Monitoring insect diversity and parasitism levels in alfalfa seed production fields in western Canada

by

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Abstract

Alfalfa, *Medicago sativa* (L.) (Fabales: Fabaceae), is an excellent source of high protein feed for livestock. Canada is the second largest producer of alfalfa seed (4.2 M kg/year) in the world, with the vast majority of production concentrated in the province of Alberta. The productivity of these fields is threatened by an invasive insect, the alfalfa weevil, *Hypera postica* (Gyllenhal) (Coleoptera: Curculionidae) as well as a myriad of other potential insect pests. Recently, insecticide resistance was confirmed in several alfalfa weevil populations in Alberta, and few other management options exist. Many natural enemies of alfalfa insect pests are present in Alberta, including biological control agents of alfalfa weevil, *Bathyplectes curculionis* (Thomson) (Hymenoptera: Ichneumonidae) and *Oomyzus incertus* (Ratzeburg) (Hymenoptera: Eulophidae); however, the distribution of these species and their parasitism levels are currently unknown. Here, we assessed the current diversity and distribution of insects in alfalfa seed production fields, with special emphasis on parasitism levels of *B. curculionis* and *O. incertus* throughout southern Alberta. A survey was conducted to collect insects, including alfalfa weevil larvae, from seed production fields in 2020 and 2021. During this survey, insect collections were taken from fields at three crop stages: bud, flower and seed, these insects were sorted to guild (pest of alfalfa or natural enemy of pests) and identified to genus and species. In addition, alfalfa weevil larval samples were collected weekly from the end of May to the beginning of July to assess the activity period of these parasitoids, finding parasitism activity throughout the month of June and into July. Parasitism levels based on the multiplex PCR assay were comparable to live rearing and ranged from 0-90% across sites. Assessing when and where these parasitoids occur, as well has how the two guilds interact will allow growers to better utilize these biological control agents and, ultimately, reduce spray applications.

Preface

This dissertation is an original, unpublished work by the author, M. Reid, with the exception of the multiplex PCR procedure (Appendix 1) in Chapter 3. It was developed by B. Mori and J. Holowachuk at Agriculture and Agri-Food Canada, in Saskatoon, Saskatchewan, CA.

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1. Literature Review

1.0 Introduction

Alfalfa (*Medicago sativa* L.) is widely grown throughout the world due to its excellent source of high protein feed for livestock and ability to fix nitrogen (Barnes et al. 1988, Lanyon and Griffith 1988, Bagavathiannan and Van Acker 2009). The origin of alfalfa is difficult to identify as it was domesticated before written records existed (Putnam et al. 2000). The domestication process was believed to have started in central east Asia. The first written records of domesticated alfalfa were from Greece where it had been brought by the Medes (ancient Iranian) armies in the time of the Roman Empire, and then spread throughout Europe. During the 15th century it was introduced to South America by the Spanish and from there was brought to California at the start of the 19th century. After 1871, winter hardy strains were established and it was then brought to Canada (Bagavathiannan and Van Acker 2009, Prosperi et al. 2015). The establishment of cold hardy germplasm for North America was difficult as alfalfa is a tetraploid, but it is easily cross pollinated and as a result hardier cultivars were established by incorporating germplasm from Europe and Russia. Breeding in North America was primarily focused on cold hardiness until bacterial wilt and other pests became large issues. As a result, Canada and USA joined together to create the *Germplasm Resources Information Project*, to create and maintain stable and diverse crop germplasm, including alfalfa (Teuber and Brick 1988).

Alfalfa is a polymorphic plant that can be diploid or tetraploid, but most commercial cultivars are tetraploid (Barnes 1980). It can also be an annual or a perennial. Perennial cultivars are most commonly grown for seed production. Alfalfa grows as a short, bushy plant with a deep taproot (Barnes 1972, Teuber and Brick 1988). The first leaf is unifoliate, the following leaves are trifoliate and grow in an alternate pattern on the stems. The stems grow in an erect manner, all originating from the crown and to an average length of 1 meter. As the plant matures it has an indeterminate growth habit which allows the plant to grow both vegetative and reproductive organs throughout the season. Alfalfa flowers are grown on a raceme typically with ten flowers per stock, the flowers range from yellow, white, blue, purple or variegated. These flowers have a tripping mechanism which ensures cross pollination by insects and works by uncovering the

pistil only once the flower has been tripped (Barnes 1980). Tripping can be done by abiotic factors, such as strong winds, in some cases, but usually only occurs after an insect lands on the flower looking for its nectaries; the pressure from the insect will open up the flower exposing the reproductive organs for pollination. Seeds start to form 2-3 days after pollination, the seeds form in a coiled fruit pod and are 1-2 mm in size (Teuber and Brick 1988).

North America is the current world leader in alfalfa seed production, producing on average over 45 million kg per year (Government of Alberta 2012). The USA is the top producer of alfalfa seed in the world with Canada coming in second (Mueller 2008). In the USA production primarily occurs in California, Idaho, Washington, and Nevada. Except for California, the northern states grow similar cultivars to those in Canada. Canada produces certified and uncertified seed and the number of hectares can fluctuate, for 2020 and 2021 there have been 16,593 and 17,293 ha in alfalfa seed production, respectively (Canadian Seed Grower's Association 2021). Canada has exported $6,220,289 and $7,345,655 CAD worth of seed in 2020 and 2021, respectively. Most of the exports are to the USA and China (Statistics Canada 2022)

1.1 Alfalfa pests

Alfalfa fields contain an abundance of arthropods with over 450 and 591 species identified in Alberta and New York fields, respectively (Harper 1988, Manglitz and Ratcliffe 1988). While the vast majority of arthropods were deemed to be accidental visitors, others were pollinators or predators and parasitoids preying on arthropods including alfalfa pests. Although, over 100 species feed on alfalfa, only four insects are major pests across most regions of the USA and Canada. These four major pests are the pea aphid, *Acyrthosiphon pisum* (Hemiptera: Aphididae), the plant bugs, *Lygus* spp., and *Adelphocoris lineolatus* (Hemiptera: Miridae), and the alfalfa weevil, *Hypera postica* (Coleoptera: Curculionidae).

Pea aphid

The pea aphid is the most common aphid found in alfalfa (Harper 1988, Manglitz and Ratcliffe 1988). Pea aphids are light green with characteristic cornicles. In the spring, overwintered eggs hatch into wingless female aphids that produce 6-7 female nymphs per day asexually; during the summer these nymphs will mature into genetically identical daughters.

Aphid offspring may be winged or wingless; winged adults occur when aphids are too crowded and need to move to find additional resources. In the fall, the shortening day length and cooler temperatures cause aphid hormones to change and produce male and female offspring. These nymphs will become adults and reproduce sexually. Females lay eggs which will overwinter on a variety of substrates including grass clumps (Sandhi and Reddy 2020).

Pea aphids originated in Europe and now have a cosmopolitan distribution (Sandhi and Reddy 2020). These aphids damage many crops with yield losses of up to 30% in peas. Damage occurs in alfalfa when infestation rates are above the economic threshold of 150-200 pea aphids per 90° sweep (Government of Alberta 2011). In alfalfa, pea aphids feed on all parts of the plant but the largest damage occurs with terminal bud feeding, this feeding restricts growth which can halt flowering and reduce seed yields (Manglitz and Ratcliffe 1988).

Plant bugs

A number of plant bugs in the family Miridae cause alfalfa yield losses (Harper 1988, Manglitz and Ratcliffe 1988). The most economically important species in the Prairies are the four *Lygus* spp.: *Lygus lineolaris* (the tarnished plant bug), *L. borealis*, *L. elisus* and *L. keltoni,* and the alfalfa plant bug *A. lineolatus. Lygus* have flattish bodies, <6 mm in length, <3 mm in width and range from pale green to reddish brown. *Lygus* have a distinctive yellowish V-shaped mark just behind the thorax. Adults overwinter in grass clumps and in litter around and on fields. In the spring, females lay eggs in host plant stems which then hatch 15 days later, and nymphs emerge and pass through five instars before reaching adulthood. Depending on location and temperature there can be more than 1 generation per year. *Lygus* have piercing-sucking mouthparts that upon feeding cause distorted plant growth and rosetting. Feeding during flowering and seed set is the most damaging as it can cause flowers to abort and seeds to shrivel (Harper 1988, Manglitz and Ratcliffe 1988). The economic threshold for *Lygus* in seed alfalfa in Alberta is 2-3 adults or 3rd and 4th instars per 90° sweep (Government of Alberta 2011).

Adelphocoris lineolatus was accidentally introduced from Europe (Harper 1988, Manglitz and Ratcliffe 1988). It has a similar body shape to *Lygus,* but adults are longer and thinner (i.e. 14 mm long and 1-2 mm wide). Adults also lack the clear V-shaped mark found on *Lygus* spp. *Lygus* and *A. lineolatus* nymphs look similar; their bodies are light green pear shaped, but *Lygus*

nymphs have black dots on their back whereas *A. lineolatus* do not. The damage and life cycle of *A. lineolatus* is similar to *Lygus*, but *A. lineolatus* can have up to 2-3 generations per year. The threshold for *A. lineolatus* is the same as the *Lygus* of 2-3 adults or 3rd and 4th instars per 90° sweep (Government of Alberta 2011).

Alfalfa weevil

In recent years, alfalfa weevil has become a large threat to the alfalfa seed industry. Alfalfa weevil is an invasive pest of Eurasian origin which was introduced to North America on three separate occasions (Radcliffe and Flanders 1998, Bundy et al. 2005). The first introduction, called the western strain, occurs west of the 100th meridian and was discovered in Utah in 1904. It is believed to have come from the France/Switzerland/Italy region. The second introduction, called the Egyptian strain, was found in the Yuma Valley on the Arizona and California border in 1939. It was thought to be of Mediterranean origin, possibly from the Nile Valley in Egypt. Initially, this population was considered a different species, but is now known to be a strain of alfalfa weevil. The third introduction, called the eastern strain, was in 1951 in Annapolis, Maryland. This strain is more damaging than the other two strains. In western Canada only the western strain is present (Erney et al. 1996, Radcliffe and Flanders 1998, Bundy et al. 2005). In 1954, alfalfa weevil was found at rates of 1/500 sweeps in southeastern Alberta (Hobbs et al. 1959, Harper 1988); however, it has since spread throughout the prairies and alfalfa seed regions of northeastern Saskatchewan and Manitoba (Soroka and Otani 2011).

Alfalfa weevil overwinters as an adult. In early spring, they enter alfalfa fields and lay eggs inside alfalfa stems (Manglitz and Ratcliffe 1988). The eggs hatch, larvae emerge out of the stems and begin feeding on alfalfa leaves. Larvae pass through four instars, and once full grown (i.e. ~9.5 mm in length), they spin a cocoon on the lower portions of the plant or on the soil surface and pupate. Seven to ten days later adults emerge. Adults are ~4.8 mm in length with a brown snout and a dark stripe down the center of the back. In Alberta there is only one generation per year, but multiple generations can occur in more southern locations. In late summer-early fall, adult weevils move out of the field into grassy hedgerows to overwinter.

Larval alfalfa weevils feed on the interveinal tissue of alfalfa leaves creating significant damage (Manglitz and Ratcliffe 1988). Almost 95% of all alfalfa weevil feeding occurs in the

third and fourth larval instar (Soroka and Otani 2011). In most fields larval feeding coincides with the most sensitive time for the alfalfa crop, during the first growth in the spring (Manglitz and Ratcliffe 1988). Feeding can result in substantial loss of foliage and has large direct and indirect impacts on alfalfa biomass and seed yield (Fick and Liu 1976, Soroka and Otani 2011). Depending on the timing of feeding and weevil density, total plant biomass can decrease including taproot reduction which can have lasting impacts on the crop (Fick and Liu 1976). The economic threshold for the alfalfa weevil larvae is 20-25 3rd or 4th instars per 90° sweep (Government of Alberta 2011).

1.2 Alfalfa pest management

Integrated pest management (IPM), which involves the use of several, harmonious strategies to reduce pest population levels below economically damaging levels, have been developed in alfalfa. The modern definition of IPM was first proposed to combat insects in alfalfa fields in California (Stern et al. 1959). Population growth models were created for economically important insects which predicted when pest populations would reach the economic threshold. An economic threshold is defined as the insect density at which management should occur to decrease population size so that the cost of yield loss with be equal to management costs (Pedigo et al. 1986). This initial program relied heavily on the knowledge of a surveyor who could identify and monitor pest populations and determine if the populations would be controlled by natural predators or if chemical intervention was required. From this initial project, IPM strategies were created and refined for other crops. IPM can include several management strategies including cultural, biological, host plant resistance and chemical (Stern et al. 1959, Pellissier et al. 2017).

Cultural control aims to make agroecosystems more conducive for natural enemies and less conducive for the pest species to survive (Mueller 2008, Pellissier et al. 2017). Cultural control can be implemented in numerous ways. Changes in growing practices can be implemented to either make the plants more robust or to make the environment hard for the insects to establish. For example, the use of fertilization and irrigation can make plant stands more robust. Both fertilization and irrigation can ensure that plant stress levels are lower and therefore can better withstand insect feeding pressure. In some perennial crops, other cultural strategies may be employed, such as the ‘clip back’ method. The clip-back method involves

clipping (cut) back the stand early in the season, when the crop is just starting to grow. A variety of methods can be used to clip the crop including machines, grazing or chemicals depending on the grower and the crop. Clipping can disrupt the growth of the crop, known as a 'setback', which encourages the crop to grow more uniformly and may change when the different crop stages are present. In California, the clip-back method has been used to successfully manage the alfalfa seed chalcid (*Bruchophagus roddi* Gussakovsky (Hymenoptera: Eurytomidae) and reduce yield losses from 16% to <0.5% in the early 1960s.

Cultural controls often lead to an increase in biological control, which is the use of other organisms to aid in the control of pest species. Biocontrol agents can be generalists (i.e. predate/parasitize a large range of pests) or specialists (i.e. predate/parasitize one preferred pest species) (Snyder et al. 2005). With IPM strategies, growers seek to increase the density and diversity of natural enemies to aid in biological control. In alfalfa, there are many generalist predators including minute pirate bugs, *Orius* spp. (Hemiptera: Anthocoridae), big-eyed bugs, *Geocoris* spp. (Hemiptera: Geocoridae), damsel bugs, *Nabis* spp. (Hemiptera: Nabidae), lacewings, *Chrysopa* spp. (Neuroptera: Chrysopidae), spiders (Araneae) and ladybeetles (Coleoptera: Chrysomelidae) (Mueller 2008). The adult and larval forms of these insects feed on eggs and small insect pests, such as thrips, mites, aphids, whiteflies, and small caterpillars. There are also more specific parasitoids/predators of the alfalfa weevil (Pellissier et al. 2017), which will be discussed below.

Breeding for host plant resistance which results in more resilient cultivars that can inhibit or withstand insect damage is becoming more common (Sorensen et al. 1988). Host resistance can be conferred in a variety of ways; in alfalfa, cultivars that have more auxiliary buds are better able to continue growing after pests damage These buds are advantageous to alfalfa production because while the pests are feeding on the terminal buds halting growth, the auxiliary buds can grow and continue to branch to produce more flowers reducing the overall impact on seed yields (Pellissier et al. 2017). Other traits must be considered when choosing a cultivar, for example, winter hardiness, dormancy requirements, as well as the cost of seed (Smith 1988).

Using the above management strategies as part of an overall IPM program can lead to a decrease in the use of chemical controls; however, chemicals are still a very useful component of IPM. Currently in Alberta, there are ten different insecticides for use in alfalfa fields, and four of

them target the alfalfa weevil. For control there are three options, and for suppression there is one. For control, there are two non-systemic synthetic pyrethroids, lambda-cyhalothrin 120 g/L (e.g. Matador® 120E, Syngenta; Silencer® 120EC, ADAMA Canada; LaBamba, Sharde CropChem Ltd.) and deltamethrin (e.g. Decis® 5EC, Bayer; Poleci 2.5 EC, Sharde CropChem Ltd.). Malathion (IPCO and Loveland Products Canada) is the one non-systemic contact organophosphate registered for use. Chlorathraniliprole (i.e. Coragen®, FMC Corporation) is for suppression of alfalfa weevil only (Alberta Crop Protection Guide 2022). One potential downfall of using insecticides is the risk of insecticide resistance building in pest populations. Resistance can develop in a number of ways and is often specific to the insect and chemical. Resistance evolution usually starts as a mutation that allows some of the population to survive and reproduce, allowing their genetics to dominate (Weston et al. 2013). Insecticide resistance in alfalfa weevil was first noted for heptachlor, a cyclodiene organochlorine insecticide in the 1960s in Virginia, USA (Bishop 1964). Recently, alfalfa weevil resistance has been found to pyrethroid insecticides in the USA and Canada (Glen 2015, "Resistant Alfalfa Weevil Project " 2019).

Scouting is an integral component of IPM programs (Stern et al. 1959). Scouting is the most important step in crop management as it gives a detailed unbiased account for what is currently occurring in the fields. Once this data is collected, growers are then able to make informed decisions. Overall management decisions are often based on economic thresholds (when developed). These thresholds are based on a cost value: what is the cost of doing nothing (e.g. cost of yield loss) compared to the cost of intervening (e.g. an insecticide application, including labour, equipment and chemical costs) (Pellissier et al. 2017). When all the aforementioned methods are used in tandem with each other, more sustainable and economically efficient production can occur.

1.3 Alfalfa weevil parasitoids

To aid in the control of alfalfa weevil in the eastern United States, several parasitoid wasps were intentionally released for biocontrol (Brunson and Coles 1968). Parasitoids that established and were later recovered included: *Bathyplectes curculionis* (Thomson) (Hymenoptera: Ichneumonidae), *Bathyplectes anurus* (Thomson) (Hymenoptera: Ichneumonidae), *Microctonus ethiopia* (News) (Hymenoptera: Braconidae), an undescribed *Microctonus* sp. "Domestic Black" (Hymenoptera: Braconidae), and, *Tetrastichus incertus*

Ratzburg (Hymenoptera: Eulophidae) (Note: *T. incertus* is currently synonymized with *Oomyzus incertus* (Ratzenberg) (Hymenoptera: Eulophidae) (CABI 2019)). *Bathyplectes* sp. "Bagged" (Hymenoptera: Ichneumonidae), *Peridesmia discus* (Walker) (Hymenoptera: Pteromalidae), *Dibrachoides druso I* (Walker) (Hymenoptera: Pteromalidae) and *Campogaster exigua* (Meigen) (Diptera: Tachinidae) were not found after release (Brunson and Coles 1968). Introducing parasitoids into an ecosystem can have varying degrees of success. Brunson and Coles (1968) considered only two species, *B. curculionis* and *B. anurus* to be successful at establishing and controlling alfalfa weevil. These two wasps parasitize early alfalfa weevil instars and were found in the field at rates up to 70% and 30%, respectively. However, the level of parasitism varied greatly between fields and regions. There are numerous other studies that released different parasitoids in various regions across North America, each with variable success (Dysart and Day 1976, Berberet and Gibson 1976, Bryan et al. 1993, Dosdall et al. 2011). In areas where three different parasitoids have become well established, alfalfa weevil is usually a manageable pest and other management strategies are usually not needed (Flanders and Radcliffe 1996).

In Alberta, two alfalfa weevil parasitoids are found: *B. curculionis* and *O. incertus* (Soroka and Otani 2011). *Microctonus aethiopoides* (Loan) (Hymenoptera: Braconidae), (synonym *M. aethiops* (Nees)), found in the western USA, was suspected to be in Alberta, but may no longer be established (Loan 1975, Rand et al. 2018).

Bathyplectes curculionis was first introduced to North America through a number of shipments from Europe to Salt Lake City from 1911-1913 (Chamberlin 1926). Initial shipments contained *B. curculionis* cocoons spun within alfalfa weevil cocoons, and in the later years shipments also contained parasitized larva. Approximately 1,500 *B. curculionis* adults were released into the fields in Utah. Six years after the introduction of this parasitoid, it was found parasitizing alfalfa weevil larvae over 320 km away from the release locations. Since then, it has followed the spread of the alfalfa weevil as it established across North America and was identified in southeastern Alberta the same year alfalfa weevil was discovered (Hobbs et al. 1959, Rand et al. 2018). It is now the only parasitoid of alfalfa weevil widely distributed throughout North America (Radcliffe and Flanders 1998).

Adult *B. curculionis* are 5-10 mm long with a mostly black body and yellowish/tan colouring on the underside of their abdomen, and their forewing areolet is closed and often

pentagonal (Soroka et al. 2020). In the spring, adults emerge from pupae within the cocoon, mate and females begin to oviposit in alfalfa weevil larvae (Chamberlin 1926). Females have been observed to oviposit into all weevil larval instars, but may have a preference for earlier instars (Chamberlin 1926, Radcliffe and Flanders 1998). Females lay one egg per weevil larva; eggs hatch within 14 days and the larva feed within the weevil larvae (Chamberlin 1926). Once *B. curculionis* larvae have finished development, they exit the weevil larva, spin a cocoon and pupate. Most will remain in these cocoons to overwinter; however, in some regions a partial second generation can occur (Chamberlin 1926, Radcliffe and Flanders 1998). In Alberta, *B. curculionis* peak flight occurs in mid-June, and only one generation is thought to occur (Soroka et al. 2020).

Oomyzus incertus is also an introduced alfalfa weevil larval parasitoid (Harcourt et al. 1984). It was first found in Europe in 1844 but little was known about its host (Chamberlin 1925). In the alfalfa weevil larval shipments to Utah in the early 1900s, some small chalcid wasps were observed. Upon closer inspection, some weevil larva were found to contain 6-17 *O. incertus* larvae and were later recognized to be a parasitoid of alfalfa weevil (Streams and Fuester 1967). In the 1960s, there was a large effort to establish *O. incertus* across the USA, but it was only successful in the eastern USA. Over time *O. incertus* has expanded its range and is now found in varying levels in most locations where alfalfa weevil is present (Chamberlin 1925, Streams and Fuester 1967, Harcourt et al. 1984, Radcliffe and Flanders 1998).

Adult *O. incertus* are small black metallic wasps that have a blue/green sheen, and dark brown antennae. Males are 1-1.15 mm long and have distinct swollen scapes, whereas females are slightly larger (1.2-1.5 mm long) and lack swollen scapes (Streams and Fuester 1967). A single female can lay many eggs within one weevil larvae; these eggs hatch within 2-3 days and complete all larval stages within the weevil larvae. The age of the weevil larvae impacts the rate at which the *O. incertus* matures. Once the weevil larvae form cocoons, the parasitoid larvae pupate. Parasitized weevil larval cocoons are brittle and brown to mahogany in colour as opposed to non-parasitized cocoons which are green in colour. There can be 3-4 *O. incertus* generations through the growing season with some of each generation entering diapause within the host pupae and will emerge the following spring (Radcliffe and Flanders 1998). The adults can be found from early spring until late fall, the peak abundance is usually during mid-summer,

but differs depending on region. In Alberta, their exact life cycle is not fully known as they are relatively newly established (Soroka et al. 2020).

The current status of *M. aethiopoides* in Alberta is unknown; however, over 54,800 individuals were released in the USA on the prairie borders (Bryan et al. 1993). *Microctonus aethiopoides* was first introduced into North America in 1948 to be used as a biocontrol of the sweet clover weevil, *Sitona cylindricollis* Faehraeus (Coleoptera: Curculionidae) (Coles and Puttler 1963). It was released in sweet clover fields in Nebraska, North Dakota, Minnesota, Washington and New Jersey from 1948-1958. In 1958 alfalfa weevil was found to also be an excellent host for this wasp. Later during parasitoid recovery projects, larvae were found within adult alfalfa weevils, but there were no tools to distinguish if it was *M. aethiopoides* or a different *Microctonus* sp.. Recently, Rand et al. (2019) assessed the rate of parasitism of *M. aethiopoides* in the Great Plains of the USA, but they were not able to collect any samples that contained this parasitoid, indicating that it may no longer be persistent in this area.

Microctonus aethiopoides parasitizes adult alfalfa weevil. The adult wasp is 2.7 mm long with a reddish head and legs and mostly black body (Loan 1975). There are two generations per year with overwintering occurring within its host as a first instar larva (Brunson and Coles 1968, Radcliffe and Flanders 1998). As they come out of overwintering, the larva develops quickly and emerges out the posterior end of the weevil. The larva spins a white cocoon in leaf litter (Coles and Puttler 1963, Dysart and Day 1976). The adults emerge 2-3 weeks after pupation and parasitize other adult weevils. The second generation does not go into diapause, instead they emerge from their adult hosts. This occurs at the same time that the new generation of adult weevils start to emerge. The second *M. aethiopoides* generation lay one egg within the new adult weevils; these eggs then hatch and the first instar larvae enter diapause and will overwinter within the adult weevils. Parasitized weevil females and most males are rendered sterile by this parasitoid, reducing weevil populations (Drea 1968).

To date, biocontrol of alfalfa weevil has not been as successful in western Canada as compared to eastern Canada. This may be due to a number of reasons, most prevalent is that western Canada has a much harsher climate than eastern Canada, decreasing the ease at which new parasitoids may establish (Radcliffe and Flanders 1998, Soroka and Otani 2011). Even though these parasitoids do not completely control the weevil in Alberta, they can still be

beneficial for alfalfa growers as parasitized larvae consume less plant matter and are killed before reproducing which reduces the population size (Duodu and Davis 1974).

1.4 Molecular detection of parasitoids

Assessment of parasitism levels has been conducted for as long as insects have been released in attempts to provide control of chronic pests. While some of the methods used to assess parasitism levels have remained constant, novel methods have been added over the past 100 years (Radcliffe and Flanders 1998). Rearing of hosts and parasitoids are often used to assess parasitism levels. In the early 1900s, alfalfa weevil parasitism levels were assessed by gathering insects or cocoons (Chamberlin 1926). A number of larvae were collected from the field and reared in cages to compare the number of *B. curculionis* cocoons to weevil larvae placed in the cage (Ashfaq et al. 2004). Other methods included collecting cocoons from the alfalfa stubble after the first crop was cut and the number of *B. curculionis* and weevil cocoons were compared to determine parasitism rates. Dissection of larvae was also used, but problems arose when determining what size of alfalfa weevil larvae were best to dissect, and *B. curculionis* larvae are very difficult to identify. Even with these issues, rearing of hosts and parasitoids to determine parasitism rates is still a commonly used technique to this day (Chamberlin 1926, (Ashfaq et al. 2004).

Currently, molecular techniques are being used to assess parasitism levels, as being able to properly identify insects is critical in the employment of biological control (Radcliffe and Flanders 1998). The introduction of molecular techniques to better estimate the level of parasitism and correctly identify immature parasitoids to species has become a valuable tool (Tilmon et al. 2000, Ashfaq et al. 2004). Distinguishing morphologically between immature parasitoids species in some cases is not possible as shown for parasitoids of *Lygus* and *Adelphocoris* (Loan and Shaw 1987). Loan and Shaw (1987) suggested that parasitized larvae should be collected later in the season due to risk of deaths caused by human rearing mishaps, and that the parasitoid larvae did not have the specific characteristics needed to distinguish them to genus or species. Being able to tell if a larva is parasitized is important for growers, but it is also important to note what species of parasitoid is involved (Mueller 2008). The use of molecular techniques that can correctly identify the species based on DNA found within a sample is very useful (Ashfaq et al. 2004). Ashfaq et al. (2004) collected *Lygus* nymphs from

alfalfa fields from Saskatchewan and compared different parasitism assessment techniques. They collected nymphs and separated them for three different assessment trials. The first trial involved rearing hosts until the parasitoid larva dropped to the bottom and spun a cocoon or until all the nymphs had died or become adults. The second trial involved dissection of nymphs and examination under the microscope to look for parasitoid larvae, when potential parasitoid larvae were found they were collected and frozen for DNA examination to confirm the species. The third trial involved freezing nymphs followed by DNA extraction. The DNA was then added into a PCR mixture with primers made for the nymphs as well as the parasitoids that they wish to detect. Comparing the parasitism rates of the three different methods showed many different parasitism rates for the same location and time that the nymphs were collected. Rearing the *Lygus* nymphs resulted in the lowest parasitism levels (29%), followed by host dissection (60%), and then the PCR estimates (78%). Currently, a multiplex PCR method including primers to identify alfalfa weevil and its two main parasitoids, *B. curculionis* and *O. incertus,* in western Canada (Appendix I) is being developed and will be explained and tested further in Chapter 2 of this thesis.

1.5 Objectives

1) Assess the insect diversity of key groups of pests and predators of seed alfalfa fields during three important crop stages: bud, flower and seed. We hypothesize that there will be an increase in diversity as the season progresses, and this will be shown in differences between the crop stages, but there also may be differences based on grower's insecticide use patterns.
2) Determine parasitism levels in alfalfa weevil population in southern Alberta. This assessment will investigate both spatial and temporal distributions of *B. curculionis* and *O. incertus.* We hypothesize that with the data collected we will have more information as to when the parasitoids are active and how they may interact with each other. This will allow growers to better time pesticide usages to avoid impacting the parasitoids populations.
3) Compare how parasitism rates detected via the multiplex PCR tool compare to the live rearing. We hypothesize that the PCR rates will be higher due to casualties while dealing with live insects.

Chapter 2

2. Monitoring seasonal diversity of insect pests and their natural enemies in alfalfa seed production fields in southern Alberta

2.1 Introduction

Insect biodiversity is important to ecosystem function and agricultural production (Altieri 1991, Cock et al. 2012, Yang and Gratton 2014). Within agriculture fields insects can cause crop damage (Oerke 2006), but also provide key ecosystem services including pest control, pollination, and nutrient cycling (Altieri et al. 1997, Losey and Vaughn 2006, Gillespie et al. 2018, Schowalter et al. 2018). To understand the importance of insect biodiversity and the roles of specific species within agricultural fields, foundational studies are needed. This is especially relevant given global insect population declines (Hallmann et al. 2017, Leather 2017, Goulson 2019, Wagner et al. 2021) and the potential effects of climate change exasperating pest issues in agricultural fields (Cannon 1998, Estay et al. 2008, Tonnang et al. 2022).

