identical resistant *L. serriola*, 13-06, 99-01 and 106-04, also showed long distance, 42.9 km, and short distance, 8.2 km, spread.

The pattern of resistant and susceptible individuals across the landscape suggests spread of resistance is likely to be sporadic. Where paired populations are compared, resistance was not necessarily apparent in both populations. For example, roadside Population 53 had resistant plants but no resistant *L. serriola* existed in the adjacent field. Therefore, plants in Population 53 are likely to have come from another source. In addition to spread by wind, it is likely that some *L. serriola* seeds will not spread far from the parent. Therefore, the combination of spread by wind and the continued use of

sulfonylurea herbicides by farmers will likely see increased resistance in *L. serriola* in the area.

The two outlier populations from the Adelaide region, about 120 km away, were the most different of the populations examined. This suggests that while seed movement occurs over a localized area, there is limited immigration from well outside the area. Most populations in the area proved to be closely related suggesting radiation from an original introduction.

5.1.2.2 The impact of seed movement on resistance spread

Given the ability of *L. serriola* seed to spread, resistant seed from roadsides or other fields could easily contaminate sites where resistance is currently absent. This spread of resistance could cause potential problems for growers, but only if they use sulfonylurea herbicides to control weeds.

5.1.2.3 Independent mutation is more important in the evolution of herbicide resistance

Although the genetic variance of resistant populations was less than susceptible populations, of the 18 resistant *L. serriola* from different sites, 11 were different to all other individuals. The other seven had three different genotypes, making 13 different resistant genotypes in total. Therefore, it is likely that resistance has evolved independently at least 13 times in the region. Given the high use of sulfonylurea

herbicides (Hawthron-Jackson *et al.* 2004) and the high susceptibility of *L. serriola* to these herbicides (Preston *et al.* 2005), it is not surprising that resistance has evolved multiple times. However, the combination of weed seed spread and continued use of sulfonylurea herbicides is likely to increase the amount of herbicide-resistant *L. serriola* in the region. It is also obvious that resistance is being spread as well as evolving *in situ*. The high number of resistant individuals in roadside populations where sulfonylurea herbicides are not used is testament to this. Adjacent resistant populations in fields and roadsides did not always have identical genotypes. For example, the genetic distance of two resistant individuals, 25-03 and 24-10, from a roadside and adjacent field was 0.069. Their origins are obviously different and they have been selected for resistance in different places.

5.1.3 Samples collected in 2004

The study of samples collected in 2004 complemented the study of 1999 samples. In the study of 1999 samples, only one individual from each population was chosen for genetic analysis. In the study of 2004 samples, more individuals from each population were used, which gave more information of genetic relationships within populations. As in 1999, paired samples, from field and adjacent roadsides, were collected in 2004.

5.1.3.1 Seed movement was responsible for distribution of herbicide resistance over a small area

Geographically close populations had close genetic relationships. All individuals except four (1-2, 1-3, 4-7 and 4-8) in Populations 1, 2, 3, and 4 were identical as determined by ISSR banding patterns. Populations 1 and 2 were located 2.7 km from Populations 3 and

4. In this small area, resistant *L. serriola* had spread to roadsides from fields and most resistant individuals were probably from the same evolutionary event. It is perhaps significant that even in this small area, several resistant genotypes could be detected.

5.1.3.2 Independent mutation is important in the evolution of herbicide resistance

Individual 10-10 had a unique genotype among the samples tested. The genetic distance between individual 10-10 and the 25 identical individuals in Populations 1, 2, 3 and 4 was 0.2177, which was the greatest genetic distance of samples collected in 2004. Populations 10 and 11 were 27.6 km from Populations 1 and 2. Therefore, resistance in 10-10 was clearly the result of an independent event.

Individuals 10-7 and 10-8 had identical banding patterns, but these were different to other individuals in Populations 1, 2, 3 and 4. Therefore the resistance mutation in these populations would also have occurred as an independent event. Individuals 11-3, 11-4 and 11-5 had identical banding patterns. These individuals had a genetic distance of 0.0984 compared with the 25 identical individuals in Populations 1, 2, 3 and 4. Individuals 10-3 and 10-4 had identical banding patterns with individuals 11-1 and 11-2. Five resistant individuals in Population 10 were from three different sources and individuals in the adjacent Population 11 were from another two sources. Individuals collected in these two populations were close to each other, within 50 m. It is possible, but perhaps unlikely that independent mutations occurred over such small distances. The most likely explanation is that some or all of the mutations occurred elsewhere and

were blown in from outside. Again this is evidence for seed movement of resistant individuals.

