Survey of T*rogoderma* spp. in Spanish mills and warehouses

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ABSTRACT

The distribution of *Trogoderma granarium* Everts has expanded steadily in recent years. This is a very destructive coleopteran species with many economic implications for the trade of grains in countries where it is present. Therefore, it is important to gather data that can confirm or reject the pest's establishment in a particular country. Spain has been cited as a location where the species is established; however, the present study demonstrates that no actual data support its stablishment in Spain. We surveyed fifteen storage premises (mills and warehouses for animal feed, wheat, and rice) distributed along the Iberian Peninsula over two years. We used traps baited with *Trogoderma* spp. sex pheromone to conduct the survey. Collected samples were morphologically and molecularly identified, since the morphological identification of this species is difficult. Although we found populations of *T. inclusum* in most of the sampled premises, and even *T. variabile* in one of them, no *T. granarium* was found in any of them. Abundant samples of other coleopterans were captured in the traps, including *Oryzaephilus surinamensis*, *Tribolium* spp., *Sitophilus* spp., *Cryptolestes ferrugineus*, *Lasioderma serricorne* and *Rhyzopertha dominica*. In conclusion, no *T. granarium* was found in our samplings in Spain.

KEYWORDS

Trogoderma granarium, *T. inclusum*, pheromone traps, morphological and molecular identification, establishment in Spain.

1. INTRODUCTION

Trogoderma granarium Everts (Coleoptera: Dermestidae) is a destructive stored products pest. Global warming and increases in the international trade of raw materials are favoring its sdistribution, which has steadily increased in recent decades (Athanassiou et al., 2019). The establishment of *T. granarium* is likely in countries with average temperatures above 20ºC for more than four months of the year (EPPO, 2020a). However, temperatures in storage facilities can be higher than those in open fields, so there is also a risk of establishment in colder climates. The European and Mediterranean Plant Protection Organization (EPPO) has listed *T. granarium* as a locally present quarantine species (EPPO, 2013), posing an important limitation to the trade of goods from countries in which this pest is established.

Trogoderma granarium is established in some countries located between 35ºN and 35ºS, which includes the southern Mediterranean region (Athanassiou et al., 2019). In many European countries, it has been intercepted and/or eradicated (Stejskal et al., 2015). *Trogoderma granarium* was reported in the south of Spain (Seville province) in 1952, as recorded in the historical files of the Department of Plant Production of the National Institute of Research and Agricultural and Food Technology (INIA) in Madrid. At that time, it was described as "common in granaries." Fernández-Gonzalez (1966) also stated that "this species is common in our granaries, pp. 141," although he did not provide any data on captures or the pest's distribution. Banks (1977), citing Fernández-Gonzalez (1966), named Spain as a location where *T. granarium* had an "uncertain status but can possibly thrive pp. 189" However, later, Rebolledo and Arroyo (1993, 1994) did some sampling around Madrid (central Spain) and concluded that no *T. granarium* was present there, since the *Trogoderma* species captured was *T. inclusum*. In spite of this, the EPPO (2020b), citing and misreading Rebolledo and Arroyo (1993, 1994), have recently listed *T. granarium* as present in Spain with a restricted distribution. Australia has named Spain as the fourth most likely country in the world to be a source of *T. granarium*. This ranking places Spain above most southern Mediterranean countries, above Saudi Arabia and Yemen, and even above India, Sri Lanka, and Pakistan (Paini and Yemshanov, 2012). This estimation was based on CABI's (2011) distribution data. Belda and Riudavets (2013) also reported finding *T. granarium* in yellow sticky traps in a survey of natural enemies of lepidopteran pests in food and feed processing companies in Spain. The most recent report of *T. granarium* in Spain appeared in Athanassiou et al. (2019), citing CABI (2018), which in turn cited EPPO bulletins. In conclusion, *T. granarium* is reported to be established in Spain despite the lack of current data to support this claim. A complicating factor is the difficulty of morphological identification of *T. granarium*, which is easily confused with other *Trogoderma* species (Athanassiou et al., 2019). This further reinforces doubts about some previous reports as in Belda and Riudavets, (2013) in which it is cited the presence of *T. granarium* in Spain. The purpose of the study of Belda and Riudavets, (2013) was to monitor Lepidoptera and this was simply a bycatch, which was not rigorously identified. In that study, only 9 specimens were captured with yellow sticky traps hung 1.5 m above ground, a difficult place for non-flying *T. granarium* adults to reach. Therefore, an expert taxonomist should be engaged to identify captured specimens; expert morphological identification along with molecular identification using a molecular diagnostic test developed by Olson et al. (2014) could be the best way to accurately identify this pest species.