The majority of Canadian field crop production occurs in the western Canadian Prairie provinces of Alberta, Saskatchewan, and Manitoba. Along with major field crops including wheat (*Triticum aestivum* L.) and canola (*Brassica napus* and *B. rapa* L.), the Prairie provinces also produce a large amount of forage seed (Statistics Canada 2022). Alfalfa (*Medicago sativa* L.) dominates seed production across Canada (Statistics Canada 2022) as it is one of the most important forage crops in temperate regions of the world, grown for use as animal forage and its nitrogen fixation qualities (Soroka and Otani 2011, Edde 2021). Many insects are found within alfalfa seed production fields (Hobbs et al. 1959, Harper 1988, Soroka and Otani 2011, Uddin 2005, Soroka et al. 2020), but a recent comprehensive study on the seasonal variation, community and diversity of pest insects and their natural enemies is lacking in western Canada.

Over 450 arthropod (mainly insect) species were previously identified on alfalfa in Alberta (Harper 1988). These species included three major guilds (i.e., herbivores, predators, and parasitoids), with a guild representing a group of organisms that use the same resources in a similar way in a specified location (Root 1967). Of those species identified in the 1980's (Harper 1988), several continue to be pests to this date. Soroka and Otani (2011) considered the major pests of alfalfa in western Canada to be: alfalfa weevil (*Hypera postica* (Gyllenhal), Coleoptera:

Curculionidae); pea aphid (*Acyrthosiphon pisum* (Harris), Homoptera: Aphididae); lygus bugs (*Lygus* spp.,: Miridae); alfalfa plant bug (*Adelphocoris lineolatus* (Goeze), Hemiptera: Miridae); alfalfa seed chalcid (*Bruchophagus roddi* (Gussakovsky), Hymenoptera: Eurytomidae); and alfalfa blotch leafminer (*Agromyza frontella* (Rondani), Diptera: Agromyzidae). In addition to pests, generalist predators including ground beetles (Coleoptera: Carabidae), damsel bugs (Hemiptera: Nabidae), big-eyed bugs (Hemiptera: Geocoridae), lacewings (Neuroptera: Chrysopidae), lady-bird beetles (Coleoptera: Coccinellidae), and spiders (Araneae) have been found across the Prairies in alfalfa fields (Harper 1988, Uddin 2005), but the impact of each predator is not known. The primary parasitoids observed in alfalfa fields are those of alfalfa weevil, *Bathyplectes curculionis* (Thomson) (Hymenoptera: Ichneumonidae) and *Oomyzus incertus* Ratzberg (Hymenoptera: Eulophidae) (Soroka and Otani 2011, Soroka et al. 2020), as well as those associated with lygus bugs, *Peristenus* spp. (Hymenoptera: Braconidae) (Goulet and Mason 2006, Soroka and Otani 2011). With the large number of insects found within alfalfa seed production fields in western Canada and the importance of the crop, more information is needed to enhance integrated pest management (IPM) practices. In particular, more information to understand insect interactions, mainly those between pests and natural enemies (i.e., predators and parasitoids).

Here, insect biodiversity in alfalfa seed production fields in southern Alberta was assessed to increase knowledge of the temporal abundance and diversity of key pests and natural enemies. Species diversity and richness was examined at three important crop stages, bud, flower, and seed, as these stages represent important time periods for crop management and production. Specific analyses were conducted on alfalfa weevil and its parasitoids, *B. curculionis* and *O. incertus*, as it is considered a major pest in seed production fields in southern Alberta and Saskatchewan.

2.2 Materials and Methods

2.2.1 Field Sites and Insect Sampling

The study was conducted in irrigated alfalfa seed production fields in southern Alberta in 2020 and 2021 (Figure 2.1). Alfalfa fields in their second year of seed production (or older) were sampled (2020: n = 8, 2021: n = 10). Growers managed their fields according to standard

agronomic practices, including insecticide application (when necessary). Insecticide application data was requested, but only a partial data set was obtained (Table 2.1).

Fields were sampled at three different crop stages during the growing season: (1) bud stage (stage 4); (2) start of flowering (stage 6); (3) and full seed production (stage 8) (Mueller and Teuber 2007) (Table 2.1). In each field, insects were sampled using a standard 38.1 cm diameter sweep net, targeting arthropods throughout the canopy. The sweeps followed a standardized transect-based method (Figure 2.2) four sets of 25 180°-sweep samples were collected (total = 100). For consistency, one person swept all the fields each year. Fields were sampled between 0800-1845 h when temperatures were above 15°C. All samples from each field were placed directly into a plastic resealable bag and placed in an insulated container on ice for transport to the laboratory where they were stored at -20°C prior to identification.

2.2.2 Sample Identification

Insects were counted and identified to order. In addition, insect pests, natural enemies, and alfalfa weevil parasitoids were identified to species (Table 2.2). The pest guild included most general and specialist pests of alfalfa. Aphids were found in most samples but were not quantified (Table 2.2). The natural enemy guild included several predators and parasitoids of alfalfa plant pests (Table 2.2). Specimens were identified to orders and families using keys in Marshall (2017). Ladybird beetles were identified to species with a key for Saskatchewan (Larson 2013). *Bathyplectes curculionis* was identified using the key published in Soroka et al. (2020). *Oomyzus incertus* was identified based on morphological comparisons to specimens provided by Dr. Julie Soroka (Agriculture and Agri-Food Canada).

2.2.3 Data Analysis

All analyses were conducted in *R* 3.6.3 (R Core Team, 2020). To investigate species diversity of the pest and natural enemy guilds between crop stages, species richness (*S*) and the Hill-Simpson diversity index (D_{Hill}) (*sensu* Roswell et al. 2021) were calculated for each field. Species richness was calculated as the number of unique species or morphospecies found per field per crop stage. The Hill-Simpson diversity index was calculated with the package *vegan* v.2.5.7 (Oksanen et al. 2020). Kruskal-Wallis rank sum tests followed by post-hoc Dunn's multiple comparison tests (Dunn 1964) (p-values adjusted with the Benjamini-Hochberg method)

were used to assess differences in species richness and diversity between crop stages by year, these were computed with *FSA* (Ogle, D.H. 2022). To further assess species diversity, rarefaction curves and extrapolation analyses were conducted using the package *iNEXT* v.2.0.20 (Hsieh et al. 2020, Chao et al. 2014). Species data was collected from both guilds and combined within each year; the numbers were then standardized to individuals per 100 sweeps. Rarefaction curves, a plot of the number of species against the number of individuals in the sample, were used to determine if a sampling was comprehensive to approximate the population it was taken from. Finally, to determine if natural enemy diversity was correlated with pest diversity at each crop stage across years, Kendall's Tau correlations were computed within the base *R* software (R Core Team, 2020).

Further analyses were conducted specifically on alfalfa weevil as it is a major pest in southern Alberta. To compare alfalfa weevil larval, *B. curculionis*, and *O. incertus* abundance by year between crop stages, Kruskal-Wallis rank sum tests were used followed by post-hoc Dunn's multiple comparison tests (Dunn 1964) (p-values adjusted with the Benjamini-Hochberg method) computed with *FSA* (Ogle 2022). In addition, to determine if alfalfa weevil larval abundance was correlated with parasitoid abundance (*B. curculionis* and *O. incertus* combined) across crop stages by year, Kendall's Tau correlations were computed within the base *R* software (R Core Team 2020). Finally, to investigate potential insecticide impacts, alfalfa weevil and *Lygus* spp. adults and larvae/nymphs population were compared between fields that had been sprayed and unsprayed during the bud collection in 2021. Kruskal-Wallis rank sum tests were used to assess differences between the sprayed and unsprayed fields for each insects and life stage, this was assessed only on fields that insecticide data was obtained. Data files were manipulated using the package *readxl* (Wickham and Bryan 2019) and *dplyr* (Wickham et al. 2020). All figures were produced using the packages *ggplot2* (Wickham 2016) and *ggpubr* (Kassambara 2020).

2.3 Results

2.3.1 Overview of the insect community

Over the two years of this study, there were five insect orders identified in the study region. Seven species were identified from the pest guild with alfalfa weevil larvae and alfalfa plant bugs dominant (Table 2.2). More varying levels of natural enemies were found across crop

stages and years (Table 2.2). Spiders, minute pirate bugs and ladybird beetles were the dominant predators found across the different crop stages sampled (Table 2.2). The two alfalfa weevil parasitoids, *B. curculionis* and *O. incertus* were found in low numbers across all crop stages sampled (Table 2.2).

2.3.2 Species diversity and richness

The diversity of alfalfa pests and their natural enemies varied across crop stage and years (Figure 2.3). In both years, there were significant difference in pest diversity between crop stage (2020: K-W = 12.005, df = 2, p = 0.0025; 2021: K-W = 13.38, df = 2, p = 0.0012). In 2020, there was no significant difference in the diversity of pests at the bud and seed stage, but both were significantly higher compared to the flower stage (Figure 2.3.A). In 2021, pest diversity increased with crop stage, but there was only a significant difference between the bud and seed stage (Figure 2.3.B).

In 2020, crop stage had no effect on natural enemy diversity (K-W = 6.125, df = 2, p = 0.047, adjusted p > 0.05, when assessing stage differences), although the seed stage had marginally lower diversity compared to the bud and flower stages (Figure 2.3.C). In 2021, crop stage had a significant effect on natural enemy diversity (K-W = 9.31, df = 2, p = 0.01). The bud stage had significantly lower natural enemy diversity compared to both the flower and seed stage (Figure 2.3.D), but there was no significant difference between the flower and seed stage. Overall, there were no significant correlations in diversity indices between the pest and natural enemy guilds across the three crop stages (2020: T = -0.12, p = 0.45; 2021: T = 0.24, p = 0.06). When looking at correlations between abundance between the two guilds there was no statistical correlation in 2020 (T = 0.124, p = 0.901) but there was a positive correlation in 2021 (T = 3.961, p = 0.000545).

In 2020, there were 774, 1,774, and 451 individuals collected and 16, 14, and 17 different species found per 100 sweeps at the bud, flower, and seed stages, respectively. When separating the two guilds, pest species richness was higher at the bud and seed stages than the flower stage (Figure 2.4.A) (pests: K-W = 12.329, df = 2, p = 0.0021). For natural enemies, there was higher species richness at the seed stage than the other two stages (Figure 2.4.B) (natural enemies: K-W = 8.65, df = 2, p = 0.0132). In 2021, there were more individuals collected per 100 sweeps (822,

615, and 823) and species (16, 20, and 21) at the bud, flower, and seed stage, respectively, than found in 2020. When separating the two guilds there were significant differences between stages (pests: K-W = 10.39, df = 2, p = 0.005; natural enemies: K-W = 10.94, df = 2, p = 0.004). For pest species richness, the seed stage had significantly more species than the flower stage, but not the bud stage. There were no significant differences between the bud and flower stages (Figure 2.4.C). For predators there was significantly higher species richness in the seed and flower stage than in the bud stage (Figure 2.4.D).

The rarefaction curves for all crop stages in 2020 and 2021 approach the horizontal asymptote (Figure 2.5) which suggests that more sampling may collect more species, but the current sampling collected many of the species within each guild.

2.3.3 Abundance of alfalfa weevil larvae and parasitoids natural enemies

Alfalfa weevil larval abundance varied significantly across crop stages sampled between the two years of this study (2020: K-W = 15.25, df = 2, p = 0.0005; 2021: K-W = 16.331, df = 2, p = 0.0003). In 2020, there was no significant difference in the number of larvae found at the bud and flower stage, but both were significantly higher compared to the seed stage (Figure 2.6.A). The flower stage had an average of 15.41 ± 6.58 (SE) larvae/sweep compared to 3.12 ± 1.28 (SE) and 0.19 ± 0.097 (SE)/sweep for the bud and seed stage, respectively. In 2021, the only significant difference in number of larvae found was between seed and the other two stages (Figure 2.6.D), with bud having an average of 3.01 ± 1.23 (SE) larvae/sweep, compared to 0.715 ± 0.32 (SE) and 0.046 ± 0.023 (SE) /sweep at the flower and seed stage, respectively.

Over the two years of this study, the two alfalfa weevil parasitoids were found in alfalfa fields at varying levels (Figure 2.6.B, C, E, F). In 2020, the abundance of *B. curculionis* and *O. incertus* varied significantly with crop stage (*B. curculionis*: K-W = 16.965, df = 2, p =0.000207; *O. incertus*: K-W = 7.389, df = 2, p = 0.025). There was no difference in the abundance of *B. curculionis* at the bud and flower stage, but both were significantly higher than the seed stage (Figure 2.6.B). There was significantly higher abundance of *O. incertus* in 2020 at the bud stage compared to the flower, but not the seed stage (Figure 2.6.C). The highest average abundance of *B. curculionis* and *O. incertus* occurred during the bud stage at 0.055 ± 0.026 (SE) and 0.045 ± 0.02 (SE) adults/sweep, respectfully. In 2021, there was no significant difference in abundance

of *B. curculionis* between stages (K-W = 4.614, df = 2, p = 0.09954), whereas there was a statistical difference on *O. incertus* abundance between crop (K-W = 10.996, df = 2, p = 0.0041). Abundance of *O. incertus* was significantly higher at the flower stage compared to both other stages (Figure 2.6.F). Average abundance was highest during the flower stage at 0.23 ± 0.11 (SE) adults/sweep. As with alfalfa weevil larvae, the abundance of parasitoids was quite variable (Figure 2.6.B, C, E, F).

There was not a significant correlation between abundance of alfalfa weevil larvae and their parasitoids during any crop stage in either year (2020: T = 0.28, p = 0.4; 2021: T = 0.26, p = 0.33).

2.3.4 Insecticide application impacts

In 2020, it is unclear if fields were sampled before or after the first set of insecticide applications as the collection dates overlapped with application dates. In 2021, the bud collection occurred after some fields were sprayed and some were not (Table 2.1). Three fields were sprayed 2-5 days before collection and four had not been sprayed. The number of alfalfa weevil and *Lygus* spp., adults and larvae/nymphs between the sprayed and unsprayed fields did not differ statistically significantly (p > 0.05) for either insect at the two life stages. However, there was a large, but non-significant difference in the number of *Lygus* spp. Nymphs (unsprayed: 156.8 ± 85.1 SE; sprayed: 4.7 ± 4.2 SE) as compared to the alfalfa weevil larvae (unsprayed: 611.8 ± 315.7 SE; sprayed 692.7 ± 114.7 SE) (Figure 2.7) between unsprayed and sprayed fields. The adults stage counts for both insects were quite small and were similar between the two field types.

2.4 Discussion

Characterizing diversity of insect pests and their natural enemies can provide important foundational knowledge on insect biodiversity and ecosystem services. Here, insects from five orders were found across southern Alberta alfalfa seed production fields over three distinct crop stages. Crop growth and development is often correlated with temporal colonization of specific insects (Helden 2010).

Overall, the diversity of alfalfa pests and their natural enemies varied over time, with no consistent trends between years (Figure 2.3). The overall diversity was higher in 2020, but there were more individuals and species found in 2021. This could be due to dominance issues, as there were more singleton species in 2021. Insect diversity of pests and natural enemies in seed alfalfa fields during the bud, flower and seed was quite variable; diversity increased as the season progressed in some guilds and years but not all (Figure 2.3). There was a positive correlation in abundance between the two guilds for 2021 but not 2020.

The rarefaction curves, based on the insects that were identified (Table 2.2), showed the species richness approached the asymptote, suggesting the sampling methods used were adequate to collect most of the species within each guild (Figure 2.5).

There were large differences in diversity and richness trends found between the years, which may have been influenced by factors including temperature, collection date, and insecticide usage. Climate plays a large role in insect development and therefore can influence insect populations between years (Champlain and Butler 1967). During this study 2021 was much warmer than 2020. June and July of 2021 had averages of 23.7°C and 25.4°C and extremes of 35.0°C and 35.5°C, respectively (Government of Canada 2022). Whereas 2020 had averages of 19.0°C and 22.6°C and extremes of 25.8°C and 29.1°C. Increases in temperature can cause insects and plants to mature faster as they can accumulate more growing degree days in a shorter time. This was not found for the alfalfa plants as the flower stage was deemed to be at stage 6 according to the Mueller and Teuber Guide (2007), two weeks earlier in 2020 than in 2021 which was the warmer year. Collection dates for bud and seed stage were within 1-6 days between the two years, June 10-12th and June 16th, and August 11th and August 10th in 2020 and 2021, respectively (Table 2.1). The flower collection sampling period was almost two weeks apart, July 4-6th in 2020 and on July 21st in 2021. These differences in sample collection date could explain some of the differences in one year and not the other, as the bud and flower stage were much closer in time in 2020 than in 2021. Lastly insecticide reports could not be collected for all the fields and nor was the timing of the applications consistent (Table 2.1). There were more insecticides reported in 2020 than 2021, but the applications for both years may have occurred before or after bud collection, which would influence the abundance and diversity of

species found (Figure 2.7). Unfortunately, this factor was outside of our control and may have impacted the data collected.

A large range of insects are found in seed alfalfa fields in southern Alberta as documented in spatial surveys by Harper (1988) and in alfalfa forage fields Sim and Meers (2017). Temporal collections were conducted by Uddin (2005) in Manitoba, Canada. Uddin (2005) focused on the three main pests *Lygu*s spp., *Adelphocoris lineolatus* and *Acyrthosiphon pisum*, which were collected weekly to assess peak populations. In the current study, the highest levels of *Lygus* spp. in 2020 were found in the bud and seed stage, coinciding with peaks reported around mid-June and again in mid-August in Manitoba (Uddin 2005), whereas in 2021, *Lygus* spp. numbers increased continually throughout the season, without the decrease in the flower stage that was seen in 2020 (Table 2.2).

The highest levels of *A. lineolatus* were in the bud and seed stages in both years. Levels were lower in the seed stage in 2020 compared to the bud stage, whereas in 2021, the values were similar in the two stages. This is similar to Manitoba where peak numbers were found in mid-June to early-July and then again mid-August (Uddin 2005). As 2020 was a cooler year the *A. lineolatus* populations may not have hit their second peak when collections were taken.

Sim and Meers (2017) concluded that most pest species stayed under the economic thresholds (*Lygus* spp.; 2-3 adults or 3rd and 4th instars per 90° sweep*, A. lineolatus*: 5 nymphs of any stage per sweep (Government of Alberta 2011)) within the fields except for alfalfa weevil (20-25 3rd or 4th instars/90° sweep (Governemnt of Alberta 2011)). Similar results were also found in our study in which only alfalfa weevil was above economic thresholds. Note that fields in this and the Sim and Meers (2017) study were managed with insecticide applications as deemed necessary by the grower.

In the natural enemy guild, ladybird beetles had the largest number of species within a family (five native and one alien species) with the most common species being the alien species *Coccinella septempunctata* (Coleoptera: Coccinellidae) (Table 2.2). Harper (1988) found 22 different species, all native to North America, whereas Sim and Meers (2017) only found the alien species *C. septempunctata. Coccinella septempunctata* was introduced to the central and eastern regions of North America and migrated west where it became established in the Rocky

Mountains by 1990 (Rice 1992). As this ladybird beetle established it displaced native ladybird beetles potentially causing the shift in ladybird beetle species collected in alfalfa fields (Alyokhin and Sewell 2004). Ladybird beetle adults and larvae are very effective predators; Uddin (2005) found a negative relationship between the number of ladybird beetles and *Lygus* species.

The other main predators found in past reports were the minute pirate bug (*Orius insidiosus* (Say), Hemiptera: Anthocoridae), lacewings (*Chrysopidae* sp., Neuroptera) and damsel bugs (*Nabidae* sp., Hemiptera) (Uddin 2005, Sim and Meers 2017) which was consistent with our findings. Lacewings, ladybird beetles and damsel bugs tend to prefer insects like lygus, aphids and plant bug nymphs, but have been recorded feeding on alfalfa weevil larvae when these other pests are not as abundant (Ouayogode and Davis 1981). Spiders and earwigs tended to be more opportunistic feeders with preferences based on size of prey rather than species. No earwigs were collected in this study as they tend to be more active at night and seek cool places during the day when our collections occurred.

There were a few common trends found across years when examining parasitoid and alfalfa weevil larval abundance in both years. Most interesting was the presence of the alfalfa weevil larvae and parasitoid wasps during all three collection periods. Finding larvae throughout the growing season was not expected as alfalfa weevil is documented as having an univoltine lifecycle in Alberta with peak populations occurring in mid-June to late July, depending on the year (Soroka and Otani 2011, Soroka et al. 2020).

Bathyplectus curculionis is documented to have a partial second generation in some US states (Chamberlin 1926, Radcliffe and Flanders 1998). Soroka et al. (2020) documented only one generation per year on the Canadian prairies with peak flight times in mid-June, usually a week or two before the alfalfa weevil larval population peaks. Collecting adult wasps throughout the season suggests that *B. curculionis* populations in southern Alberta may differ and exhibit a partial second generation. *Oomyzus incertus* can have 3-4 generations per year in the US with peak abundance generally occurring in mid-summer, depending on region (Radcliffe and Flanders 1998). The exact number or timing of peak flight is unknown in Alberta, and they are still considered a recently established species in the prairies (Soroka et al. 2020). The varying number found per 100 sweeps could have been impacted by collection timing and insecticide

sprays, especially during the flower stage (Figure 2.6). Here, almost no wasps were collected in 2020, whereas it was the highest collection period in 2021 at 20 wasps per 100 sweeps.

Although *B. curculionis* and alfalfa weevil were found throughout the season, the peak populations align with those reported by Soroka et. al (2020). This suggests there could be more than one generation for these insects, or alternatively, there could also be prolonged emergence occurring which would cause different pockets of insects to be collected at different crop stages. Insect emergence can be impacted by climate change as warmer temperatures can increase insect development, as observed in some Lepidoptera species, which have changed from having one generation per year to having two or three (Altermatt 2010). Some insects experience disruptions during their dormant phase where diapause induction cues can become confused if paired with high temperatures. This could cause some insects to go into diapause or emerge at different times and can cause prolonged emergence, not a true second generation (Forrest 2016).

There was a slight correlation between larval abundance and total number of parasitoids during the bud stage in 2021. This is important to note as this stage is the most critical for growers, if they need to spray an insecticide it must be applied before the flower stage when pollinators are added to the fields. Rand (2013) found correlations between parasitism rates and larval densities in North Dakota and Montana, USA. They showed that the relationship between parasitism rates for *B. curculionis* and larval density changed from a positive relationship one year to a negative the next year. Given the differences in abundance and population trends across years and locations, there is a need to continue to investigate the life cycle of *B. curculionis* and *O. incertus* to fully assess their ability as biocontrol agents.

2.5 Tables and figures

Table 2.1 Insecticide usage and sweep net sample collection dates in alfalfa seed production fields for 2020 and 2021 in southern Alberta.

		Insecticide			
Year	Field	Name of Product	Active Ingredient	Date Applied	Control/Suppression[a]
2020[b]	Y	Matador	Lambda-cyhalothrin	June 10th	N/A
	Y	Coragen	Chlorantraniliprole	June 10th	S
	W	Decis	Deltamethrin	June 10-15th	C
		June 10-12th bud stage collection			
	V	Cygon	Dimethoate	June 18th	S
	U	Malathion	Malathion	June 18th	C
	T	Coragen	Chlorantraniliprole	June 19th	S
	W	Lorsban	Chlorpyrifos	June 20-25th	N/A
	S	Coragen	Chlorantraniliprole	June 22nd	S
	U	Voliam Xpress	Lambda-cyhalothrin, Chlorantraniliprole	June 22nd	N/A
	V	Lorsban	Chlorpyrifos	June 26th	N/A
	T	Decis	Deltamethrin	July 6th	C
		July 4-6th flower stage collection			
		August 10-11th seed stage collection			
2021[c]	E	Matador	Lambda-cyhalothrin	June 11th	N/A
	C	Assail	Acetamiprid	June 11th	N/A
	D	Corgen	Chlorantraniliprole	June 14th	S
	D	Beleaf	Flonicamid	June 14th	N/A
	E	Malathion	Malathion	June 14th	C
		June 16th bud stage collection			
	H	Corgen	Chlorantraniliprole	June 18th	S

G	Matador	Lambda-cyhalothrin	June 18th	N/A
B	Malathion	Malathion	June 18-26th	C
A	Corgen	Chlorantraniliprole	June 21st	S
A	Beleaf	Flonicamid	June 21st	N/A
A	Matador	Lambda-cyhalothrin	June 21st	N/A
		July 21st flower stage collection		
		August 11th seed stage collection		

[a] Control or suppression of alfalfa weevil larvae based on label

[b] Insecticide usage was reported for 6 of the 8 fields

[c] Insecticide usage was reported for 7 of the 10 fields

Table 2.2 Abundance of the major pest and natural enemy species collected in alfalfa seed production fields in southern Alberta, 2020-2021. Values are means of individuals per 100 sweeps.

Guild	Crop Stage	Order	Family	Species	2020 [c]		2021 [c]	
					Mean	SE	Mean	SE
Pest	Bud	Coleoptera	Curculionidae	*Hypera postica* (adults)	59.38	24.69	21.36	8.57
				Hypera postica (larvae)	312.22	128.45	366.10	129.87
		Hemiptera	Cicadellidae	*Empoasca* sp.	17.38	7.38	2.24	0.96
			Miridae	*Adelphocoris lineolatus*	58.91	25.31	75.36	24.20
				Lygus sp. (adults)	18.8	2.02	37	8.8
				Lygus sp. (nymphs)	59.1	16.1	60	35.2
				Other Miridae sp. [a]	0.88	0.40	0.96	0.38
			Rhopalidae	*Harmostes sp.*	3.00	1.22	0.70	0.24
		Thysanoptera [a]			35.38	16.04	28.56	10.68
	Flower	Coleoptera	Curculionidae	*Hypera postica* (adults)	4.06	1.87	33.40	11.72
				Hypera postica (larvae)	1541.13	658.78	68.40	24.41
		Hemiptera	Cicadellidae	*Empoasca* sp.	1.66	0.74	7.74	3.04
			Miridae	*Adelphocoris lineolatus*	2.91	0.89	14.49	5.82
				Lygus sp. (adults)	16.4	7.7	55.1	16.8
				Lygus sp. (nymphs)	14	9.9	120.5	39.8
				Other Miridae sp. [a]			0.70	0.24
			Rhopalidae	*Harmostes* sp.	0.66	0.38	1.16	0.41
		Thysanoptera [a]			45.50	18.46	723.36	341.18
	Seed	Coleoptera	Curculionidae	*Hypera postica* (adults)	73.78	31.39	6.74	2.71
				Hypera postica (larvae)	19.44	9.65	6.10	2.22
		Hemiptera	Cicadellidae	*Empoasca* sp.	26.97	14.16	27.40	10.98
			Miridae	*Adelphocoris lineolatus*	18.56	6.84	71.80	24.15
				Lygus sp. (adults)	30.9	12.8	155.1	41.5
				Lygus sp. (nymphs)	96.	34.1	196.3	37.5

				Other Miridae sp. [a]			5.56	2.43
			Rhopalidae	*Harmostes* sp.	2.56	1.08	2.34	0.99
		Thysanoptera [a]			36.50	20.09	161.20	59.69
Natural Enemies	Bud	Araneae [a]			27.13	15.54	4.80	1.85
		Coleoptera	Coccinellidae	*Coccinella septempunctata* [b]	4.97	2.34	5.60	2.22
				Hippodamia convergens	0.63	0.27	0.18	0.09
		Hemiptera	Miridae	*Orius insidiosus*	1.63	0.75	49.00	18.31
			Nabidae	*Nabis* sp.	3.00	1.25	1.56	0.61
		Hymenoptera	Eulophidae	*Oomyzus incertus*	4.47	2.00	2.50	1.26
			Ichneumonidae	*Bathyplectus curculionis*	5.47	2.56	1.24	0.42
		Neuroptera	Chrysopidae	*Chrysoperla carnea*	0.61	0.22	1.12	0.46
	Flower	Araneae*			11.25	4.86	16.00	5.62
		Coleoptera	Coccinellidae	*Coccinella septempunctata* [b]	12.63	6.53	2.50	1.00
				Coleomegilla maculata			0.18	0.09
				Hippodamia parenthesis	0.63	0.27	1.00	0.33
				Hippodamia convergens			1.16	0.45
				Hippodamia tredecimpunctata			0.18	0.09
				(larvae) [a]			7.94	3.18
		Hemiptera	Miridae	*Orius insidiosus*	3.44	1.81	137.60	45.67
			Nabidae	*Nabis* sp.	2.84	1.20	13.16	5.15
		Hymenoptera	Eulophidae	*Oomyzus incertus*			20.16	8.14
			Ichneumonidae	*Bathyplectus curculionis*	1.13	0.59	2.88	0.99
		Neuroptera	Chrysopidae	*Chrysoperla carnea*	0.81	0.33	11.45	4.41
	Seed	Araneae [a]			5.88	2.56	9.40	3.67
		Coleoptera	Coccinellidae	*Coccinella septempunctata* [b]	5.50	2.36	17.46	8.82
				Coccinella transversoguttata			0.48	0.19
				Hippodamia parenthesis	1.00	0.42	1.40	0.63
				Hippodamia convergens			4.86	1.80
				(larvae) [a]	68.31	34.26	41.92	18.29

Hemiptera	Miridae	*Orius insidiosus*	27.38	13.16	59.12	25.21
	Nabidae	*Nabis* sp.	6.38	3.51	18.60	6.29
Hymenoptera	Eulophidae	*Oomyzus incertus*	1.00	0.46	0.70	0.28
	Ichneumonidae	*Bathyplectus curculionis*	0.22	0.13	0.72	0.24
Neuroptera	Chrysopidae	*Chrysoperla carnea*	4.13	1.42	10.25	3.68

[a] Individuals were not identified to genus and species and were not used in the species richness assessments

[b] Alien ladybird beetle species

[c] In 2020 nine fields were sampled, in 2021 ten fields were sampled

SE = standard error

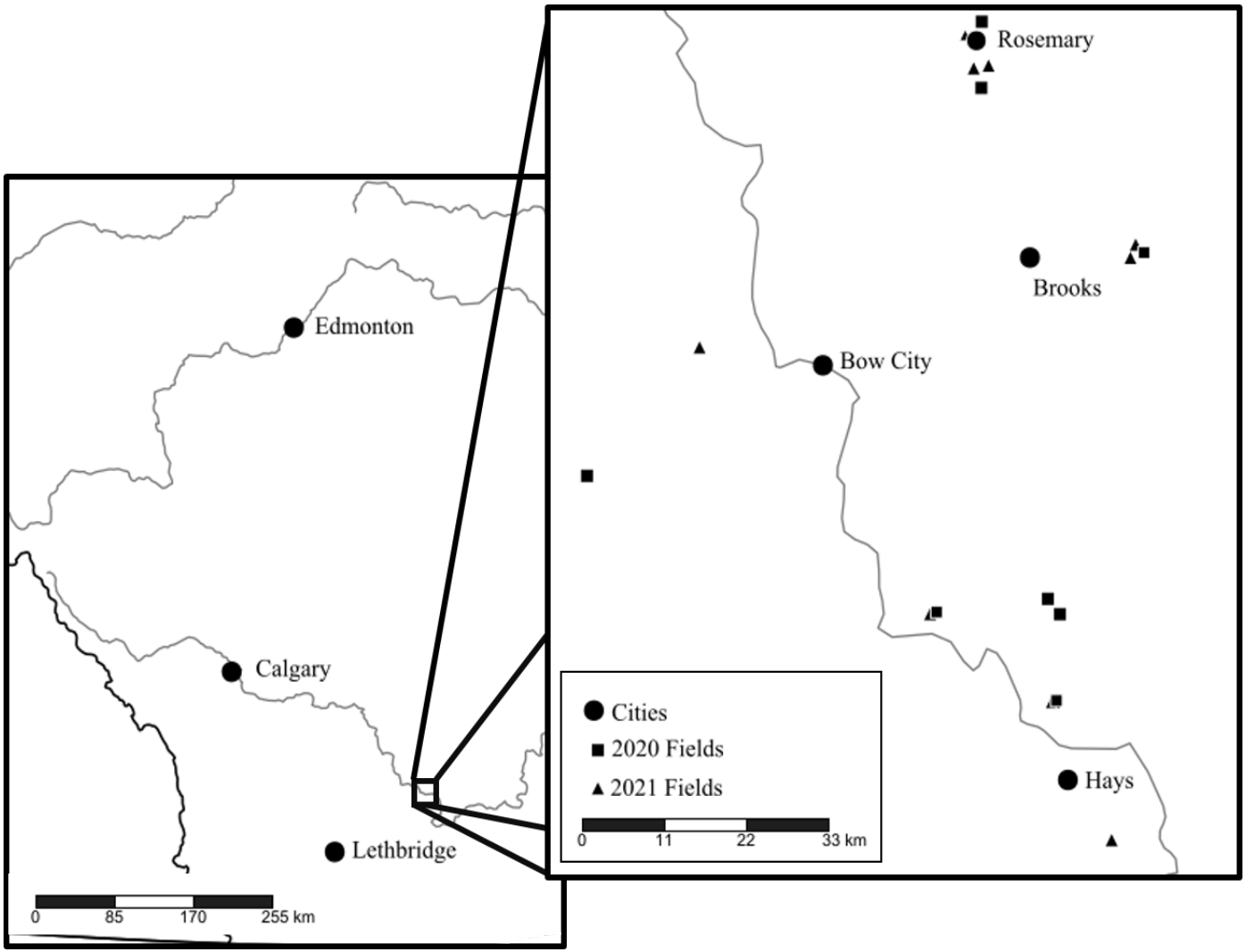

Fig. 2.1 Location of alfalfa seed production fields surveyed in 2020 and 2021 in southern Alberta. The grey line in the inset map represents the South Saskatchewan River.

