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Trichogramma Westwood, 1833 (Hymenoptera, Trichogrammatidae) collected in orchards in Rio Grande do Sul State, Brazil

Trichogramma *Westwood, 1833 (Hymenoptera, Trichogrammatidae) coletado em pomares no estado do Rio Grande do Sul, Brasil*

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ABSTRACT
The collection and identification of the egg parasitoid *Trichogramma* Westwood, 1833 (Hymenoptera: Trichogrammatidae) is the first step towards a successful application in the field. This is because, in a biological control program, it is necessary to select the parasitoid species most adapted to the cultivation and climatic conditions of the region where they will be used. Therefore, the objective of this work was to collect *Trichogramma* in orchards in the State of Rio Grande do Sul, aiming to identify it at the species level by the morphological method and to characterize it by the molecular method of the ITS2 region (internal transcribed space) of rDNA (Ribosomal Deoxyribonucleic Acid). The collection was carried out with traps made with micro-tuller fabric, randomly distributed in orchards. The capture occurred at all collection points. The identification resulted in *Trichogramma pretiosum* Riley, 1879 (Hymenoptera: Trichogrammatidae) for all collections. The DNA fragments present in the agarose gel were around 500 bp.
Keywords: biological control; egg parasitoid; fruit trees; region ITS2.

RESUMO
A coleta e a identificação do parasitoide de ovos *Trichogramma* Westwood, 1833 (Hymenoptera: Trichogrammatidae) consistem no primeiro passo para uma aplicação bem sucedida em campo. Isso porque, em um programa de controle biológico, é necessário selecionar as espécies de parasitoides mais adaptadas às condições de cultivo e climáticas da região onde serão utilizadas. Portanto, o objetivo deste trabalho foi coletar *Trichogramma* em pomares no estado do Rio Grande do Sul, visando identificá-lo ao nível das espécies pelo método morfológico e caracterizá-lo pelo método molecular da região ITS2 (espaço interno transcrito) de rDNA (ácido desoxirribonucleico ribossomal). A coleta foi realizada com armadilhas confeccionadas com tecido micro-tule, distribuídas aleatoriamente em pomares. A captura ocorreu em todos os pontos de coleta. A identificação resultou em *Trichogramma pretiosum* Riley, 1879 (Hymenoptera: Trichogrammatidae) para todas as coletas. Os fragmentos de DNA presentes no gel de agarose estavam em torno de 500 pb.
Palavras-chave: árvores frutíferas; controle biológico; parasitoide de ovos; região ITS2.

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INTRODUCTION

The fruticulture has economic expression in all regions of Rio Grande do Sul State, in Brazil. It stands out with the fourth largest fruit offering in the country, with 2.718 million tons (CARVALHO *et al.*, 2018). Fruit production in Rio Grande do Sul State is distinguished by its quality and the possibility of production in off-season, which allows good prices and trade, for table consumption, throughout the country. The diversity of soils and the existence of regions with different climatic conditions, added to the plurality of ethnicities, have favored the cultivation of a great number of species, providing fruit supply during all the months of the year.

However, one of the major problems in fruit production is outbreaks of insects considered as agricultural pests. Biological control is one of the alternatives for this problem. As an alternative, stand the egg parasitoids of genus *Trichogramma* Westwood, 1833 (Hymenoptera: Trichogrammatidae) that have been used worldwide as biological control agents because they have a wide geographic distribution, are highly specialized and efficient in the regulation of populations of several species of Lepidoptera.

The collection and identification of *Trichogramma* is the first step to a successful field use (HASSAN, 1997). This happens because, in a biological control program, it is necessary to select the species of the parasitoid most adapted to the crop and climatic conditions of the region where it will be used. Traditionally, *Trichogramma* is taxonomically identified at species level through the morphology of the genitalia of the male (PINTO & STOUTHAMER, 1994). However, in some cases, identification is hampered by the reduced size of the individual (0.25 mm) and/ or the presence of cryptic species (BORBA *et al*., 2005).

