



## EVALUATING POPULATION DYNAMICS, PHYLOGENETIC AND BIOCHEMICAL VARIABILITY IN THE GERMAN (*BLATTELLA GERMANICA*) AND AMERICAN (*PERIPLANETA AMERICANA*) COCKROACHES

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### ABSTRACT

The present study is the first one from Pakistan which has been devised with an objective to unearth population dynamics and molecular phylogeny of German (*B. germanica*) and American (*P. americana*) cockroaches using mitochondrial cytochrome oxidase genes subunit I and II (COI and COII). Furthermore, it also presents a comparative analysis of biochemical variability within these two species. Cockroaches (n=1500) were captured from various residential buildings and population dynamics was ascertained through morphologic identification. The conventional PCR was used for COI and COII gene analysis in both species. Results revealed that from a total of 1500 adult and nymph cockroaches captured, significantly ( $P \leq 0.05$ ) higher (810, 54%) population was of the *P. americana* species whereas 690 (46%) were of *B. germanica* species. Adults were significantly ( $P \leq 0.05$ ) dominant both for *B. germanica* (437, 54%) and *P. americana* (351, 51%) species. Similarly, females had significantly ( $P \leq 0.05$ ) dominant population (945, 63%) as compared to male cockroaches (555, 37%). The PCR products of the isolates of both species in the present study revealed 710bp DNA for COI and 750bp DNA for COII in both species. All the studied biochemical attributes (total protein, total glucose, alanine transaminase and aspartate transaminase) were significantly ( $P \leq 0.05$ ) higher for *B. germanica* as compared to *P. americana*. In conclusion, the *B. germanica* cockroaches have a dominant population as compared to the *P. americana* in Pakistan. Furthermore, the phylogenetic results on cockroaches in our study though showed diversity in isolates but are suggestive of their polyphyletic nature. The biochemical analytes in these cockroach species can be utilized as reference values. Future research directions need a detailed population dynamics of Pakistan in relation to genetic variability.

**Keywords:** Phylogenetics, *P. americana*, *B. germanica*, Pakistan

### 1. Introduction

Cockroaches (also known as 'roaches') basically belong to the order Blattodea (Blattaria) and superorder (Dictyoptera). Apart from cockroaches, this superorder also includes termites and mantids (Inward et al., 2007). About 4600 species of cockroaches have been reported worldwide. However, from these 4600 species, only 30 species have close encounter and association with humans. The three species mostly prevalent globally (including Pakistan), in order of prevalence, are the American cockroach (*Periplaneta americana*), the German cockroach (*Blattella*

*germanica*) and the Oriental cockroach (*Blatta orientalis*) (Booth et al., 2011; Memona et al., 2014). Owing to their uniqueness in feeding, living, breeding, exploitative nature, physiology, and morphology, they are considered as most diverse pests with economic and medical importance. Though mostly dwelling in caves, crevices, under the rocks and in dark places, they are also abundant in almost every human-made facility such as buildings, apartments, hospitals, pantries, restaurants *etc.* In general, unsanitary, warm and moist environments are their most favorite dwelling sites thus being one of the major vectors of many protozoan, viral and bacterial diseases (Tatfeng et al., 2005). In addition, they may cause allergic dermatitis, asthma, typhoid fever and food poisoning. Research studies have revealed that cockroaches can transmit about 50 species of pathogenic bacteria mostly including *Salmonella*, *Campylobacter* and Shiga-toxin producing *E. coli*. Mechanical transmission by cockroaches occurs through their saliva, faeces and nutrients of their eggs (Liu et al., 2024; Tatfeng et al., 2005).

Human thought and behavior towards pests such as cockroaches mainly depend upon human knowledge and type of interaction between humans and pests. Factors such as traditions, religious inclination and rituals greatly influence these behaviors (Naqqash et al., 2014). A few life style studies regarding perception of Pakistani people towards various pests have reported that cockroaches are the most abundant pests in Pakistan. They are considered filthy insects and are killed instantly upon sight. Excessive cleaning and maintenance of hygiene are the only coping mechanisms used in general by the Pakistanis to eradicate and control cockroach populations (Naeem et al., 2014; Rasheed et al., 2024). Furthermore, scanty studies regarding the vector potential of cockroaches have also been conducted in Pakistan and it has been reported that the cockroaches are main vectors for *Pseudomonas*, *Escherichia*, *Salmonella*, *Klebsiella*, and *Shigella* (Arif et al., 2017; Naqqash et al., 2014). Presence of bacterial load in the biological samples of patients as well as on the body of cockroaches have confirmed cockroaches as major source of nosocomial infections in another Pakistani study (Memona et al., 2014).