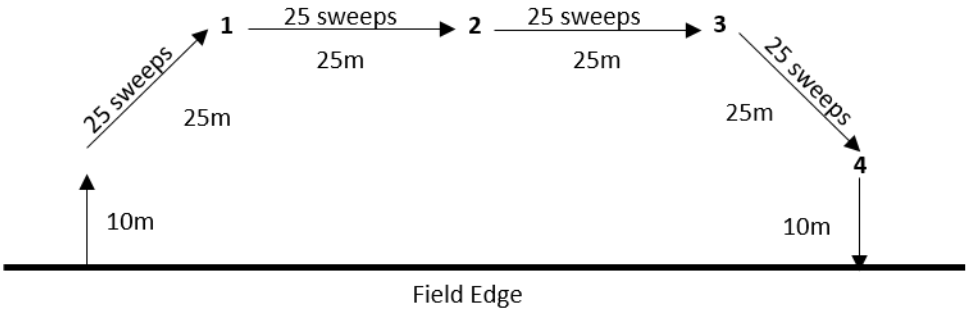

Fig. 2.2 Biodiversity sampling collection transect. Using a 38.1 cm diameter sweep net four sets of 25 180°-sweep samples were collected (total = 100). Each sample collection is represented with a bold number. All samples were combined together for each field

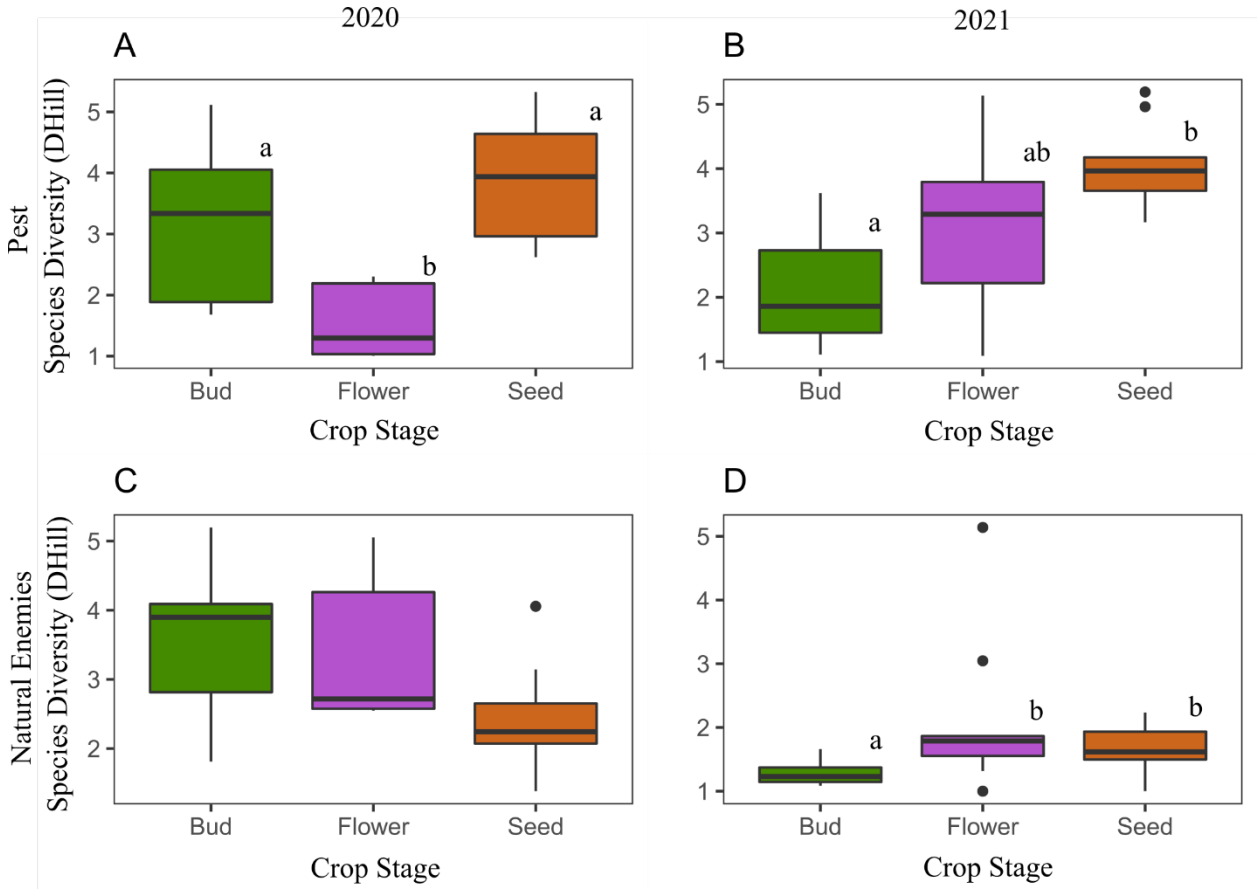

Fig. 2.3 Species Diversity (D_{Hill}) of the insect pest and natural enemy guilds at each crop stage sampled in southern Alberta, 2020-2021. The index was calculated for each alfalfa seed production field in southern Alberta and are per 100 sweeps. Stages with difference letters are significantly different [$p < 0.05$; Dunn test with p-values adjusted with the Benjamini-Hochberg method]. The columns separate years (A, B) 2020, (C, D) 2021. The rows separate guilds (A, C) pests (A: K-W = 12.005, df = 2, p = 0.0025; C: K-W = 13.38, df = 2, p = 0.0012), (B, D) predators (B: K-W = 6.125, df = 2, p = 0.047; D: K-W = 9.31, df = 2, p = 0.01).

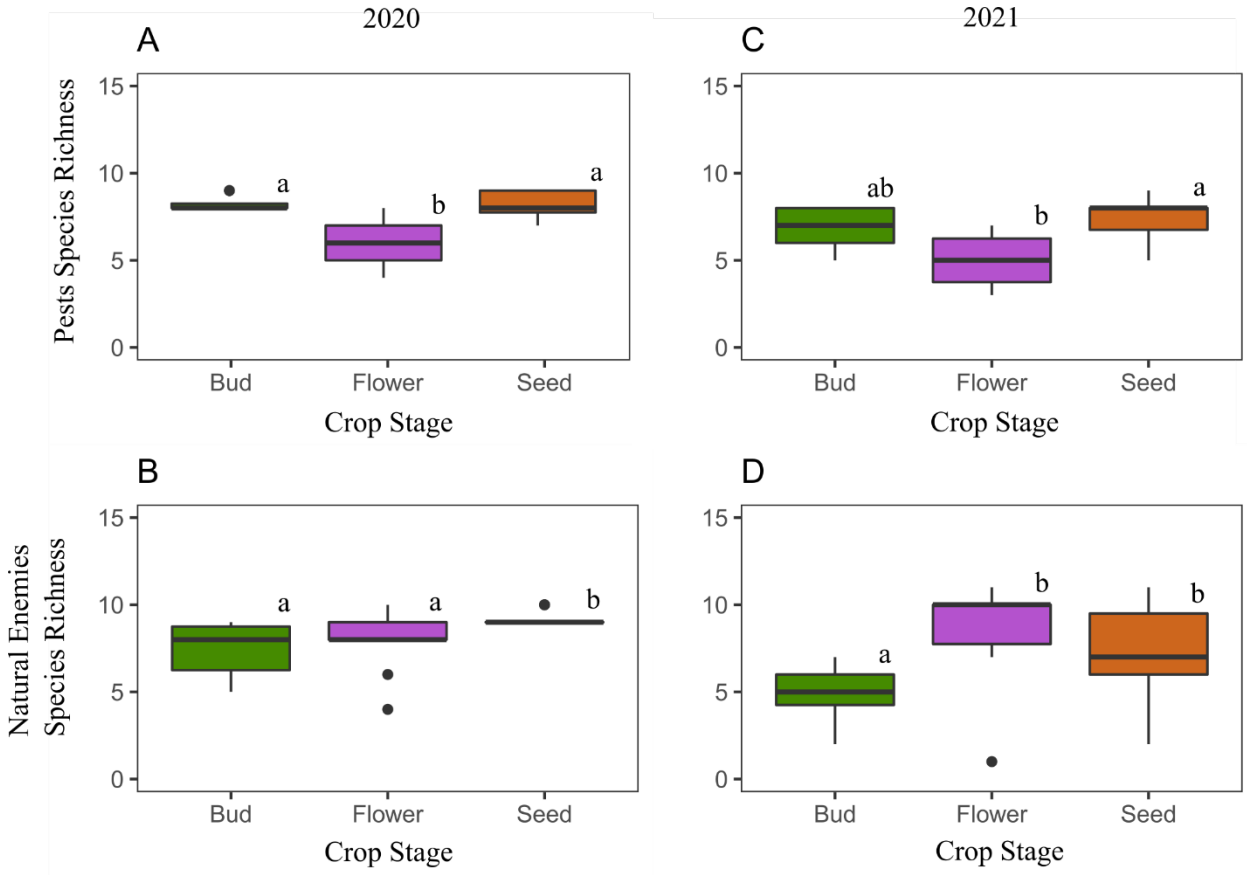

Fig. 2.4 Species richness of the insect pest and natural enemy guilds at each crop stage sampled in southern Alberta, 2020-2021. The value was calculated for each alfalfa seed production field in southern Alberta and are per 100 sweeps. Stages with difference letters are significantly different [$p < 0.05$; Dunn test with p-values adjusted with the Benjamini-Hochberg method]. The columns separate years (A, B) 2020, (C, D) 2021. The rows separate guilds (A, C) pests (A: K-W = 12.329, df = 2, p = 0.0021; C: K-W = 10.39, df = 2, p = 0.005), (B, D) predators (B: K-W = 8.65, df = 2, p = 0.0132; D: K-W = 10.94, df = 2, p = 0.004).

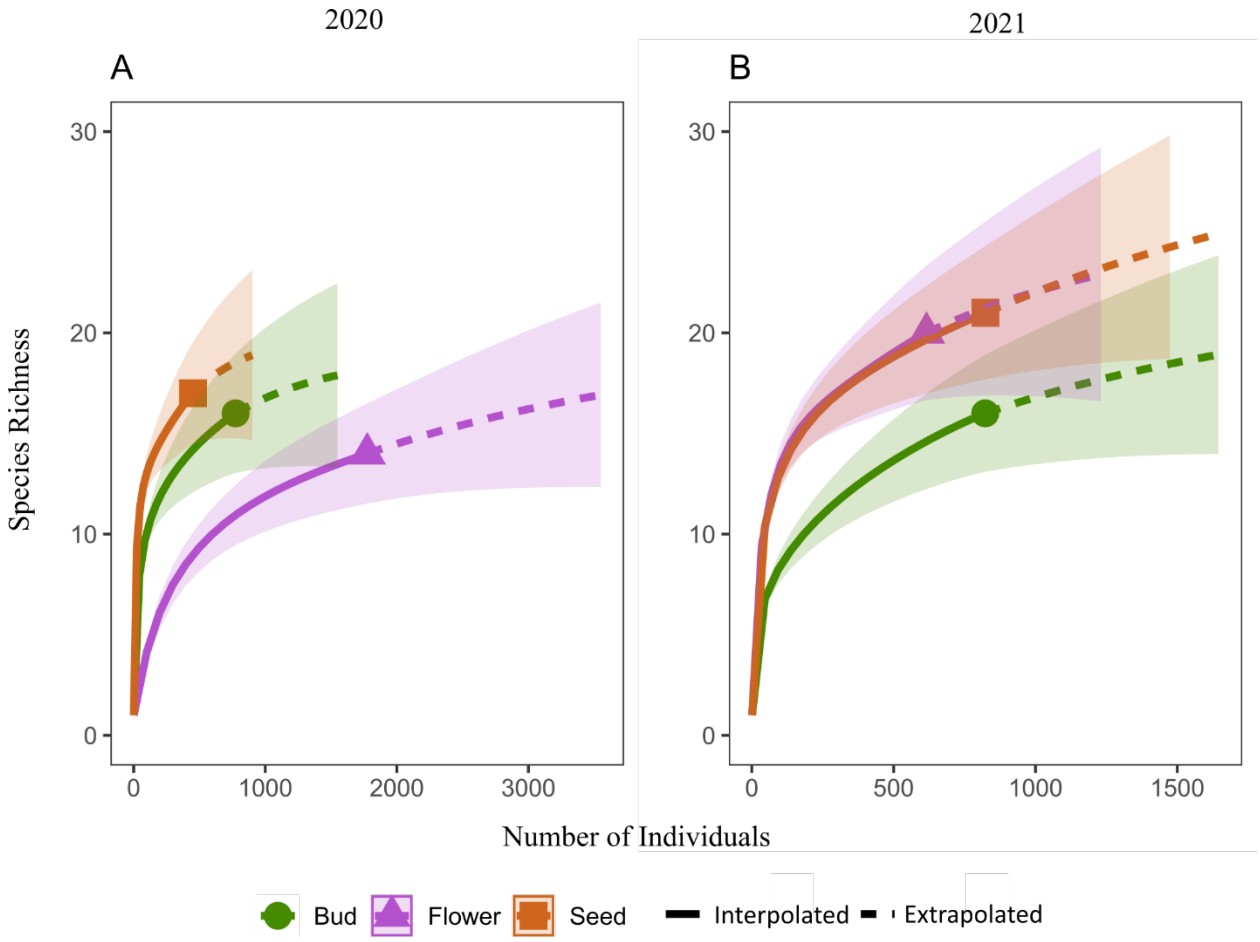

Fig. 2.5 Rarefaction curves of the combined species abundance data for each stage in alfalfa seed production fields in southern Alberta and are per 100 sweeps. The species abundance is calculated across both guilds. The two graphs are separated by years (A) 2020, (B) 2021. The shaded areas represent the 95% confidence interval.

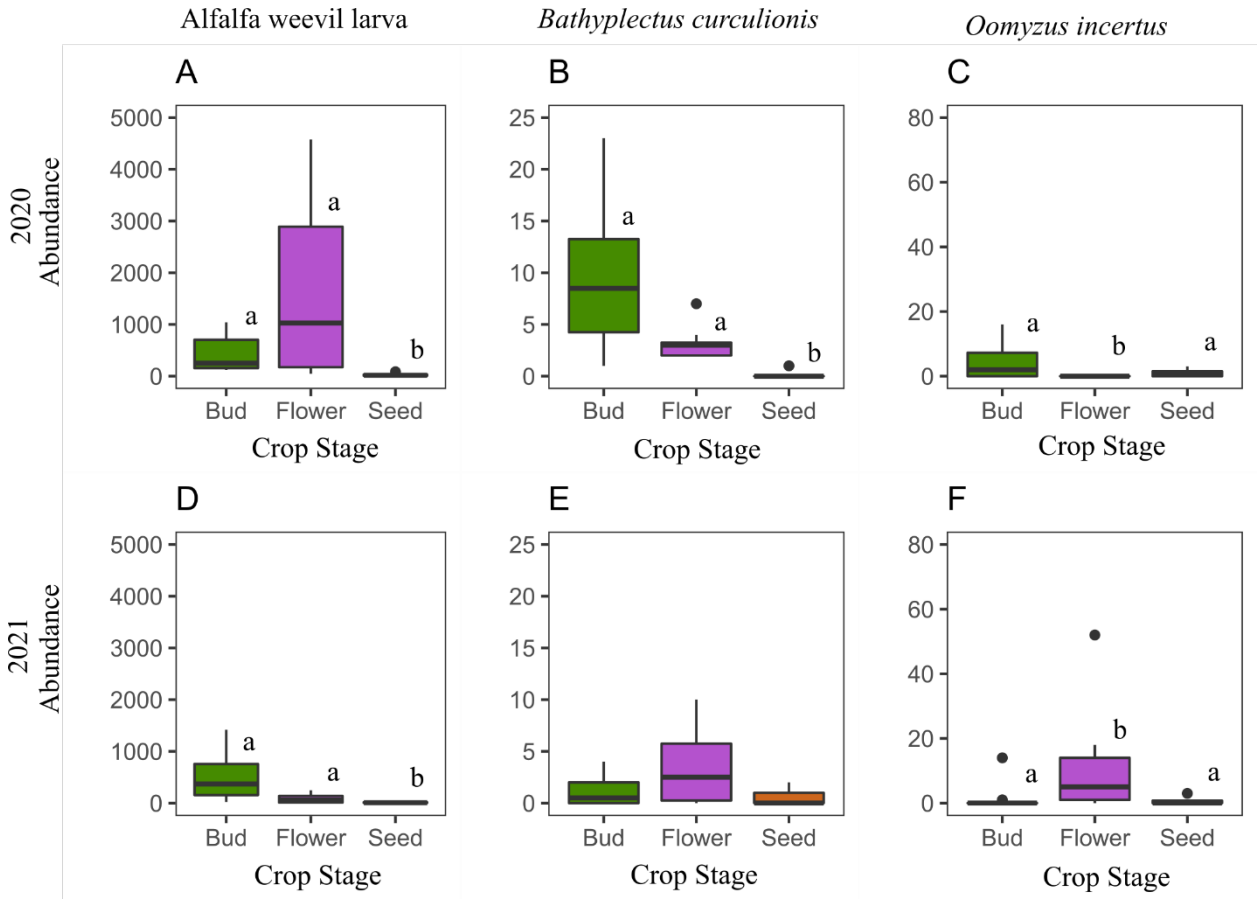

Fig. 2.6 Abundance of parasitoid wasps and alfalfa weevil larvae in 2020 (A, B, C) and 2021 (D, E, F) across crop stages. The counts are per 100 sweeps in alfalfa seed production fields in southern Alberta. Stages with different letters are significantly different [$p < 0.05$; Dunn test with p-values adjusted with the Benjamini-Hochberg method] (A: K-W = 15.25, df = 2, p = 0.000489; B: K-W = 16.97, df = 2, p = 0.000207; C: K-W = 7, df = 2, p = 0.0249; D: K-W = 16.33, df = 2, p = 0.000284; E: K-W = 4.6144, df = 2, p = 0.0995; F: K-W = 10.996, df = 2, p = 0.004095).

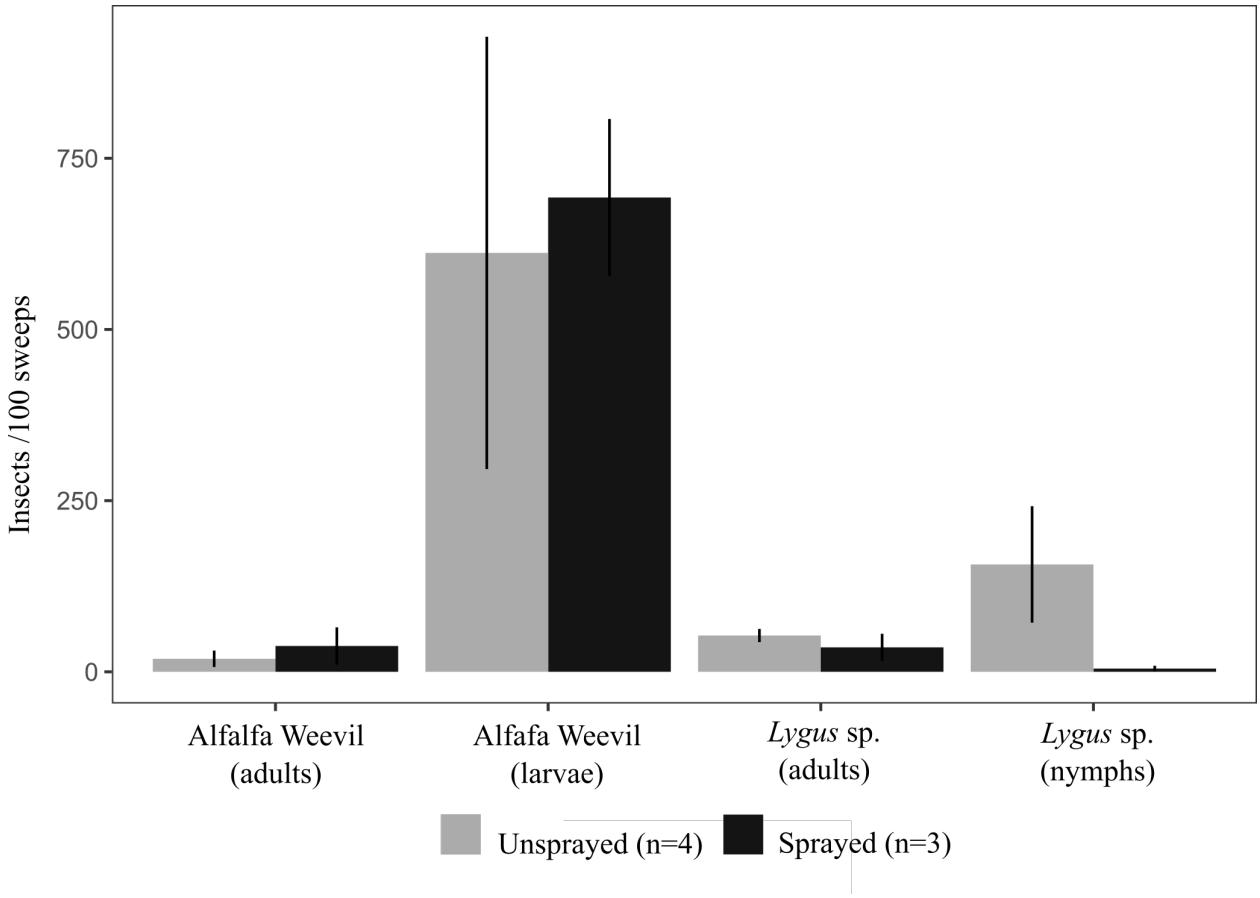

Fig. 2.7 Abundance of alfalfa weevil and *Lygus* spp. adults and larvae/nymphs in 2021 during the bud stage. The counts are per 100 sweeps in unsprayed (n = 4) and sprayed (n = 3) alfalfa seed production fields in southern Alberta. Error bars denote standard error.

Chapter 3

3. Assessing parasitism rates of alfalfa weevil larvae in southern Alberta alfalfa seed production fields

3.1 Introduction

Alfalfa (*Medicago sativa* L.) is the most valuable forage crop across the world because it is high in protein, low in fiber and is able to fix its own nitrogen and improve soil quality (Barnes et al. 1988, Bagavathiannan and Van Acker 2009). Alfalfa weevil, *Hypera postica* (Gyllenhal) (Coleoptera: Curculionidae), is an invasive insect found in most alfalfa fields in North America (Bundy et al. 2005, Soroka et al. 2020). Although both adults and larvae can damage alfalfa, most damage occurs by larvae feeding on developing alfalfa buds and expanding terminal foliage (Soroka and Otani 2010). In regions where there is no first cut of alfalfa prior to seed harvest, alfalfa weevil poses an increased issue as the stands are not harvested during the season and the weevils life cycle is not as disturbed as in forage alfalfa fields (Harper et al. 1990). Many different integrated pest management (IPM) strategies, including cultural, chemical, biological control, and plant resistance, have been used to mitigate weevil damage; however, ultimately chemical control is most often used (Mueller 2008, Dosdall et al. 2011, Pellissier et al. 2017, Alberta Crop Protection Guide 2022). Recent issues with insecticide resistance in North America (Glen 2015, Alexander 2016) necessitate exploration of other management techniques.

Since alfalfa weevil was first found in North America, various parasitoid wasps (Hymenoptera) have been released for classical biological control starting as early as 1911 with shipments to Utah (Chamberlin 1926). Several wasps can parasitize specific life stages of alfalfa weevil (Bryan et al. 1993). The parasitoids released either kill their host or leave them sterile and unable to reproduce. Various wasps do well in different regions based on the local climate and ecological communities. Generally, when three or more parasitoids have established in the region there is little economic damage caused by alfalfa weevil (Radcliffe and Flanders 1998).

In Alberta, three alfalfa weevil parasitoids have been found: *Bathyplectes curculionis* (Thomson) (Hymenoptera: Ichneumonidae), *Oomyzus incertus* (Ratzenberg) (Hymenoptera: Eulophidae) and *Microctonus aethiopoides* (Loan) (Hymenoptera: Braconidae) (synonym *M. aethiops* (Nees)) (Soroka and Otani 2011, Soroka et al. 2020). *Microctonus aethiopoides* is

suspected to be in Alberta as one adult was found during a survey in 2019 (Soroka et al. 2020), but during a survey in 2018 across the Northern Great Plains of the USA no adults were found (Rand et al. 2018), despite over 54,800 individuals being previously released (Bryan et al. 1993). It appears *M. aethiopoides* is not widely established, but small populations may be present in isolated locations.

Bathyplectes curculionis appears to be a very adaptive species as they are one of the only parasitoids to have established throughout North America (Radcliffe and Flanders 1998). Adult *B. curculionis* are 5-10 mm long with a mostly black body and yellowish/tan colouring on the underside of their abdomen (Soroka et al. 2020). In the spring adult wasps emerge from their overwintering cocoons and mate (Chamberlin 1926). The females then lay their eggs within the alfalfa weevil larva, which will hatch 14 days later. *Bathyplectes curculionis* larva develop within the weevil larva until it is ready to pupate, to do this, the *B. curculionis* larva will emerge, killing the weevil larva and cocoon itself. Most *B. curculionis* will stay in this cocoon until the next season, but in some regions of USA there is a partial second generation (Chamberlin 1926, Radcliffe and Flanders 1998). In Alberta, the peak flight occurs in mid-June and no second generation has been recorded (Soroka et al. 2020).

Oomyzus incertus (syn. *Tetrastichus incertus* Ratzburg (Hymenoptera: Eulophidae)) is smaller than *B. curculionis* at 1.2-1.4 mm in length and can be found in most alfalfa growing regions of North America (Chamberlin 1925, Streams and Fuester 1967). The adults are black with a blue/green sheen to them. Females lay multiple eggs within the weevil larvae, these eggs hatch within 2-3 days and will stay within the host even after pupation. The *O. incertus* pupae will enter diapause or will emerge as an adult, this parasitoid can have 3-4 generations per growing season (Radcliffe and Flanders 1998). In Alberta the life cycle is not fully known, but they have been collected throughout the season (Soroka et al. 2020).

Determining if alfalfa weevil larvae are parasitized is important for growers, as it may have implications for management of alfalfa weevil; however accurately assessing parasitism rates can be difficult. Live rearing requires access to adequate labour, rearing facilities, and host plant material for food. In addition, premature death may occur due to a variety of reasons and may skew estimated parasitism rates (Ashfaq et al. 2004). Dissection of host tissues can eliminate the need to rear insects, but it is difficult to properly identify the parasitoid species

(Loan and Shaw 1987), especially if multiple species may be present. Molecular tools offer an alternative to the traditional rearing or dissection techniques as they are developed for species specific identification, usually based on PCR with species-specific primers (Gariepy et al. 2007).