Ciociola Junior *et al*. (2001), aiming to identify two close species of *Trichogramma*, *T. rojasi* Nagaraja & Nagarkatti and *T. lasallei* Pinto, used the sequencing of the region ITS2 (internal transcribed space) of Ribosomal Deoxyribonucleic Acid (rDNA) and verified that the rDNA sequence of the two species differed in number and position of the nucleotides, showing that they are distinct. The study of this region was chosen because it has been little modified through the evolution (ORREGO & AGUDELO-SILVA, 1993). Thus, species of difficult identification, by the use of traditional taxonomic techniques, can have their identities clarified by the polymorphism of the length of sequences showed by the techniques of molecular markers.

Therefore, the objective of this work was to collect *Trichogramma* in orchards in Rio Grande do Sul State, aiming to identify them by morphological and molecular method.

MATERIAL AND METHODS

COLLECTION OF *TRICHOGRAMMA*

The sampling of *Trichogramma pretiosum* specimens was carried out in commercial orchards in different locations of Rio Grande do Sul state, Brazil (figure 1). The studied crops were peach, apple, pear, *uvaia (Eugenia uvalha,* Myrtaceae*),* strawberry guava fruit-*araça* (*Psidium cattleianum*, Myrtaceae), orange, plum, *pitanga* (*Eugenia uniflora,* Myrtaceae), persimmon, grape, guava, blackberry, fig and bergamot.

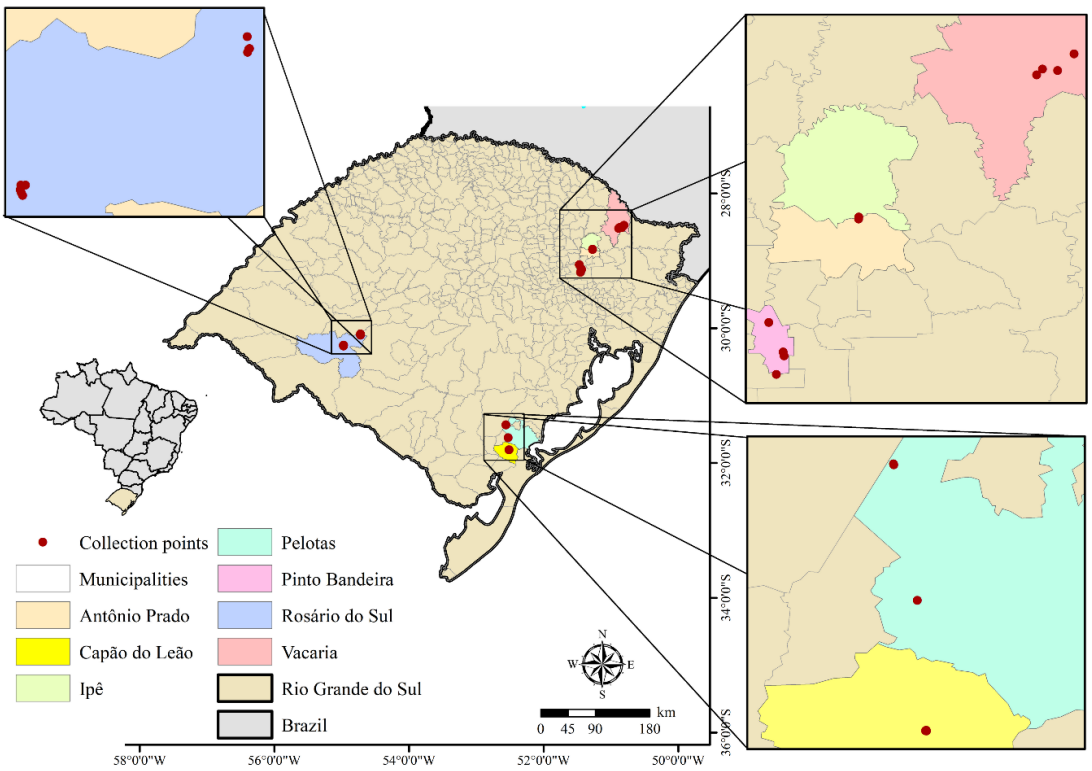

Figure 1 – Location of *Trichogramma pretiosum* sampling in commercial orchards in Rio Grande do Sul State, Brazil.