Regarding genetic variability and phylogenetic studies on various species of cockroaches, literature is rife with research studies which have mainly emanated from USA (Murienne et al., 2008), China (He et al., 2022; Xiao et al., 2012; Zhu et al., 2021), Turkey (Cevahir et al., 2023) and Iran (Hashemi-Aghdam et al., 2017). However, to the best of knowledge, there is no such study for cockroaches in Pakistan. The present study is thus the first one from Pakistan which has been devised with an objective to unearth population dynamics and molecular phylogeny of German (*B. germanica*) and American (*P. americana*) cockroaches using mitochondrial cytochrome oxidase genes subunit I and II (COI and COII). Furthermore, it also presents a comparative analysis of biochemical variability within these two species.

## 2. Materials and methods

### 2.1. Insects

The nymph and adult cockroaches (n=1500) were collected from various residential buildings and apartments of Lahore, Pakistan from May to August, 2023, during the night time. Food-baited pitfall traps were used for the capture. Each cockroach was carefully captured and transferred in sterile glass jars for transport to the Entomology Research Laboratory of the Lahore College for Women University, Lahore, Pakistan. All cockroaches were anaesthetized through freezing at 0°C for 5mins and examined under dissection microscope for taxonomic characteristics (Harwood and James, 1979). Identification of the two species (*B. germanica* and *P. americana*) of cockroaches and sexual dimorphism was carried out through morphological features as prescribed and keeping in perspective the external pattern, color, supination and setation on bodies of the collected cockroaches (Fotedar et al., 1991).

Prior to phylogenetic and biochemical analyses, the cockroaches were washed thoroughly with normal saline. After external washing, they were soaked in 70% ethyl alcohol for 2-3mins and dried in room temperature in order to attain external decontamination (Harwood & James, 1979). Later they were frozen in ice till further analyzed.

## 2.2. Sampling and DNA extraction

The genomic DNA extraction was carried out from the legs of the cockroaches using Qiagen AMP DNA mini-extraction kits (Hilden, Germany, CAT# 51306). The prescribed protocol for extraction was implied (Gilbert and Vance, 1998) with minor modification. The sample was homogenized with dissection scissors in microcentrifuge tubes. For lysis, about 30-50mg sample was lysed through 180 $\mu$ L of ATL buffer (Qiagen, Holden Germany) having 20 $\mu$ L of proteinase K. This solution was incubated at 56°C overnight in a shaking water bath. Post-incubation, a 50 $\mu$ g of RNase A (Boeringer Mannheim, Germany) was added to the solution. After multiple chloroform extractions, the DNA was extracted with an equal volume of isopropanol. It was stored at -20°C till further analyzed.

## 2.3. PCR amplification and sequencing

A 658bp segment of mitochondrial COI gene, and 601 bp segments for COII and COII genes were amplified for present study. Universal primers for insects were used as in previous studies (Simon et al., 1994; Yue et al., 2014; Zhang et al., 2010) and are given below:

COI Primers: 1. LCO1490 (GGTCAACAAATCATAAAGATATTGG)

2. HCO2198 (TAAACTTCAGGGTGACCAAAAATCA)

COII Primers: 1. CO1DL (CCWCGWCGWTAYTCWGAYTAYCCWGA)

2. CO2DL (WGAATARRCATAWSWTCARTATCATTG)

PCR was conducted using a thermocycler (Applied Biosystems GeneAMP PCR System, 2700) and the PCR conditions for COI and COII are given in Table 1. Products were examined through gel electrophoresis and all the attained fragments were sequenced in both directions. The amplified and purified PCR amplicon were sequenced by Apical Scientific SDN, BHD. The nucleotide sequences of COI and COII of German and American cockroaches were retrieved from the NCBI GenBank repository and compared to sequences of this study isolates.

