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The ground beetle *Pseudophonus rufipes* reveals as predator of *Ceratitis capitata* in citrus orchards

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Abstract

The Mediterranean fruit fly *Ceratitis capitata* (Wiedemann) is a major citrus pest in Spain. Although medfly is being controlled chemically, alternative biorational strategies should be developed, like those based on conservation biological control of polyphagous predators. The ground beetle *Pseudophonus rufipes* (De Geer) is the most abundant carabid inhabiting the ground of citrus orchards in eastern Spain. However, little is known about its activity-density and predatory role against *C. capitata* in the citrus agroecosystem. Here we report on its predatory potential on the medfly's developmental stages that take place in the citrus ground. This carabid species preyed efficiently on *C. capitata* third-instar larvae and especially on pupae but not on teneral adults. Moreover, predation under field conditions was evaluated by detecting *C. capitata* DNA remains using PCR-based gut-content analysis. Half-life DNA detectability of *C. capitata* was of 32.33 h. *Pseudophonus rufipes* specimens were field-collected after *C. capitata* sterilized pupae were deployed in a commercial citrus orchard. Thereafter, the carabids re-captured by pitfall traps were analyzed, being DNA-remains of *C. capitata* detected in 22.9% of them. Data reported here clearly suggest that *P. rufipes* could play an important role in regulating medfly populations in citrus orchards. This information is particularly useful when biological control conservation strategies are being considered to control this pest.

Keywords: Mediterranean fruit fly, carabids, predators, functional response, DNA prey detection.

1. Introduction

The Mediterranean fruit fly or medfly *Ceratitis capitata* (Wiedemann) (Diptera: Tephritidae) is one of the most devastating fruit pests worldwide. Hitherto, medfly control has primarily been based on aerial and ground applications of organophosphate insecticides and, more recently, on the use of the naturally derived compound spinosad (Chueca et al*.,* 2007; Urbaneja et al., 2009). Nevertheless, chemical approaches might affect adversely the environment, and induce resistance (Magaña et al., 2007, 2008). In recent years, research efforts have focused on developing environmentally friendly methods to control medfly. To this end, different biological control approaches are currently being implemented in Spanish citrus orchards, one of which involves the identification and conservation of polyphagous ground-dwelling predators of the medfly (Urbaneja et al., 2006; Monzó, 2009).

Generalist predators play a major role in biological control of agricultural pests (Legaspi et al., 1996; Morris et al., 1999; Symondson et al., 1996, 2002a,b; Sheppard et al., 2004; Foltan et al., 2005). Ground-dwelling beetles (Coleoptera: Carabidae) are considered one of the most important generalist predators having a long-standing tradition in pest management strategies in Central European agriculture. Indeed, there are many reviews assessing their role as biological control agents (Luff, 1987; Lövei and Sunderland, 1996; Kromp, 1999). Most of these works describe carabids as aphid predators, although a few species have been identified as predators of larvae and pupae of Diptera (Allen, 1990; Tolonen, 1995 and Lys, 1995).

Citrus orchards in the Mediterranean basin afford the potential to maintain semi-permanent ground habitats that host a rich and abundant complex of ground-dwelling natural enemies (Monzó et al., 2005; Urbaneja et al., 2006; Aguilar-Fenollosa et al., 2009). *Pseudophonus rufipes* De Geer (Coleoptera: Carabidae) has been described as the most abundant carabid on the ground surface of Spanish citrus orchard floor, comprising half of all the ground-dwelling beetles captured (Monzó et al., 2005). This

species, able to adapt to a wide range of environments, is one of the dominant ground-dwelling beetles in numerous crops in different regions of the Northern Hemisphere (Coaker and Williams, 1963; Jones, 1976b; Lövei and Sárospataki, 1990; Farinós et al., 2009; Miñarro et al., 2009). *Pseudophonus rufipes* has previously been reported as a pest control agent of different pests (Kromp, 1999). Holopainen and Helenius (1992) concluded that this carabid contributed to the suppression of *Rhopalosiphum padi* L. (Hemiptera: Aphidae) populations in a spring barley field in Finland. It has also been documented as an effective predator of the potato beetle *Leptinotarsa decemlineata* (Say) (Coleoptera: Chrysomelidae) and the cereal leaf beetle *Oulema melanopus* L. (Coleoptera: Chrysomelidae) (Sorokin, 1981; Bartl, 1997). However, its role as a biological control agent in citrus remains unknown.