For sampling, traps were used, made with micro-tuller tissue in the size of 5 x 3 cm, in which cards of blue sky paperboard (2 x 4 cm) were inserted, with a central area of 4 cm^2. Each card contained, on average, 200 eggs from the alternative host *Anagasta kuehniella* Zeller, 1879 (Lepidoptera: Pyralidae), glued with 20% arabic gum and unfeasible by exposure under a germicidal lamp (STEIN & PARRA, 1987). The traps were distributed randomly in the orchards, fixed to the branches at a median height through a ribbon, remaining for a period of 48 h. After, the traps were collected and taken to the Entomology Laboratory of Embrapa Clima Temperado. The cards were then cataloged, describing the code, culture, collection site, city, coordinates and altitude. After this procedure, the cards were insulated in glass tubes (8.5 x 2.5 cm), closed with PVC plastic film and kept in air conditioned room, regulated with temperature of 25 ± 1ºC, relative humidity of 60 ± 10% and photophase of 14 h, being watched during five days for the observation of parasitism (STEIN & PARRA, 1987).

At the emergence of adults, some of the individuals were kept in the laboratory for multiplication in eggs of *A. kuehniella* and the other part was packed in 70% alcohol solution. After, one group was identified through the morphological characters of the male and the other group was used to produce isofemale for molecular identification (CIOCIOLA JUNIOR *et al.*, 2001).

MORPHOLOGICAL IDENTIFICATION

The male specimens, present in the 70% alcohol solution, were transferred separately to containers previously identified with the code of each card, containing 10% KOH solution, where they remained for about 1 to 3 hours at 30°/40°C, for the insects' clarification (QUERINO *et al.*, 2002). After this period, the insects were transferred to distilled water where they remained for about 10 minutes (QUERINO *et al.*, 2002). To cease the clarification process, the insects were transferred to glacial acetic acid for another 10 minutes (QUERINO *et al.*, 2002).

At assembly of the slides, each individual was placed alone, in a dorsoventral position, on a slide containing a droplet of Hoyer's medium (QUERINO *et al.*, 2002). After the slides were prepared, they were taken to a greenhouse for a period of one week at 45 ± 5ºC (QUERINO *et al.*, 2002). The identification was based on the terminology used by Pinto (1998).

MOLECULAR CHARACTERIZATION

The glass slides (8 x 2 cm) were placed in the freezer for 5 minutes. After, eggs of the alternative *A. kuehniella* host were sifted onto these slides and offered to newly emerged *Trichogramma* collected in each culture. After 24 hours of parasitism, the slides were packed in glass tubes (3 x 9 cm), kept in in air conditioned room at 25°C, relative humidity of 70 ± 10% and photoperiod of 14 hours. After 5 days, 50 eggs of the previously parasitized host from each population were individualized in test tubes (2.5 x 7.5 cm). Each tube contained a droplet of pure honey for adult feeding. These tubes were sealed with PVC film, in order to avoid the escape of parasitoids. After the first stage and with the emergence of adults and confirmation of sex, one female and one male, virgin of each lineage, were individualized in test tubes (2 x 10 cm). A period of 24 hours was allowed for copulation. After this period, a carton with 40 previously unavailable *A. kuehniella* eggs was offered for each couple. Adults of *Trichogramma* were offered pure honey as feeding. Of the couples obtained, only the couple with the highest parasitism was maintained. The emerged individuals were packaged in eppendorf tubes containing 100% alcohol. All procedures described in this paragraph were carried out in accordance with Ciociola Junior *et al*. (2001).

In DNA extraction, five individuals from each strain of isofemale were placed in 5µl microtubes and 100 µL of Chelex 100® (5%) (m/v), 4 µL proteinase K (29 mg/ml) and incubated for at least six hours at 56°C, followed by 10 minutes at 95°C in thermocycler, according to the technique of Ciociola Junior *et al*. (2001).

