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Abstract In classical biocontrol programs a rapid and correct identification of the introduced antagonist is a key issue during both the release and establishment monitoring phases. It is often difficult to distinguish morphologically cryptic species or immature stages, and, in this case, an accurate diagnosis can be provided by molecular diagnostic methods. Among the conventional and real-time PCR based methods, loop-mediated isothermal amplification (LAMP) is a particularly suitable technique as it allows a rapid amplification of target DNA directly in the field. During the program implemented in Italy against the Asian Chestnut Gall Wasp (ACGW) *Dryocosmus kuriphilus*, we developed a real-time LAMP assay, combined with a simple DNA extraction, for rapid in-field identification of larvae, pupae and adults of the biocontrol agent, the parasitoid *Torymus sinensis*. Validation of the assay comprised adults as well as preimaginal stages of parasitoids obtained from ACGW galls collected from different localities and results confirmed the effectiveness of the LAMP assay to rapidly and specifically identify the target parasitoid in the field. This assay will be a valuable tool for quick on-site checking of the parasitism rate.

Key words: *Torymus sinensis*, *Dryocosmus kuriphilus,* LAMP (Loop Mediated Isothermal Amplification), ACGW, biocontrol, parasitism rate,

Introduction

The measure of the success of classical biocontrol programs is largely determined by whether and to what extent the target pest's density changes after the establishment of introduced natural enemies (Guit, Wratten, & Barbosa, 2000; Hoddle, 2004; Mahr, Whitaker, & Ridgway, 2008). Identification, preliminary safety testing and release of control agents are undoubtedly matters of great concern (Van Driesche & Hoddle, 2000). Nonetheless, once the agent is approved for release, an important issue is quantifying post-release the effectiveness of parasitoids and predators in reducing pest abundance (Stiling & Cornelissen, 2005; Furlong & Zalucki, 2010). To objectively assess the effectiveness of biocontrol in terms of costs and benefits relative to conventional control, standardized measures of success are needed in post-release surveys repeated over time (Sweetman, 1935; Van Driesche & Hoddle, 2000). The evaluation of parasitism rate can be performed following ‘rearing’ or ‘dissection’ methods applied to field-collected insects or plant material if host species feed in concealed or semi-concealed situations (i.e. leaf miners, gallmakers, borers). The former method can take a long time and could be delayed by many factors (i.e. diapause, weather conditions), whereas the latter is relatively quick and can guide decisions on whether more or different agents should be released (Day, 1994; Guit et al., 2000).

Whichever method is adopted, a correct identification of the parasitoids obtained is the critical step in the evaluation phase of the biocontrol program implemented, as it is a decisive factor when assessing the efficacy and host specificity of control agents and their possible interactions with the pest/native natural enemy complex (Delucchi, Rosen, & Schlinger, 1976; Agustí et al., 2005; Gariepy, Kuhlmann, Gillott, & Erlandson, 2008). Strong support in systematics and taxonomy is essential to correctly identify parasitoids at the species level (Van Driesche & Hoddle, 2000). However, using morphological features to distinguish closely related taxa or members of cryptic species complexes is often difficult, if not impossible, especially for immature stages (Dawah & Rothfritz, 1996; Agustí et al., 2005; Mathé-Hubert, Gatti, Poirié, & Malausa et al., 2013). Several molecular diagnostic methods that have been developed and largely implemented over the last 20 years can assist, complement and even replace morphologically based approaches (Agustí et al., 2005; Gariepy et al., 2008; Jenkins, Chapman, Micallef, & Reynolds, 2012).

In particular, the loop-mediated isothermal amplification (LAMP) is based on specific amplification of target DNA without the need for thermal cycling steps, thus allowing reactions to be performed in a portable heating block (Notomi et al., 2000; Tomlinson, Barker, & Boonham, 2007). The possibility of using LAMP directly in the field, with short reaction time and no need for a long training period for the staff involved in the survey, gives considerable advantages compared to other conventional or real-time PCR based methods (Jenkins et al., 2012; Tomlinson, Dickinson, & Boonham, 2010; Tomlinson et al., 2013;). As the DNA polymerase in LAMP reactions is not influenced by the co-presence of inhibitors or non-target DNA (Kogovšek et al., 2015; Lenarčič, Morisset, Mehle, & Ravnikar, 2013; Notomi et al., 2000) crude DNA extracts can be used, thus increasing the usefulness and portability of the method in the field (Danks & Boonham, 2007).