## 2.4. Phylogenetic analysis

Maximum likelihood (Tamura Nei+1) and neighbor-joining phylogenetic trees for both COI and COII in German and American cockroaches were estimated using freeware Molecular Evolution and Genetic Analysis (MEGA X, Pennsylvania State University, USA). Non-parametric bootstrapping was used for ascertaining the robustness. Models with lowest Akaike Information, Criterion Correction (AIC) values were used for devising the phylogenetic trees.

## 2.5. Biochemical analysis

For determining the biochemical analytes in the hemolymph of studied cockroaches, the cockroaches were thawed with a washing of 90% ethyl alcohol, brought to room temperature and hemolymph was attained as prescribed (Smith, 1994). Total proteins (TP) were determined as per Bradford (Bradford, 1976) whereas the total glucose (TG) was determined through the acid extract of the study sample by phenol-sulphuric acid reaction (DuBois et al., 1956), Alanine Transaminase (ALT) and Aspartate Transaminase (AST) were determined through commercially available kits using spectrophotometer.

## 2.6. Statistical analysis

The Statistical Package for Social Sciences (Windows Version 12, SPSS Inc, Chicago, USA) was used for the statistical tests. Values for the studied biochemical attributes were given as mean ( $\pm$ SE). Normality of data was tested through Shapiro-Wilk tests and homogeneity of variance. For attaining the difference between the biochemical analytes of *B. germanica* (n=100) and *P. americana* cockroaches (n=100), t-test was implied on the means, with a difference being significant at  $P \leq 0.05$ . Chi square test was implied on percentages for population dynamics.

### 3. Results and discussion

Pakistan belongs to the Warm Climate Zones of the world with hot and humid weather as its mainstay. The climatic conditions hence are conducive to various pests and insects. Though a few studies related to the vector potential of cockroaches have been published (Naeem et al., 2014; Naqqash et al., 2014), however there is a complete paucity of research work regarding the population dynamics, phylogeny and biochemical analyses of cockroaches prevalent in Pakistan. The present study hence the first of its kind which presents a preliminary data regarding the population dynamics, phylogeny and biochemical analyses of the cockroaches prevalent in Pakistan. Results regarding morphological identification of cockroaches revealed that from a total of 1500 adult and nymph cockroaches captured, significantly ( $P \leq 0.05$ ) higher (810, 54%) population was of the *P. americana* species whereas 690 (46%) were of *B. germanica* species. Adults were significantly ( $P \leq 0.05$ ) dominant both for *B. germanica* (437, 54%) and *P. americana* (351, 51%) species. Similarly, females had significantly ( $P \leq 0.05$ ) dominant population (945, 63%) as compared to male cockroaches (555, 37%). There is a paucity of literature from Pakistan regarding population dynamics of cockroaches hence comparisons of our results cannot be conducted. However, comparing our results with those from other countries it was revealed that Turkey has reported a dominant population of *B. germanica* (58%) followed by *B. orientalis* (32%) and *P. americana* (10%) (Cevahir et al., 2023) The species of cockroaches in this Turkish study was confirmed through Restriction Fragment Length Polymorphism (RFLP) and nucleotide sequence. In another study conducted on cockroach population dynamics of Turkish hospitals, *B. germanica* was dominant in population (98.2%) followed by *B. orientalis* (1.1%) (Kutrup, 2003). A study from Nigeria has reported results similar to ours with higher population dynamics for *P. americana* (73%) as compared to *B. germanica* (27%) (Ejimadu et al., 2015). On the contrary, the European countries mostly are abundant in *B. germanica* cockroaches (Tang et al., 2019). A study conducted to assess vector potential of cockroaches in Pakistan has reported that isolates from *P. americana* had highest contamination (45%) followed by that in *B. orientalis* (32%) and *B. germanica* (23%) with females showing highest contamination (65%) as compared to their counterpart males (35%) (Arif et al., 2017). These results if tallied with ours make sense that higher population of *P. americana* results in higher vector potential. It is worth mentioning that in our study, the species identification of collected cockroaches was solely conducted through morphologic features. And it has been elaborated that this method of species identification and nymph/adult identification is an ambiguous method because of supination, setation and coloration of the insects (Cevahir et al., 2023; Kutrup, 2003). While studying population dynamics of cockroaches, it is inevitable their living habitat must also be kept in perspective. The *B. orientalis* and *P. americana* have a liking for wet and humid environments whereas the *B. germanica* tends to live mostly in kitchens and basements (Kutrup, 2003). In our study, all the cockroaches were collected from residential buildings and apartments and during rainy months (monsoon), hence this could be a plausible justification for dominant population of *P. americana* as compared to the *B. germanica* cockroaches.