The polymerase chain reaction (PCR) reaction was done in a total volume of 50 µL. For each reaction, it was used: 5 µL of sample of DNA; 5 µL (10x reaction buffer); 3 µL of dNTP (10 mM); 2.5 µL of primer ITS2 F (5'-TGTGAACTGCAGGACACATG-3') to 5 mM, located in the region 5.8S of the rDNA; 2.5 µL of primer ITS2 R (5'-GTCTTGCCTGCTCTGAG-3') to 5 mM, located in the region 28S of the rDNA; 0.2 µL of *Taq* DNA polimerase, 1.5 µL of $MgCl_2$ [50 mM] and 30.3 µL of doubly distilled and autoclaved water. The thermal cycler program used was: a cycle of 3 minutes at 94°C, 33 cycles of 40 seconds at 94°C, 45 seconds at 55°C and 45 seconds at 72°C, with a final cycle of 5 minutes at 72°C after the 33° cycle.

The buffer used was the TBE 0.5X. The voltage used in the run was 40 volts in the period when the DNA was in the channel. After DNA output, the voltage was increased to 60 volts. The characterization was performed by comparing the DNA amplified bands length of the specimens with that of the molecular marker.

RESULTS AND DISCUSSION

Sampling of *Trichogramma* occurred in all collection points (table 1). The altitudes from the orchards ranged from 59 to 966 m.

Table 1 – Collections of *Trichogramma* spp. made in orchards in Rio Grande do Sul State, Brazil.

Code	Culture	Locality	Municipality	Coordinates	Altitude (m)
*TRS1	Peach	District of Pinto Bandeira	Bento Gonçalves	S 29° 7' 10,8'' WO 51° 26' 8,15''	690
TRS2	Khaki	District of Pinto Bandeira	Bento Gonçalves	S 29° 7' 9,8'' WO 51° 26' 7,4''	696
TRS3	Organic Peach	District of Pinto Bandeira	Bento Gonçalves	S 29° 10' 5,7'' WO 51° 27' 0,7''	574
TRS4	Peach	-	Pinto Bandeira	S 29° 03' 18,2'' WO 51° 27' 59,9''	613
TRS5	Grape	Embrapa Uva e Vinho	Bento Gonçalves	S 29° 07' 39,9'' WO 51° 26' 36,7"	677
TRS6	Apple	Nova Escocia	Vacaria	S 28° 28' 16,5'' WO 50° 48' 9,2''	939
TRS7	Apple	Rasip V	Vacaria	S 28° 30' 26,2'' WO 50° 50' 18,2''	948

Continua...>

Continuação da tabela 1

Code	Culture	Locality	Municipality	Coordinates	Altitude (m)
TRS8	Organic Apple	Embrapa Uva e Vinho	Vacaria	S 28° 31' 0,7'' WO 50° 53' 1,8''	966
TRS9	Peach	Embrapa Uva e Vinho	Vacaria	S 28° 30' 15,1'' WO 50°52' 16,0''	960
TRS10	Apple	-	Ipê	S 28° 49' 33,3'' WO 51° 16' 15,8''	864
TRS11	Blackberry	-	Ipê	S 28° 49' 48'' WO 51° 16' 16,9''	876
TRS12	Peach	Farm Palma	Capão do Leão	S 31° 48' 08,7'' WO 52° 30' 41,1''	59
TRS13	Fig	Farm Palma	Capão do Leão	S 31° 48' 12,4'' WO 52° 30' 39,4''	63
TRS14	Pear	Farm Palma	Capão do Leão	S 31° 48' 11,2'' WO 52° 30' 44,2''	71
TRS15	Peach	-	Pelotas	S 31° 25' 55,5'' WO 52° 33' 24,9''	227
TRS16	Peach	-	Pelotas	S 31° 26' 0,3'' WO 52° 33' 23,6''	267
TRS17	Orange	Embrapa Cascatinha	Pelotas	S 31° 37' 19,2'' WO 52° 31' 25,2''	186
TRS18	Bergamot	Orchard Arerunguá Field 4	Rosário do Sul	S 30° 15' 41,5'' WO 54° 58' 11,9''	128
TRS19	Bergamot	Orchard Arerunguá Field 12	Rosário do Sul	S 30° 15' 31,1'' WO 54° 58' 16,9''	132
TRS20	Bergamot	Orchard Arerunguá Field 17	Rosário do Sul	S 30° 15' 30,2" WO 54° 58' 18,2"	138
TRS21	Orange	Orchard Arerunguá Field 19	Rosário do Sul	S 30° 15' 24,6" WO 54° 58' 18,7''	138
TRS22	Bergamot	Orchard Arerunguá Field 22	Rosário do Sul	S 30° 15' 18,9'' WO 54° 58' 21,4''	133
TRS23	Orange	Orchard Tono Field 25	Rosário do Sul	S 30° 06' 02,7'' WO 54° 43' 02,0''	159
TRS24	Orange	Orchard Tono Field 6	Rosário do Sul	S 30° 05' 47,9'' WO 54° 42' 54,1''	144
TRS25	Bergamot	Orchard Tono Field 4	Rosário do Sul	S 30° 05' 50,9'' WO 54° 42' 57''	131
TRS26	Bergamot	Orchard Tono Field 1	Rosário do Sul	S 30° 05' 54" WO 54° 43' 3''	137