There are three medfly developmental stages that are susceptible to being preyed upon by ground dwelling predators: late third instars larvae, that jump from the fruit to pupate underground, pupae and teneral adults, which remain on the soil until they are able to fly. Although *P. rufipes* is known to feed upon *C. capitata* pupae under laboratory conditions (Urbaneja et al., 2006), there are no studies assessing whether *P. rufipes* actually feeds on *C. capitata* in the field, and more importantly, if this carabid can contribute to the control of this pest.

Here, we report on the functional response of *P. rufipes*, to the three developmental stages of *C. capitata* that can be found on the citrus-grove ground. Moreover, we were able to assess whether *P. rufipes* preys on *C. capitata* under field conditions when alternative preys are available by using a *C. capitata*-specific pair of primers to detect medfly predation by PCR analysis of the gut content of *P. rufipes* field specimens.

2. Materials and Methods

Functional response on *C. capitata*

The functional response assays were performed using adult specimens of *P. rufipes* collected from citrus fields close to the Instituto Valenciano de Investigaciones Agrarias (IVIA). The carabids were captured by empty pitfall traps. The traps were checked every 24 h, early in the morning to avoid long permanence in the traps due to its nocturnal activity. The captured specimens were transported to the laboratory and once there, to standardize their hunger, they were individually placed in 100 ml plastic containers and starved for 48 h but provided with water supplied on soaked cotton. The plastic containers were placed in a climatic chamber at 25 ± 2ºC, 60 ± 5% RH, and 16:8 (L:D) h photoperiod. All the functional response assays were performed under the same environmental conditions. The sex ratio of the predators used was 1:1 in order to simulate the sex ratio that could be expected under field conditions.

The functional response of *P. rufipes* was obtained for third instars larvae, pupae and teneral adults of *C. capitata*. Medfly individuals were obtained from a laboratory colony, maintained at the IVIA (Valencia, Spain) since 2002 (Monzó et al., 2009a). For each developmental stage treatment, different prey densities (2, 4, 8, 12, 20, 40 and 60) were exposed to starved *P. rufipes* with eight replicates per density, in an arena consisting of a plastic Petri dish (14 cm in diameter and 1.6 cm high) with thin layer of moistened pearlite (Floreal, Agroperlita F-13®; Semillas Diago S.L. Picassent, Valencia SP) to facilitate predator movements. After 24 h, the predator was removed from the arena and the number of individuals preyed on was recorded. Prey was not replaced during the experiment. In the treatment with third-instars larvae, all the individuals that pupated during the experiment were not taken into account for the statistical analysis. In all treatments, water was supplied on soaked cotton. In the treatment with adult medfly, food consisting of a mixture of sugar and hydrolyzed yeast (4:1; w/w) was provided to minimized prey mortality. Finally, to assess natural prey mortality of third instars-larvae and teneral adults, two control treatments (with eight replicates) were performed at a density of 20 preys and without the presence of the predator.

Tracking medfly predation

Detection period

Live adult field specimens of *P. rufipes* were collected in individual 150 ml containers using the same pitfall traps as in the functional response assays. Carabids were starved (water was supplied daily on soaked cotton) for a two-day period, at 25ºC and 16:8 h (L:D) photoperiod. After starvation, one medfly pupae from the IVIA colony was offered to each carabid. Predators were allowed to feed on pupae for a 15-minute period. Thereafter, any remaining prey was withdrawn and digestion time set to zero. Carabids were divided into sets and maintained in starvation (given only water) at 25ºC and 16:8 h L:D each set with a different digestion time: 0 h (n = 15 individuals), 1 h (n = 16), 6 h (n = 15), 12 h (n = 17), 24 h (n = 16), 48 h (n = 18) and 72 h (n = 16). At the end of each digestion time, carabids were frozen at -80ºC for subsequent molecular assay. Additional carabids (n = 11) were starved for two days and frozen for use as negative controls in the PCR.

DNA from each *P. rufipes* specimen was tested in triplicate by PCR (Agustí et al., 2003a). Each sample scored as zero was tested with the universal pair of primers 18S (Monzó et al., 2009b) to assess whether PCR failure was due to a lack of *C. capitata* DNA.