The aim of the present study was to determine the current status of *T. granarium* in Spain so that the necessary measures for its eradication or management can be implemented. To accomplish this, we sampled different types of grain storage facilities (feed, rice and wheat mills, and wheat warehouses) with traps baited with *Trogoderma* spp. pheromone. These facilities store cereal grains, as wheat and rice that are primary host of the pest. The sampled facilities were located in the eastern and southern parts of the Iberian Peninsula, warm areas where *T. granarium* is most likely to be found. Several facilities are located near the area where *T. granarium* was recorded in 1952. Other dermestids and the most abundant coleopteran pests captured were also identified.

2. MATERIALS AND METHODS

2.1. Storehouse characteristics.

Fifteen storehouses with different structures that store different commodities were surveyed for *Trogoderma* spp. (Table 1). Mills 1 and 2 produce feed for pets, pigs, poultry, and other animals. They are concrete/steel structures with 4 to 5 connected floors in a large, unique space. Warehouses 3 and 4 are located at seaports; warehouse 3 is a concrete silo, and warehouse 4 is a flat storeroom. They store mostly durum wheat but occasionally store other grains for short periods of time. Warehouses 5 to 9 are one-floor storerooms where locally produced durum wheat is kept in the spring and summer months; warehouse 10 stores paddy rice. Mills 11 to 13 are rice mills; mills 14 and 15 are flour mills. Preliminary inspections revealed some grain debris around all the surveyed facilities except the rice and flour mills, which had stricter cleaning protocols in place. Facilities were located in southern and eastern Spain along the Mediterranean coast, including one in Portugal near Lisbon and one in northern Spain in La Rioja province (Fig. 1).

2.2. Insect sampling.

Mills and warehouses were sampled monthly for 1 year (facilities 1 to 10) from March 2016 to May 2017, or for 5 months (facilities 11 to 15), from May 2 to October 2017. A shorter sampling period was done in 2017 since most of the captures of 2016 were produced during spring and summer with very low captures during autumn and winter. Five PC floor traps (Agricultural Supply Services, Gloucester, UK) were used in each location, and three STORGARD ® WB Probe II ® traps (Agricultural Health Econex, Murcia, Spain) were also used in warehouses 3 to 10. Floor traps are good for evaluating coleopterans that contaminate facilities where commodities are stored and probe traps are appropriate for estimating arthropod contamination in grain bulk. Both traps were baited with commercially available female sex pheromone lure (Agricultural Health Econex, Murcia, Spain) for *Trogoderma* spp. The pheromone was impregnated on rubber septa and attached to both types of traps. Lures were replaced every four weeks. This sex pheromone is not specific to *T. granarium*; it also captures other *Trogoderma* species, such as *T. variabile* Ballion, *T. inclusum* LeConte, and *T. glabrum* Herbst (Cross et al., 1976) and also some other non-target storage beetles. A rapid visual scouting method was used to estimate the densities of other coleopteran pest species captured in the floor and probe traps (Boll et al., 2007). This method evaluates abundant classes. Class boundaries were defined approximately as powers of the square root of 10, as follows: **1** (1-3); **2** (4-10); **3** (11-32); **4** (33-100); **5** (101-316); **6** (317-1000); **7** (1001-3162); **8** (3163-10000).