* TRS = Trichogramma Rio Grande do Sul.

Similarly to what was done in the present study, Pratissoli *et al.* (2002) verified the collection of *Trichogramma* at different altitudes in Espirito Santo State, Brazil. In their work, in plantings located at lower altitudes (200 and 380 m), the average number of samples with parasitism were higher and the lowest indices were obtained in regions with altitudes between 750 and 1050 m. The characteristics of the collection points in orchards in Rio Grande do Sul State, Brazil may have provided conditions that interfered in the adaptability and, consequently, in the dispersion of *Trichogramma* strains in each agroenvironment, as it has occurred in different species (MACIEIRA & PRONI, 2004; FORREST & CHISHOLM, 2017; STUCKI *et al.*, 2017).

The identification resulted in the occurrence of *Trichogramma pretiosum* Riley, 1879 (Hymenoptera: Trichogrammatidae) for all the captures of this work. The diagnostic characters used for the identification were: long flagellate setae with uniformly tapered apex; long back blade, reaching the apex of the volselas; short ventral carina not reaching half of the genital capsule; ventral processes near the base of the intervolsell process and long intervolselar pointed process, not reaching the apex of the volselas (figure 2).

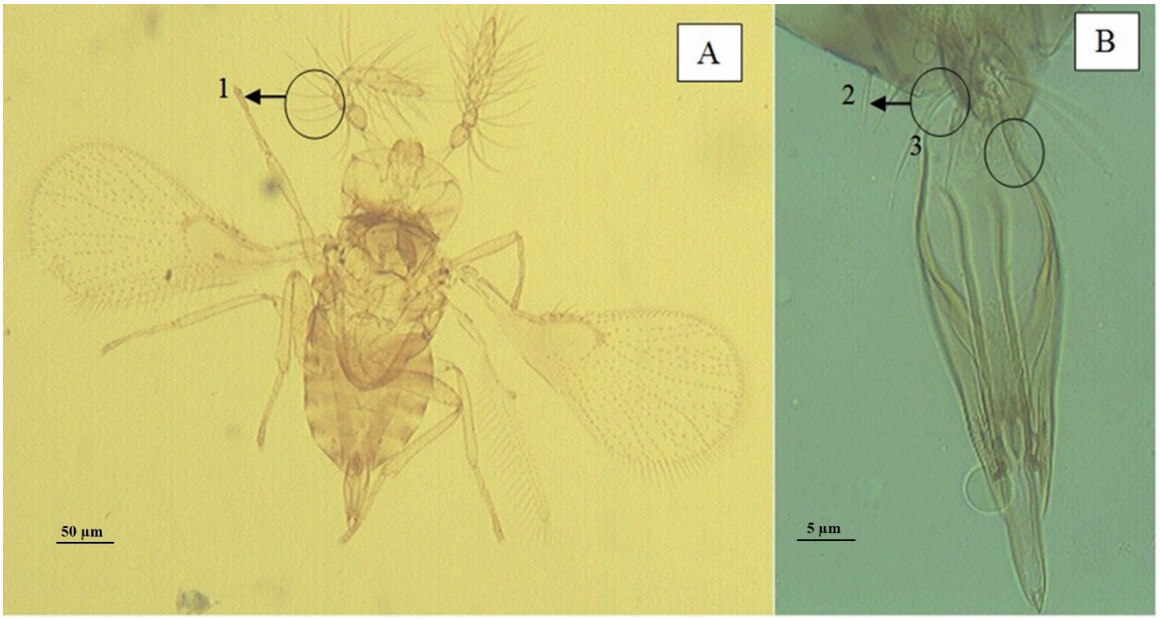

Figure 2 – A) *Trichogramma pretiosum* mounted on Hoyer's medium; B) male genitalia: 1 – long flagellate bristles, very thin at their apexes; 2 – dorsal blade with basal recess; 3 – long intervolselar process, reaching or extending beyond half the length of the volselas.

