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RED FLOUR BEETLE *TRIBOLIUM CASTANEUM* (TENEBRIONIDAE, COLEOPTERA): POPULATION DYNAMICS, SCREENING OF *WOLBACHIA* IN DIFFERENT REGIONS AND CEREAL FOODS OF PAKISTAN.

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ABSTRACT

Red flour beetle (*Tribolium castaneum*) is considered as an important pest causing significant economic loss of stored grain foods. Present study was performed to scrutinize the screening of *Wolbachia* on red flour beetle for the pathogen potential as tool to integrate in future pest management programs. Study was conceded in three crops i.e., Wheat (*Triticum aestivum*), Rice (*Oryza sativa*) and Lentils (*Lens culinaris*) to evaluate population dynamics of the stored grain beetle from different districts and hosts along with natural and artificial diets comparison. The statistical analysis based on different parameters of observed populations of *T. castaneum* from wheat showed low population in the months of May and June though steady increase for the month of July whereas in September the population start to decline. The Hafizabad and Sheikhupura districts exhibited nearly equal population in wheat. *T. castaneum* populations of Sargodha exhibited lowest trend in rice though maximum for Sheikhupura and nearly equal with Hafizabad. The increasing trend in population of *T. castaneum* in rice was same as in July and August there was increasing trend though decline in September. The monthly analysis in lentils showed that minimum population of *T. castaneum* recorded in July and August as compared to wheat and rice. In district Sargodha, population of *T. castaneum* in lentils was uppermost though district Sheikhupura showed least population. Genetic analysis through mitochondrial DNA markers showed very promising positive results whereas for *Wolbachia* (*wsp* gene) in *T. castaneum* contagion remained Hafizabad 24%, Sheikhupura 25%, Sargodha 17%, Peshawer 15% and Hyderabad 10%, however *Wolbachia* infection density is quite low which will further be exploited through sequences and MLST markers.The objective of this study was to describe the population dynamics and screening of potential *Wolbachia* infection in Stored grain beetle.

Keywords: Foods sources, Pakistan, Screening, *Tribolium castaneum, Wolbachia*

INTRODUCTION

Wheat, Rice and lentils are main cereal crops with the major area under cultivation in Pakistan which plays a vital role in improving the economic growth of the country (GOP, 2019). These three cereal crops (Wheat, Rice and lentils) provides nearly 55% of the carbohydrates and 20% of the food calories consumed globally (Breiman and Graur, 1995). It is recorded that there are around 1660 insect pest species which attack on stored agricultural products during storage period (Hagstrum and Subramanyam, 2006). Ten species are well identified and habitually harmful to grain products (damage from excrement, living and dead insect, egg shells, pupal cases, cocoons and complex (Lohar, 2001). Estimations of the damage are as much as 5-10% of the world production whereas in the some tropical countries these losses are as high as 30% Post harvest losses in wheat, rice and lentils are mainly due to red flour beetles. *T. castaneum* attack stored grain products such as flour, cereals, meal, crackers, beans, spices, pasta, cake mix, dried pet food, dried flowers, chocolate, nuts, seeds, and even dried museum specimens (Weston and Rattlingourd, 2000; Pugazhvendan *et al.*, 2009). *T. castaneum* found as a major stored grain pest in home and stores secured places, particularly where there is a favorable ambient

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temperature (Tripathi *et al.*, 2001). Red flour beetle (*T. castaneum*) causes some major economic and ecological losses of billion dollars every year to stored grain foods, affected product becomes contaminated with faeces and auxiliary with increasing humidity stimulates molding (Danahaye *et al.*, 2007). Red Flour beetle has attained the status as a major pest (Danahaye *et al.*, 2007) and also developed into an imperative insect model of developmental biology and evolution biology studies (Strobl, *et al.*, 2017)