2.3. Morphological and molecular identification of *Trogoderma granarium* and other *Trogoderma* species.

Once we separated members of the genus *Trogoderma* from the other trapped specimens, all *Trogoderma* were identified based on previously published taxonomic keys. Characteristics such as the color of the elytra, the morphological features of the eyes, antennal club morphology, and male genitalia were critical for identification (Green, 1979; EPPO, 2013; IPPC, 2016). To distinguish *T. granarium* from the other captured *Trogoderma* species, we also used a PCR-based method incorporating a pair of *T. granarium*-specific primers (der16SF4 and der16SR1) (Olson et al., 2014). DNA was extracted from each *Trogoderma* spp. individual using the SpeedTools Tissue DNA extraction kit (Biotools, Madrid, Spain) according to the manufacturer instructions. The total DNA was eluted in 100 µl of AE buffer (provided with the kit) and stored at -20°C. One negative control without insect DNA was added to each DNA extraction set. PCR was performed in 20 µl reaction volumes using 2 µl of DNA template, 0.2 µM of each primer, and 10 µl of DNA Amplitools Master Mix (Biotools, Madrid, Spain). Samples were amplified for 40 cycles at 94ºC for 45 s; at 58ºC for 45 s; and at 72ºC for 60 s. The first cycle of denaturation was conducted at 95ºC for 15 s, and a final extension was done at 72ºC for 5 min.

To ensure the presence of DNA in specimens that were not amplified with specific primers, we double-checked with a pair of universal arthropod primers (16S LR-J-12961 and 16S LR-N-13398) (Simon et al., 1994; Olson et al., 2014). For this amplification, PCR reaction volumes (25 µl) contained 2 µl of DNA template, 0.2 µM of each primer, 1.25 U of Taq DNA polymerase (Invitrogen), 0.2 mM dNTPs (Promega) and 2.5 mM of $MgCl_2$ in the manufacturers' reaction buffer. Samples were amplified for 40 cycles at 95ºC for 30 s; at 45ºC for 30 s; and at 72ºC for 60 s. The first cycle of denaturation was done at 95ºC for 15 s, and a final extension was done at 72ºC for 5 min. If the expected fragment was not amplified with the universal pair of primers, the specimen was discarded from the analysis.

Amplifications were conducted in a 2720 thermal cycler (Applied Biosystems, CA, USA). The target DNA and water were always included as positive and negative controls, respectively. Each individual was tested up to 3 times; a single positive test was considered a positive result. PCR products were separated by electrophoresis using 2.4% agarose gels stained with GelRed™ and visualized under UV light.

To ensure that the amplified bands were obtained exclusively from *T. granarium* and not from other species potentially present in stored cereals in Spain, the specificity of the primers der16SF4 and der16SR1 was tested on two to five individuals of *T. granarium* and on fifteen other non-target species, including other *Trogoderma* species (Table 2). Negative samples were also amplified with 16S LR-J-12961 and 16S LR-N-13398 universal primers to double-check the results.

Finally, the identification of ninety two adults from all *Trogoderma* morphologically identified (a sample of three individuals trapped in each facility on each date) was confirmed using PCR with der16SF4 and der16SR1 specific primers and double-checked with 16S LR-J-12961 and 16S LR-N-13398 universal primers.

3. RESULTS

3.1. Trap captures of *Trogoderma* spp.

A total of 4,418 *Trogoderma* spp. adults were captured in twelve of the fifteen facilities sampled. Only very few larvae were captured. The primary species captured was *T. inclusum* (4,377 individuals). Most of these individuals were males, as expected from traps baited with female sex pheromone. The highest number of *T. inclusum* individuals were captured in feed mill 1 (1,273) and warehouse 6 (1,094). Between 273 and 389 *Trogoderma* spp. individuals were captured in facilities 5, 9, 11, 12, and 13; fewer than 273 or even cero *Trogoderma* spp. were captured in the rest of the facilities (Figure 1). Most of the *T. inclusum* captures were done in floor traps (4,299); only 78 individuals were captured in probe traps. Forty-one *T. variabile* individuals were also captured, all in feed mill 1.