The Asian Chestnut Gall Wasp (ACGW) *Dryocosmus kuriphilus* Yasumatsu (Hymenoptera: Cynipidae), native to China is an invasive species and included in the quarantine list of European Union (EC, 2000). After being accidentally introduced into Japan, Korea, North America, and Nepal (in the forties, fifties, seventies, and nineties, respectively), it arrived in north-western Italy in 2002 and then spread rapidly throughout Italy and several European countries (Slovenia: 2005; France: 2005; Hungary and Switzerland: 2009; Spain, Croatia and the Netherlands: 2010; Czech Republic: 2012; Austria and Germany: 2013; Portugal and Turkey: 2014; United Kingdom: 2015; Belgium: 2016) (EPPO, 2015; EPPO, 2016a, 2016b). ACGW represents a very serious threat to chestnut stands in all the invaded countries as high numbers of galls on leaves and shoots reduces tree vigour and severely affects nut and timber productivity (Kato & Hijii, 1997; Maltoni, Mariotti, & Tani, 2012; Battisti, Benvegnù, Colombari, & Haack, 2014). Management of ACGW infestations, after the unsuccessful attempts to reduce pest densities by other measures, currently relies only on classical biological control methods (Moriya, Inoue, & Mabuchi, 1989). The release of the parasitoid *Torymus sinensis* Kamijo (Hymenoptera: Torymidae) from the native region of the gall wasp has been shown to reduce damage below a tolerable threshold level in Japan (less than 30%; Gyoutoku & Uemura, 1985; Moriya, Shiga, & Adachi, 2003) as well as in the USA, where a decline of pest population density has been also reported (Cooper & Rieske, 2007). In Italy, adults of *T. sinensis* were released in the first introduction site of ACGW ~~starting~~ in 2005, and later in all the other invaded areas (Quacchia, Moriya, Bosio, Scapin, & Alma, 2008; MiPAAF, 2010).

We developed a real-time LAMP assay combined with a crude DNA extraction for the identification directly in the field of larvae, pupae and adults of *T. sinensis*. The method was developed in order to quickly identify the preimaginal stages of the parasitoid and to evaluate the parasitism rate, so as to aid prompt management decisions regarding possible further releases of the parasitoid. Moreover, it may help the quality assessment of the parasitoid rearing program.

Materials and methods

The real-time LAMP assay was first developed using pure DNA extracted from voucher specimens by a salting out protocol. Then, the assay was validated using crude DNA extracted with a simple procedure from adults emerged from the galls (rearing method) and from preimaginal stages obtained by dissecting collected galls from the field.

Insect material

For the LAMP protocol development, 13 *T. sinensis*, 29 other chalcid adult parasitoids belonging to the same genus, family, and superfamily (21, 1, and 7 species, respectively; Table 1) as well as two *D. kuriphilus* were used as voucher specimensfor molecular characterization . All the specimens were stored in 70% alcohol after their emergence from chestnut or oak galls (i.e. *T. geranii* Walker emerged from galls of *Biorhiza pallida* Olivier) collected in pure or mixed chestnut stands inVeneto (Crespano del Grappa - TV), Piemonte (Avigliana - TO), and Campania (Serino - AV, Sicignano degli Alburni - SA) regions.

Validation of the LAMP protocol was first tested on 30 adults of *T. sinensis* and 15 adults of other native chalcid parasitoids belonging to Torymidae (*Megastigmus dorsalis* Fabricius and *Glyphomerus stigma* Fabricius), Eurytomidae (*Eurytoma pistaciae* Rondani and *E. brunniventris* Ratzeburg) and Eupelmidae (*Eupelmus urozonus* Dalman, *E. annulatus* Nees, *E. rostratus* Ruschka). Three adults of *D. kuriphilus* were also tested. All these specimens emerged from chestnut galls collected during the summer and winter at the rearing centre of the Veneto Region (Crespano del Grappa); *G. stigma* emerged from galls of *Diplolepis rosae* Linnaeus. In addition, a sample of 10 to 13 adults of *T. sinensis,* obtained from each of 8 Italian and 1 French population were further tested (N=101), including a few individuals from Korea (N=6) and Japan (N=3). All the specimens were first identified using various diagnostic morphological characters (Kamijo, 1982; de Vere Graham & Gijswijt, 1998).