Wolbachia is a complex group of intracellular, gram- negative bacteria which has capability to transfer through maternally. Alpha-proteobacteria observed in numerous species of arthropods (Inaki *et al.,* 2011; Ravikumar *et al.*, 2011; Dobson *et al.*, 2002). Through electron microscopic examination it is revealed that *Wolbachia* is surrounded by three layers; two internal layers and one external layer. The outer most layers have a vacuole (Taylor and Hoerauf, 1999). In 1924, *Wolbachia* was firstly observed in the ovaries of *Culex pipientis* (Bourtzis, 2008). Until the late of nineteen century *WoIbachia* were members of an unusual and insignificant genus of bacteria. But now *Wolbachia* are considered richest group of bacteria infecting approximately 40% to 60% insects, 40% of mosquito species and 90% of filarial nematodes (Vasquez *et al.*, 2011; Hilgenboecker *et al.*, 2008; Xi *et al.*, 2006). At present, it has got importance due to its massive abundance and possible applicants in pests and vector disease control of invertebrates (Bourtzis, 2008). Generally, *Wolbachia* is occurred in the reproductive tissues of host induce a range of reproductive abnormalities such as male killing, parthenogenesis, feminization and most commonly cytoplasmic incompatibility observedin four different arthropod orders: CoIeoptera, Lepidoptera, Diptera and Pseudoscorpiones (Werren *et al.*, 2008; Jiggins *et al.*, 2001). Feminization; mostly reported from terrestrial isopods species and in some insect species also (Bouchon *et al.*, 1998; Rigaud *et al.*, 1999; Michel-Salzat *et al.*, 2001; Negri *et al.*, 2006; Narita *et al.*, 2007). It is one of the valuable strategies for *Wolbachia* to ensure its double transmission to the descendants. In infected male, *Wolbachia* cause androgenic gland hypertrophication which results in malformation of androgenic hormone (Azzouna *et al.*, 2004).

It was reported that parthenogenesis was observed in mites, wasps and thrips, in case of parthenogenesis male individual develops from unfertilized eggs (Stouthamer *et al.*, 1990; Arakaki *et al.*, 2001) and cytoplasmic incompatibility (CI); mostly detected in arachnids, isopods and insect orders. This diverse feature was first documented in mosquito *C. pipiens* and now has been described in each order of insects (Harris and Braig, 2003). An understanding of the factors responsible for the populations of *T. castaneum* is essential for effective integrated control of pests. The aims of present study were to investigate the screening of *Wolbachia pipientis* on the *T. castaneum* which may provide a useful tool to integrate pest control management practices for future.

MATERIALS AND METHODS

The experiment was conducted in the Department of Zoology, Faculty of Life Sciences, Government College University, Faisalabad during 2017-18 on wheat (*Triticum aestivum*), rice (*Oryza sativa*) and lentils (*Lens culinaris*) which were collected from gowdowns and grain markets of three cities of Pakistan (Hafizabad, Skeikhupura and Sargodha) for determining the population dynamics of *Tribolium castenauem*. However for screening of *Wolbachia* collection was conducted from five cities (Hafizabad, Sheikhupura, Sargodha, Hyderabad and Peshawar). Collected samples of red flour beetle were reared in semi- field condition on natural diet and in lab on artificial diets (powder flours, yeasts, vitamins and essential elements). After collection, the insect population was kept in the jars in laboratory and was covered with muslin cloths. Insect populations were regularly investigated for study of different life stages and was sieved and transferred to new natural and artificial sterilized hosts diets at temperature 30±2ºC and relative humidity at 70% for maximum insect growth. Different parameters were studies for the population dynamics (count the number of eggs, larvae, pupae and adults) for quality and survival potential studies. Approximately 1200-1400 adults of *T. castaneum* were taken from collected samples and reared in laboratory

Different cage trials studies (20-100 pairs of *T. castaneum*,) were introduced to each of the test resource. The data for number of hatched larvae, larvae survived to pupate and enclosed adult insects were counted to estimate the population dynamics. Population was counted during the optimum conditions in June, July, August and September. Analysis of Variance (ANOVA) of data conducted with Duncan's multiple range tests to determine the significance of differences at 0.05 probability level. Comparisons between means of treatments for various parameters were made by standard error calculation (Gomez and Gomez, 1984; Steel and Torrie, 1980).