Trogoderma inclusum, the main species trapped, was found to be more abundant in spring, summer, and early autumn than in late autumn and winter (Figure 2A, 2B, 2C). In feed mills and seaports, grain or processed feed was present throughout the year. However, in warehouses 5 to 9, grain was present only in spring and summer. These warehouses were empty in winter until the grain was loaded again in May. Rice mills 11, 12, and 13 were more active in spring and summer. Therefore, the abundance of *T. inclusum* correlated not only with the presence of grain, but also with the higher temperatures of spring, summer, and early autumn.

3.2. Morphological and molecular identification of *Trogoderma granarium*.

None of the adult specimens collected could be identified as the regulated pest *T. granarium* according to the diagnosis protocols of the EPPO (EPPO, 2013) and the IPPC (IPPC, 2016). When sixteen insect species were tested with der16SF4 and der16SR1 primers using PCR, only the target species showed the expected band, demonstrating the high specificity of this pair of primers (Table 2). When species that gave negative results with the specific primers were tested with the universal primers, they all showed positive amplification, indicating the presence of insect DNA.

The PCR analysis of all *Trogoderma* adults from every storehouse and date using der16SF4 and der16SR1 primers gave negative results, confirming that none of the individuals were *T. granarium*. When we double-checked with 16S LR-J-12961 and 16S LR-N-13398 universal primers, all were amplified, indicating the presence of insect DNA in all samples.

3.3. Trap captures of other coleopterans.

Other coleopterans that are pests of food storage areas were also collected with the *Trogoderma* lures in both the floor and the probe traps, and some of these were captured in very high numbers (Table 3). The main species captured were *Oryzaephilus surinamensis* L., *Tribolium* spp., and *Sitophilus* spp., followed by *Cryptolestes ferrugineus* Stephens, *Lasioderma serricorne* F., and *Rhyzopertha dominica* F.

The two feed mills and the durum wheat warehouses showed the highest infestations, while these insects were barely present in the rice and flour mills. We also observed a high amount of psocids (Psocoptera) in feed mill 2 and in all the warehouses, although we did not quantify the abundance of this pest.

4. DISCUSSION

Although we conducted exhaustive sampling in some of the warmest regions of the Iberian Peninsula (Andalusia), including the region where *T. granarium* was first detected in the 1950s (Seville), we did not detect any *T. granarium* specimens. We also sampled other parts of the Iberian Peninsula where grain is stored (as in the case for most of the eastern Mediterranean coast), with the same negative results. Furthermore, the Spanish Plant Protection Services have not indicated the presence of any exceptionally damaging grain pest, as would be the case if *T. granarium* was present and established in any region of the country. We therefore conclude that *T. granarium* may not currently be established in Spain, even though it was detected in the 1950s. If it is present, it may exist sporadically. Although *T. granarium* has been intercepted in Europe multiple times, there are no data indicating that the species is established in the EU (Stejskal et al., 2015). Reports of the pest's presence by international organizations, such as the EPPO or CABI, do not seem to reflect the current situation. Oddly, according to Athanassiou et al. (2019), citing CABI, *T. granarium* is present in Europe (Spain and Switzerland). We have provided our arguments regarding Spain. However, some importing countries (i.e., Russia) request to issue phytosanitary certificates guaranteeing that a commodity is free of *T. granarium*. For this reason, to determine the status of this pest in the country is important, and a continued and sustained monitoring would be advisable.

We can confirm that *T. granarium* was present in the south of Spain in the sixties because the lab colony established at that time in the ETSIAAB-Madrid, which was maintained until recently, was morphologically and molecularly identified by us as *T. granarium*.

This study used the PCR-based diagnostic assay for *T. granarium* described by Olson et al. (2014). However, we were forced to slightly modify the PCR conditions because Olson et al.'s conditions were not specific enough and detected other *Trogoderma* species. This was resolved by increasing the annealing temperature from the proposed 45ºC to 58ºC. We also conducted a new specificity test with some European populations of *T. granarium* that was not included in their study (all populations tested by Olson et al. (2014) originated in America and Asia). Because this molecular diagnostic method could also be used to further analyze grain samples for the presence of immature stages of the pest, other species potentially present in stored cereals in Spain were tested for specificity (Table 1). Eggs and other immature stages of insect species are impossible or very difficult to detect and identify, particularly those that are internal pests of grain kernels (Solà et al., 2018). The analysis of grain samples is very useful when there is a recent infestation, because some grain samples may not yet contain adults, but eggs and other immature stages could be present.