Here, parasitism levels of alfalfa weevil in several alfalfa seed production fields in southern Alberta were assessed across two growing seasons. We used a recently developed molecular tool based on a species-specific multiplex PCR to identify alfalfa weevil, *B. curculionis* and *O. incertus* (Appendix I). Results from the molecular tool were compared to live rearing of alfalfa weevil to improve the estimates of parasitism rates in southern Alberta. Ultimately, this study will provide growers more information as to when parasitoid wasps are active, and when most parasitism is taking place in southern Alberta.

3.2 Materials and methods

3.2.1 Weekly parasitoid and parasitism survey, 2020 and 2021

Alfalfa seed production fields (second year of production or later) were surveyed weekly from week 22 to week 25 in 2020 and until week 27 in 2021. Three and eight fields were surveyed in 2020 and 2021, respectively. These sampling periods coincided with late vegetative to the beginning of flowering crop stages in 2020 and late vegetative to late flowering in 2021. Fields were managed by individual growers according to standard agronomic practices. This includes insecticide applications, when necessary. Insecticide application dates were requested, but only a partial set was obtained (Table 3.1). In each field, 3 yellow sticky cards were attached to wooden stakes (2.54 x 5.08 x 121.92 cm) at crop canopy height 25 m apart along the field edge. Cards were collected and replaced weekly, and the height adjusted to the crop canopy. In the laboratory, yellow sticky cards were examined for *B. curculionus* and *O. incertus. Bathyplectes curculionis* was identified using the key in Soroka et al. (2020). *O. incertus* was identified based on a sample specimen provided by J. Soroka, and to further confirm identification, DNA was extracted from a subset of individuals and compared to confirm identification (methods below). In 2021, an addition of 100 180°-sweeps were taken with a standard 38.1 cm diameter sweep net to collect larvae at each field (Figure 3.1). One hundred larvae were taken from the sweep sample collected and placed in a refrigerated container for

transport to the laboratory. In the laboratory, larvae were frozen at -80°C prior to DNA extraction and parasitism rate determination.

3.2.2 Regional parasitism rate survey, 2020 and 2021

In 2020, during week 27, 8 fields were surveyed across the alfalfa seed producing region in southern Alberta (Figure 3.2). In each field, 100 180°-sweeps were collected with a standard 38.1 cm diameter sweep net. The contents of the sweep net were placed into a resealable plastic bag, and then placed in a refrigerated container for transport to the laboratory. In the laboratory, samples were then frozen at -20°C until DNA was extracted.

In 2021, during week 26, 11 fields were sampled (Figure 3.3). Two 100 180°-sweep samples were taken from 7 of the fields, and only one 100 sweep sample taken from 4 of the fields. The second sample from the 7 fields were used for the live rearing study (Section 3.2.2.1). The field samples were transferred from the sweep net into resealable plastic bags and transported in a refrigerated container to the laboratory. In the laboratory, the first 100 sweep sample was frozen at -20°C until DNA extractions occurred to assess the parasitism rate based on a multiplex PCR assay. The second 100 sweep sample was placed into a cage in a growth chamber (21°C, 70% relative humidity, 16:8 h light:dark) for at least 12 hours to acclimatize the larvae to the laboratory. These larvae were then transferred individually into cups to determine the parasitism rate based on live rearing.

3.2.3 Parasitism rate - live rearing, 2021 only

The live rearing protocol was based on Brewer et al. (1997) with some modifications. Clear lidded plastic cups (30 ml) (ULINE model no. S-20778, Edmonton, AB) were prepared with 12-14 small holes in the lids to increase ventilation to prevent moisture buildup and increase air flow. Two layers of slightly moistened paper towel were placed on the bottom of each cup, to which alfalfa foliage was then added. Larvae were collected as stated above (Section 3.2.2). From each field, 200 third and fourth instar larvae were placed individually into each cup and then into a growth chamber (21°C, 70% relative humidity, 16:8 h light:dark). The larvae were monitored every three days. Mortality was checked by assessing movement when prodded with a laboratory spatula and live larvae were provided fresh alfalfa unless the larva had started to cocoon. The status of the larva was then recorded. Sixteen days after the larvae were placed in

cups, a final assessment took place. Larvae were recorded as no parasitism: adult weevil, dead larva, pupae, half pupated, larval cocoon, or parasitized: *B. curculionis* cocoon, *B. curculionis* adult, or *O. incertus* adults. *Oomyzus incertus* cocoons were not counted as they form a cocoon within the alfalfa weevil cocoon (Streams and Fuester 1967) and no dissections were performed.

3.2.4 Parasitism testing - DNA extractions, 2020 and 2021

For samples collected for both the weekly and the regional survey, we used a multiplex PCR assay (Appendix I) to identify larvae parasitized by either *B. curculionis* or *O. incertus*. From each field, 20 larvae were subsampled from the 100 larvae collected. DNA was extracted from individual larvae using a DNeasy® Blood and Tissue kit (Qiagen, Mississauga, ON, CA) following the manufacturer's suggested protocol with a final elution of 25 µl. DNA concentration and quality was assessed with a NanoDrop 2000c spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA).

DNA extracts were then used in a multiplex PCR to determine the parasitism rates at each site (Appendix I). Each 25.5 µl reaction contained 9.3 µl ultrapure water, 2.5 µl 10x PCR buffer (200 mM Tris-HCl pH 8.4, 500 mM KCl), 2 µl $MgCl_2$ (50 mM), 1 µl BSA (1 mg/ml), 1 µl dNTP mix (10 mM), 5 µl of the forward primer (10 µM), 1 µl of each reverse primer (10 µM), 0.2 µL Taq DNA polymerase (5 U/µl), and 1.5 µl of template DNA. The forward primer was 5.8SF (TGTGAACTGCAGGACACATGAAC), and the reverse primers used were AW-R (ACCTGCTCTGAGGTCGAAAG) for alfalfa weevil, Bc-R (CGCAAACCATTCGGCGTTAT) for *B. curculionis*, and Oi-R (ATGCGTGTGCTCGTACTCTG) for *O. incertus*. Samples were run on a SimpliAmp Thermal Cycler (ThermoFisher Scientific, Waltham, MA, USA) with the following conditions: initial denaturation at 94°C for three minutes; 30 cycles of 94°C for 30 seconds, 58°C for 30 seconds, 72°C for one minute; and a final extension at 72°C for five minutes. The PCR products were electrophoresed on 1% TAE agarose gel containing Syber-Safe (ThermoFisher Scientifc, Waltham, MA, USA), at 90 V for 1 hour, each gel was run with positive controls, one for each species and a negative control, containing the PCR master mix without DNA. DNA products were visualized by ultraviolet transillumination with ChemiDoc™ Imagining System (Bio-Rad, Hercules, CA, USA) The gels were then scored for parasitism by presence of parasitoid DNA bands (Appendix II). The multiplex PCR assay was designed to produce an amplicon size of 465 bp for alfalfa weevil (*H. postica*), 685 bp for *B. curculionis* and

265 bp for *O. incertus*. Band checks were removed from the agarose gel with a sterile blade, these gels were cleaned with QIAquick® PCR & Gel Cleanup Kit (Qiagen, Mississauga, ON, CA) following the manufacturer's suggested protocol and sequenced at the Molecular Biology Service Unit at the University of Alberta on an ABI 3730 Genetic Analyzer following standard protocols (Appendix III).

3.2.5 Data analysis

Differences in parasitism were assessed between the two years. The weekly survey data was checked for normality visually with histograms, and q-q plots, and with the Shapiro-Wilk's test. As the data were not normally distributed, a Kruskal-Wallis rank sum test was used to determine if there were significant differences between years. A generalized linear mixed effects model (GLMM) with a binomial distribution was used to determine if parasitism level varied by week using the package *lme4* (Bates et al. 2015). The proportion of alfalfa weevil larvae parasitized by *B. curculionis*, *O. incertus* and total parasitism was the response variable with week as the explanatory variable and field as a random effect. Finally, parasitism rates were compared between the live rearing and the multiplex PCR using a Kruskal-Wallis rank sum test as the data did not meet all assumptions of normality.

All analyses were conducted in R 3.6.3 (R Core Team, 2020). Data files were manipulated using the R package *readxl* (Wickham, H. and Bryan, J. 2019) and *dplyr* (Wickham, H. et al. 2020). Figures were produced in R using the package *ggplot2* (Wickham, H. 2016) and *ggpubr* (Kassambara, A. 2020).

3.3 Results

3.3.1 Weekly survey

The number of adult parasitoids found on yellow sticky cards was extremely low across the weeks sampled and precluded further statistical analyses. *Bathyplectus curculionis* adults were collected each week in both years (averages in 2020: 1.36 ± 0.34 (SE)/sticky card, 2021: 0.71 ± 0.1.4 (SE)/ sticky card) (3.2). Fewer *O. incertus* adults, compared to *B. curculionis*, were found on yellow sticky cards in both years (2020: 0.2 ± 0.1 (SE)/ sticky card, 2021: 0.3 ± 0.2

(SE)/ sticky card). They were not found in all weeks during collections, but parasitism rates in 2021 indicate that they were present in the fields.

In 2021, sweep sampling to assess biodiversity of arthropods reported in Chapter 2, occurred during week 24 (June 16); the number of parasitoids found in the 100-sweep net sample averaged 1.24 ± 0.42 (SE) for *B. curculionis* and 2.50 ± 1.26 (SE) for *O. incertus* compared to the yellow sticky cards which where 0.97 ± 1.8 (SE) and 0.04 ± 0.4 (SE), respectively (Table 2.2).

In 2021, the rates of parasitism were quite variable over the period sampled with no significant differences between each week (*B. curculionis*: $\chi^2 = 0.002$, df = 1, $p > 0.05$; *O. incertus*: $\chi^2 = 0.01$, df = 1, $p > 0.05$; total parasitism: $\chi^2 = 0.001$, df = 1, $p > 0.05$) (Figure 3.4). The percent of larvae parasitized by *B. curculionis* ranged from 0 to 60 % (Figure 3.4.A), with the highest rate occurring during week 27 (July 6). Parasitism by *O. incertus* was more variable between fields and weeks, parasitism ranged from 0 to 70% (Figure 3.4.B). Larvae found with both parasitoids DNA were more uncommon and ranged from 0-25% (Figure 3.4.C). Overall parasitism ranged throughout the sample period from 0-90% with week 27 (July 6) being on average the highest parasitism rate at 65 ± 8 (SE) % (Figure 3.4.D). Even though there was no significant difference between weeks, visually the parasitism rates for *B. curculionis* were fairly flat across weeks 23-26 (June 3 – 29) with an increase in week 27 (July 7). Whereas with *O. incertus,* there could be two potential peak flight times, one at week 24 (June 16) and a second may have been building at week 27 (July 7).

3.3.2 Regional survey

The regional survey samples were taken during week 27 (July 4-6) in 2020 and one week earlier at week 26 (June 29) in 2021. Parasitism rates in the regional survey were lower in 2020 (Figure 3.5.A) compared to 2021, at averages of 6.9 ± 5.5 (SE) % compared to 48.6 ± 8.7 (SE) %, respectively ($\chi^2 = 10.04$, df = 1, $p = 0.002$). In 2020, only 3 of the 8 fields had parasitism, two at rates of 5% and 5.2% (only *B. curculionis* found*)* with the third at 45% (both species found) (Figure 3.2). In 2021, all 11 fields had some parasitized larvae ranging from 10-90% (Figure 3.3) and the parasitism rates between the two species was fairly even (*B. curculionis:* 12.4 ± 5.1 (SE) %, *O. incertus*: 16.3 ± 6.3 (SE) %), with an average of 7.4 ± 2.9 (SE) % of the larvae having

DNA from both parasitoids (Figure 3.5). Five of the 11 fields in 2021 had double parasitism, whereas in 2020 only one field had double parasitism.

3.3.3 Parasitism testing

Insects for live rearing were collected during week 27 (July 7), and there was a significant difference between the estimated parasitism rate from live reared and DNA extracted rates ($\chi^2 = 7.597$, df = 1, p = 0.00585). The average was 39.6 ± 6.4 (SE) % for live rearing and 67.4 ± 7.2 (SE) % when using the multiplex PCR technique (Figure 3.6.A). Parasitism rates were determined by the presence of an adult parasitoid or their cocoon for live rearing, whereas for the DNA assessment, the rates were determined by the presence of DNA from at least one parasitoid species. Due to difficulties in obtaining enough larvae for rearing, parasitism rates in Fields F and G are only based on 39 and 26 larvae, respectively. All other fields were based on 200 larvae. When looking at the differences found between the two techniques in each of the fields there are large discrepancies, fields C and D had the same live rearing rates of 29% but the DNA-based parasitism rates differed by 28.4% (Figure 3.6.B).

3.4 Discussion

Since the 1950's, 13 European parasitoid wasp species have been released in North America (Brunson and Coles 1968). Two have been found consistently in southern Alberta and parts of Saskatchewan where the alfalfa weevil has established, *B. curculionis* and *O. incertus* (Harper 1988, Soroka et al. 2020). Their life cycles are not fully known in the prairies, for instance whether *B. curculionis* is univoltine, or how many *O. incertus* generations occur or their peak flight times. To assess biological control potential of these parasitoids these elements of their life cycle are crucial to know.

The weekly survey determined when parasitism was occurring for both parasitoids. While molecular techniques do have the downfall of not knowing what stage the parasitoid wasp is at when they are collected, they did show that parasitism was occurring in the first week of collections. This shows that both species could be flying as early as mid-May and parasitizing early alfalfa weevil instars. This was also confirmed with their presence in Chapter 2 during the biodiversity assessment (Table 2.2) and to a lesser extent on the yellow sticky cards (Table 3.2). Yellow sticky cards are a great tool for assessing some insects that fly, but based on this studies

results they are not attractive enough for these parasitoid wasps and are not recommended to monitor them.

The average live rearing parasitism rate from this study (39.6 ± 6.4 SE %) was similar to the report in Wyoming, USA (Brewer et al. 1997). The Wyoming study found only *B. curculionis*, *Bathyplectus anura* (Thomson) (Hymenoptera: Ichneumonidae) and *Bathyplectus stenostigma* (Thomson) (Hymenoptera: Ichneumonidae) and no *O. incertus* (Brewer et al. 1997). The studies were conducted approximately 1,000 km and 24-26 years apart but still concluded similar parasitism rates. A more recent study with live rearing on the Montana-North Dakota border, US (Rand 2013) found only *B. curculionis* and *O. incertus*. The two years the study was conducted *B. curculionis* had parasitism rates of 57 % and 23.7 % whereas *O. incertus* had lower rates at 1.4 % and 4.9 %. They were able to correlate the density of alfalfa weevil larvae with parasitism rates, but the correlations were in opposite directions over the two-year study. In the Alberta population only 5 of the 1070 larvae reared had *O. incertus*, whereas the molecular techniques showed that 39 of the 121 larvae tested in the same time period were parasitized.

Using molecular techniques to determine parasitism has become more popular in recent years. As many parasitoids are morphologically similar during larval development, waiting for adults to emerge during a live rearing assessment was the only way to identify parasitoids to species. Molecular techniques are being developed to asses many parasitoid - prey interactions in a wide range of agronomically important pest species (Ashfaq et al. 2005, Gariepy et al. 2005, Traugott et al. 2006, Levi-Mourao et al. 2022).

A molecular protocol for assessing parasitism in alfalfa weevil populations has been published recently (Levi-Mourao et al. 2022). The study focused on the two prominent local parasitoids in Spain: *B. curculionis* and *B. anura*, the latter is not found in the western prairies (Soroka 2013). They showed parasitism rates from live rearing to be 3.4 % in 2020 and 0.9 % in 2019, with the new molecular protocol producing rates of 12.4 % in 2020 and 18% in 2021 (Levi-Mourao et al. 2022). Overall, the parasitism rates are much lower in Spain than in Alberta, but the same trends appeared, higher parasitism rates with molecular techniques rather than with live rearing and high variability between years.

While parasitism rates were quite high in 2021 in the current study, the 2020 parasitism rates were very low and almost non-existent. These differences may be due to several factors: samples were not taken by the same person both years, in the same field locations, or during the same week number. Many of the 2020 samples were taken from field edges whereas the 2021 samples were taken 10 m into the fields. The 2020 samples were taken one week later (week 27) than the 2021 samples. Collections later in the season increase the chance that the fields could have been treated with an insecticide the week before and/or during the collection period (Table 3.1). Insecticide usage in 2021 indicates that there may have been a potential decrease in parasitism rates following an application (Table 3.1, Figure 3.4). There were applications of various insecticides in the different fields during weeks 23-24 (June 9-16), collections in the following weeks 24-25 (June 16-23) had the lowest parasitism rates of the collection period. In addition to this 2021 was a much hotter summer than 2020, which can change how insects interact and develop.

In summary, we determined parasitism levels in alfalfa weevil population in southern Alberta both spatially over a wide range and temporally in a few fields. The data collected shows that the parasitoids may be active during the whole bud stage, which is an economically important stage for the growers. During this stage the growers must decide on which chemical control options to use before the crop flowers, as some of the insecticides that control alfalfa weevil cannot be used during bloom to protect pollinators. The data from this study showed that live rearing had lower parasitism rates than using a molecular technique as found in other such comparisons (Ashfaq et al. 2004, Levi-Mourao et al. 2022).

3.5 Tables and figures

Table 3.1 Insecticide usage and collection dates for parasitism rates in fields for 2020 and 2021 in southern Alberta.

		Insecticide			
Year	Field	Name of Product	AI	Date Applied	Control/Suppression[a]
2020[b]		Collection 1 Week 22 (June 2)			
		Collection 2 Week 23 (June 8)			
	Y	Matador	Lambda-cyhalothrin	June 10th	N/A
	Y	Coragen	Chlorantraniliprole	June 10th	S
	W	Decis	Deltamethrin	June 10-15th	C
		Collection 3 Week 24 (June 15)			
	V	Cygon	Dimethoate	June 18th	S
	U	Malathion	Malathion	June 18th	C
	T	Coragen	Chlorantraniliprole	June 19th	S
	W	Lorsban	Chlorpyrifos	June 20-25th	N/A
	S	Coragen	Chlorantraniliprole	June 22nd	S
	U	Voliam Xpress	Lambda-cyhalothrin, Chlorantraniliprole	June 22nd	N/A
		Collection 4 Week 25 (June 22)			
	V	Lorsban	Chlorpyrifos	June 26th 0	N/A
	T	Decis	Deltamethrin	July 6th	C
2021[c]		Collection 1 Week 22 (June 3)			
		Collection 2 Week 23 (June 9)			
	E	Matador	Lambda-cyhalothrin	June 11th	N/A
	C	Assail	Acetamiprid	June 10-15th	N/A
	D	Corgen	Chlorantraniliprole	June 14th	S
	D	Beleaf	Flonicamid	June 14th	N/A
	E	Malathion	Malathion	June 14th	C
		Collection 3 Week 24 (June 16)			
	H	Corgen	Chlorantraniliprole	June 18th	S
	G	Matador	Lambda-cyhalothrin	June 18th	N/A
	B	Malathion	Malathion	June 18-26th	C
	A	Corgen	Chlorantraniliprole	June 21st	S
	A	Beleaf	Flonicamid	June 21st	N/A
	A	Matador	Lambda-cyhalothrin	June 21st	N/A

Collection 4 Week 25 (June 23)
Collection 5 Week 26 (June 29
Collection 6 Week 27 (July 6)

[a] Control or suppression of alfalfa weevil larvae

[b] Insecticide usage was reported for 6 of the 8 fields

[c] Insecticide usage was reported for 7 of the 10 fields

Table 3.2 Sticky card counts and parasitism levels for 2020 and 2021 in southern Alberta.

			Sticky cards[a]		Parasitism (%)[c]		
Year	Collection Week	Field	*B. curculionis*	*O. incertus*	*B. curculionis*	*O. incertus*	*Double*
2020	22	Z	22	1	[d]	[d]	[d]
	22	X	16	1			
	22	Y	11	0			
	23	Z	10	1			
	23	X	4	0			
	23	Y	14	0			
	24	Z	18	0			
	24	X	1	0			
	24	Y	8[b]	0			
	25	Z	2	0			
	25	X	1	0			
	25	Y	9	0			
2021	22	A	11	0			
	22	B	5	0	[d]	[d]	[d]
	22	C	10	1			
	22	D	2	3			
	22	E	0	0			
	22	F	33	0			
	22	G	0	0			
	22	H	6	17			
	22	I	10[b]	0			
	23	A	3	0	14.28	28.57	0
	23	B	1	0	0	0	0

23	C	2	0	33.33	0	0
23	D	0	1	18.75	31.25	6.25
23	E	0	0	10	35	5
23	F	4	0			
23	G	0	0	0	0	0
23	H	2	0			
23	I	1	0			
24	A	2	1	10	10	5
24	B	6	0	10	15	0
24	C	4	0	15	55	10
24	D	2	0	10	5	0
24	E	1	0	5	40	15
24	F	6	0			
24	G	9	0	6.25	43.75	18.75
24	H	1	0			
24	I	2	0			
25	A	2	0	5	60	0
25	B	0	0	5	10	0
25	C	2	0	15	20	0
25	D	2	0	0	0	0
25	E	0	0	5	5	0
25	F	8	0			
25	G	15	0	0	0	0
25	H	2	0			
25	I	2	0			
26	A	2	0	0	0	0
26	B	0	0	0	15	0
26	C	3	0	0	0	0
26	D	1	0	0	10	0

26	E	1	0	5	30	0
26	F	6	0			
26	G	1	0	10	25	5
26	H	3	0			
26	I	9	0			
27	A	3	0	0	70	10
27	B	0	0	60	15	0
27	C	1	0	26.31	5.26	0
27	D	0	0	20	35	5
27	E	0	0	35	30	25
27	F	2	0			
27	G	0	0	12.5	37.5	25
27	H	0	0			
27	I	1	0			

[a] Total sum from all three cards per field

[b] Total sum for 2 of the three cards

[c] Parasitism assessed through DNA extractions

[d] Larvae were not collected in 2020 and only in some fields in 2021

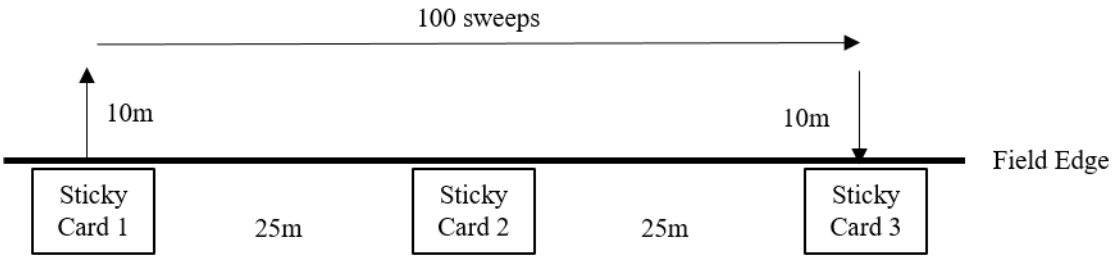

Fig. 3.1 Sampling collection transect. Using a 38.1 cm diameter sweep net a 100, 180°-sweep sample was collected. Along the field edge 3 yellow sticky cards were attached to wooden stakes (2.54 x 5.08 x 121.92 cm) and placed 25 m apart. Each week the cards were removed and reattached at canopy height.

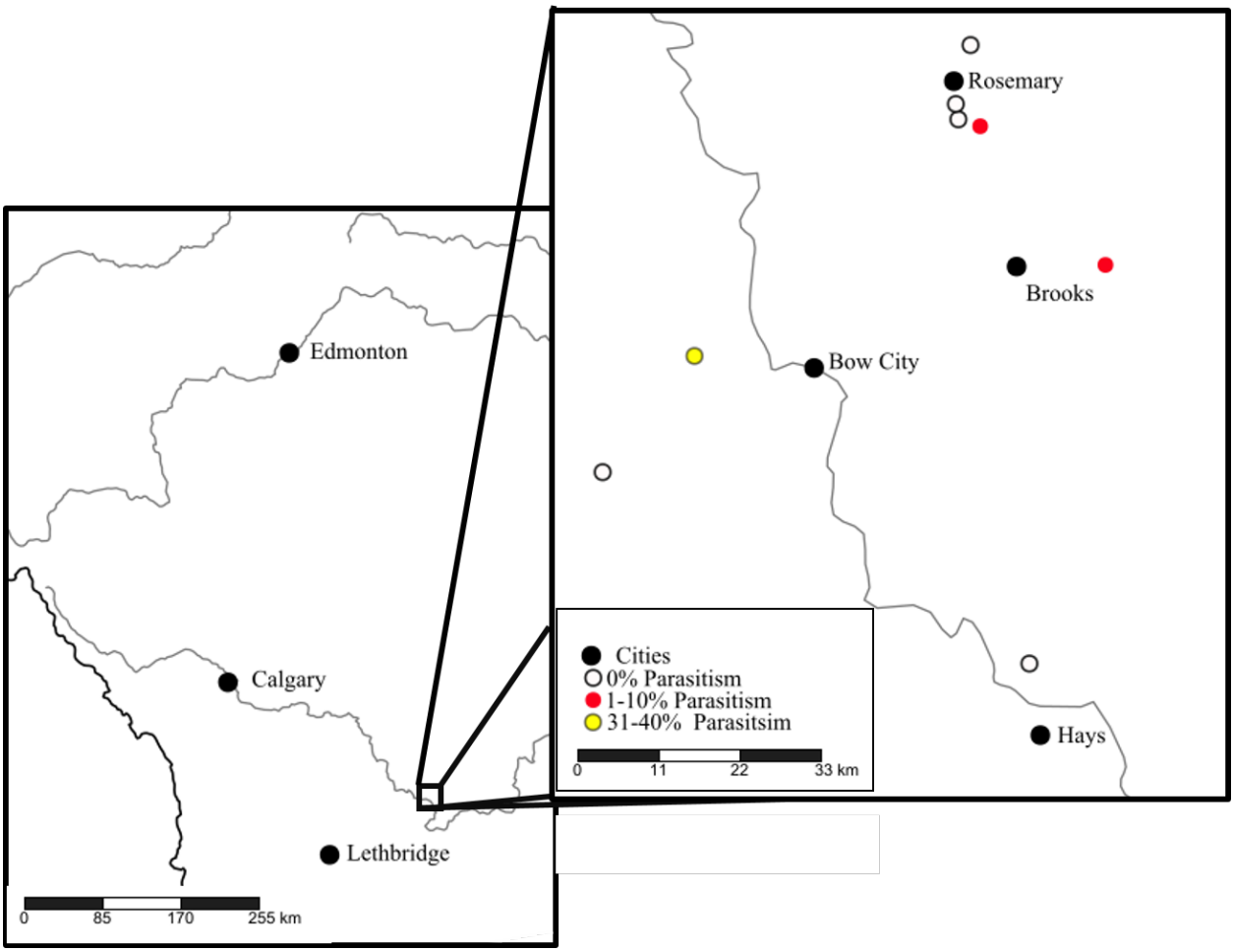

Fig. 3.2 Parasitism rates as determined by the multiplex PCR tool and location of alfalfa seed production fields surveyed in 2020 in southern Alberta. The grey line in the inset map represents the South Saskatchewan River.

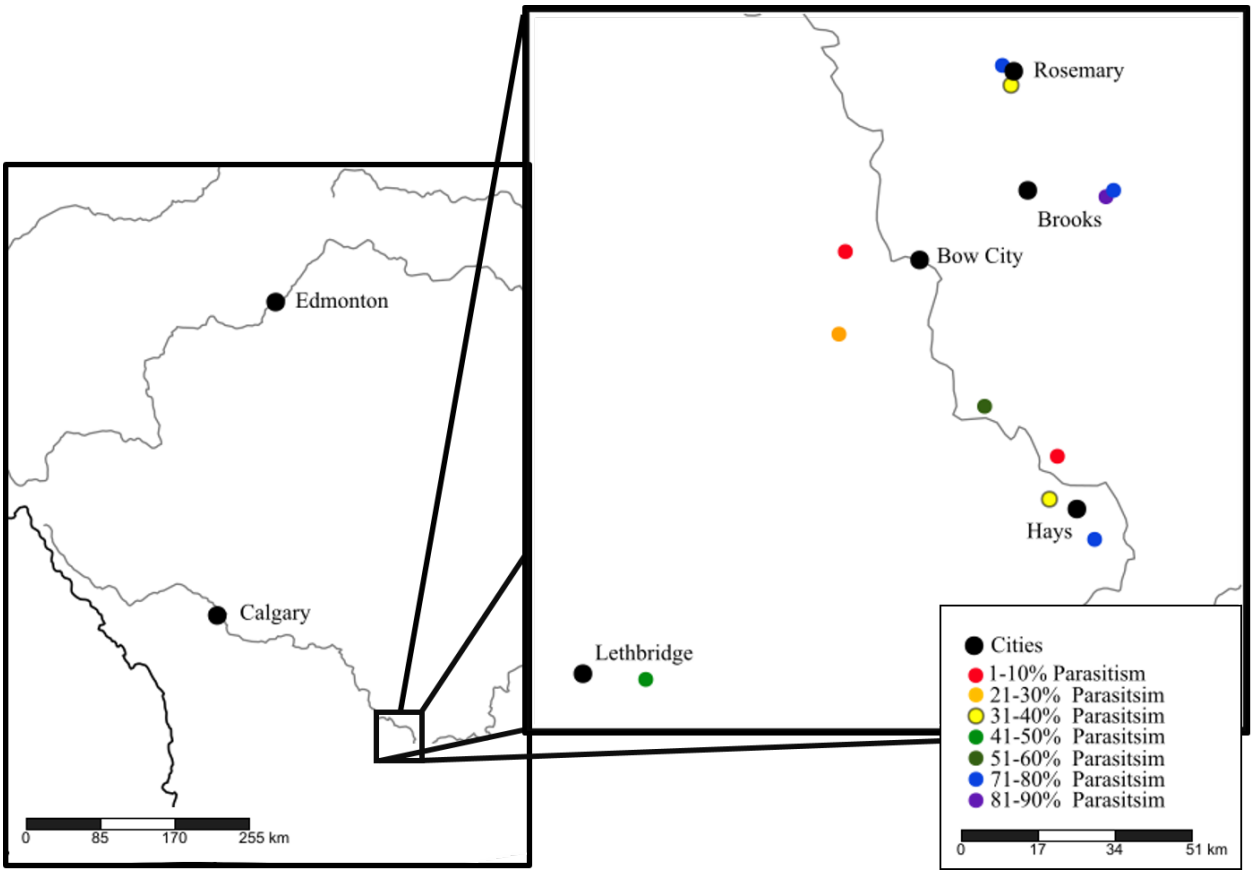

Fig. 3.3 Parasitism rates as determined by the multiplex PCR tool and location of alfalfa seed production fields surveyed in 2021 in southern Alberta. The grey line in the inset map represents the South Saskatchewan River.