A few resistant individuals were only slightly different to other resistant individuals. This raises a number of issues with respect to the evolution of herbicide resistance in *L. serriola*. Individuals 4-7 and 4-8 had a genetic distance of only 0.0157 from the 25 identical individuals in Populations 1, 2, 3 and 4, suggesting a different, but closely related, parent of these two individuals with the other individuals. The genetic distance between individuals 10-3, 10-4 and 11-1, 11-2 was also only 0.0157. Independent mutation could be responsible for herbicide resistance in these populations. However, because the genetic distances are small, a possibility is that the mutation endowing resistance happened some time ago and populations have diverged since.

5.1.4 Comparison of samples collected in 1999 and 2004

In 2004, five years after the initial survey, samples were collected along one of the main roads in the 1999 survey area. A higher proportion of resistant *L. serriola* was collected in 2004 than in 1999. This is probably because the continuing use of sulfonylurea herbicides in the area has continued selection for herbicide resistance in the area. At the time of collecting in 2004, most of the populations had more than eight individuals growing. However in 1999, there were nine (out of the 36) resistant populations that had less than 4 individuals at the time of collection. This suggests that the resistant populations might have also grown larger over that time.

In 2004, there was one site in which more than 14 *L. serriola* in the field were resistant while the adjacent roadside population was susceptible. This site was very close, within 100 m, to where Population 83 was collected in 1999. In 1999, at this site, there were 16 resistant individuals collected along the roadside, but there were no *L. serriola* found in the adjacent field. These dramatic shifts in resistance over the course of a few years point to interesting population dynamics of herbicide resistant *L. serriola*. *L. serriola* is a high seed producer (Prince *et al.* 1985, Amor 1986) with a relatively short-lived seedbank (Marks and Prince 1982). The genetic analysis presented here would be consistent with a situation where *L. serriola* populations are transient at any one locality. That is, populations rapidly become locally extinct but the locality is repopulated from outside. Therefore, as use of sulfonylurea herbicides continues in fields, it will be expected that the frequency of resistant individuals will increase across the whole area, not just within farmed fields.

5.1.5 Comparison of the spread of resistant *L. serriola* with other species

There has been some interest in documenting the spread of herbicide resistant genes in the environment. Research on the spread of herbicide resistance of *Avena fatua*, indicated the importance of seed movement (Andrews *et al.* 1998). *Avena fatua* is an autogamous plant like *L. serriola*. Unlike *L. serriola*, the seed of *A. fatua* does not have structures that assist spread by wind. The seed of *A. fatua* was most likely spread by harvesting machinery in the research reported by Andrews *et al.* (1998). One consequence of spread by farm machinery is that seed will not move to fields or other sites where the machinery does not go. This makes the spread characteristics different to

L. serriola, where spread occurs by wind. In contrast, research on localizing the origins of herbicide resistant *Alopecurus myosuroides* suggested no obvious spread between patches (Cavan *et al.* 1998). *A. myosuroides* also does not have structures that allow spread of seed by wind. Cavan *et al.* (1998) suggested that the lack of evidence for resistance spread by seed movement meant that farmers should focus on seed banks and herbicide use rather than putting extra effort into seed movement control. Research on genetic variation in triazine resistant and susceptible *Solanum nigrum* populations indicated that both independent mutation and seed movement contributed to the spread of resistant *S. nigrum*. In addition, *S. nigrum* could be spread over short and long distances, most likely by birds (Stankiewicz *et al.* 2001). *S. nigrum* is almost completely autogamous (Stankiewicz *et al.* 2001), and the seed is spread by birds (Burgert and Bumside 1973, Weller and Phipps 1978/79). *S. nigrum* collected in different locations were more related to some susceptible populations than to other resistant groups, while other resistant populations from widely separated areas had identical genotypes. This indicates the independent evolution of resistance occurring in this species. The factors endowing the spread of resistance of *S. nigrum* are similar to those for *L. serriola* found in this research, except that birds are likely to spread seed over a much larger area than wind.

5.1.6 Suggestions for managing the spread of herbicide resistance *L. serriola*

The herbicide use patterns by farmers are significant factors in the evolution of herbicide resistance (Gressel and Segel 1978, Gressel 1982). This is also true of *L. serriola* in this area, despite the weed's ability to move via seed dispersal. The fairly

large number of independent mutation events in herbicide resistant *L. serriola* detected here point to rapid selection for resistance through the use of herbicides.

The widespread adoption of sulfonylurea herbicides across the area by farmers in the late 1980s and early 1990s has resulted in significant selection pressure for resistance, not only in *L. serriola*, but also in *Lolium rigidum* (Hawthorn-Jackson *et al.* 2004); Preston, unpublished) and *Sisymbrium orientale* (Preston, unpublished). Clearly continued use of these herbicides will increase resistance.