Abundant populations of *T. inclusum* were found in feed mill 1. This facility contained significant amounts of debris due to the continuous movement of different types of grains and other materials for producing pet feed. *Trogoderma inclusum* was also found in warehouse 6, another facility that was not particularly clean. However, only few individuals of this species were captured with probe traps, indicating that they were not present in the grain. This species is a secondary pest that has been found on a wide range of raw agricultural products, processed foods, mixed animal feeds, and natural hosts. It is more likely to infest processed dry foods, animal feeds, and processing plants, being present around storage facilities, than to be present as a serious pest of stored grains (Partida and Strong, 1975). Furthermore, *T. inclusum* is less damaging than *T. variabile*, with which it can co-occur (Gerken and Campbell, 2018).

As mentioned by Barak (1989) (cited in Athanassiou et al., 2019), floor traps baited with *Trogoderma* pheromone also capture other coleopteran species, as we observed. Possibly, insects were trapped by chance and afterwards they were not able to come out of the traps. Also, if traps capture some individuals, perhaps these ones attract more of their species. The most abundant species captured were *O. surinamensis* and *Tribolium* spp. These are secondary pests that cause significant damage to stored grain. The list of species found in our study do not differ from those reported in Spain in previous surveys (Pascual Villalobos and del Estal 2004; Pascual Villalobos et al., 2006; Riudavets et al., 2002; Belda and Riudavets, 2013; Castañé and Riudavets, 2015), but species abundance varied in some of these surveys. This variation is probably related to the type of commodity, facility, or method of measurement. One noteworthy point is the high number of *O. surinamensis* captured in our survey, a finding that aligns with the observations of some pest controllers reporting an increase in numbers of this species in recent years.

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6.- AUTHOR'S CONTRIBUTIONS

Cristina Castañé, Conceptualization, Formal analysis, Investigation, Writing, Review & Editing, Funding acquisition.

Nuria Agustí, Conceptualization, Methodology, Investigation.

Pedro del Estal, Methodology, Investigation.

Jordi Riudavets, Conceptualization, Methodology, Investigation, Writing, Review & Editing, Funding acquisition.

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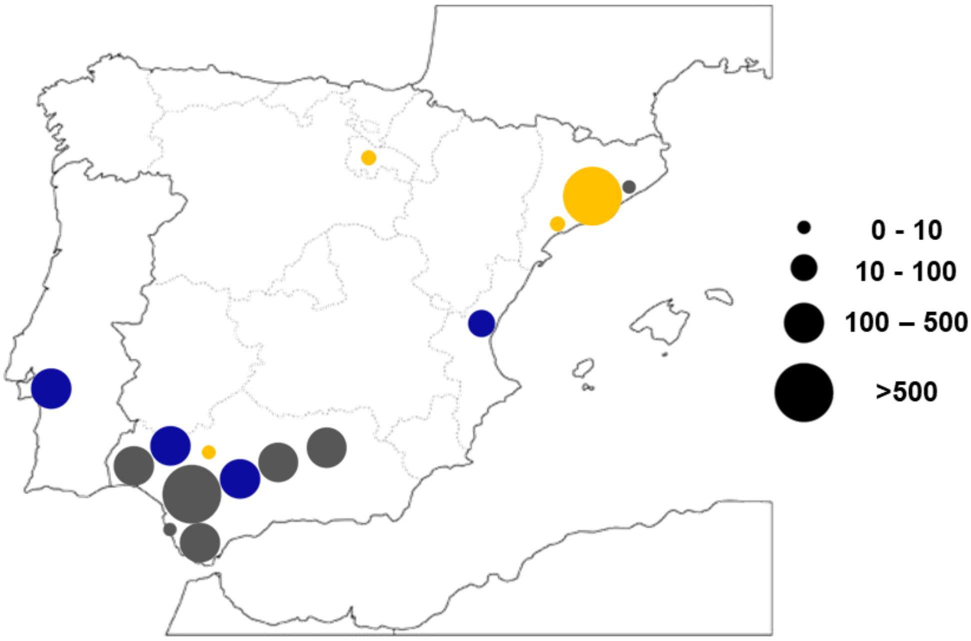

Figure 1. Abundance (number of adults) of *Trogoderma* spp. captured in mills and warehouses sampled in the Iberian Peninsula, according to the type of grain processed: durum wheat (grey), soft wheat and feed (yellow), and rice (blue).