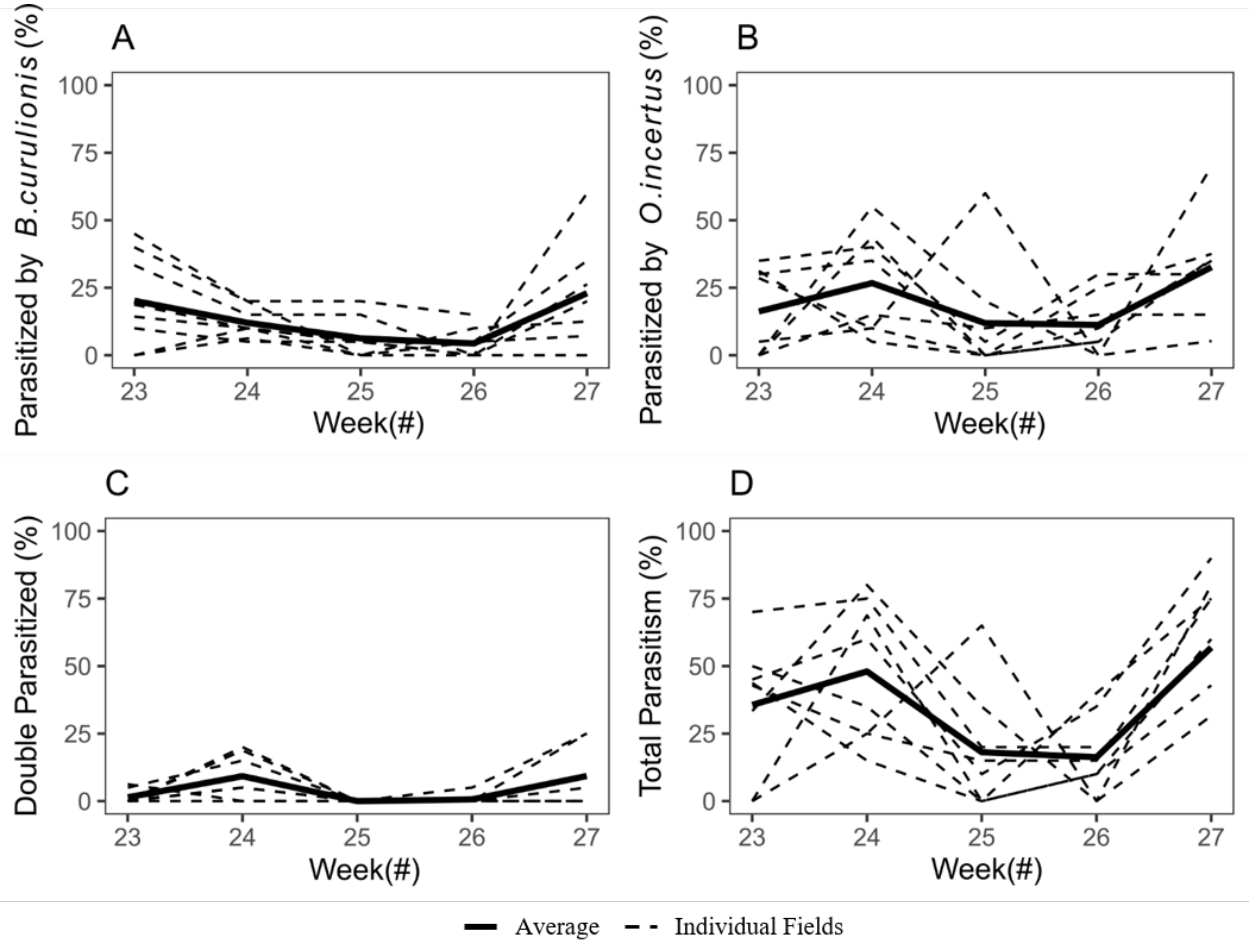

Fig. 3.4 Alfalfa weevil parasitoid parasitism rates during 2021 through weeks 23-27. Parasitism rates were determined by a multiplex PCR tool. Number of parasitoids are counted per 20 larva, (A, B) show the number of larvae with only one of the parasitoids, (C) shows the number of larvae that had DNA from both parasitoids. Total parasitism rates (D) from each of the fields. Dotted lines show each field individually, solid line show the average across all fields.

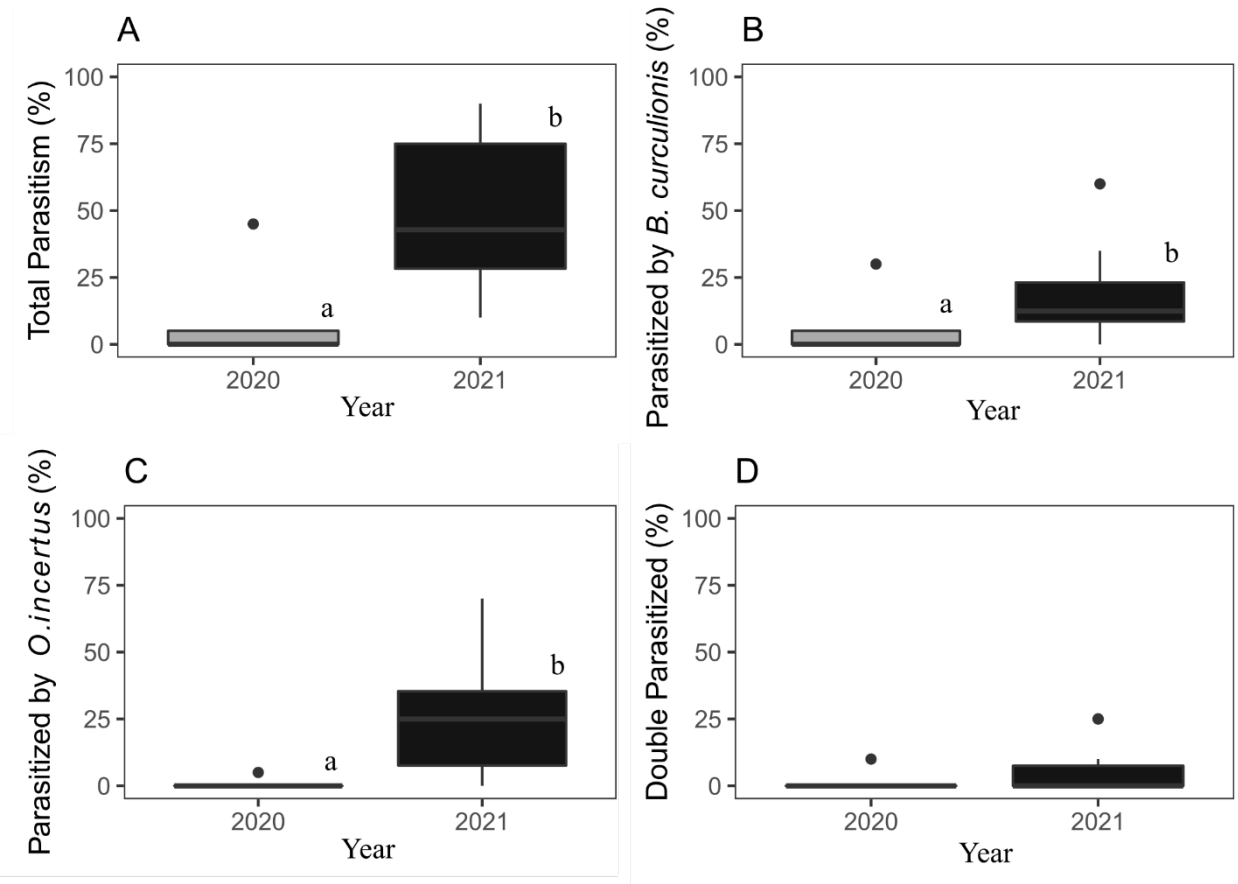

Fig. 3.5 Alfalfa weevil parasitism rates in 2020 and 2021 during week 27 (July 7). Parasitism rates were determined by a multiplex PCR tool. Total parasitism rates (A) are pooled from each of the fields (B, C) the number of larvae with only one parasitoid detected, (D) number of larvae that had DNA from both parasitoids. All estimates are based on 20 larvae. Years with different letters are significantly different [(K-W = (A): 10.04; (B): 5.93; (C): 9.33, df =1, $p < 0.05$]

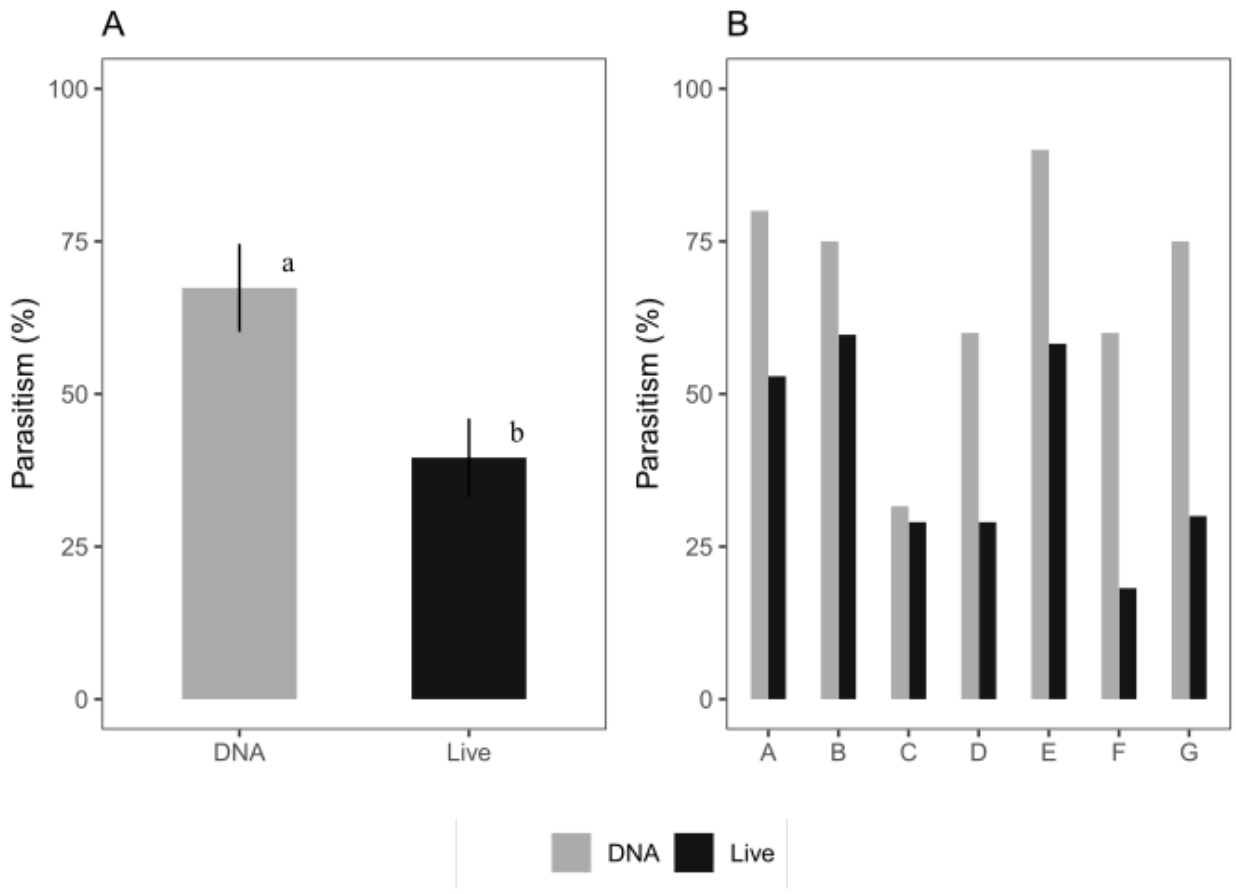

Fig. 3.6 Parasitism rates based on live rearing and the multiplex PCR tool during week 27 (July 7) in 2021. (A) Average parasitism rates for each method, different letters are significantly different [K-W = 7.597, df =1, p = 0.00585]. (B) Individual parasitism rates for each method by field. Fields A-E live rearing rates are based on 200 larvae in individual cups. Fields F and G only 39 and 26 larvae were used, respectively. Error bars denote standard error.

4 General Discussion

4.1 Introduction

This project was developed to study the insect diversity and parasitism levels in alfalfa seed production fields in southern Alberta. From this study more temporal information was obtained about the insect interactions in these fields. This chapter contains results from both studies and will discuss and summarize some of the key findings, as well as propose some future research directions.

4.2 Summary

Alfalfa is an important agronomic and economic crop for North America. It is the top forage for livestock as it adds nitrogen into the soil and is very high in protein (Mueller 2008). Alfalfa is also used in human markets, such as alfalfa sprouts and in herbal medicines (Price 1988). In order to maintain and increase production of this crop, insect interactions need to be further studied. This study found variable pest and natural enemy populations with no consistent trends between years or crop stages. Insect diversity and parasitism levels had large trend differences between the two years of collections. Some of these could be due to timing of collections, in some years insecticides had been sprayed on some of the fields and not on others, as well as the flower collections were done almost 2 weeks apart. Weather was also very different between the two years with 2021 having hotter and longer heat waves (Government of Canada 2022). All of these factors may have influenced and contributed to the variability found across the two study years.

Although there were no significant trends in the insect populations, the insects collected were consistent with previous diversity studies conducted in seed and forage alfalfa fields (Harper 1988, Uddin 2005, Sim and Meers 2017). The main pest species found in Uddin (2005) and Sim and Meers (2017) study were *Lygu*s spp., *Adelphocoris lineolatus* and *Acyrthosiphon pisum*. Although *A. pisum* were not quantified in this current study they were found in most of the samples collected. The common natural enemies between the studies were the ladybeetles (Coleoptera: Coccinellidae), minute pirate bug (*Orius insidiosus* (Say), Hemiptera: Anthocoridae), lacewings (Neuroptera: Chrysopidae) and damsel bugs (Hemiptera: Nabidae). Most of these natural enemies tend to prefer soft bodied prey, such as aphids and plant bug

nymphs some have been seen to prey on alfalfa weevil larvae (Ouayogode and Davis 1981). Alfalfa weevil parasitoids were not identified in these diversity studies, but some have been documented in more specialized studies of alfalfa weevil in the Canadian prairies (Soroka et al. 2020).

Insect collections from fields can be done in a variety of ways, and different techniques can lead to a bias in certain insects over others (Uddin 2005). Sweep nets collect insects found within the foliage, leaving ground dweller insects unsampled. Yellow sticky cards also tend to bias more toward foliage dwellers and are not attractive to all insect species (Table 3.2) as found when sampling for the parasitoid wasps, *Bathyplectes curculionis* (Thomson) (Hymenoptera: Ichneumonidae) and *Oomyzus incertus* (Ratzenberg) (Hymenoptera: Eulophidae). These two wasps were found throughout the collection periods, with no consistent trend within fields or time periods. Soroka et al. (2020), stated that *B. curculionis* was univoltine in the Canadian prairies, but that in warmer regions may have a partial second generation. As climates continue to warm this insect may increase the range it can survive as well as the number of generations that it can have in one year as other insects have done (Altermatt 2010). *Oomyzus incertus* had different trends during this study as it was found during all crop stages and parasitism rates were high when assessed with the multiplex PCR but during the live rearing experiment only five larvae were confirmed to be parasitized by this parasitoid. Low live rearing parasitism rates for *O. incertus* as compared to *B. curculionis,* was also found in in the US states south of the Canadian prairies (Rand 2013).

4.3 Conclusion

Many insects are found within alfalfa seed production fields in the Canadian prairies (Harper 1988). These insect populations change throughout the season as well as between years. For example, there were 22 ladybird beetle species found in 1988 (Harper 1988), but only 6 in the current study. These populations may have disappeared or decreased to such low number that in 2015-2016, Sim and Meers (2017) only found one species of lady beetle, *Coccinella septempunctata* (Coleoptera: Coccinellidae). This study conducted 5 years after Sim and Meers (2017), found 6 species with the most abundant being *C. septempunctata* (Table 2.2). Changes in insect populations are important to note for growers so that they can better utilize their natural enemies and predict when pest populations will reach economic levels and need interventions to

decrease populations below economic injury levels. Therefore, further assessments of insect interactions and trends are critical for assessing whether management is needed. This is particularly important for parasitoid wasps, as they are very small and spend much of their lifecycle within their host, so their populations are hard to assess through regular means.

4.4 Future Directions

To better assess how diversity changes in these fields, a different approach may be needed. Uddin (2005) collected samples every two weeks throughout the season and focusing on a few specific insects to better assess their temporal differences, rather than the three time periods. In doing this more general trends could be found. Another aspect to consider would be assessing diversity in different agricultural fields that have an established alfalfa crop in the vicinity and compare it to other fields that do not. Alfalfa is a perennial crop and may act as a reservoir for insects to harbor over winter and while other crops are being harvested.

For future work to fully understand how the parasitism rates change, a longer collection period may be need as the average parasitism rates were the highest during the last week, week 27. Using different collection methods may also be helpful to better assess when the parasitoids are at their peak flights. Secondly, it remains to be determined what parasitism rate is high enough to keep alfalfa weevil populations below the economic threshold. Assessing parasitism in non-sprayed and sprayed locations on a temporal scale may be able to give the growers the information needed to make these decisions. This study showed there was decreased parasitism rates found during week 25 and 26, this was 1-2 weeks after spray applications occurred (Table 3.1). Being able to control this variability will determine whether the decrease in parasitism was in fact due to the insecticides used or due to the parasitoids' lifecycles. Using these methods of molecular detection is slow, and cannot be readily done in a field, therefore investigation of alternative, faster, detection techniques to attribute parasitism rates to a tangible field practice would be ideal, for example an LAMP test.

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Appendix I

Development of a multiplex PCR to identify alfalfa weevil, *Hypera postica*, larvae parasitized by *Bathyplectes curculionis* and *Oomyzus incertus*

Identification of voucher specimens

Voucher specimens *Bathyplectes curculionis* (Hymenoptera: Ichneumonidae) and *Oomyzus incertus* (Hymenoptera: Eulophidae) were collected from parasitized alfalfa weevil, *Hypera postica* (Coleoptera: Curculionidae) larvae from several locations in Manitoba in 2017 by Manitoba Agriculture, Food and Rural Development staff. DNA was extracted from 10 of each parasitoid species using a QIAamp DNA extraction (Qiagen, Mississauga, ON) kit following the manufacturer's suggested protocol with a final elution of 25 µl.

The whole insect was suspended in 180 µl of ATL buffer and the tissues homogenised in 1.5 mL tubes using a nylon pestle. Samples were incubated with 20 µl proteinase K overnight at 56°C. The following morning, 200 µl AL buffer and 1 µl carrier RNA was added to each sample tube followed by 200 µl of ethanol (99.8%). The samples were transferred to a QIAamp MinElute column and spun at 6000 × g for one minute. The columns were washed twice with 500 µl of wash buffers (AW1 and AW2) and the DNA eluted from the column with 25 µl of elution buffer.

Parasitoid identity was confirmed using customized primers for Hymenoptera NewParF and NewParR (Santos et al. 2008). The PCR mix for each sample contained 16.5 µl ultrapure water, 2.5 µl 10x PCR buffer (200 mM Tris-HCl pH 8.4, 500 mM KCl), 2 µl $MgCl_2$ (50 mM), 0.25 µl dNTP mix (10 mM), 1.25 µl of each primer (10 µM), 0.2 µl Taq DNA polymerase (5 U/µl), and 1 µl of template DNA for a total reaction volume of 25 µl. Amplification was performed in Bio-Rad C1000 Thermal Cycler with the following cycle: initial denaturation at 94°C for one minute; 5 cycles of 94°C for 30 seconds, 45°C for one minute, 72°C for 90 seconds; 35 cycles of 94°C for 30 seconds, 47°C for 40 seconds, 72°C for 90 seconds; and a final 72°C for ten minutes. PCR products were electrophoresed on a 0.9 % TAE agarose gel containing Gel-Red (Biotinium – Cedarlane, Burlington, Ontario, Canada) at 90 V for 1 hour. DNA products were visualized by ultraviolet transillumination. Amplicons from all haplotypes

were purified using QIAquick purification kit and Sanger sequenced. Sequences were compared with the BOLD barcode database for species identification.

The voucher specimens for the alfalfa weevil were collected from Alberta in May 2019. DNA was extracted from the whole adult body using a Qiagen DNAeasy Blood and Tissue extraction kit following the manufacturer's suggested protocol for insect tissue. Final elution in 75 µl.

The whole insect was suspended in 180 µl of ATL buffer and the tissues homogenised in 1.5 mL tubes using a nylon pestle. Next 20 µl proteinase K was added to each sample tube followed by one hour incubation at 56°C. After incubation, 4 µl RNase A (100 mg/ml) was added followed by incubation at room temperature for 2 minutes. Next 200 µl AL buffer was added to each sample tube followed by 200 µl of ethanol. After a quick vortex the samples were transferred to a column and spun at 6000 × g for one minute. The columns were washed twice with 500 µl of wash buffers (AW1 and AW2) and the DNA eluted from the column with 75 µl of elution buffer.

Species identification was confirmed using the universal CO1 barcode primers LCO1490 and HCO2198 (Folmer et al. 1994) (Table 1). The PCR mix for each sample contained 18.25 µl ultrapure water, 2.5 µl 10x PCR buffer (200 mM Tris-HCl pH 8.4, 500 mM KCl), 1.25 µl $MgCl_2$ (50 mM), 0.3 µl dNTP mix (10 mM), 0.7 µl of each primer (10 µM), 0.2 µl Taq DNA polymerase (5 U/µl), and 1 µl of template DNA for a total reaction volume of 25 µl. Amplification was performed in a Bio-Rad C1000 Thermal Cycler with the following cycle: initial denaturation at 94°C for one minute; 5 cycles of 94°C for 40 seconds, 45°C for 40 seconds, 72°C for one minute; 35 cycles of 94°C for 40 seconds, 51°C for 40 seconds, 72°C for one minute; and a final 72°C for five minutes. PCR products were electrophoresed on a 0.9% TAE agarose gel containing Gel-Red (Biotinium – Cedarlane, Burlington, Ontario, Canada) at 90V for 1 hour. DNA products were visualized by ultraviolet transillumination. Amplicons were purified using QIAquick purification kit and Sanger sequenced. Sequences were compared with the BOLD barcode database for species identification.

Multiplex PCR design

The ITS2 region was amplified in several confirmed voucher specimens for each of the three species using ribosomal primers 5.8SF and inDNA-44 (Table 1). Amplification was performed in a Bio-Rad C1000 Thermal Cycler with the following cycle: initial denaturation at 94°C for two minutes; 30 cycles of 94°C for one minute, 55°C for one minute, 72°C for 90 seconds; and a final 72°C for five minutes. Amplicons were purified using QIAquick purification kit and Sanger sequenced. The sequences were aligned and a consensus sequence was generated for each species. Sequences were BLASTED to confirm species identification. The consensus sequence for each species were aligned in CLC Genomics Workbench (Qiagen) and unique species-specific reverse primers were designed based on differences in the consensus sequences. The universal 5.8SF primer was the forward primer for all 3 species (Table 2). The amplicons were designed to be different sizes to confidently identify the results (*B. curculionis* = 685bp, *H. postica* = 465 bp, and *O. incertus* = 265 bp). The primers were tested for cross priming and non-specific annealing.

The multiplex PCR mix for each sample contained 9.3 µl ultrapure water, 2.5 µl 10x PCR buffer (200 mM Tris-HCl pH 8.4, 500 mM KCl), 2 µl $MgCl_2$ (50 mM), 1 µl BSA (1 mg/ml), 1 µl dNTP mix (10 mM), 5 µl of the forward primer (10 µM), 1 µl of each reverse primer (10 µM), 0.2 µl Taq DNA polymerase (5 U/µl), and 1.5 µl of template DNA for a total reaction volume of 25.5 µl. Amplification was performed in a Bio-Rad C1000 Thermal Cycler with the following cycle: initial denaturation at 94°C for three minutes; 30 cycles of 94°C for 30 seconds, 58°C for 30 seconds, 72°C for one minute; and 72°C for five minutes. PCR products were electrophoresed on a 0.9% TAE agarose gel containing Gel-Red (Biotinium – Cedarlane, Burlington, Ontario, Canada) at 90V for 1 hour. DNA products were visualized by ultraviolet transillumination.

Table S1. PCR primers for ITS2 and CO1 sequencing

Primer	Sequence
NewParF	TAAGWTTAATTATTCGRTTAGAATTARG
NewParR	TAAACTTCWGGATGACCAAAAAATCA
LCO1490	GGTCAACAAATCATAAAGATATTGG
HCO2198	TAAACTTCAGGGTGACCAAAAAATCA

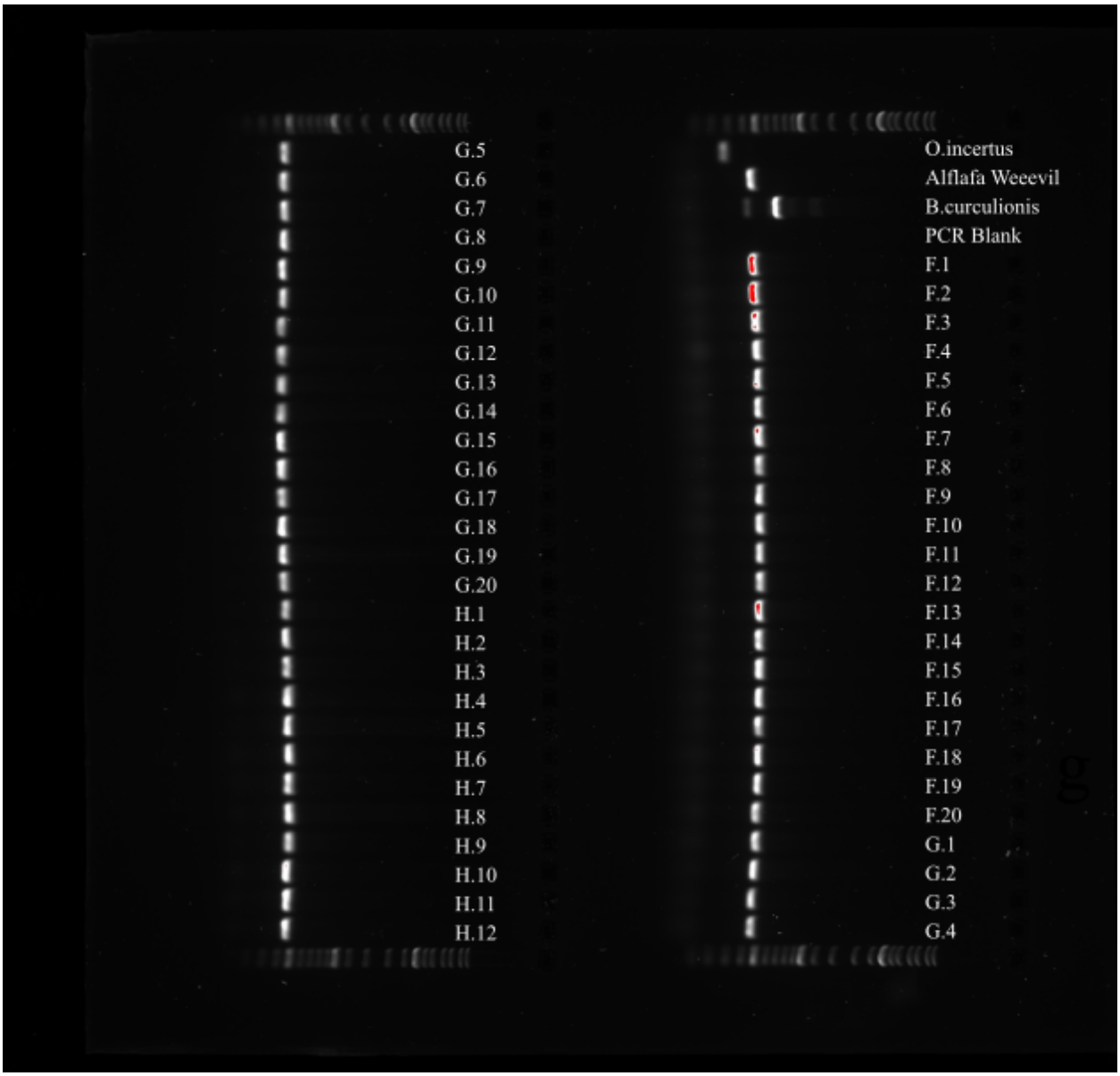
Table S2. Multiplex PCR primers designed to identify alfalfa weevil (*H. postica*) larvae parasitized by *Bathyplectes curculionis* and *Oomyzus incertus.*

Primer	Sequence
inDNA44	TCCTCCGCTTATTGATATGC
5.8SF	TGTGAACTGCAGGACACATGAAC
AW-R (*H. postica* reverse primer)	ACCTGCTCTGAGGTCGAAAG
Bc-R (*B. curculionis* reverse primer)	CGCAAACCATTCGGCGTTAT
Oi-R (*O. incertus* reverse primer)	ATGCGTGTGCTCGTACTCTG