Primers

The *C. capitata* specific pair of primers CcITS737 that amplifies a fragment size of 130 bp (Monzó et al., 2009b) was used to check whether *P. rufipes* resorted to medfly pupae as prey under field conditions. This pair of primers has previously been demonstrated not to react with a wide range of alternative prey, both predators and phytophagous, and to be highly sensitive (Monzó et al., 2009b).

DNA extraction

All *P. rufipes* specimens were frozen (-80ºC, 20 minutes). Guts were removed by

dissection, and total DNA was extracted from the gut contents following the "Salting-out" protocol (Sunnucks & Hales 1996), adding fresh Proteinase-K at 100 µg/ml after tissue homogenization. Total DNA was finally dissolved in 100 µl LTE-R (10mM Tris-HCl pH 8.0, 0.1mM EDTA pH 8.0, 6 µg/ml RNase A). DNA integrity was verified by gel electrophoresis in 1% agarose gel and concentration adjusted to 5-10 ng/µl for PCR amplification.

Amplification conditions

Each primer pair was used in 20 µl volume reactions, containing: 300 nM dNTPs (Eppendorf AG, Hamburg, Deutschland), 1x DNA pol buffer (Biotools B&M labs S.A., Madrid, Spain), 3 mM MgCl2 (Biotools), 0.75 u DNA polymerase (Biotools), 10 pmol each primer, and 10 ng of total DNA. Amplification profile was: one denaturation step at 94ºC for 2 min, 40 cycles at 94ºC for 30 sec, 50ºC for 30 sec, and 72ºC for 15 sec;, followed by a final extension at 72ºC for 2 min. Amplification was performed in a Mastercycler® ep gradient-S thermal cycler (Eppendorf). PCR products were run in a 2% agarose D-1 low EEO (Pronadisa, Sumilab S.L., Madrid, Spain) gel in 0.5x TBE buffer, stained with ethidium bromide and visualized under UV light.

Field assay

The field trial was conducted in the 1-ha clementine citrus orchard located in Bétera described above, where high populations of *P. rufipes* had previously been documented. About 40,000 two-day-old Vienna-8 tsl sterile male pupae were deployed in the orchard to simulate a high natural medfly infestation (Monzó et al., 2009b). The test was performed on September 17th 2007, when this carabid shows a peak of activity (Monzó, 2010). Eight pitfall traps, not baited with the water-ethanol mixture, were installed in the central area of the plot, and were checked 1, 2, 3, 4, 6, 7 and 8 days after medfly pupae release. *Pseudophonus rufipes* adults were collected early in the morning, immediately taken to the laboratory and frozen for later DNA extraction. DNA from each carabid gut content was tested by PCR using the CcITS737 primer pair in

triplicate. Samples that gave a faint positive detection were tested in another PCR screening, adjusting DNA concentrations. The percentage of samples testing positive by PCR was obtained for the whole assay.

Data analysis

Functional response

In the functional response experiments, a logistic regression of the relative proportion of prey killed was performed (Trexler et al., 1988; Juliano, 2001) in order to discriminate between Type II and Type III functional responses. The data were fitted to a polynomial function with intercept, linear and quadratic coefficients using the maximum likelihood method (SPSS, 1999). A positive linear coefficient and a negative quadratic coefficient imply that the data fit a type III functional response whereas a negative linear coefficient means a better adjustment to type II. Once this analysis was performed, data were fitted to the corresponding functional response equation. Because the species used in these assays were predators and there was no prey replacement, we used the “random-predator” equation (Rogers, 1972; Royama, 1971) for a type II functional response equation, for those densities in which not all the prey was consumed before the end of the assay. Therefore, the two-prey density was excluded from the analysis in the treatment using pupae. The data were fitted through a non-linear least-squares regression by means of the Levenberg-Marquardt iterative estimation procedure. The functional response parameters, attack rate and handling time, were extracted from this regression.

Detection period

To determine detection periods under laboratory conditions, data of positive detections were subjected to Probit analysis using Proc Probit in PC SAS version 9.1.3. A Chi-square (χ^2) test was performed to determine the data fit to the Probit model. Detectability half-life (post-ingestion time during which 50% of positives were still detectable) was obtained (Chen et al., 2000).