The LAMP assay was then tested on larvae and pupae of the populations for which they were available (Table 2). At least 1,000 galls were collected during late winter at 7 Italian release sites in 4 regions (Veneto, Valle d'Aosta, Piemonte, Liguria) and one site in France. After collection, a subsample of 300 galls for each site was stored at 4° C to arrest the development of gall inhabitants at larval or pupal stages. Galls randomly chosen from each subsample were then dissected. Dissections were carried out until between 12 and 16 immature individual parasitoids were obtained and the number dissected recorded.

DNA extraction

Two types of DNA extractions were performed. For the LAMP assay development, a pure DNA extraction was carried out on 42 adult parasitoids (Table 1) using a salting out protocol (Patwary, Kenchington, Bird, & Zouros, 1994). Conversely, for the LAMP assay validation, a crude DNA extraction was performed following a simple protocol intended for applications under field settings on: i) the adult specimens reported in the previous section i.e. 30 adults of *T. sinensis* and 18 adults of other chalcid parasitoids; 110 adults of *T. sinensis* from 11 different populations; ii) the immatures dissected from galls (Table 2). Whole insect bodies were individually placed into 1.5-ml Eppendorf tubes containing 200 µl of double-distilled water and ground with a plastic sterile pestle to obtain a crude homogenate. Tubes were then shaken by hand without vortexing for few seconds before taking the volume to be tested. Approximate DNA concentrations were determined at 260 nm using the Nano-drop 2000 spectrophotometer (Nano-drop Technologies, Wilmington, DE, USA).

Design of LAMP primers

Two sets of primers satisfying LAMP requirement criteria were designed (Notomi et al., 2000). A first set of primers, specific for *T. sinensis*, was developed on the sequences of internal transcribed spacer 2 (ITS2) in the nuclear ribosomal region. Primers were designed on the *T. sinensis* sequences retrieved from GenBank (accession numbers AB200273, AB200274, and AB200275), and aligned with homologous sequences of other species of Torymidae and Pteromalidae (i.e., *T. geranii*, GenBank accession number AB200280; *T. flavipes*, GenBank accession numbers HM574233 and HM574237; *Mesopolobus xanthocerus* (Thomson), GenBank accession number HM573972). In preliminary analyses (results not shown), some falsepositives with *T. affinis* were obtained when loop primers only were included in the reactions, although the use of these primers is usually recommended to improve both the specificity and the speed of the reaction (Nagamine, Hase, & Notomi, 2002). These primers wereexcluded from the primer set.

To avoid falsenegative results (Tomlinson et al., 2010), and to check quality of both DNA and reagents, a set of primers, hereafter referred to as external amplification control (EAC) primer set (D'Agostino, 2013), was designed on the 28S region of other chalcid wasps using sequences retrieved from Munro et al. (2011). Preliminary tests showed a wider inclusiveness of this primer set. Nonetheless, this was not a matter of concern because the purpose of the design was to ensure reliability of the assay by excluding the presence of inhibitors or suboptimal reaction conditions (i.e. not sufficient DNA template).

All the sequences alignments were edited using the ClustalW algorithm in the software MEGA, version 6, (Tamura, Stecher, Peterson, Filipski, & Kumar, 2013). Primers were designed using LAMP Primer Explorer software (version 4; Fujitsu System Solutions Ltd., Tokyo, Japan) and synthesized by Invitrogen.

LAMP development and diagnostic performance

The DNA extracted by the salting out method from the specimens listed in Table 1 was used for the evaluation of the sensitivity and specificity of both the primer sets. The reaction mixture for the *T. sinensis* LAMP test contained 15 µl of Isothermal MasterMix 1× (OptiGene), 0.2 µM of each external primer, 2 µM of each internal primer, and 1 µl of template DNA. The same conditions were used for the EAC assay, with a further addition of two loop primers, 1 µM each. Total volume for both reactions was 25 µl. LAMP reactions were performed on a Genie II device (OptiGene).