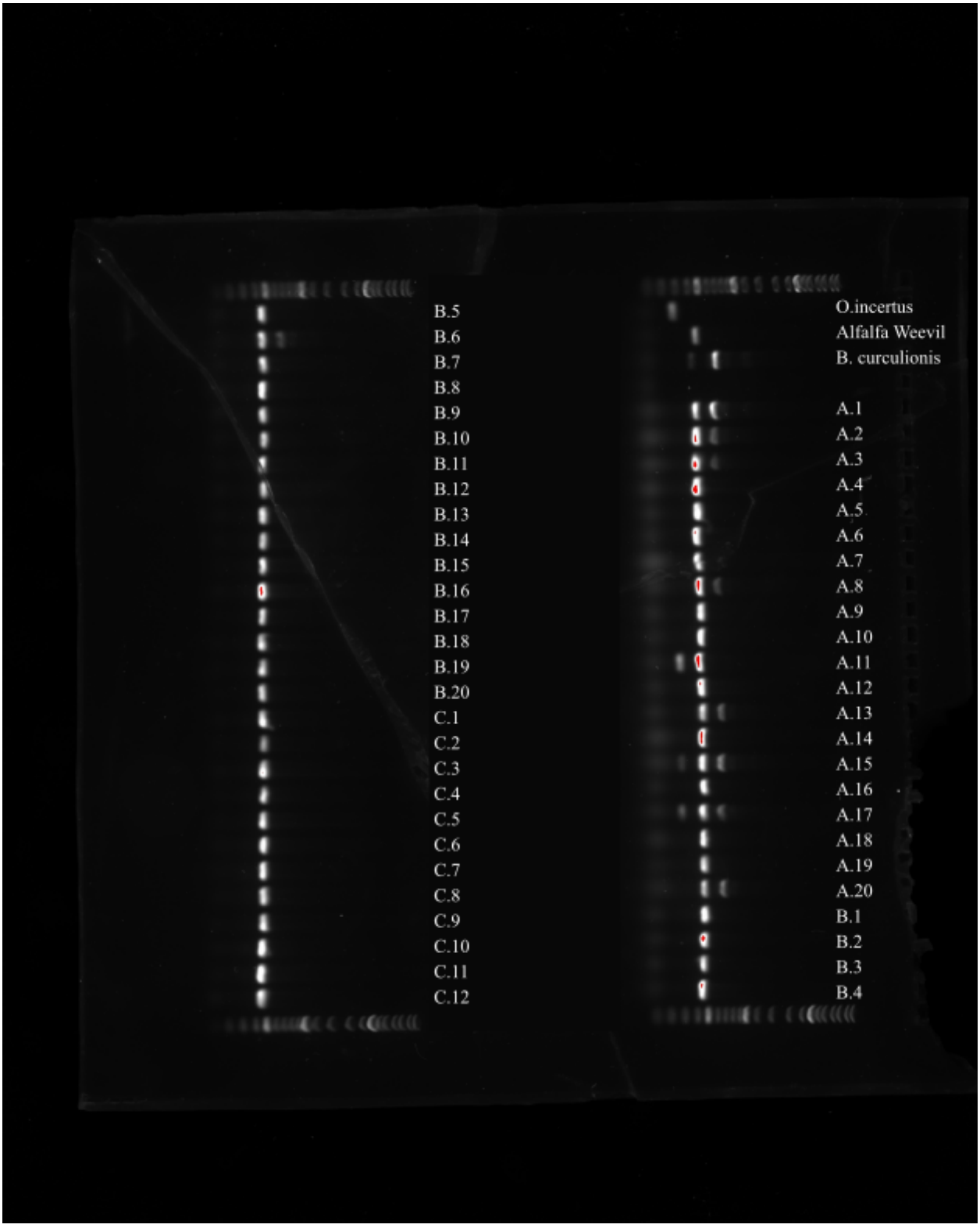
Appendix II

DNA products visualized by ultraviolet transillumination with ChemiDoc™ Imagining System (Bio-Rad, Hercules, CA, USA) on a 1% TAE agarose gel containing Syber-Safe (ThermoFisher Scientifc, Waltham, MA, USA)

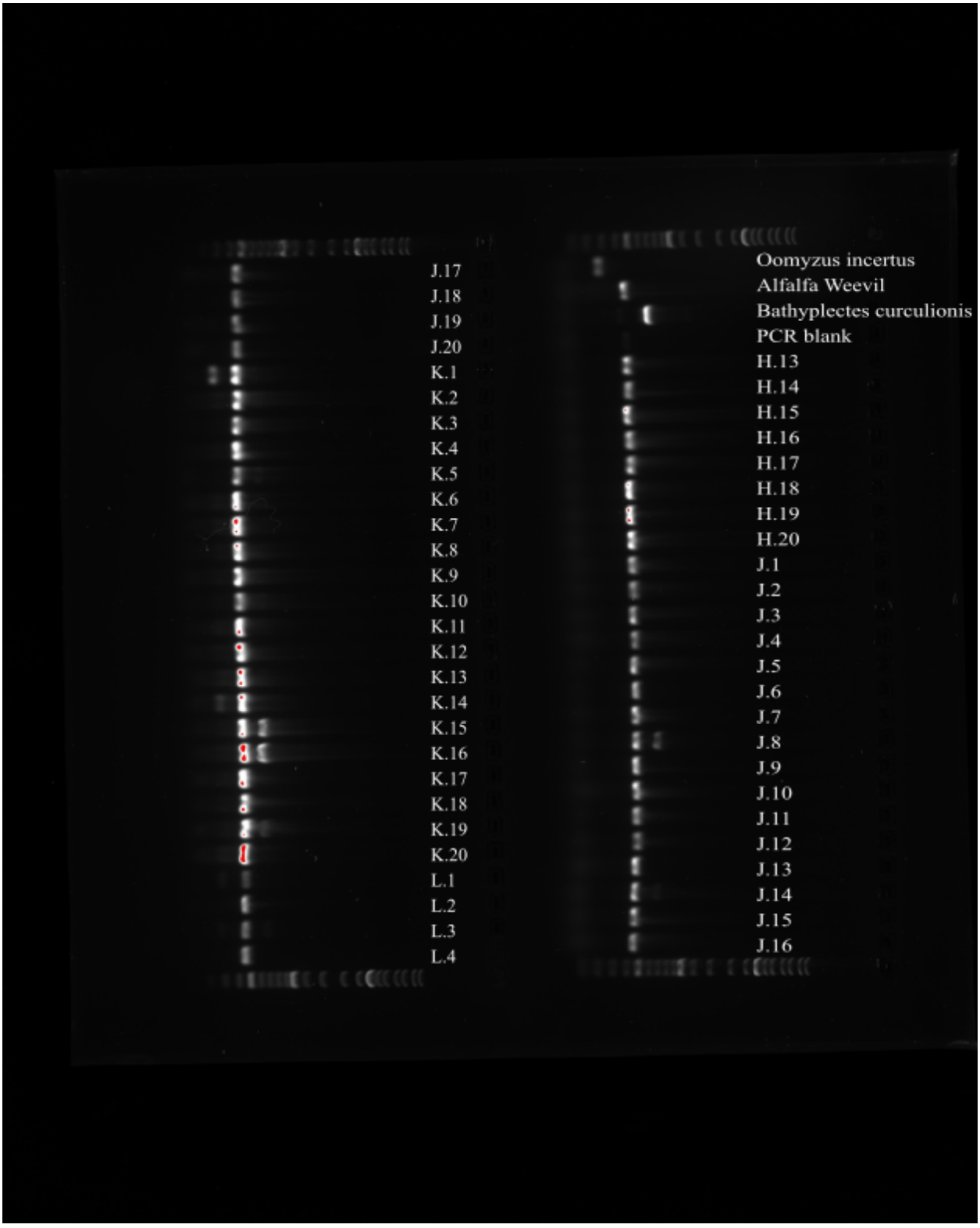
Gel 1



Gel 2


B.5
B.6
B.7
B.8
B.9
B.10
B.11
B.12
B.13
B.14
B.15
B.16
B.17
B.18
B.19
B.20
C.1
C.2
C.3
C.4
C.5
C.6
C.7
C.8
C.9
C.10
C.11
C.12
O.incertus
Alfalfa Weevil
B. curculionis
A.1
A.2
A.3
A.4
A.5
A.6
A.7
A.8
A.9
A.10
A.11
A.12
A.13
A.14
A.15
A.16
A.17
A.18
A.19
A.20
B.1
B.2
B.3
B.4

Gel 3


J.17
J.18
J.19
J.20
K.1
K.2
K.3
K.4
K.5
K.6
K.7
K.8
K.9
K.10
K.11
K.12
K.13
K.14
K.15
K.16
K.17
K.18
K.19
K.20
L.1
L.2
L.3
L.4
Oomyzus incertus
Alfalfa Weevil
Bathyplectes curculionis
PCR blank
H.13
H.14
H.15
H.16
H.17
H.18
H.19
H.20
J.1
J.2
J.3
J.4
J.5
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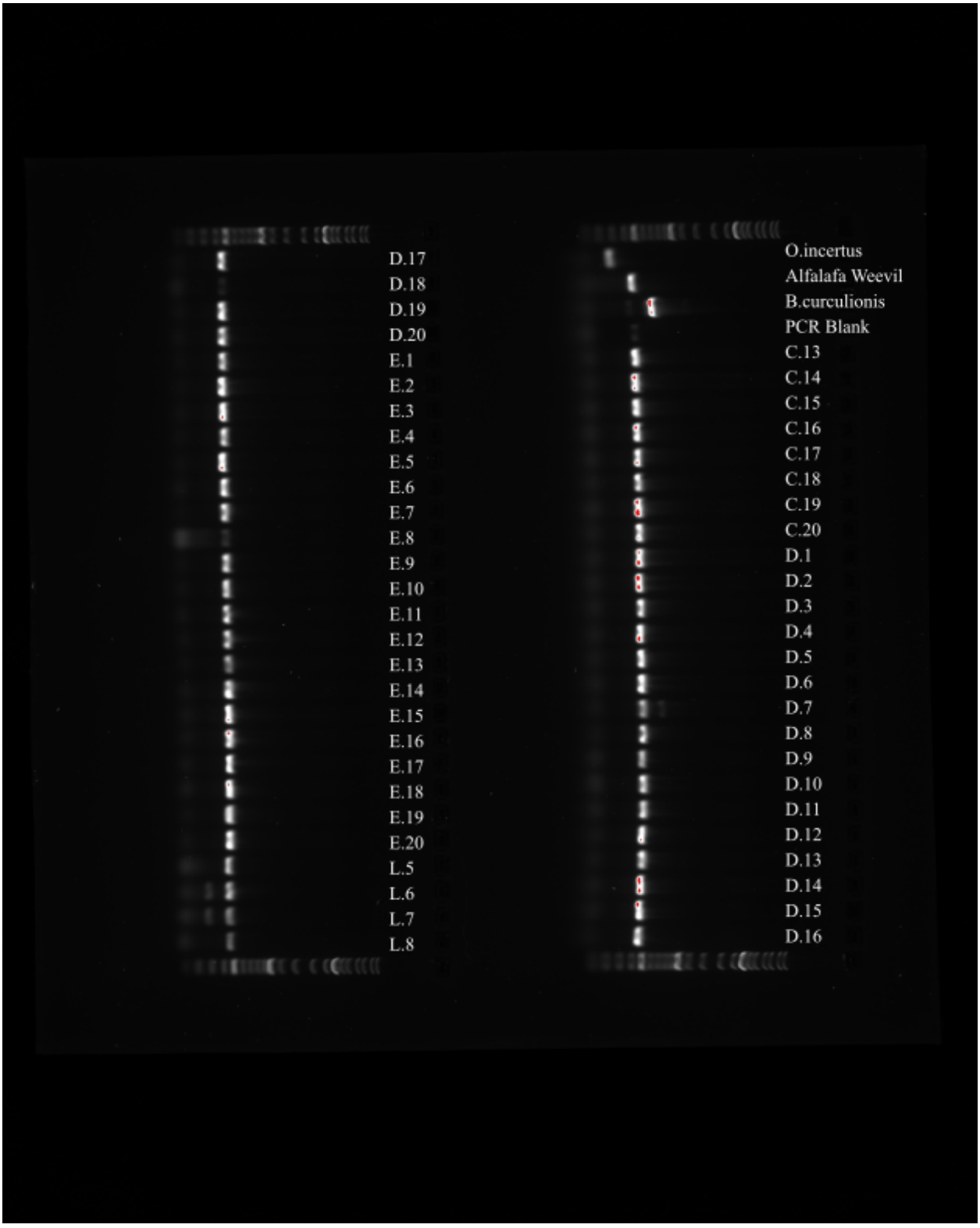
J.12
J.13
J.14
J.15
J.16

Gel 4


D.17
D.18
D.19
D.20
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E.2
E.3
E.4
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E.6
E.7
E.8
E.9
E.10
E.11
E.12
E.13
E.14
E.15
E.16
E.17
E.18
E.19
E.20
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L.6
L.7
L.8
O.incertus
Alfalafa Weevil
B.curculionis
PCR Blank
C.13
C.14
C.15
C.16
C.17
C.18
C.19
C.20
D.1
D.2
D.3
D.4
D.5
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D.12
D.13
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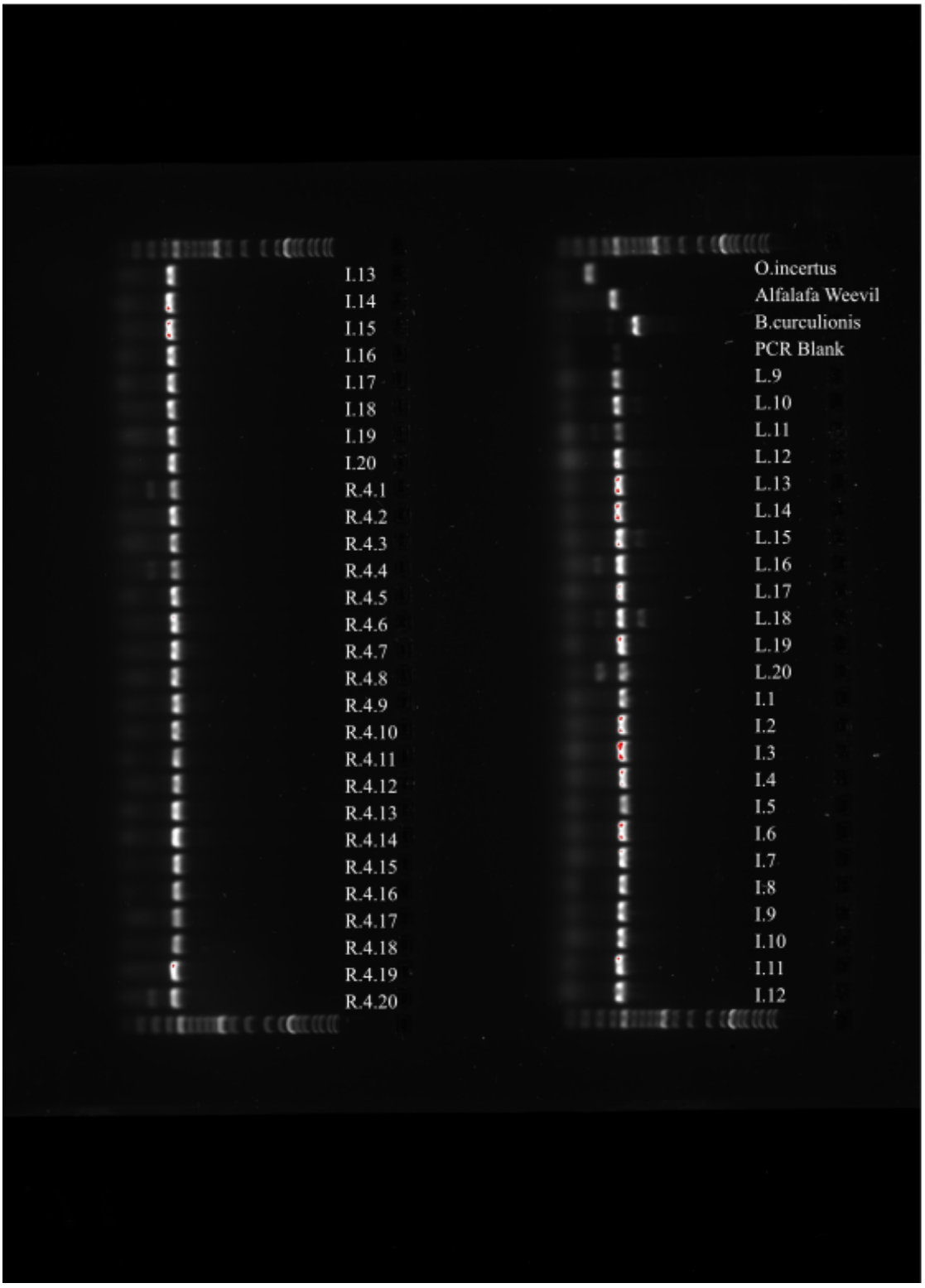
D.15
D.16

Gel 5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Alfalafa Weevil
B.curculionis
PCR Blank
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L.10
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L.12
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L.14
L.15
L.16
L.17
L.18
L.19
L.20
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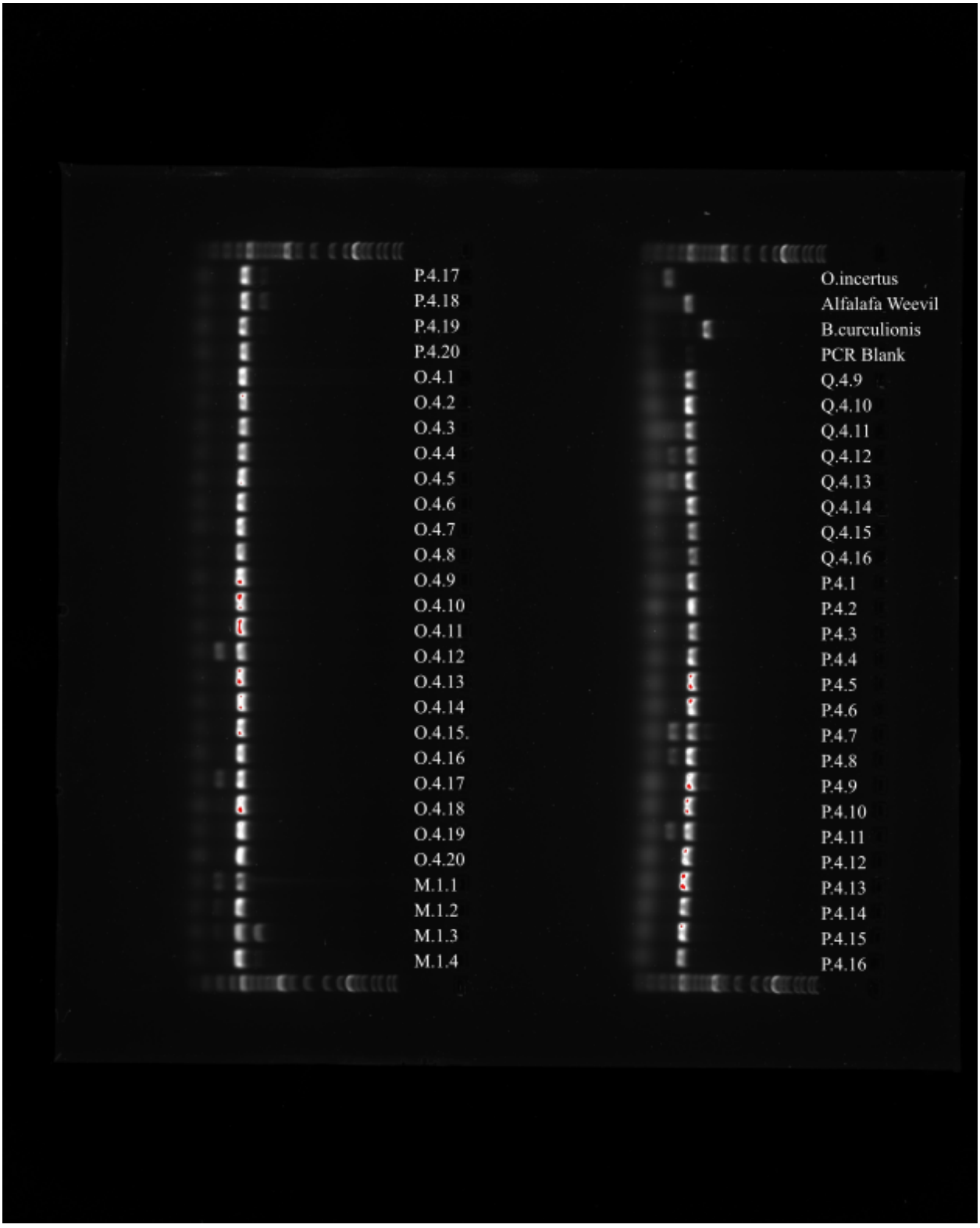
Gel 6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O.4.14
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O.4.16
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O.4.18
O.4.19
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M.1.4
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Alfalafa Weevil
B.curculionis
PCR Blank
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Q.4.14
Q.4.15
Q.4.16
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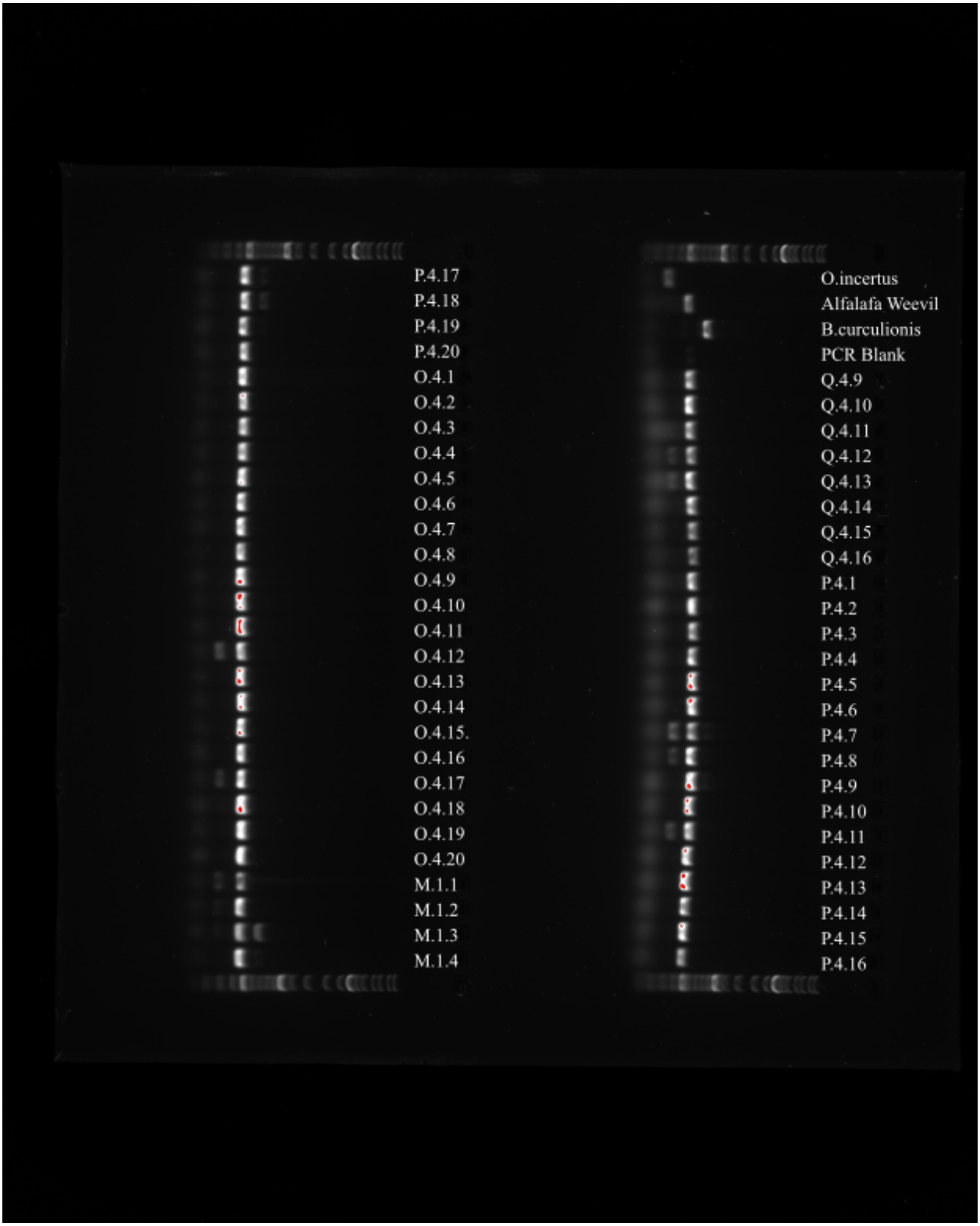
P.4.15
P.4.16

Gel 7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O.4.18
O.4.19
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Alfalafa Weevil
B.curculionis
PCR Blank
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Q.4.14
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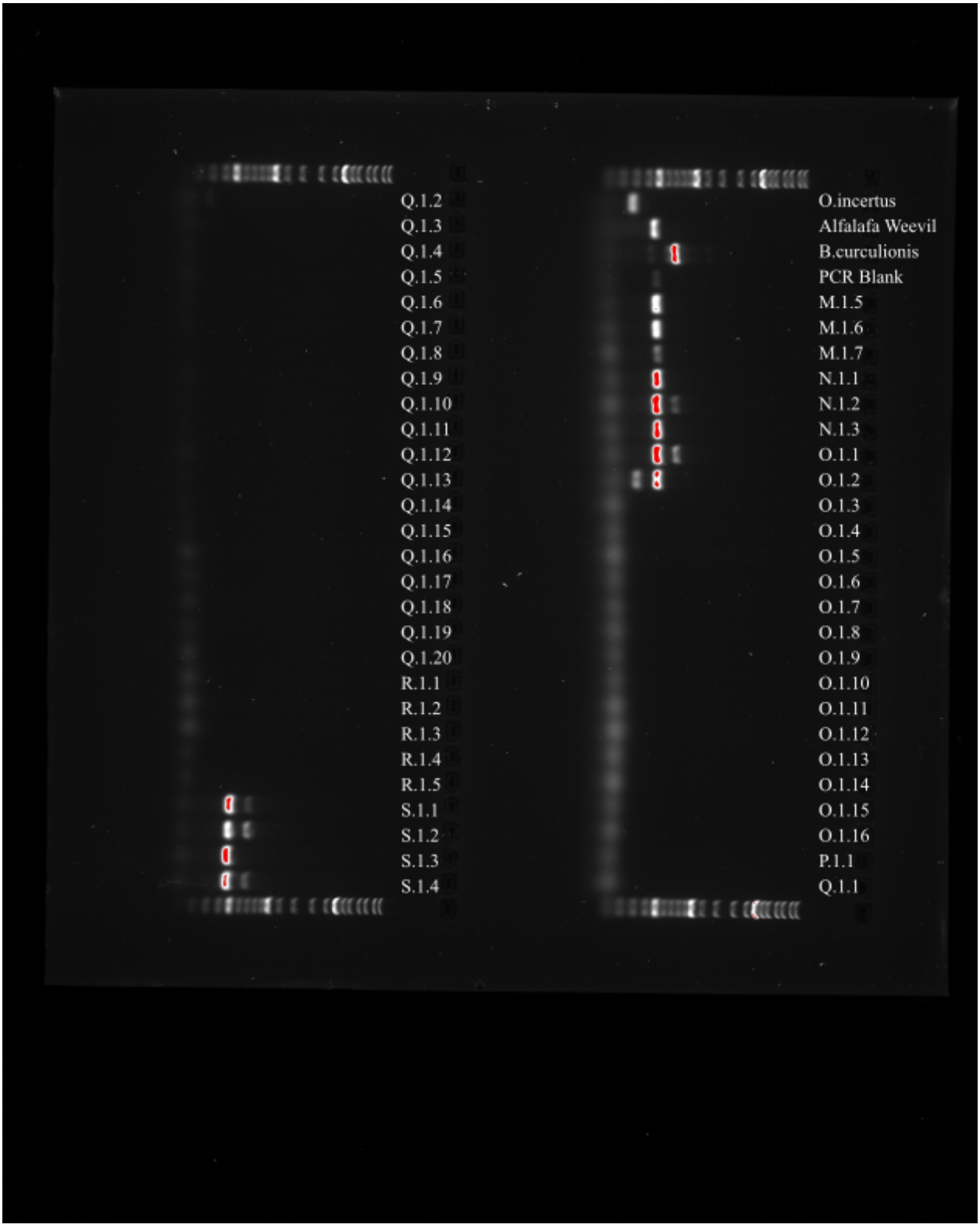
P.4.16

Gel 8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Q.1.17
Q.1.18
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Q.1.20
R.1.1
R.1.2
R.1.3
R.1.4
R.1.5
S.1.1
S.1.2
S.1.3
S.1.4
O.incertus
Alfalafa Weevil
B.curculionis
PCR Blank
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M.1.6
M.1.7
N.1.1
N.1.2
N.1.3
O.1.1
O.1.2
O.1.3
O.1.4
O.1.5
O.1.6
O.1.7
O.1.8
O.1.9
O.1.10
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O.1.15
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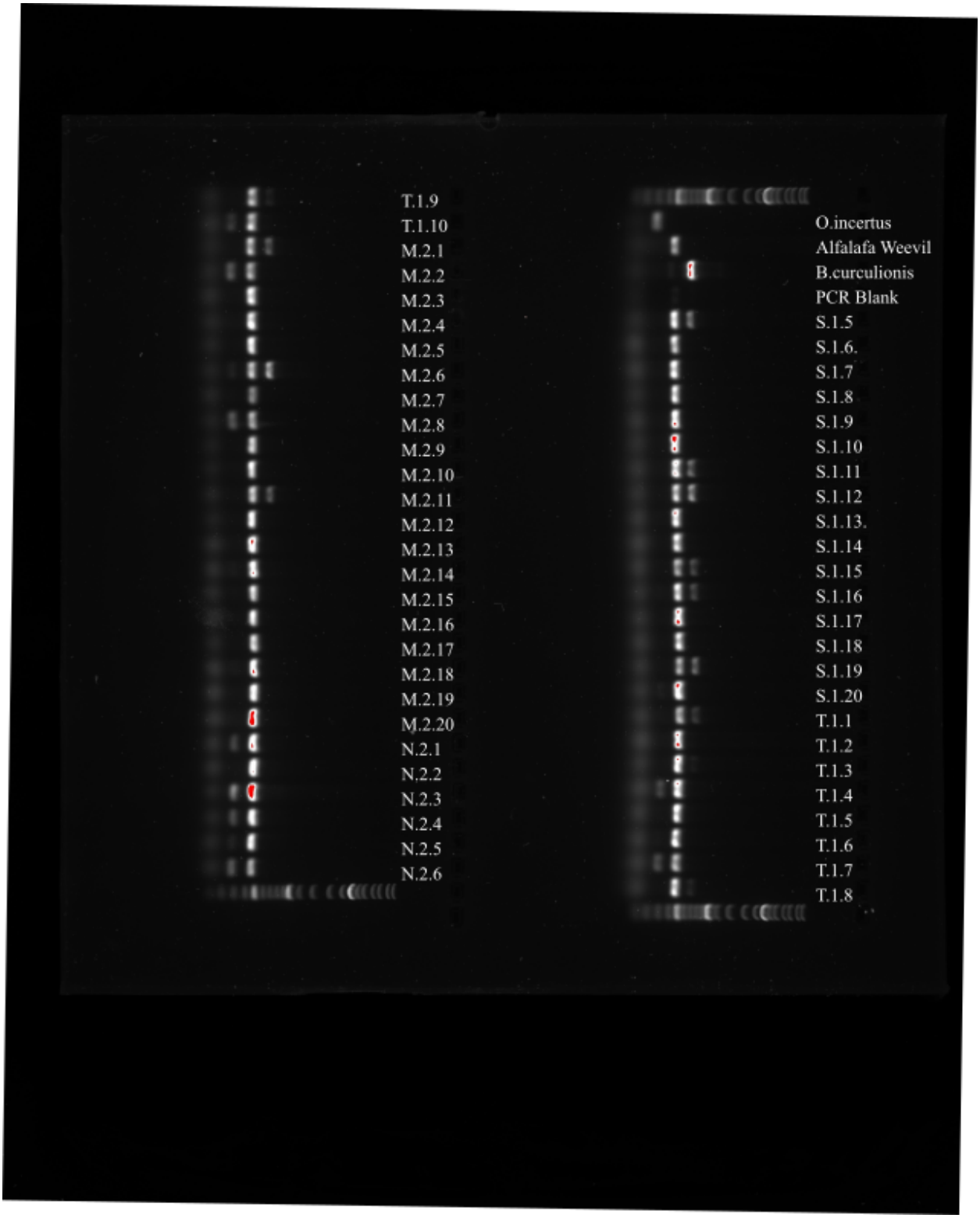
P.1.1
Q.1.1