3. Results

Functional response

Control mortalities were 1.9% and 0% for adults and third-instar larvae, respectively. In the treatment with larvae, the linear and quadratic coefficients of the logistic regression of the proportion of eaten prey were -0.081 ± 0.015 and 0.0008 ± 0.0002, respectively. Both parameters were significant (*df* = 55; linear: χ^2 = 28.99, *P* < 0,001; quadratic: χ^2 = 714.95, *P* < 0.0001). In the treatment with pupae the estimated values were -0.169 ± 0.021 and 0.0015 ± 0.0003, both parameters proving significant too (*df* = 57; linear: χ^2 = 83.60, *P* < 0.001; quadratic: χ^2 = 35.92, *P* < 0.001). In the adult treatment the estimated values were -0.082 ± 0.024 and 0.0009 ± 0.0003, respectively. Both parameter were significant (*df* = 55; linear: χ^2 = 12.24, *P* < 0.001; quadratic: χ^2 = 6.74, *P* < 0.001). Because all the linear coefficients estimated were negative and the quadratic coefficients positive, a type II functional response was obtained from the logistic regression (Figure 1). The highest attack rate was obtained when pupae were offered, whereas the shortest handling times were obtained in the treatments using larvae and pupae as prey (Table 1). Because the estimated attack rate (± SE) value obtained with adults includes 0, predation on this *C. capitata* stage can be considered as negligible.

Tracking medfly predation

The proportion of positive detection data fitted the assumptions of the Probit model for pair of primers used (χ^2 = 5.3756, *df* = 4, *P* = 0.2509) (Figure 2). Detectability half-life was of 32.33 h. A total of 36 *P. rufipes* individuals were captured and analyzed in the field experiment. The proportion of positive detections was 22.2% for the entire assay (Figure 3).

4. Discussion

Our laboratory studies have demonstrated that *P. rufipes* may be an efficient predator of pupae and third-instars larvae but not of teneral adults, being especially voracious when predating on pupae. Carabids often feed on low activity preys. This would explain, in part, their strong preference for the less mobile developmental stages of *C. capitata*. In addition, medfly pupae are similar in size and shape to the seeds preferred by this ground-dwelling beetle species (Honek et al., 2003). When comparing the handling times and attack rates estimated in this study with the ones obtained for *P. cribata* and *F. auricularia* assayed under similar laboratory conditions and using similar prey (Monzó, 2010; Monzó et al., 2009a), *P. rufipes* is the most efficient medfly predator, presenting the highest attack rate estimates and the shortest handling times, both for pupae and larvae. On the other hand, developmental stage preferences differ amongst these ground-dwelling predators. *Pardosa cribata* was the most efficient predator of teneral adults while *F. auricularia* preferred third-instars larvae as prey (Monzó, 2010; Monzó et al., 2009a).

By using PCR-based prey detection analysis we were able to track *C. capitata* predation by the ground beetle *P. rufipes* in citrus orchards, where a wide range of alternative preys and food resources are also present. The calculated DNA detectability half-life for the ITS737 primer pair (32.3 h) is in the range of those reported (18 h to 88.5 h) for other carabid species (Sheppard et al., 2005; Harper et al., 2005; Juen and Traugott, 2007). This value is shorter than that obtained by the lycosid *P. cribata* feeding on *C. capitata* adults, using the same primer pair (72 h) (Monzó et al., 2009b). Due to starvation adaptations, the spiders' gut system is prepared to store ingested food long-term (Harwood et al., 2001). This feature also means that DNA of the prey can be detected for longer (Greenstone et al., 2007).

The good rates of medfly predation under field conditions obtained in this work indicate that *P. rufipes* is able to play an important role in multi-tactic strategies, currently required to control *C. capitata*. Besides, the action of this generalist predator against

this pest should be considered in conjunction with that exerted by other components of the ground-dwelling predatory complex inhabiting Spanish citrus orchards, such as the lycosid *P. cribata* or the earwig *F. auricularia*. All these predators showed different seasonal and circadian activities, medfly developmental stage preferences and foraging strategies (Riechert and Lawrance, 1997; He et al., 2008; Monzó, 2010; Monzó et al., 2009a), therefore complementing their action as medfly control agents. The next step in developing *C. capitata* biocontrol strategies should consider the conservation and augmentation of these ground-dwelling natural enemies in the citrus ecosystem, for example via cover-crop management, thereby improving their potential as biological control agents.