DNA Molecular amplification performed after DNA extraction from the sample through extraction kits were used. Later on DNA quantification was performed by both UV Spectrophotometer and Nano drop method. For barcoding of individuals of *T. castaneum* populations from Pakistan, PCR with mitochondrial primers Pat and Dick and S12 were steered. PCR was carried out in a 25 µl volume with two sets of mitochondrial primers following the protocols of the manufacturer (Sigma-Aldrich). DNA extractions from the collected samples were performed through extraction kits (Qiagen). Quantification of DNA was performed by UV Spectrophotometer and Nano drop methods. 5 µl of each isolated DNA was used for quality by running through Gel electrophoresis. Barcoding of individuals of T. castaneum populations was performed through PCR in a 25 µl volume with two sets of mitochondrial primers. To investigate the samples for Wolbachia infection, the wsp primers 81F (5tggtccaataagtgatgaagaaac-3) and 691R (5-aaaaattaaacgctactcca-3) were used. The reactions were set up in 25 µl volumes containing, 1x NH4 buffer (Fermentos), 2mM MgCl2, 100 µM dNTPs, 0.2 µM of each primer, 0.5 U Taq polymerase (Fermentos) and 2 µl of the template DNA. PCR were started for 2 minutes at 95°C and followed by 32 cycles at 94°C for 30 seconds, 60°C for 45 seconds, 72°C for 1 minute and a final extension at 68°C for 15 minutes. The products of the PCR with mitochondrial primers were purified following a slightly adapted Fermentos DNA extraction-pure kit protocol. The purified DNA samples were stored at 4°C. The quantitative analysis for the detection of Wolbachia infection in T. castaneum population was completed after PCR. Aldrich). For evaluation of proficient result and sequences generation some specimens were

purified via ethanol precipitation followed by quantification. Further optimization of PCR condition and Taq concentrations were standardized. Screening of Wolbachia was performed through molecular markers using the wsp general primers (Sigma-

RESULTS AND DISCUSSION

Population dynamics of the *T. casteneum*

Studies were carried out to evaluate population dynamics in the model stored grain insect, screening of the *Wolbachia* endosymbionts for the role of host microbe interaction and tangentially incorporation for effective implementation in the pest management programmes.

Population dynamics of the *T. casteneum* on wheat samples for all three localities (Hafizabad, Sheikhupura and Sargodha) showed the high population trends in Sargodha as compared to the population of Hafizabad and Sheikhupura. Relatively high population was recorded during the month of July and August and population was decreased gradually from the last weeks of September (Fig. 1a). High population of *T. castenaum* was recorded on rice in Sheikhupura district as compared to Hafizabad and Sargodha (Fig. 1b). Low population of *T. castaneum* was recorded on lentils because of its thick seed coat. Red flour beetle showed least population on lentils among three stored grains crops. The lentils provide least nutrition food to the red flour beetle and less adequate environment to increase population (Fig. 1c).