Gel 9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M.2.8
M.2.9
M.2.10
M.2.11
M.2.12
M.2.13
M.2.14
M.2.15
M.2.16
M.2.17
M.2.18
M.2.19
M.2.20
N.2.1
N.2.2
N.2.3
N.2.4
N.2.5
N.2.6
O.incertus
Alfalafa Weevil
B.curculionis
PCR Blank
S.1.5
S.1.6.
S.1.7
S.1.8
S.1.9
S.1.10
S.1.11
S.1.12
S.1.13.
S.1.14
S.1.15
S.1.16
S.1.17
S.1.18
S.1.19
S.1.20
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T.1.4
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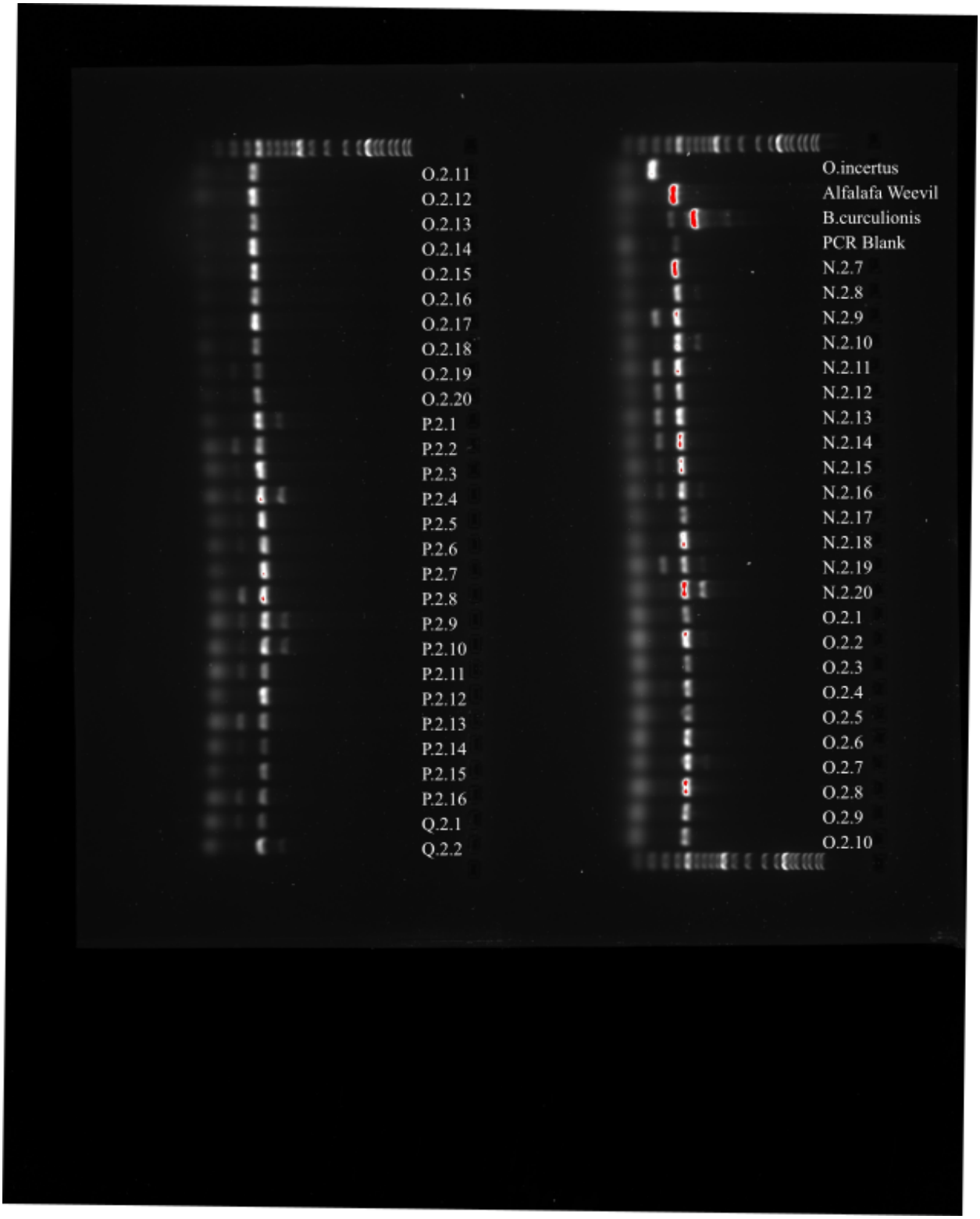
T.1.6
T.1.7
T.1.8

Gel 10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O.2.20
P.2.1
P.2.2
P.2.3
P.2.4
P.2.5
P.2.6
P.2.7
P.2.8
P.2.9
P.2.10
P.2.11
P.2.12
P.2.13
P.2.14
P.2.15
P.2.16
Q.2.1
Q.2.2
O.incertus
Alfalafa Weevil
B.curculionis
PCR Blank
N.2.7
N.2.8
N.2.9
N.2.10
N.2.11
N.2.12
N.2.13
N.2.14
N.2.15
N.2.16
N.2.17
N.2.18
N.2.19
N.2.20
O.2.1
O.2.2
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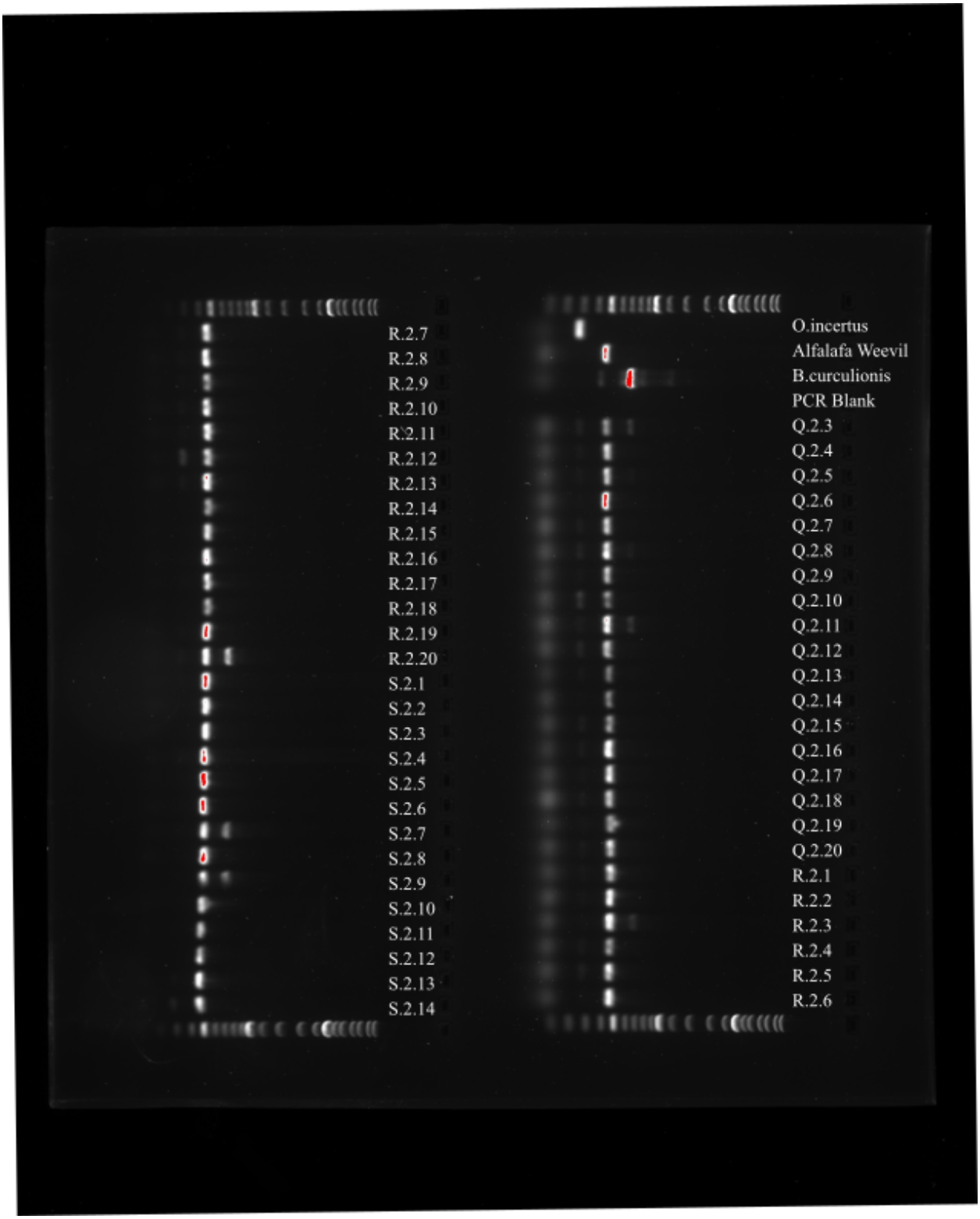
O.2.5
O.2.6
O.2.7
O.2.8
O.2.9
O.2.10

Gel 11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R.2.14
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R.2.16
R.2.17
R.2.18
R.2.19
R.2.20
S.2.1
S.2.2
S.2.3
S.2.4
S.2.5
S.2.6
S.2.7
S.2.8
S.2.9
S.2.10
S.2.11
S.2.12
S.2.13
S.2.14
O.incertus
Alfalafa Weevil
B.curculionis
PCR Blank
Q.2.3
Q.2.4
Q.2.5
Q.2.6
Q.2.7
Q.2.8
Q.2.9
Q.2.10
Q.2.11
Q.2.12
Q.2.13
Q.2.14
Q.2.15
Q.2.16
Q.2.17
Q.2.18
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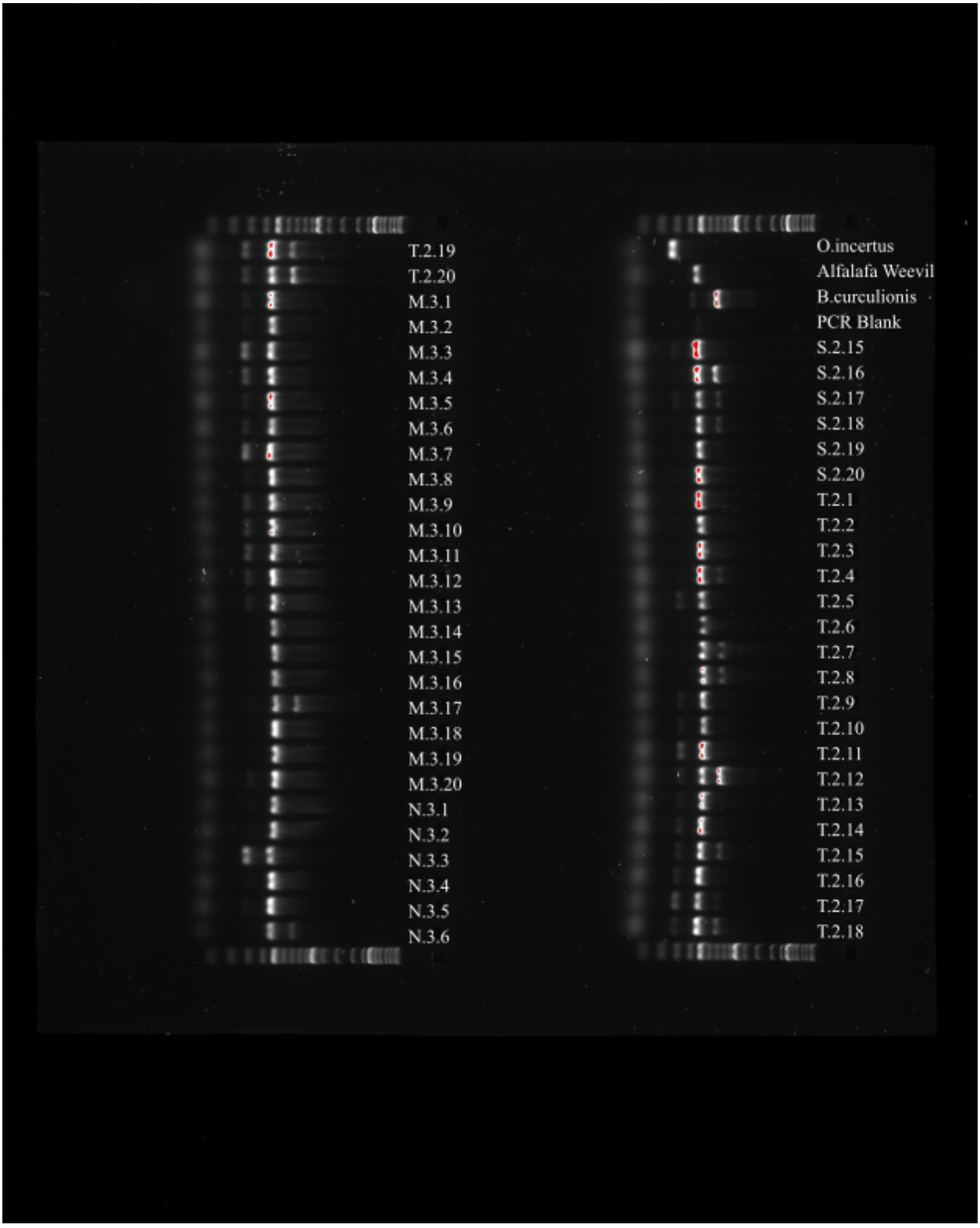
R.2.4
R.2.5
R.2.6

Gel 12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M.3.8
M.3.9
M.3.10
M.3.11
M.3.12
M.3.13
M.3.14
M.3.15
M.3.16
M.3.17
M.3.18
M.3.19
M.3.20
N.3.1
N.3.2
N.3.3
N.3.4
N.3.5
N.3.6
O.incertus
Alfalafa Weevil
B.curculionis
PCR Blank
S.2.15
S.2.16
S.2.17
S.2.18
S.2.19
S.2.20
T.2.1
T.2.2
T.2.3
T.2.4
T.2.5
T.2.6
T.2.7
T.2.8
T.2.9
T.2.10
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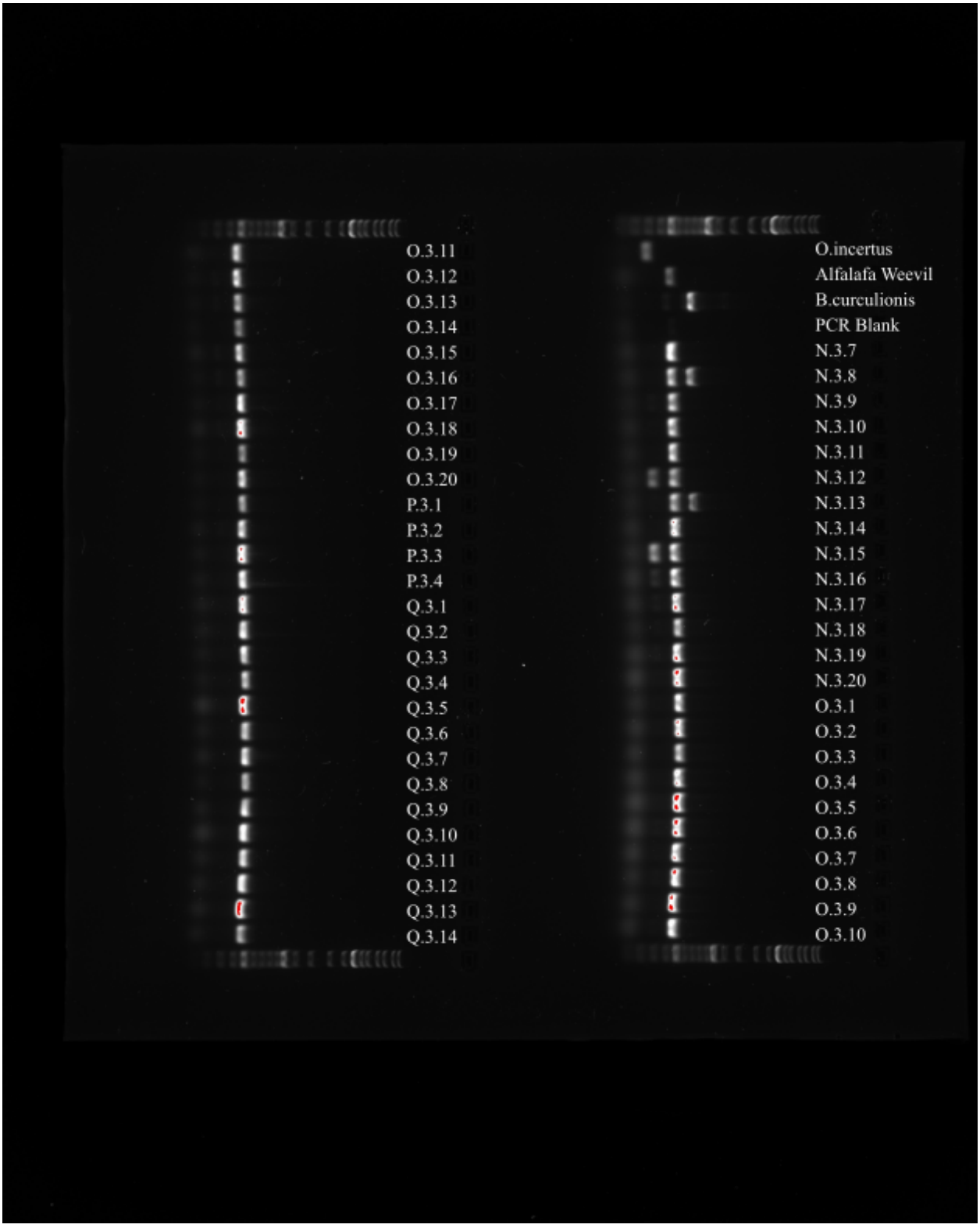
T.2.16
T.2.17
T.2.18

Gel 13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O.3.18
O.3.19
O.3.20
P.3.1
P.3.2
P.3.3
P.3.4
Q.3.1
Q.3.2
Q.3.3
Q.3.4
Q.3.5
Q.3.6
Q.3.7
Q.3.8
Q.3.9
Q.3.10
Q.3.11
Q.3.12
Q.3.13
Q.3.14
O.incertus
Alfalafa Weevil
B.curculionis
PCR Blank
N.3.7
N.3.8
N.3.9
N.3.10
N.3.11
N.3.12
N.3.13
N.3.14
N.3.15
N.3.16
N.3.17
N.3.18
N.3.19
N.3.20
O.3.1
O.3.2
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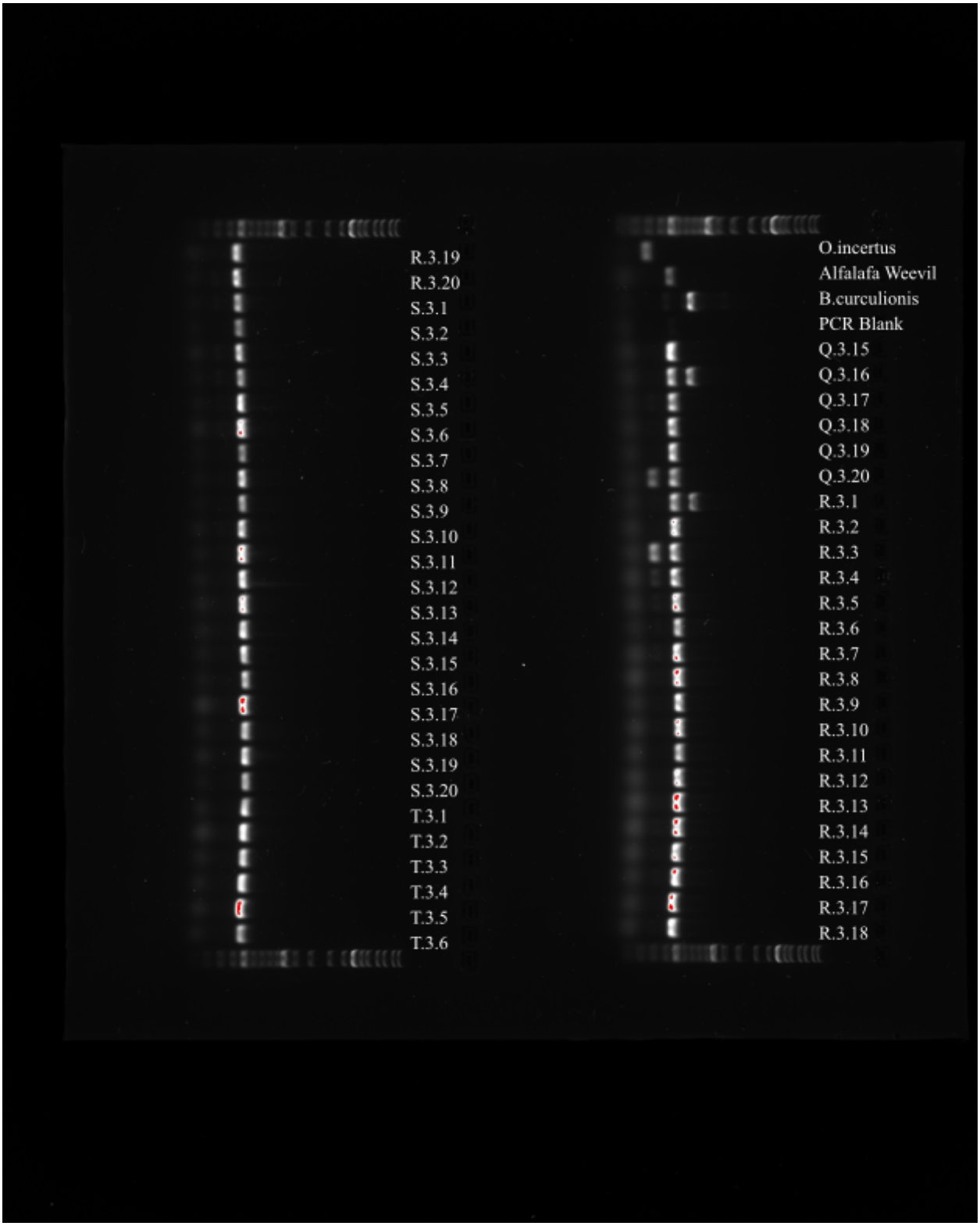
O.3.6
O.3.7
O.3.8
O.3.9
O.3.10

Gel 14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S.3.10
S.3.11
S.3.12
S.3.13
S.3.14
S.3.15
S.3.16
S.3.17
S.3.18
S.3.19
S.3.20
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T.3.2
T.3.3
T.3.4
T.3.5
T.3.6
O.incertus
Alfalafa Weevil
B.curculionis
PCR Blank
Q.3.15
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Q.3.18
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R.3.6
R.3.7
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R.3.9
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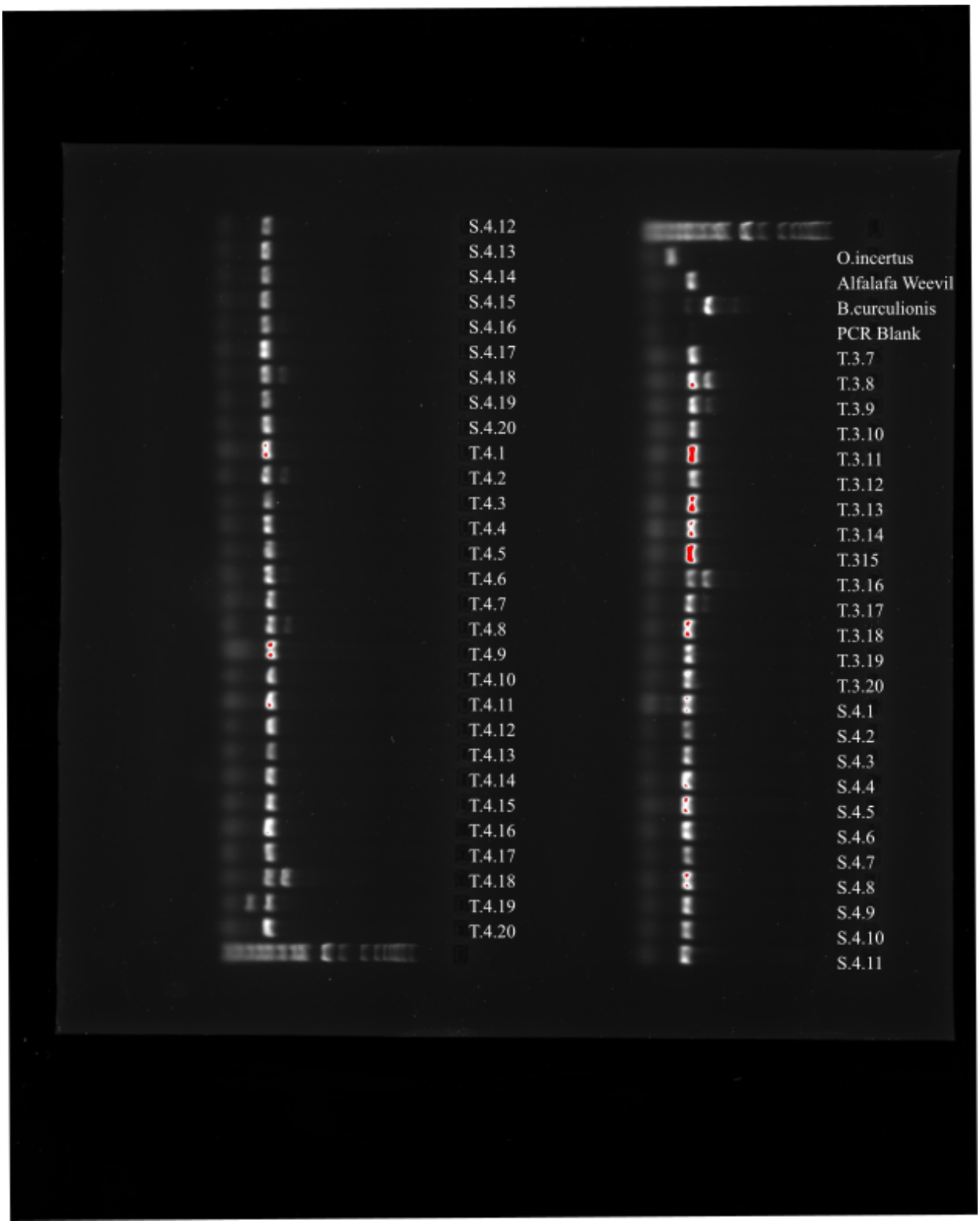
R.3.17
R.3.18

Gel 15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T.4.6
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T.4.9
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T.4.11
T.4.12
T.4.13
T.4.14
T.4.15
T.4.16
T.4.17
T.4.18
T.4.19
T.4.20
O.incertus
Alfalafa Weevil
B.curculionis
PCR Blank
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T.3.11
T.3.12
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T.3.14
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T.3.16
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T.3.18
T.3.19
T.3.20
S.4.1
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S.4.3
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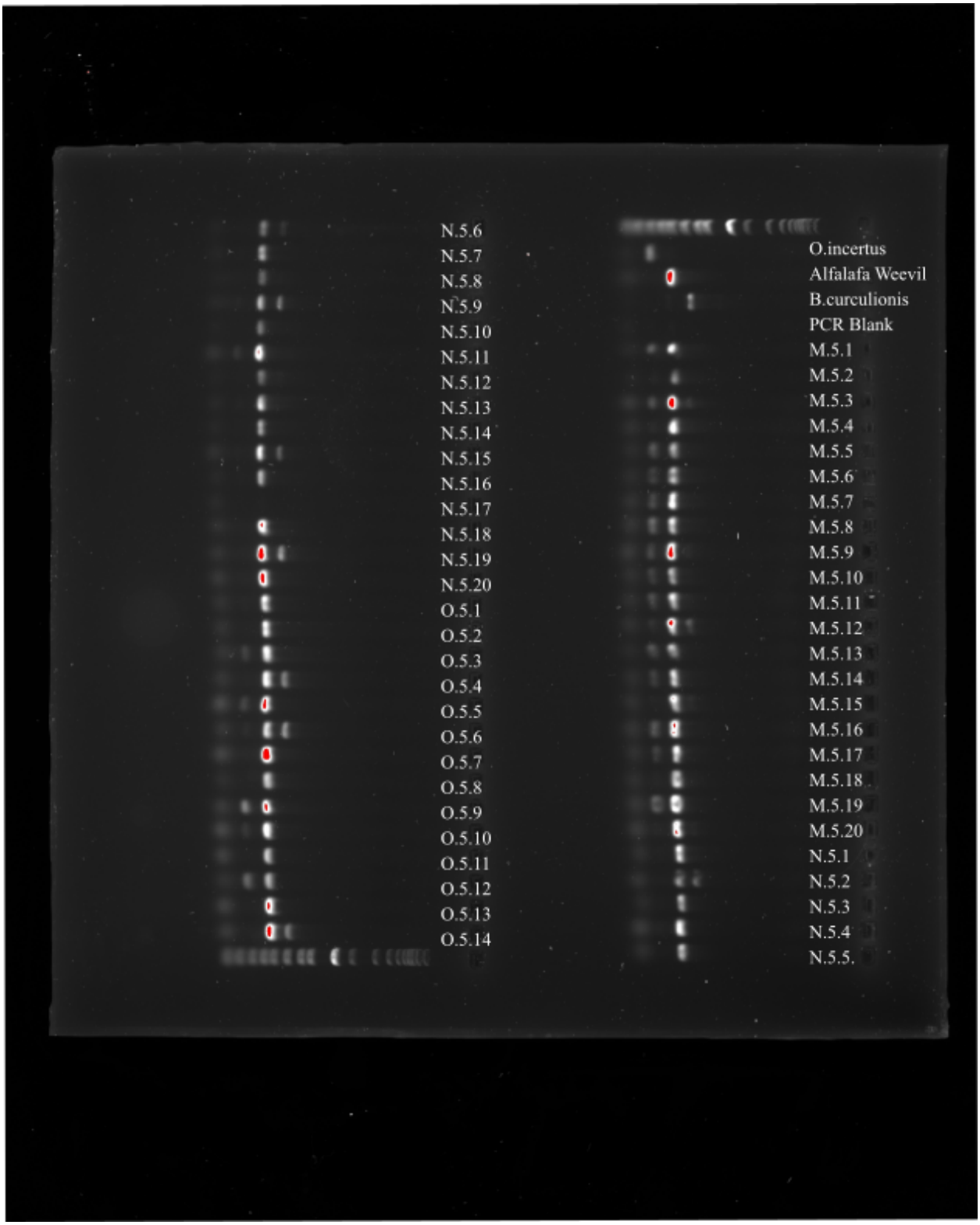
S.4.11