The PCR products of the isolates of *B. germanica* and *P. americana* cockroaches in present study examined through gel electrophoresis revealed 710bp DNA for COI and 750bp for COII in both species (Fig 1 and 2). While using COI as a marker of species determination in cockroaches, approximately 700bp sequence was attained having a barcode region of 658bp (Şeyda and Pektaş, 2023). An interesting study directed towards assessing the origin of cockroaches through various biomarkers including COI and COII has reported 1280bp for COI and 650bp for COII, respectively (Legendre et al., 2015). On this basis, the Devonian origin of cockroaches was nullified whereas the Permian origin was endorsed. Results similar to ours have been reported in a study directed towards phylogeny of cockroaches which reported amplification and sequencing of 3' and 5' ends of COI and COII in cockroaches, respectively (Djernaes et al., 2012). A lower range of 679bp to 687bp has been reported for COII gene while studying phylogenetic relationship between five families of cockroaches (Maekawa and Matsumoto, 2000). A unique 710bp DNA in five species of cockroaches (*B. germanica*, *P. americana*, *S. lateralis*, *B. orientalis* and *S. Longipalpa*) has been

reported using mtDNA barcode in concordance to our results (Hashemi-Aghdam et al., 2017). The COI and COII genes have ultimately been endorsed as vital biomarkers for species identification in cockroaches (Fan et al., 2022; Hashemi-Aghdam et al., 2017).

The nucleotide sequences for COI and COII in *B. germanica* and *P. americana* cockroaches of the present study were submitted to the International Nucleotide Sequence Database Collaboration via NCBI GenBank and are given in Table 2 and 3, respectively. The phylogenetic associations for COI and COII in *P. americana* and *B. germanica* are given in Fig 4 and 5, respectively. Various studies have globally been published regarding phylogenetic analysis of various cockroach species and have reported results different than ours. The superorder dictyoptera which includes orders of Blattodea (cockroaches and termites) and Mantodea (mantises). The mantises and termites are monophyletic whereas the cockroaches are considered as polyphyletic. The phylogenetic results on cockroaches in our study though showed diversity in isolates but are suggestive of their polyphyletic nature, and are close to the ones reported while assessing origin of cockroaches earlier (Legendre et al., 2015). Similar topology of phylogenetic trees for these two species of cockroaches (*B. germanica* and *P. americana*) have been reported with minor difference in few alignments (Cheng et al., 2016; Hashemi-Aghdam et al., 2017).

The present study, for the first time, included the comparative analysis of various biochemical attributes (TP, TC, TG, ALT and AST) in the hemolymph of *B. germanica* and *P. americana* cockroaches which can be used as reference intervals for cockroaches, and the results are given in Table 4. All the studied biochemical attributes were significantly ( $P \leq 0.05$ ) higher for *B. germanica* as compared to *P. americana*. Mean ( $\pm$ SE) values for the studied attributes of present study are way higher than those reported for *B. germanica* in a study conducted on assessing the effect of silver nanoparticles on this specie (Abd El-Raheem and Eldafrawy, 2016). Difference in geo-location of the study could be attributed to this difference. Furthermore, higher values of the present study than those reported earlier could be an inherent property of cockroaches dwelling in Pakistan or could be an adaptive mechanism. While studying the carbohydrate and fat metabolism in *P. americana* cockroaches, it has been reported that this species has a high metabolism for lipid and thus enhanced than normal lipogenesis and gluconeogenesis which cause elevation of biochemical analytes (Michitsch and Steele, 2008; Oguri and Steele, 2003).