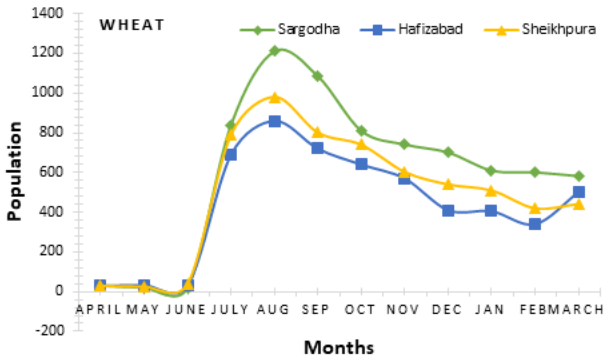

Fig. 1a

Population of red flour beetle on wheat in study arena

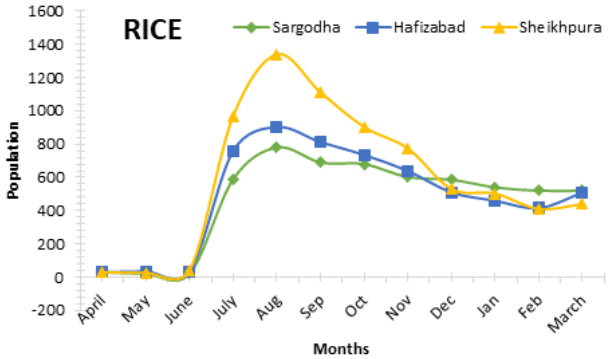

Fig. 1b

Population of red flour beetle on rice in study arena

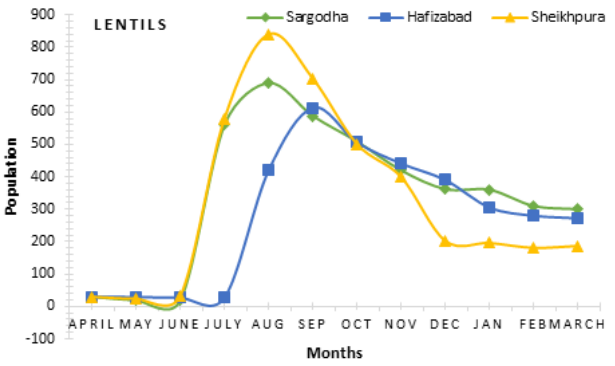

Fig. 1c

Population of red flour beetle on lentils in study arena

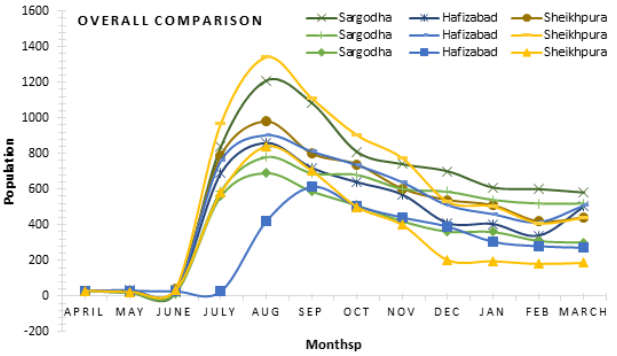

Fig. 2

Overall comparison of red flour beetle in all localities during the year

The statistical analysis of pragmatic populations of *T. castaneum* from wheat showed that in first two months (May and June) *T. castaneum* population was low but from the third month July, there was steady increase in *T.castaneum* population in while from the second week of September the population start to decline. The Hafizabad and Shaikhupura showed the nearly equal population in wheat. For rice, district Sargodha, showed lowest population of *T. castaneum* in rice though Sheikhupura exhibited maximum in rice grains. Population of *T. castaneum* in Hafizabad is nearly equal to district Sheikhupura. The increasing trend in population of *T. castaneum* in rice was same as in July and August, there was increasing trend but after second week of September there was decline. The monthly analysis in lentils showed that minimum population of *T. castaneum* recorded in July and August as compared to wheat and rice. In district Sargodha, population of *T. castaneum* in lentils was maximum but district Sheikhupura showed minimum population. The Analysis of Variance (ANOVA) showed that there was significant ($P<0.05$) difference in population of red flour beetle among different localities and hosts and diets during all observational dates (Table 1a, 1b, 2 and 3)

Table 1a.
Analysis of variance table for all population.

Source of variation	Degrees of freedom	Sum of squares	Mean squares	F-value
Month	3	3033965	1011322	
Crop	2	437737	218868	12.16**
District	2	205605	102803	5.71**
Crop x District	4	263925	65981	3.66*
Error	24	432153	18006	
Total	35	4373385		

* = Significant (P<0.05); ** = Highly significant (P<0.01

Table 1b.
Comparison for mean values of insect population in all localities.