Optimal conditions for LAMP reaction were determined by performing runs in a range of temperatures for different time periods. The identity of amplification products was evaluated in terms of annealing temperature determined through the measure of fluorescence during a slow annealing step (0.05°C/s) starting at 98°and ending at 84°C.

The sensitivity of both the assays was tested through 10-fold serial dilutions of template DNA in double-distilled water, starting from a higher concentration of 10 ng μl^{-1} to a lower concentration of 100 fg μl^{-1}. Each run contained double-distilled water as negative control (Tomlinson et al., 2013). The specificity of both the *T. sinensis* and the EAC primer sets was evaluated using the specimens reported in Table 1. Both sensitivity and specificity were tested on pure DNA extracted by the salting out method.

Validation of LAMP with crude DNA

Evaluation of the specificity of the LAMP assay on crude DNA was first performed on the 48 specimens (30 adults of *T. sinensis* and 18 adults of other chalcid parasitoids) emerged at the rearing centre. Reactions were repeated three times for each specimen to confirm the diagnosis. For those specimens that were not amplified by the *T. sinensis*-specific set of primers, a EAC primer set test was performed in order to ensure quality of both DNA and reagents. The assay was then tested on a further 110 adults of *T. sinensis* belonging to 11 different populations and 80 larvae and 25 pupae obtained by the dissection method from 8 populations (Table 2).

To confirm the results obtained by LAMP assays, 20 specimens (10 immature and 10 adults), randomly selected among the above mentioned 263 samples, were identified by sequencing of the DNA barcode region (Hebert, Cywinska, & Ball, 2003). For each specimen, 100 μl of the crude DNA extract was used for a further DNA extraction using Qiagen DNeasy Plant mini kit (Valencia, CA, USA). The DNA obtained was then eluted in 50 μl of AE buffer and directly used to amplify and sequence the barcode region of the cox1 gene (Hebert et al., 2003), using the universal primers and the amplification condition as reported in Folmer, Black, Hoeh, Lutz, & Vrijenhoek (1994). PCR products were checked through electrophoresis on 1.0% agarose gels stained with SYBR® (Invitrogen) and then purified using exonuclease and antarctic phosphatase (GE Healthcare). PCR products were then sequenced at BMR Genomics Service (Padova, Italy).

Statistical analyses

Throughout the text, temperature values are expressed in degrees Celsius and time values in minutes and seconds. All average values are reported as mean ± standard deviation (SD), unless otherwise specified.

One-way analysis of variance (ANOVA), followed by a Tukey's HSD (Honest Significant Differences) test, was used to compare the mean annealing temperatures of the 110 *T. sinensis* adults from the eleven different populations. An alpha level of 0.05 was considered statistically significant. Statistical analyses were performed using STATISTICA, version 8 (Statsoft Inc., Tulsa OK, USA).

Results

LAMP primers design

The designed *T. sinensis* primer set contained twoexternal primers (TS_F3 and TS_B3) and two inner primers (TS_FIP and TS_BIP).The EAC primer set was composed of two external (CH_F3 and CH_B3) and two internal primers (CH_FIP and CH_BIP), with the addition of two loop primers (CH_Fl and CH_BL) in order to accelerate the amplification reaction. Primer sequences for both the primer sets are reported in Table 3.

LAMP development and diagnostic performance

Optimal temperature and running time for both the LAMP reactions were an isothermal condition of 67°C maintained for 19 and 23 min. for the *T. sinensis* and the EAC LAMP assays, respectively. The specificity and the sensitivity of both primer sets and the diagnostic performance of the LAMP assays were then tested on the DNA extracted by the salting out method.

Specific primers worked exclusively on *T. sinensis* DNA and did not amplify any of the non-target sequences. All the 13 *T. sinensis* DNA samples were correctly identified while EAC primers amplified all the 44 DNA samples analyzed. Amplification was not observed in the negative control reactions. The serial dilutions of DNA tested with both the assays, showed an analytical sensitivity of 10 pg μl^{-1}. The annealing temperatures of the amplification products were 88.82 ± 0.09°C and 87.51 ± 0.18°C for *T. sinensis* and EAC LAMP assays, respectively.