In order to manage resistance across the area farmers will need to change their herbicide use patterns and use alternative controls for these weeds. These may include other herbicides, herbicide mixtures or other non-chemical weed control practices. However, there is little farmers can do about the introduction of resistant seed blown by wind, although the introduction of windbreaks may help. The fact that herbicide resistance has spread rapidly across the area could compromise control techniques. However, introduction of resistant seed from outside will be of no consequence if that herbicide is not going to be used. On the other hand, it could lead to more rapid evolution of resistance when the herbicide is used (Hidayat *et al.* 2004).

5.2 ISSR primers and results

Eight primers were screened and four were chosen for use in ISSR fingerprinting of the samples. If more primers were used, it is likely that the number of polymorphic bands detected would increase. This would not affect the assessment that independent mutation events had occurred in the area. Individuals scored as genetically different

would still be different. However, individuals scored as identical may separate with more bands scored. If this were the case, the importance of independent mutation events would become greater.

In some other research of population genetic variation using ISSRs, the number of primers used varied, from several to even 20 (Prevost and Wilkinson 1999, McRoberts et al. 2005, Zhang et al. 2005, Aga et al. 2005). Although only four primers were used in this research, the total number of bands scored and the proportion of polymorphic bands reflect other published researches in the study of population genetics using ISSR and, more importantly, produced enough differentiation between and within populations to allow relevant conclusions to be reached. Prevost and Wilkinson (1999) used four primers out of the nine screened to ISSR fingerprint potato cultivars. McRoberts et al. (2005) used five ISSR primers that produced a total of 35 markers over 189 individuals. In cotton (Liu and Wendel 2001) used three primers $(GA)_9T$, $(GA)_9C$, $(GA)_9A$ and double primer combinations. In some other examples, while more primers were used, fewer bands and polymorphic loci were recorded compared to here. For example, Zhang et al. (2005) used 17 primers for the detection of genetic variation in *Pinus squamate*, but only a total of 73 bands in 31 individuals were recorded and nine were polymorphic in the population.

5.3 Future research

5.3.1 Sequencing the ALS gene in different populations

This research has established that a number of different genotypes occur among resistant individuals of *L. serriola* in the Yorke Peninsula. Previous work has

established that resistance is due to a mutation within the ALS gene (Preston *et al.* 2005a). At least two *L. serriola* populations collected 25 km apart had the same mutation. Given that resistance can occur both by seed movement and independent mutation, it would be interesting to know which mutations within ALS are represented in the samples examined in the current study. Such a study may elucidate how resistance has evolved in the area. For example, do individuals that are identical in the ISSR screen have the same mutations in ALS? Do non-identical, but closely related individuals have the same mutations? Such studies would provide information on when the resistance alleles appeared, before or after divergence of the ISSR loci.

Preston *et al.* (2005a) showed that two resistant populations not only had the same mutations in ALS, but that the mutant enzymes had the same enzymatic properties. This included resistance to herbicides, altered affinity for pyruvate and altered feedback inhibition. One of the unknown issues with regard to resistance to sulfonylurea herbicides is why it is so common in populations (Preston and Powles 2002). Understanding the impact of mutations in ALS on plant performance in the field is a way of obtaining this information. Having a collection of individuals with varying levels of genetic diversity, but with the same mutation within ALS could be useful in this endeavour.

5.3.2 Determine the initial gene frequency of resistance in *L. serriola*

Knowing the initial gene frequency of ALS-inhibiting herbicide resistance in *L. serriola* could help to understand the rapid evolution of herbicide resistance in this species. Although there are 90 species with ALS-inhibiting herbicide resistant populations in

the world, few of these have had the initial resistant gene frequency determined. The rapid evolution of herbicide resistance in *L. rigidum* was in some degree related to the high initial frequency of the resistance (Preston and Powles 2002). The initial frequency of resistance may vary between different species. The frequency of resistance in *L. rigidum* to sulfometuron-methyl was 4.6×10^{-5} to 1.2×10^{-4}, and to imazapyr was 1×10^{-5} to 5.8×10^{-5} (Preston and Powles 2002), which was much higher than the frequency of resistance in *Arabidopsis thaliana* (10^{-8}-10^{-9}) (Jasieniuk *et al.* 1996), cotton (10^{-7}) (Rajasekaran and Grula 1996), and tobacco (5×10^{-8}) (Harms and Dimaio 1991). Unlike *L. rigidum*, *L. serriola* is an obligate self-pollinated species. The initial gene frequency for resistance is a major driver of selection (Preston and Powles 2002) for resistance. This may be different in self-pollinated species compared to out crossing species. Given that *L. serriola* has wind borne seed, it may be difficult to find populations not exposed to ALS-inhibiting herbicides. However, understanding the evolution of resistance in this species to ALS-inhibiting herbicides could be valuable in planning strategies to avoid resistance to other herbicides in self-pollinated weeds.

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