District	Crop			Mean
	Wheat	Rice	Lentils	
Hafizabad	600.5±160.5 c	655.0±172.5 bc	321.8±119.1 d	525.8±091.0 B
Sargodha	813.8±243.7 ab	545.5±146.4 c	504.5±111.7 cd	621.3±101.0 AB
Shaikhupura	678.0±184.9 bc	890.8±261.0 a	563.8±153.4 c	710.8±114.5 A
Mean	697.4±107.5 A	697.1±112.8 A	463.3±74.4 B	

Table 2.
Means of analysis of variance for three cities (Hafizabad, Sargodha and Sheikhupura).

Source of variation	df	Hafizabad		Sargodha		Sheikupura	
		MS	F-value	MS	F-value	MS	F-value
Month	3	229532	9.30*	334455	17.27**	476752	35.94**
Crop	2	127818	5.18*	112850	5.83*	110163	8.30*
Error	6	24690		19362		13265	
Total	11						

NS = Non-significant (P>0.05); * = Significant (P<0.05); ** = Highly significant (P<0.01)

Table 3.
Comparison for mean values of insect population in all localities (districts).

Diet	District			Mean
	Hafizabad	Sargodha	Shaikhupura	
Natural	301	202	280	261.0±30.1 A
Artificial	270	170	240	226.7±29.6 B
Mean	285.5±15.5 A	186.0±16.0 C	260.0±20.0 B	

Data depicted that during the studied samples growth and development of the red flour beetle, *T. castaneum* on wheat, rice and lentil flour at 30±2ºC and 75 % R.H are more Significantly ($P \leq 0.05$) different for different localities and hosts (Hassan and Khan, 1988; Ajayi and Rahman, 2006). It is concluded during the studies that the rate of larval, pupal and adult development of *T. castaneum* considerably varied for the provisions of different kind of food and also other biological factors (Singh *et al.*, 1992; White and Loschiavo, 1988). It is evident that the results obtained in this study are in agreement with those obtained by Hassan and Khan (1988), Singh *et al.* (1992) and White and Loschiavo (1988) population growth of *T. castaneum* is much more higher on wheat and rice as compared to lentils (Hassan and Khan, 1988; Singh *et al.*, 1992) whereas the comparisons of the natural and artificial diets found potential results on natural diets for all foods in all localities (Ajayi and Rahman, 2006). Abusharma *et al.* (1987) examined that the *T. castaneum* adults developed in wheat, more significantly ($P \leq 0.05$) whereas, the rice cultivars grain borer found that there was a significant difference in number of eggs and weight loss percentage between cultivars. Hassan and Khan (1988) studied growth and development of the red flour beetle, *T. castaneum* on wheat and lentil flour found that the larval period was much shorter for the insects reared on wheat flour than those on lentil flour. Singh *et al.* (1992) studied the biology of *T. castaneum* on wheat. Different parameter of correlation of red flour beetle and wheat were significant and positive kept at different laboratory conditions and diets. Highly significant differences were obtained in pupal populations between rice and lentils when *T. castaneum* was reared at different laboratory conditions. Hence, these results confirm the conclusions of Good (1936) and Howe (1956) who stated that larval food had no effect on the pupal period of *T. castaneum*. The results obtained showed that both food and laboratory conditions had significant effects on the total developmental duration of *T. castaneum*. The results presented here are generally in agreement with those of other workers. Abusharma *et al*. (1987) reported that whole wheat flour and semolina were more suitable than whole rice flour and broken (crushed) wheat for the development of the different stages of the *T. castaneum*. Growth and development of *T. castaneum* improved well on cereal seeds (rice, wheat and maize) and their products. Sattigi *et al.*, (1995) investigated the biology of *T. castaneum* on whole flour of rice, wheat, maize and sorghum under uncontrolled laboratory conditions at 24 −29°C and 70% R.H and concluded that with regard to development, wheat flour was the most suitable while rice flour was the least, which agreed with the results of the current investigation. Similar More adults failed to develop and survive in lentils as compared to wheat and rice which demonstrates the better nutritive value of wheat and rice. Similar results were observed in whole meal and artificial diets suggesting high nutritive value of wheat and rice for *T. castaneum* development and effective against pests.