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Tables

Table 1. Estimated attack rates and handling times (± SE) resulting from the fitting to the empirical functional response equations of the *Pseudophonus rufipes* predation values obtained with different prey densities. The parameter estimates were obtained for the three *Ceratitis capitata* stages that are present in the citrus ground.

	Attack rate (d^{-1})		**Handling time (d)**	
	Estimated	± SE	Estimated	± SE
Third-instars larvae	0 .855	± 0 .213	0 .044	± 0 .008
Pupae	3 .07	± 1 .134	0 .048	± 0 .003
Adults	0 .055	± 0 .101	0 .095	± 0 .043

Figure captions

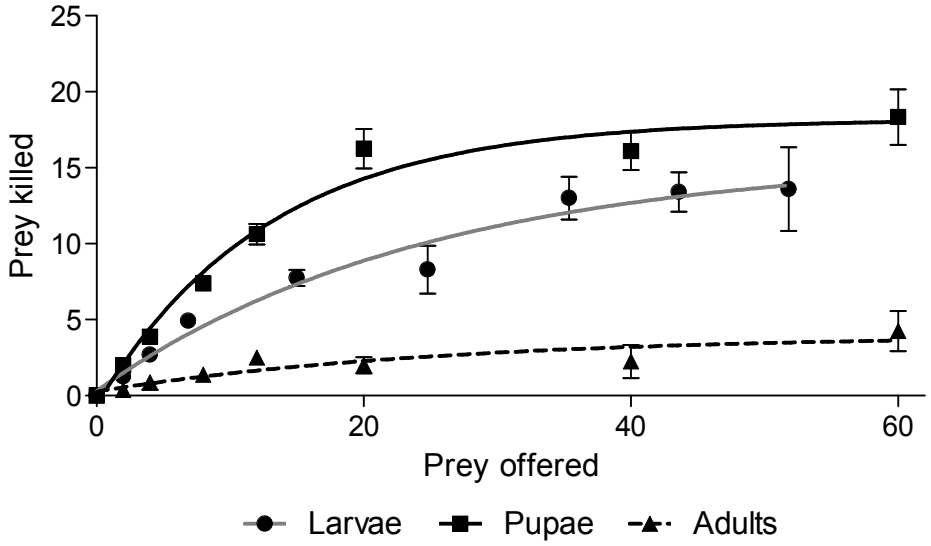
Figure 1. Observed number of *Ceratitis capitata* third-instars larvae, pupae and teneral adults killed by *Pseudophonus rufipes* during 24h and their functional response curves (Type II) fit by non-linear least square.

Figure 2. The *Ceratitis capitata* DNA detection probability curves in *Pseudophonus rufipes* samples after feeding, using CcITS737 primer pair. Line is fitted Probit model with 95% fiducial limits.

Figure 3. *Ceratitis capitata* positive amplifications using CcITS737 primer pair on the gut content of 36 *Pseudophonus rufipes* specimens captured in a Clementine citrus orchard after an artificial medfly pupae infestation. Carabids were captured 1, 2, 3, 4, 6, 7 and 8 days after the infestation. Each sample is named with the day of capture and specimen number. 100pb ladder has been used; C_{-i}, PCR negative controls, *Samples1.8 and 7.1 were tested at two DNA concentrations. **A)** PCR1, **B)** PCR2, **C)** PCR3 and **D)** PCR4.

Figures

Figure 1. Observed number of *Ceratitis capitata* third-instar larvae, pupae and teneral adults killed by *Pseudophonus rufipes* during 24h and their functional response curves (Type II) fit by non-linear least square.