Validation of LAMP with crude DNA

Validation of the method on 48 adult parasitoids showed that 30 *T. sinensis* individuals were positive to the *T. sinensis* assay, whereas all tested insects were positive to the EAC assay. Average positive reaction times of crude DNA were 16:34 ± 00:42 for *T. sinensis* assay, and 14:14 ± 1:43 for EAC assay. Annealing temperatures were 88.98 ± 0.06°C and 87.40 ± 0.10°C for *T. sinensis* and EAC assays, respectively. There was a slight variation in the starting average DNA concentrations of crude extracts (1.17 ± 0.17 ng μl^{-1}), reaction times (16:28 ± 1:47), and mean annealing temperatures (88.84°C ± 0.14°C) among the 11 populations of *T. sinensis*. Interestingly, the mean annealing temperature of *T. sinensis* adults differed significantly among populations ($F_{(10, 99)}$ = 2.04, $p < 0.05$), with the Korean specimens showing the highest average value, although the Tukey's test was not significant for any pair of means.

Forpreimaginal stages (larvae and pupae) from 8 available populations (Table 2), average DNA concentrations of crude extracts , reaction times, and mean annealing temperatures were: 2.72 ± 1.27 ng μl^{-1}, 16:47 ± 1:40, 88.84°C ± 0.17°C for larvae; 2.05 ± 0.07 ng μl^{-1}, 15:21 ± 1:52, 88.76°C ± 0.16°C for pupae. At some sites, it was recorded a low number of larval samples that generated a positive amplification out of the total number of samples tested. DNA barcoding of the 20 samples confirmed the accuracy of the LAMP identification. Positive LAMP results always corresponded to *T. sinensis*, whereas negative results corresponded to species belonging to the genera *Eupelmus* (Hymenoptera: Eupelmidae) and *Mesopolobus* (Hymenoptera: Pteromalidae).

Discussion

The LAMP assay enabled real-time detection of *T. sinensis*, the biocontrol agent of the ACGW, through a simple procedure designed to give rapid on-site results for samples without the need for time-consuming analyses or rearing in the laboratory. The use of a quick technique, together with the simple equipment required and the possibility to discriminate insect material obtained from various life stages, make the method valuable for making decisionswhen evaluating effectiveness of biocontrol programs directly in the field.

The whole procedure developed in this study, from sample preparation to the evaluation of amplification products, was completed in less than 30 minutes. The specific LAMP primer set showed high specificity to *T. sinensis*, with no positive reactions when other species were tested. However, we cannot exclude that the primer set designed specifically for *T. sinensis* works for the closely related *T. beneficus*, which cannot be discriminated on the basis of morphological characters (Yara, 2004). Unfortunately, it has not been possible to retrieve and test any specimen of *T. beneficus* to better validate the *T. sinensis* primer set. For this purpose, it would be interesting to look at the possibility to obtain an annealing temperature specific for the DNA region of the target species, as a way to unambiguously identify the samples and, concurrently, to detect possible variants (Ririe, Rasmussen, & Wittwer, 1997). In our case, the Korean population showed the highest mean annealing temperature, although this result needs to be supported by a higher number of samples. If confirmed, this result may reveal the occurrence of two different strains of *T. sinensis* in Korea and Japan, as the European populations came from Japan, given their introduction history (Murakami, Ohkubo, Moriya, Gyoutoku, Kim, & Kim, 1995; Yara, 2004; Quacchia et al., 2008).

The diagnostic sensitivity level of the assay was high enough to correctly identify the parasitoid in any of the life stages considered, without the need for complex DNA extraction and quantification. Successful outcomes were easily obtained by simply grinding the insects in double distilled water and by directly adding these crude homogenates to pre-prepared strips containing isothermal field stable reagents. Results were positive when specimens both stored in alcohol and freshly collected were used, demonstrating that even DNA of relatively low purity gives positive amplification products (Huang, Hsu, Haymer, Lin, & Wu, 2009). We found that the negative results were successfully amplified by the EAC primer set, minimizing the risk of false negative results.