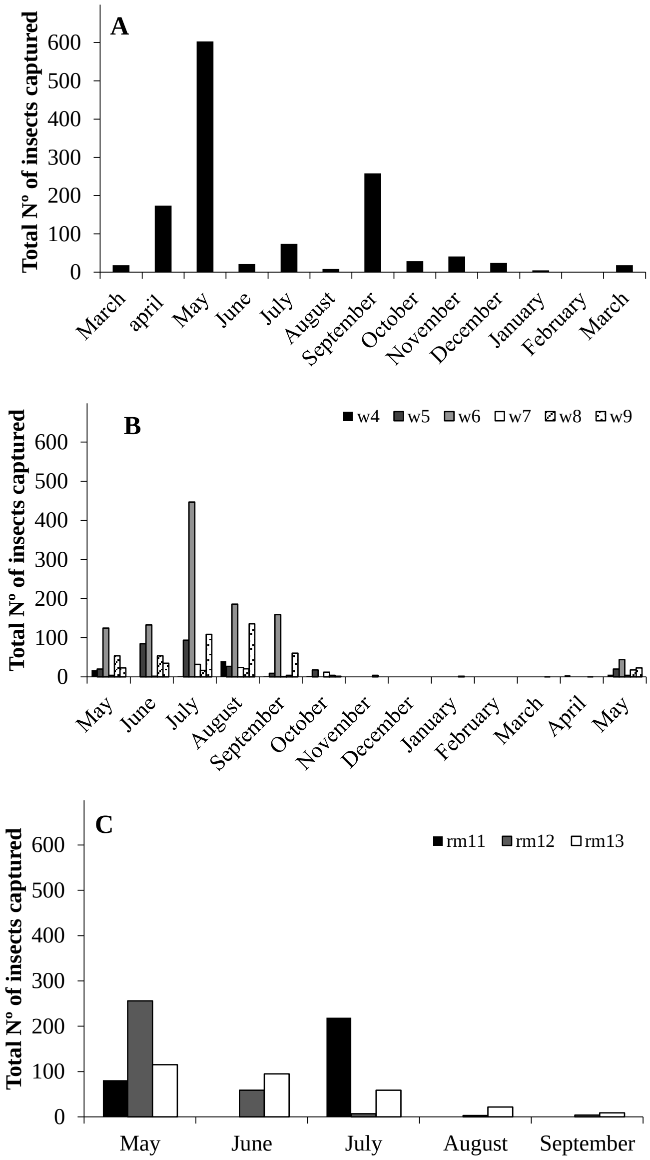

Figure 2. Total captures per month of *Trogoderma inclusum* in floor traps in the sampled facilities: A) Feed mill 1; B) Warehouses 4-9; C) Rice mills 11-13. No *T. inclusum* was captured in feed mill 2, warehouse 3, or flour mills 14 and 15.

Facility	Location	Storage structure	Commodity	No. & type of traps
1	Barcelona	mill	feed	5 floor
2	Tarragona			5 floor
3	Barcelona harbour	warehouse	durum wheat	5 floor +3 probe
4	Cadiz harbour			5 floor +3 probe
5	Cadiz			5 floor +3 probe
6	Cadiz			5 floor +3 probe
7	Huelva			5 floor +3 probe
8	Cordova			5 floor +3 probe
9	Seville			5 floor +3 probe
10	Valencia		rice	5 floor +3 probe
11	Seville	mill	rice	6 floor
12	Seville			5 floor
13	Lisbon			5 floor
14	Seville	mill	soft wheat	5 floor
15	Navarra			5 floor

Table 1. Location and characteristics of the surveyed storehouses. They were two feed mills, seven durum wheat warehouses, one rice warehouse, three rice mills and two soft wheat mills. Five to six floor traps were located in each facility and also three probe traps were also located in the bulk grain of the durum wheat and rice warehouses. Samples were monthly collected from March to May in facilities 1 to 10 during 2016, and from May to October during 2017.