The occurrence of *T. pretiosum* in the studied orchards was expected, since the species is widely distributed throughout the South American continent, being associated with 26 hosts, corresponding to 63% of those parasitized by species of this genus (ZUCCHI & MONTEIRO, 1997; GOULART *et al*., 2008; QUERINO *et al*., 2017).

Querino & Zucchi (2012), studying the geometric morphometry of *T. pretiosum*, observed that the main variations were in the dorsal lamina, as individuals could present a dorsal lamina with a broad base and the apex of the posterior extension of the dorsal lamina could vary from round to slightly pointed.

The DNA fragments of *T. pretiosum*, present on the agarose gel of the present work, were around 500 bp (figure 3).

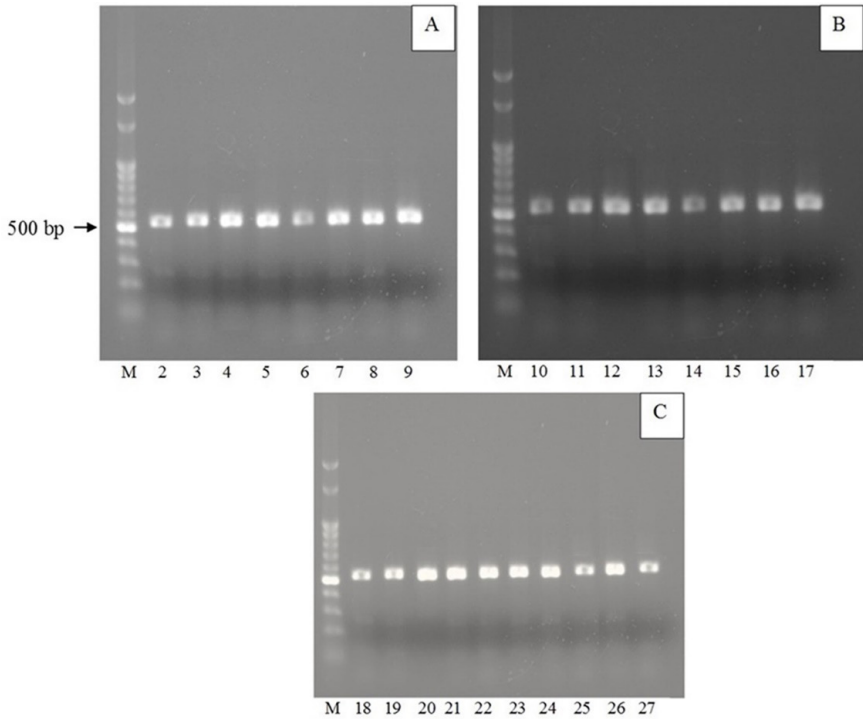

Figure 3 – 1.5% agarose gel showing the PCR products. M: molecular markers. 2-27: sample identification with *Trichogramma pretiosum* DNA.

When comparing populations of *T. pretiosum* from several regions of Brazil, Ciociola Junior *et al.* (2001) observed that the difference between them is very small, showing that there is a pattern in the sequencing of these populations, with a variation of 522 to 528 bp, by analyzing the size of the PCR products, producing a molecular key. However, this key is valid only for species sequenced in their work. Thus, collections of other populations must be carried out to construct a more complete and comprehensive key for the *Trichogramma* species existing in Brazil. Small differences in the position of some nucleotides of these sequencing do not alter the identification of the species whereas, in other sequenced species, visible differences were found in relation to the populations of *T. pretiosum*, both in the size of the sequencing and in the positioning of the nucleotides (ALMEIDA & STOUTHAMER, 2015).

Thus, through new studies with the sequencing of the ITS2 region, it becomes possible to verify the composition of bases present in the DNA of these parasitoids and to differentiate the populations collected at species level.

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