Detection of endosymbiont *Wolbachia*

To investigate the potential of *Wolbachia* on mitochondrial DNA of *T. castaneum,* PCR amplification with mitochondrial primers showed 70-90% amplification of *T. castaneum* for both CO1 and S12 from Hafizabad, Shaikhupura, Sargodha, Peshawar and Hyderabad districts. DNA bands patterns of approximately 600 bps were obtained (Fig. 3, Table 4). Genetic analysis of the mitochondrial markers (data not shown) of *T. castaneum* showed very promising results and after purification of the samples sequences will be generated in future phylogenetic experiments.

PCR with *Wolbachia* general primers

Polymerase Chain Reaction is used for the exposure of *Wolbachia* in *T. castaneum* population in different districts of Pakistan. First, good quality of genomic DNA was extracted from collected samples (10 from each locality) and then analysis has been done through molecular techniques using *Wolbachia* general primers *wsp* 81F and *w*sp 691R.

Some populations of *T. castaneum* were found infected with *Wolbachia* but the density of *Wolbachia* infection is quite low. However, the *T. castaneum* population of Hafizabad and Sheikhupura exhibited high degree of *Wolbachia* infection than population of Sargodha with a diversified frequency of infection 24%, 25% and 17% respectively. Red Flour Beetle population of Peshawar and Hyderabad showed no sign of *Wolbachia* infection during the preliminary screening however some samples of these localities were again steered and found positive amplicons with quite low density of infection 15% and 10% respectively (Fig. 4, Fig. 5). DNA bands patterns of approximately 600 bps were obtained during the amplification. The confirmatory test results of qPCR datasets showed that there were positive amplicons of *Wolbachia* in *T. castaneum* samples, collected from different districts of Pakistan though density of *Wolbachia* infection was quite low (data not shown).

Wolbachia has the ability to alter the reproductive capacities of its host, due to this ability in most of countries huge efforts have been going on role of *Wolbachia* as biological control component against many arthropod species. With the help of *Wolbachia* general primers *w*sp 81F and *w*sp 691R, *Wolbachia* was found dominant among the examined species like reported for other arthropods species (Wang *et al.*, 2009; Arthofer *et al.*, 2009; Selivon *et al.*, 2002).

We studied for the first-time screening of *T. castaneum* population of Pakistan for the mitochondrial diversification which may help for detail study of possible infection of the endosymbiont *Wolbachia* through CO1 mitochondrial gene primers in the selected populations of *T. castaneum. Wolbachia* and mitochondrial DNA have the same way of transmission i.e. cytoplasmic pathway. *Wolbachia* influence the host mitochondrial DNA evolution and results in its diversification. Ming *et al.* (2015) reported *Wolbachia* Infection dynamics in *Tribolium* species and their effects on the mating behavior and reproduction of the host. During these findings *Wolbachia* general primers (*w*sp primers) were used for screening of *Wolbachia* and most of the *T. castaneum* populations from the selected regions of Pakistan were found infected. Potential of selected *w*sp primers for strain characterization was found limited due to frequent recombination's (Malloch and Fenton, 2005). The *Wolbachia* infection rate was quite low may be due to high density of its associated bacteriophages. Arthofer *et al.* (2009) reported the high *Wolbachia* diversity in *Rhagoletis cerasi* from eight European countries. *T. castaneum* population of Pakistan also

showed higher rate of amplification through mitochondrial gene (CO1) primers. *Wolbachia* has ability to lessen the suitability of their host and change the reproductive strategies of its hosts and this phenomenon could be used as another method to control the agricultural insect pests (McMeniman *et al.*, 2009; Moreira *et al.*, 2009; Zabalou *et al.*, 2009). Scientists have focused on the possible application of *Wolbachia* through biological control programmes (Zabalou *et al.*, 2009; Rasool *et al.,* 2017) and incorporation in integrated pest management of insect pest including agricultural pests and vectors of human diseases and also can be avoided. Different strategies have been projected including discharge of properly raised transinfected males directly in the field to reduce the natural population of insect pest or using these symbionts to transfer the genes of desire all over the pest population (Saridaki and Bourtzis, 2010)