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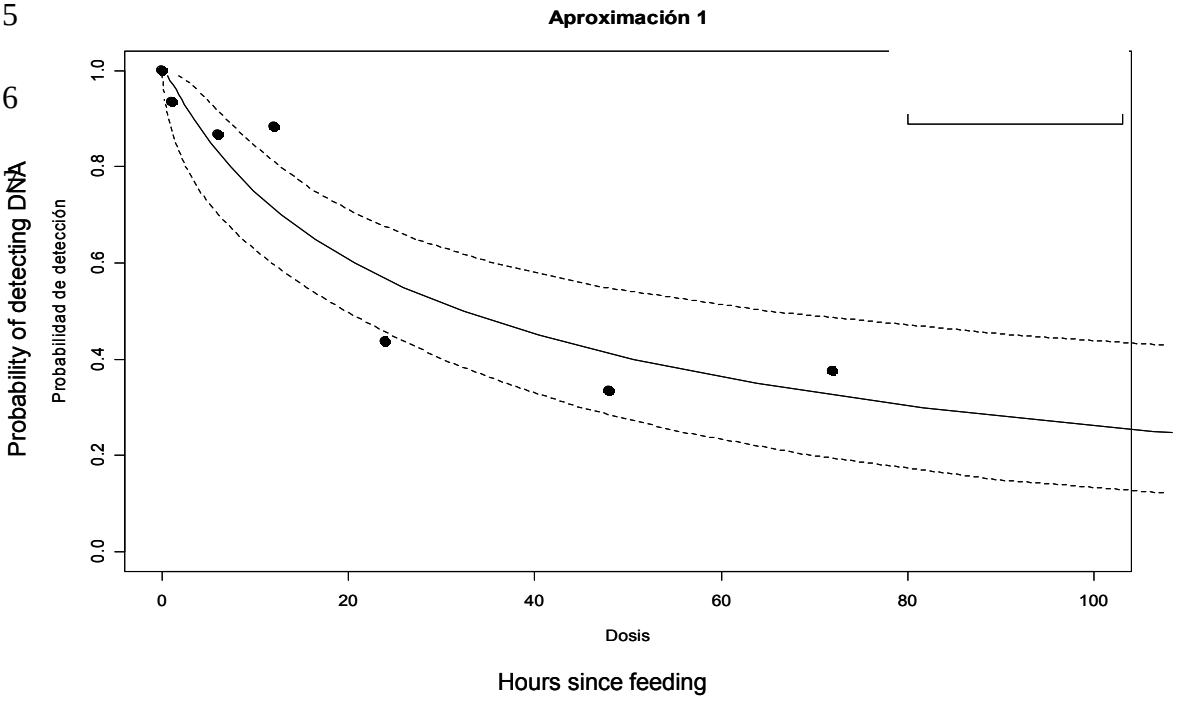