Gel 16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O.5.3
O.5.4
O.5.5
O.5.6
O.5.7
O.5.8
O.5.9
O.5.10
O.5.11
O.5.12
O.5.13
O.5.14
O.incertus
Alfalafa Weevil
B.curculionis
PCR Blank
M.5.1
M.5.2
M.5.3
M.5.4
M.5.5
M.5.6
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M.5.8
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M.5.12
M.5.13
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M.5.15
M.5.16
M.5.17
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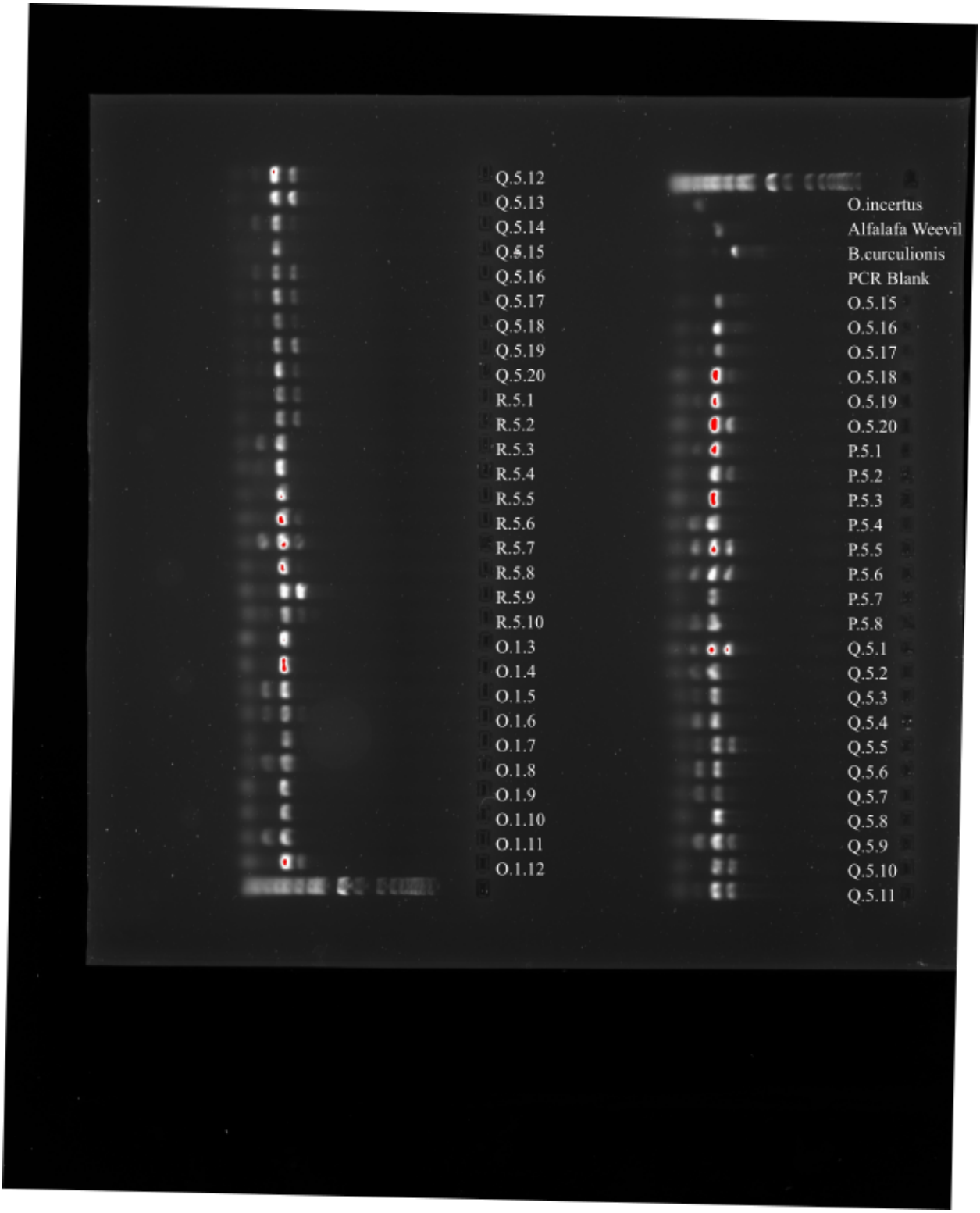
N.5.5.

Gel 17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R.5.7
R.5.8
R.5.9
R.5.10
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O.1.4
O.1.5
O.1.6
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O.1.10
O.1.11
O.1.12
O.incertus
Alfalafa Weevil
B.curculionis
PCR Blank
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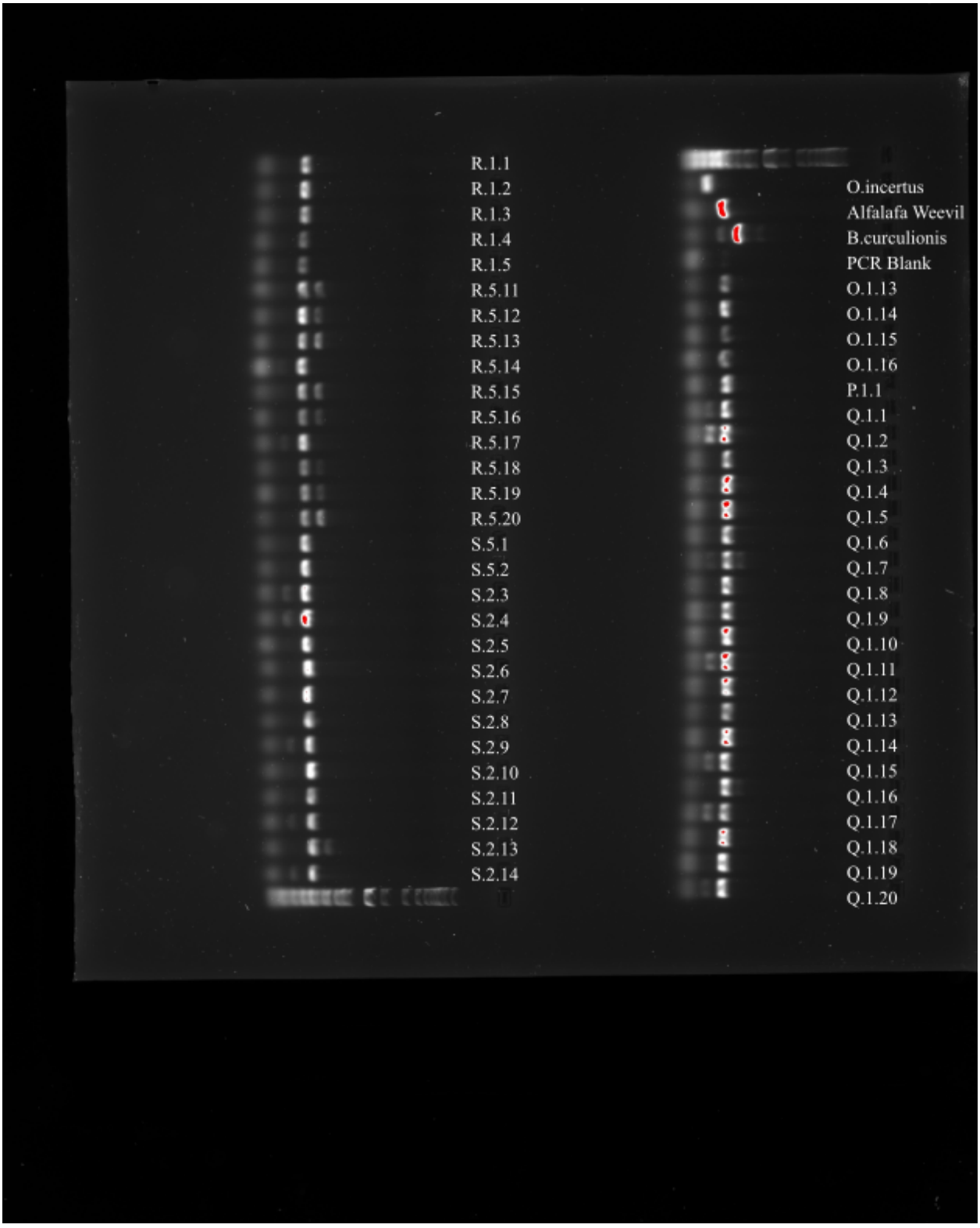
Q.5.11

Gel 18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R.5.16
R.5.17
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S.2.5
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S.2.9
S.2.10
S.2.11
S.2.12
S.2.13
S.2.14
O.incertus
Alfalafa Weevil
B.curculionis
PCR Blank
O.1.13
O.1.14
O.1.15
O.1.16
P.1.1
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Q.1.2
Q.1.3
Q.1.4
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Q.1.12
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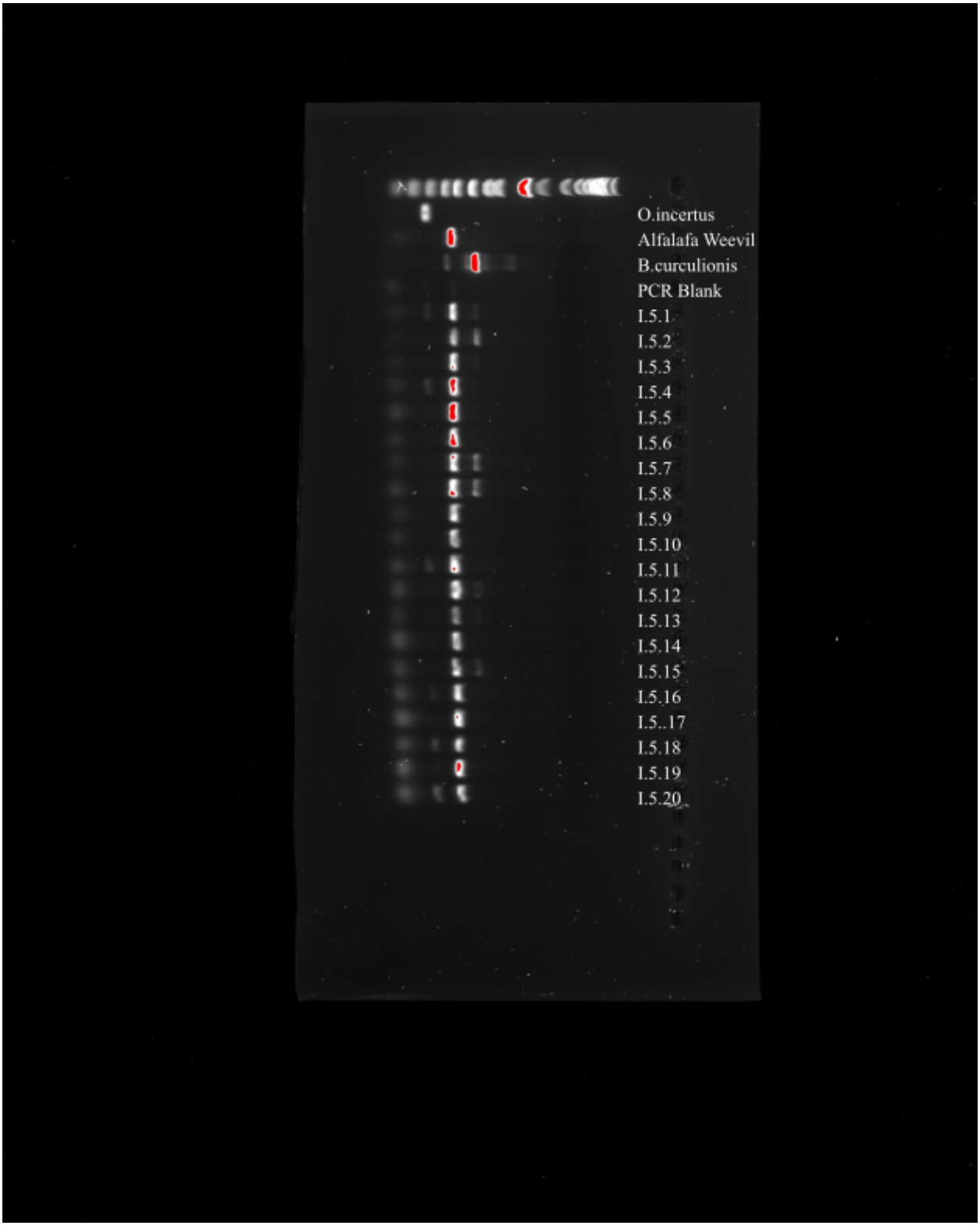
Q.1.20

Gel 19


O.incertus
Alfalafa Weevil
B.curculionis
PCR Blank
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I.5.8
I.5.9
I.5.10
I.5.11
I.5.12
I.5.13
I.5.14
I.5.15
I.5.16
I.5..17
I.5.18
I.5.19
I.5.20

Appendix III

Confirmation of species identity of amplicons from excised bands sequenced at the Molecular Biology Service Unit at the University of Alberta on an ABI 3730 Genetic Analyzer

R.4.4 Gel 5. *Oomyzus incertus* Location: 50.598755, -111.799141 Collector: M. Reid

CGGACCNCNCCTGGCTGAGGGTCGTTCTTAAGNNNANCCAGACTGCTCGTCTTCGTG
GCGAGCGTATACCTGANNGTTCGTCGGGGNCCNCCTCGCGTGGTTGCCTCGGTGTCG
CTCTAAACNAATCNAACGANTTGTGTTGTATCGAACGT

L.6 Gel 4. *Oomyzus incertus* Location: 50.097190, -111.800336 Collector: M. Reid

TGCGGTNNTCGGNNNCGATTCCCGGACCNCGCCTGGCTGAGGGTCGTTCTTAAGTTC
AAACCAGACTGCTCGTCTTCGTGGCGAGCGTATACCTGAGCGTTCGTCGGGGCCCAC
CTCGCGTGGTTGCCTCGGTGTCGCTCTAAACGAATCGAACGACTTGTGTTGTATCGA
ACGTTCGCGAAAAAACGAACGAGCGAGAATAGAGTACACAGAGTACGAGCACACG
C

Q.4.7 Gel 6. *Oomyzus incertus* Location: 50.585833, -111.803377 Collector: M. Reid

CNNGNCGTTCGATTCGTTTAGAGCGACACCGAGGCAACCACGCGAGGTGGGCCCCG
ACGAACGCTCAGGTATACGCTCGCCACGAAGACGAGCAGTCTGGTTTGAACTTAAG
AACGACCCTCAGCCAGGCGTGGTCCGGGAATCNTATCCGAGGACCGCAATGTGCGT
TCGAAATGTCGATGTTCATGTGTCCTGCAGNTCAC

M.1.2 Gel 7. *Oomyzus incertus* Location: 50.759777, -112.105883 Collector: M. Reid

AAGTCGTTCGATTCGTTTAGAGCGACACCGAGGCAACCACGCGAGGTGGGCCCCGA
CGAACGCTCAGGTATACGCTCGCCACGAAGACGAGCAGTCTGGTTTGAACTTAAGA
ACGACCCTCAGCCAGGCGTGGTCCGGGAATCGTATCCGAGGACCGCAATGTGCGTT
CGAAATGTC GATGTTCATGTGTCCTGCAGTTCACA

P.4.17 Gel 17. *Bathyplectus curculionis* Location: 50.052900, -111.679921 Collector: M. Reid

TTNCGNAACACGACGCGCGCGCTCACGGANNNNNCGCATCTGTCCTTAAAACTAAG
TGATAGCTCCGGGGACTCGATCGACCGGCCGTTGAGCAGCGGCGTTCGCTGTTCAAT
CGTGTGCGCGACTGAATTACGCACGAGAAGCGAAGCGAGGACCAACGCGGTTCGGG
GTCATGCCCTTCCATCANCGNACNNTCACGACACTTGCGCACGCGCGNTCACGCACA
AATGNCAATGAN

L.7 Gel 5. *Oomyzus incertus* Location: 50.097190, -111.800336 Collector: M. Reid

CACAAGTCGTTAGAGCGACACCGAGGCAACCACGCGAGGTGGGNCCCGACGAACG
CTCA

I.7 Gel 5. *Bathyplectus curculionis* Location: Collector: M. Reid

ANTTGTGTTGTAATTCTTGCGCAACACGACGCGCGCGCTCACGGATCGNTNNNNNNN
NNNCTNAAAACTAAGTGATAGC
TCCGGGGACTCGANNNNCNNNCCGNTGAGCAGCGGCGTTCGCTGTTCAATCGTGTG
CGCGACTGAATTA

T.1.8 Gel 9. *Bathyplectus curculionis* Location: Collector: M. Reid

CGCGCGCTCACGGATCGGTCGCATCTGTCCTTAAAACTAAGTGATAGCTCCGGGGAC
TCGATCGACCGGCCGTTGAGCAGCGGCGTTCGCTGTTCAATCGTGTGCGCGACTGAA
TTACGCACGAGAAGCGAAGCGAGGACCAACGCGGTTCGGGGTCATGCCCTTCCATC
AACGCACGCTCACGACACTTGCGCACGCGCGTTCACGCACAAATGTCAATGAATGT
GGTTGTTTGTCTCTCGGTTTTTCCGCTCAGCTCAAATTTTGGCTCAACTCGATTCTCAT
AAAATCTTGCGCGCTCGTACNCGAAANTCCTNGNGTAAATAAAGGATACGCGTAAC
GGGCGCTCCTCNNNGTCCGGATCNAGANACTCGACCTCCNNGACCNGCNATTA

T.1.9 Gel 9. *Bathyplectus curculionis* Location: 49.70430, -112.75877 Collector: M.Reid

CCCGGACCNCGCCTGGCTGAGGGTCGTTTACGCATAAAATTAAGACTGCTCTTGCGA
TTGTTTCGCGAGCGAATGTATTGGGCGTTCGTCGATGGCGTATAATACCGGCCGTTG
CGCCGGTGAGCAATCGGCGTCGCTTGAAATAACGTAATCGCTGGTCACGGAGGTCG
AGTCTCTCGATCCGGACCGCGAGGAGCGCCCGTTACGCGTATCCTTCATATACACAA
GGATTTTCGCGTACGAGCGCGCAAGATTCTATGAGAATCGAGTTGAGCCAAAATTTG
AGCTGAGCGGAAAAACCGAGAGACAAACAACCACATTCATTGACATTTGTGCGTGA
ACGCGCGTGCGCAAGTGTCGTGAGCGTGCGTTGATGGAAGGGCATGACCCCGAACC
GCGTTGGTCCTCGCTTCGCTTCTCGTGCGTAATTCAGTCGCGCACACGATTGAACAG
CGAACGCCGCTGCTCAACGGCCGGTCGATCGAGTCCCCGGAGCTATCACTTAGTTTT
AAGGACAGATGCGACCGATCCGTGAGCGCGCGCGTCGTGTTGCGCAAGAATTACAA
CACNNNTACGAGAATGCTGAGCTGACGAGACTCGAATAACGCCGAATGGTTTGCGA
A

N.2.5 Gel 10. *Oomyzus incertus* Location: 50.730869, -112.084387 Collector: M. Reid

ATTCCCGGACCNCGCCTGGCTGAGGGTCGTTCTTAAGTTCAAACCAGACTGCTCGTC
TTCGTGGCGAGCGTATACCTGAGCGTTCGTCGGGGCCCACCTCGCGTGGTTGCCTCG
GTGTCGCTCTAAACGAATCGAACGACTTGTGTTGTATCGAACGTTCGCGAAAAAACG
AACGAGCGAGAATAGAGTACACAGAGTACGAGCACACGC

N.2.8 Gel 10. *Bathyplectus curculionis* Location: 50.730869, -112.084387 Collector: M. Reid

TTNCGCAACACGACGCGCGCGCTCACGGATCGGTCGCATCTGTCCTTAAAACTAAGT
GATAGCTCCGGGGACTCGATCGACCGGCCGTTGAGCAGCGGCGTTCGCTGTTCAATC
GTGTGCGCGACTGAATTACGCACGAGAAGCGAAGCGAGGACCAACGCGGTTCGGGG

TCATGCCCTTCCATCAACGCNCGCTCACNACACTTGCGCACGCGCGNTCACGCACAA
AT

O.2.2 Gel 10. *Bathyplectus curculionis* Location: 50.227368, -112.003781 Collector: M. Reid

CTGAGGGTCGTATCTATTTCAAAGACTGCTCGGNTTTCGTCGGGCGCCCGTAAAACG
GTGCCGGACATAACGTCAGAGCGAGTTGGATGTTTTACGCGTTGTCGTCGTAAGATG
ACGATGCGACATCTTAAAACGCGAAAGCGGCCTGTAAACTAACGAATTTATTCGGA
GGTTTTCCGGCNCAGCAAACGTCNAACGTGCNAGTGTAAACTTGTACGCGAGGTAT
ATATATACNNTTGCGCNNGNNTATATANNNATCGNCATGTCCGANNTTTNTATTGAA
AANNNNGAAACAGACNNNNGATCNTTANNCTTNNANNAAGAACGTTCTAGAA

O.2.7 Gel 10. *Bathyplectus curculionis* Location: 50.227368, -112.003781 Collector: M. Reid

GTTCCCGGACCACTCCTGGCTGAGGGTCGTATCTATTTCAAANACTGCTCNGTTTTCN
TCNGGCGCCCGTAAAACGGTGCCGGACATAACGTCNNAGCGAGTTGGATGTTTTAC
NCGTTGTCGTCNTAAGATGACGATGCNACATCTTAAAACGCGAAAGCGGCCTGNNN
NCTAACGAATTTATTCGGAGGTTTTCCGGCACANCAAACGTCGAACGTGCGAGTGTA
AACTTGTNCGCGAGGTNTNTNTATATATNTNNNCTCNNGCNCNNTTNTATANNGNNA
NNNCGTGNNANATATNTCTATNN

P.2.6 Gel 10. *Oomyzus incertus* Location: 50.052900, -111.679921 Collector: M. Reid

CGGACCACGCCTGGCTGAGGGTCGTTCTTAAGTTCAAACCAGACTGCTCGTCTTCGT
GGCGAGCGTATACCTGAGCGTTCGTCGGGGCCCACCTCGCGTGGTTGCCTCGGTGTC
GCTCTAAACGAATCGAACGACTTGTGTTGTATCGAACGTTCGCGAAAAAACGAACG
AGCGAGAATAGAGTACACAGAGTACGAGC

Q.2.1 Gel 10. *Oomyzus incertus* Location: 50.585833, -111.803377 Collector: M. Reid

GGNTNCGATTCCCGGACCNCGCCTGGCTGAGGGTCGTTCTTAAGTTCAAACCAGACT
GCTCGTCTTCGTGGCGAGCGTATACCTGAGCGTTCGTCGGGGCCCACCTCGCGTGGT
TGCCTCGGTGTCGCTCTAAACGAATCGAACGACTTGTGTTGTATCGAACGTTCGCGA
AAAAACGAACGAGCGAGAATAGAGTACACAGAGTACG

Q.2.3 Gel 11. *Oomyzus incertus* Location: 50.585833, -111.803377 Collector: M. Reid

ANTCCCGGACCACGCCTGGCTGAGGGTCGTTCTTAAGTTCAAACCAGACTGCTCGTC
TTCGTGGCGAGCGTATACCTGAGCGTTCGTCGGGGCCCACCTCGCGTGGTTGCCTCG
GTGTCGCTCTAAACGAATCGAACGACTTGTGTTGTATCGAACGTTCGCGAAAAAACG
AACGAGCGAGAATAGAGTACACAGAGTACGAGCACACGNNNNNNGTGCGAGTGTA
A

Q.2.8 Gel 11. *Oomyzus incertus* Location: 50.585833, -111.803377 Collector: M. Reid

GANACNATTCCCGGACCNCGCCTGGCTGAGGGTCGTTCTTAAGTNNNNNNNNNANN
GCTCGTCTTCGTGGCGAGCGTATACCTGAGCGTTCGTCGGGGCCCACCTCGCGTGGT
TGCCTCGGTGTCGCTCTAAACGAATCGAACGACTTGTGTTGTATCGAACGTTCGCGA
AAAAACGAACGAGCGAGAATAGAGTACACAGAGTACGAGCACACGCATAA

Q.2.10 Gel 11. *Oomyzus incertus* Location: 50.585833, -111.803377 Collector: M. Reid

GANTCCCGGACCACGCCTGGCTGAGGGTCGTTCTTAAGTTCAAACCAGACTGCTCGT
CTTCGTGGCGAGCGTATACCTGAGCGTTCGTCGGGGCCCACCTCGCGTGGTTGCCTC
GGTGTCGCTCTAAACGAATCGAACGACTTGTGTTGTATCGAACGTTCGCGAAAAAAC
GAACGAGCGAGAATAGAGTACACAGAGTACGAGCACACGNNNNNNGTGCGAGTGT
AAACTTGTACGCGAGGTATATATATANNTATNCTCTTGCGCAGNTNTATATAGCGAT
CGNCGNGNCCNATATTTNTATTGNAAAAACGGANACANAC

Q.2.8 Gel 11. *Bathyplectus curculionis* Location: 50.585833, -111.803377 Collector: M. Reid

CCTCGGNTTCTGTTCCCGGACCNCTCCTGGCTGANGGTCGTATCTATTTCNANACTG
NTCNGNTTTCNTCNGGCGCCCGTNAAACGGTGCCGGACNTAACGTCNNAGCGAGTT
GGATGTTTTACNCGTTGTCGTCNTAAGATGACGATGCNACNTCTTAAAACNCNAAAG
CGGCCTGTNNACTAACGAATTTATTCNGAGGTTTTCCGGCNCANCNAACGTCGAACG
TGCGAGTGTAAACTTGTNCNCGAGGTAT

Q.2.11 Gel 11. *Bathyplectus curculionis* Location: 50.585833, -111.803377 Collector: M.Reid

GGANCACGTCCTGGCTGAGGGTCGTTTNNNCATNAAATTANTGACTGCTCTTGNNAT
TGTTTCCCGAGCGAATGTATNGGGNNNNCGTCNNNGNCNNATNNNACNNGNCGNTG
CNCCGGTNANCNATNNNCNNNGCTTGANNTAACNTAATCACTGGTCNCGGAGGNCN
AGTCTCTCGATCCNGANNNNNAGGANCGCCCGTTACNCGTATCCTTNNNNTNNNCA
NNGATTTTCNCNNNNNANCGCNCAANATTCTATGANAATCNAGTTGANCCAAAATT
TGANCTGANNNNANNAACCGANAGACNAACAACCACATTCNTTGACNTTTGTGCGT
GAACGCGCGTGCGCNAGTGTCGTGAGCGTGCGTTGATGGAAGGGCNTGACCCCNAA
CCGCNTTNNNCCTCGCTTCGCTTCTCGTGCGTAATTCAGTCGCGCACACGATTGAAC
AGCGAACGCCGCTGCTCAACGGCCGGTCGATCGAGTCCCCGGAGCTATCACTTAGTT
TTAAGGACAGATGCGACCGATCCGTGAGCGCGCGCGTCGTGTTGCGCAAGAATTAC
CACACAAGTACGAGAATGCTGAGCTGACGAGACTCGAATAACGC

R.5.7 Gel 17. *Bathyplectus curculionis* Location: 50.598755, -111.799141 Collector: M. Reid

GCGCGCTCACGGATCGGTCGCATCTGTCCTTAAAACTAAGTGATAGCTCCGGGGACT
CGATCGACCGGCCGTTGAGCAGCGGCGTTCGCTGTTCAATCGTGTGCGCGACTGAAT
TACGCACGAGAAGCGAAGCGAGGACCAACGCGGTTCGGGGTCATGCCCTTCCATCA
ACGCACGCTCACGACACTTGCGCACGCGCGTTCACNNNCAAA

R.5.6 Gel 17. *Oomyzus incertus* Location: 50.598755, -111.799141 Collector: M. Reid

ACGATTCCCGGACCNCGCCTGGCTGAGGGTCGTTCTTAAGTTCAAACCAGACTGCTC
GTCTTCGTGGCGAGCGTATACCTGAGCGTTCGTCGGGGCCCACCTCGCGTGGTTGCC
TCGGTGTCGCTCTAAACGAATCGAACGACTTGTGTTGTATCGAACGTTCGCGAAAAA
ACGAACGAGCGAGAATAGAGTACACAGAGTACGAGCACACGCATA