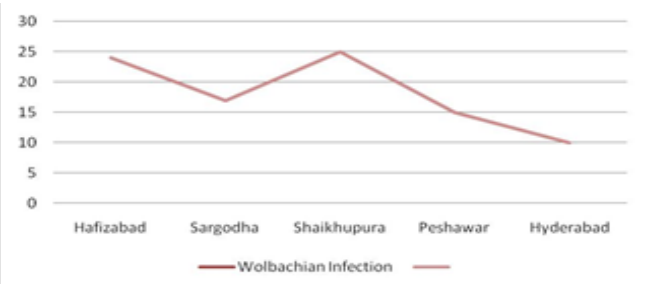

Fig. 4
Amplification of *Tribolium castaneum* samples through *wsp Wolbachia* gene

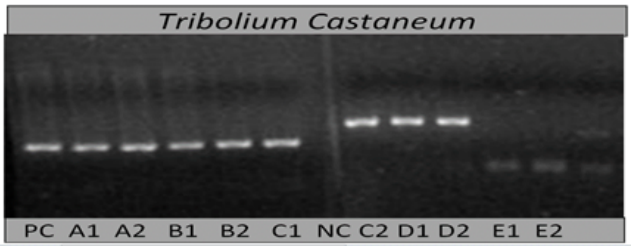

Fig. 3
PCR amplications of individuals of *Tribolium castaneum* from Hafizabad [A1,A2], Sheikhupura [B1,B2], Peshawar [C1,C2], Hyderabad [D1,D2] and Sargodha [E1,E2] using CO1 mitochondrial primer, Positive control [PC], Negative control [NC].

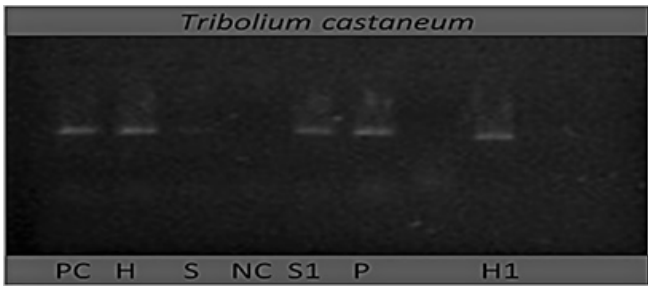

Figure. 5
PCR amplicons of individuals of *T. castaneum* from Hafizabad [H], Sargodha [S], Sheikhupura [S], Peshawar [P] and Hyderabad [H1] using *wsp* gene, Positive control [PC], Negative control [NC].

Table 4.
Amplification of *Tribolium castaneum* samples through mitochondrial (CO1 and S12) genes.

Sr. No.	Location	Number of samples	Results CO1	Results S12
1	Hafizabad	10	++++++++ --	+++++++++ -
2	Shaikhupura	10	+++++++++ -	+++++++++ -
3	Sargodha	10	+++++++ ---	+++++++ ---
4	Peshawar	10	+++++++++ -	+++++++++ -
5	Hyderabad	10	++++++++ --	+++++++++ -

CONCLUSION

The total developmental period of *T. castaneum* reared on wheat, Rice and Lentils both on natural and artificial diets varied considerably according to the prevailing environmental conditions dietary ingrdients 2. Food type different cereals served as food had significant effect on the total developmental period and body weight of the *T. castaneum* which indicates its suitability. Natural diets of Rice and wheat was the most favorable for the development of the different biological stages of the beetle; 3. The potential mitochondrial DNA markers of *T. castaneum,* from all studied localities are very much promising which may help to study its phylogenetics, evolution and speciation. 4. Screening of *Wolbachia* in *T. castaneum* found positive amplicons and further its strain diversification which may be integrated in future pest management strategies and open new horizons.

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