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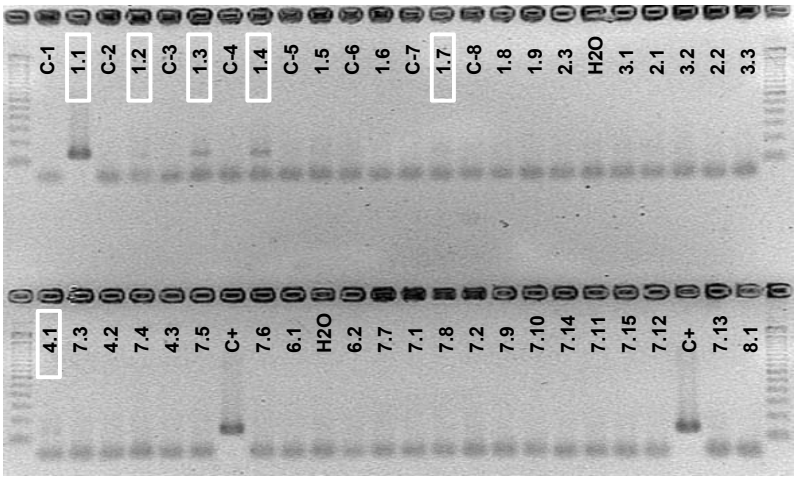
A)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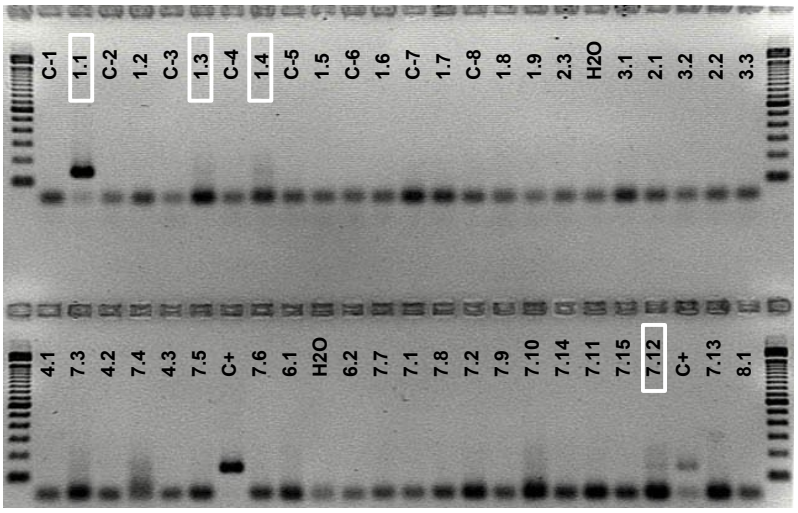
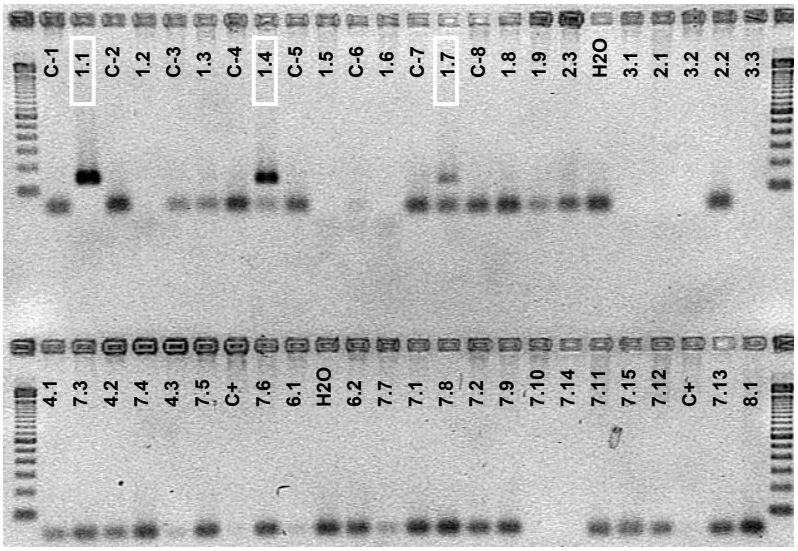
4.1 7.3 4.2 7.4 4.3 7.5 C+ 7.6 6.1 H2O 6.2 7.7 7.1 7.8 7.2 7.9 7.10 7.14 7.11 7.15 7.12 C+ 7.13 8.1

B)


C-1 1.1 C-2 1.2 C-3 1.3 C-4 1.4 C-5 1.5 C-6 1.6 C-7 1.7 C-8 1.8 1.9 2.3 H2O 3.1 2.1 3.2 2.2 3.3
4.1 7.3 4.2 7.4 4.3 7.5 C+ 7.6 6.1 H2O 6.2 7.7 7.1 7.8 7.2 7.9 7.10 7.14 7.11 7.15 7.12 C+ 7.13 8.1

C)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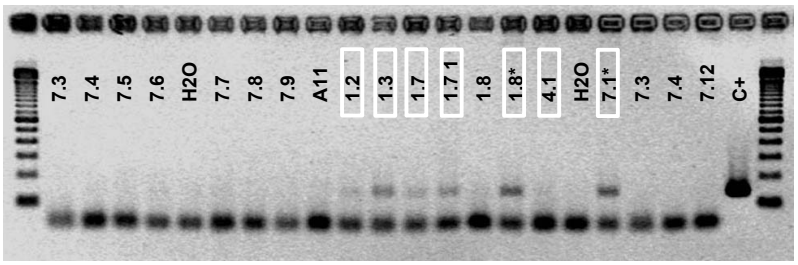
4.1 7.3 4.2 7.4 4.3 7.5 C+ 7.6 6.1 H2O 6.2 7.7 7.1 7.8 7.2 7.9 7.10 7.14 7.11 7.15 7.12 C+ 7.13 8.1

D)


7.3 7.4 7.5 7.6 H2O 7.7 7.8 7.9 A11 1.2 1.3 1.7 1.7.1 1.8 1.8* 4.1 H2O 7.1* 7.3 7.4 7.12 C+