Family	**Species**	**Origin of the sample**	**n**	**der16SF4 / der16SR1**	**16S LR-J-12961 / 16S LR-N-13398**
Dermestidae	*Trogoderma granarium* Everts	Spain*	5	+	+
	Trogoderma granarium Everts	Iran	5	+	+
	Trogoderma granarium Everts	Turkey	4	+	+
	Trogoderma granarium Everts	Greece	3	+	+
	Trogoderma inclusum LeConte	Spain	5	-	+
	Trogoderma variabile Ballion	Spain	5	-	+
	Trogoderma variabile Ballion	USA	5	-	+
	Trogoderma angustum Solier	Germany	5	-	+
	Trogoderma sternale Jayne	USA	5	-	+
	Dermestes maculatus De Geer	Spain	3	-	+
	Dermestes haemorrhoidalis Küster	Spain	4	-	+
Tenebrionidae	*Tribolium confusum* Jacquelin du Val	Spain	5	-	+

	Tribolium castaneum Herbst	Spain	5	-	+
	Sitophilus oryzae L.	Spain	5	-	+
Dryophthoridae	*Sitophilus zeamais* Motschulsky	Spain	5	-	+
	Sitophilus granarius L.	Spain	5	-	+
Anobiidae	*Rhyzopertha dominica* F.	Spain	5	-	+
Silvanidae	*Oryzaephilus surinamensis* F.	Spain	5	-	+

*This colony originated with individuals collected in the sixties in the south of Spain; it has been kept in culture in the ETSIAAB-Madrid since then.

n, number of individuals tested

Table 2. Species and geographical samples tested for specificity of the *T. granarium*-specific primer pair der16SF4 and der16SR1 with the universal pair of primers 16S LR-J-12961 and 16S LR-N-13398.

Location	Structure	Facility	*O. surinamensis*		*Tribolium spp.*		*Sitophilus spp.*		*C. ferrugineus*		*L. serricorne*		*R. dominica*	
			Floor	Probe	Floor	Probe	Floor	Probe	Floor	Probe	Floor	Probe	Floor	Probe
Barcelona	feed mills	1	3	.	7	.	2	.	0	.	0	.	0	.
Tarragona		2	4	6	4	6	4	5	2	2	0	3	5	3
Barcelona	warehouses	3	7	.	3	.	4	.	0	.	.	.	1	.
Cadiz		4	4	8	2	3	1	3	0	0	0	2	1	3
Cadiz		5	6	7	3	2	3	3	0	0	3	5	2	0
Cadiz		6	7	8	6	7	3	5	5	5	5	4	3	4
Huelva		7	7	7	7	7	0	5	0	6	3	4	0	2
Cordova		8	5	7	5	6	5	5	5	8	0	0	4	0
Seville		9	7	8	6	7	4	6	6	5	5	4	0	3
Valencia		10	0	2	0	0	0	4	0	5	0	0	0	0
Seville	rice mills	11	0	.	0	.	0	.	0	.	0	.	0	.
Seville		12	4	.	0	.	0	.	0	.	0	.	0	.
Portugal		13	5	.	0	.	4	.	3	.	0	.	3	.

Seville	flour mills	14	0	.	0	.	0	.	0	.	0	.	0	.
Navarra		15	0	.	0	.	0	.	0	.	0	.	0	.

Table 3. Total adult captures of the main coleopteran pest species in five floor and/or three probe traps lured with *Trogoderma* pheromone located in the sampled facilities. Estimations of the number of captured specimens used the following class boundaries: **1** (1-3); **2** (4-10); **3** (11-32); **4** (33-100); **5** (101-316); **6** (317-1000); **7** (1001-3162); **8** (3163-10000).