The samples tested were not sufficient to give reliable estimates of parasitism. However, the proportion of positive larval samples roughly reflects the parasitoid success at the different sites. A higher occurrence of *T. sinensis* was indeed recorded where the parasitoid was released first. Alternatively, the high proportion of positive pupal samples can be explained by the period of gall collection (i.e. late winter). In this period only *T. sinensis* occupies the galls as the other parasitoids generally use other hosts for overwintering (Luo, Huang, & Liao, 1987; Shiga, 2009). A proper identification of *T. sinensis* using a reliable on-site molecular technique is of great importance as the release of the parasitoid is, at present, the only viable long-term management option against *D. kuriphilus* in the invaded areas (Yara, 2006; Gibbs et al., 2011). Although the biocontrol of ACGW is successful in those areas where *T. sinensis* has been introduced (Gyoutoku & Uemura, 1985; Moriya et al., 2003; Cooper & Rieske, 2007; Ferracini et al., 2015a), many recently invaded regions have now to deal with this problem (EPPO, 2014; EPPO, 2015). Consequently, a correct identification of both preimaginal and adult stages of the parasitoid is fundamental during all the phases of the biological control program (Danks, 1988; Gordh & Beardsley, 1999). This is particularly true if we consider that other parasitoid species, such as local oak gall wasp parasitoids, are rapidly recruited as enemies by ACGW once it arrives in a new area and that *T. sinensis* may cause unwanted non-target effects (Aebi et al., 2006; Quacchia et al., 2012; Matošević & Melika, 2013; Panzavolta et al., 2013; Palmeri et al., 2014; Ferracini et al., 2015b).

The LAMP assay we developed is suitable for diagnostic as well as research use (Lenarčič et al., 2013) as it can be used both for a rapid quality check of the released stock in new areas and a quick in-field monitoring of the parasitism rate of the biological control agent. Moreover, this method bypasses the need for specialist knowledge or a long period of training for the staff involved (Jenkins et al., 2012) avoiding, in particular, the long procedure in the morphological identification of preimaginal stages.

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Table 1. Insect material tested for the development of the LAMP assays with pure DNA extracted by the salting out protocol.

Superfamily	**Family**	**Species**		**Number of tested samples**
Chalcidoidea	Torymidae	*Torymus*	*sinensis*	13
			*affinis**	4
			auratus	2
			*cyaneus**	1
			erucarum	2
			*favardi**	2
			flavipes	2
			*formosus**	1
			geranii	2
			notatus	4
			scutellaris	1
		Megastigmus	*dorsalis*	1
	Eupelmidae	*Eupelmus*	*urozonus*	1
			annulatus	1
	Eurytomidae	*Eurytoma*	*pistaciae*	1
			brunniventris	1
	Ichneumonidae	*Orthopelma*	*mediator*	1
	Pteromalidae	*Mesopolobus*	*tibialis*	1
	Ormyridae	*Ormyrus*	*nitidulus*	1

* Indicates congeneric species of *T. sinensis* emerged from cynipid galls on oak.

Table 2. Individuals of *T. sinensis* from different populations tested for the validation of the LAMP method with crude DNA. For each sample the number of samples that generated a positive amplification out of the total number of samples tested is reported.

Country	**Region**	**Site**	**Larvae**	**Pupae**
France		St. Dalmas de Tende	8/10	2/2
Italy	Valle d'Aosta	Forte di Bard	4/7	5/5
	Piedmont	Avigliana	2/8	3/4
	Liguria	Millesimo	7/11	1/1
	Veneto	Cavaso del Tomba	2/14	-
		San Mauro di Saline	1/12	-
		Pianezze	2/15	-
		Seren del Grappa	2/3	11/13
Total			**80**	**25**

Table 3. Primers used for the loop-mediated isothermal amplification (LAMP) assays.

LAMP assay target	Primer	Sequence (5' – 3')
Torymus sinensis	TS_F3	CGCAAGATGGATGAGAGAGAG
	TS_B3	GCAAACAGAGAGCTCCGG
	TS_FIP	TCAAAACACTCACGAGGCGCGTCGC-TCGAAACAATGGCG
	TS_BIP	TACGCACACGCACACGCTACTCGAC-GCAAACAACACG
Chalcid wasps	CH_F3	GGTGAACTATGCCTGGTCAG
	CH_B3	TTCGCTTTACCAGATGAGACTC
	CH_FIP	CCGACGATCGATTTGCACGTCAGAC-GAAGTCAGGGGAAACC
	CH_BIP	ACTGGGTATAGGGGCGAAAGACTAA-TCAAGCGAGTGCCAGCTATC
	CH_FL	CGCTACGGACCTCCATCAG
	CH_BL	GAACCATCTAGTAGCTGGTTCC