#### 4. Conclusion

In a nutshell, the *P. americana* cockroaches have a dominant population as compared to the *B. germanica* in Pakistan. Furthermore, the phylogenetic results on cockroaches in our study though showed diversity in isolates but are suggestive of their polyphyletic nature. The biochemical analytes in these cockroach species are far higher than reported elsewhere and can be utilized as reference values. Future research directions need a detailed population dynamics of Pakistan in relation to genetic variability. Furthermore, methods regarding analyses of analytes in hemolymph of the cockroaches need avid attention.

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#### Credit authorship contribution statement

**Safoora Riaz:** Supervision, Project Administration

**Ayesha Zafar:** Investigation, Validation, Data Analysis

**Farkhanda Manzoor:** Conception of Idea

**Mushtaq Hussain Lashari:** Write up and Review

#### Declaration of competing interest

The authors have no conflict of interest to declare.

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**TABLES**

**TABLE 1** PCR conditions for cytochrome oxidase genes subunit I and II (COI and COII) in *B. germanica* and *P. americana* cockroaches

Stages	Cycle	Temperature	Time
<b>COI</b>			
<b>Stage 01</b>	Initial Denaturation	94°C	5min
	Denaturing	94°C	30sec
<b>Stage 02</b> 35 Cycles	Annealing	50°C	35sec
	Extension	72°C	40sec
<b>Stage 03</b>	Final Extension	72°C	5.0min
<b>Stage 04</b>	Hold	4°C	Infinity
<b>COII</b>			
<b>Stage 01</b>	Initial Denaturation	94°C	5min
	Denaturing	94°C	30sec
<b>Stage 02</b> 35 Cycles	Annealing	55°C	35sec
	Extension	72°C	40sec
<b>Stage 03</b>	Final Extension	72°C	5min
<b>Stage 04</b>	Hold	4°C	Infinity

**TABLE 2** The isolates and their published accession numbers for *B. germanica*

#	Isolates	Accession #	
		COI	COII
1	<i>B. germanica</i> LCWU11 (Pakistan 2023)	PP087114	PP101122
2	<i>B. germanica</i> LCWU12 (Pakistan 2023)	PP087115	PP101123
3	<i>B. germanica</i> LCWU13 (Pakistan 2023)	PP087116	PP101124
4	<i>B. germanica</i> LCWU14 (Pakistan 2023)	PP087117	PP101125
5	<i>B. germanica</i> LCWU15 (Pakistan 2023)	PP087118	PP101126
6	<i>B. germanica</i> LCWU16 (Pakistan 2023)	PP087119	PP101127
7	<i>B. germanica</i> LCWU17 (Pakistan 2023)	PP087120	PP101128
8	<i>B. germanica</i> LCWU18 (Pakistan 2023)	PP087121	PP101129
9	<i>B. germanica</i> LCWU19 (Pakistan 2023)	PP087122	PP101130
10	<i>B. germanica</i> LCWU20 (Pakistan 2023)	PP087123	PP101131

**TABLE 3** The isolates and their published accession numbers for *P. americana*

#	Isolates	Accession #	
		COI	COII
1	<i>P. americana</i> LCWU1 (Pakistan 2023)	PP087104	PP101112
2	<i>P. americana</i> LCWU2 (Pakistan 2023)	PP087105	PP101113
3	<i>P. americana</i> LCWU3 (Pakistan 2023)	PP087106	PP101114
4	<i>P. americana</i> LCWU4 (Pakistan 2023)	PP087107	PP101115
5	<i>P. americana</i> LCWU5 (Pakistan 2023)	PP087108	PP101116
6	<i>P. americana</i> LCWU6 (Pakistan 2023)	PP087109	PP101117
7	<i>P. americana</i> LCWU7 (Pakistan 2023)	PP087110	PP101118
8	<i>P. americana</i> LCWU8 (Pakistan 2023)	PP087111	PP101119
9	<i>P. americana</i> LCWU9 (Pakistan 2023)	PP087112	PP101120
10	<i>P. americana</i> LCWU10 (Pakistan 2023)	PP087113	PP101121

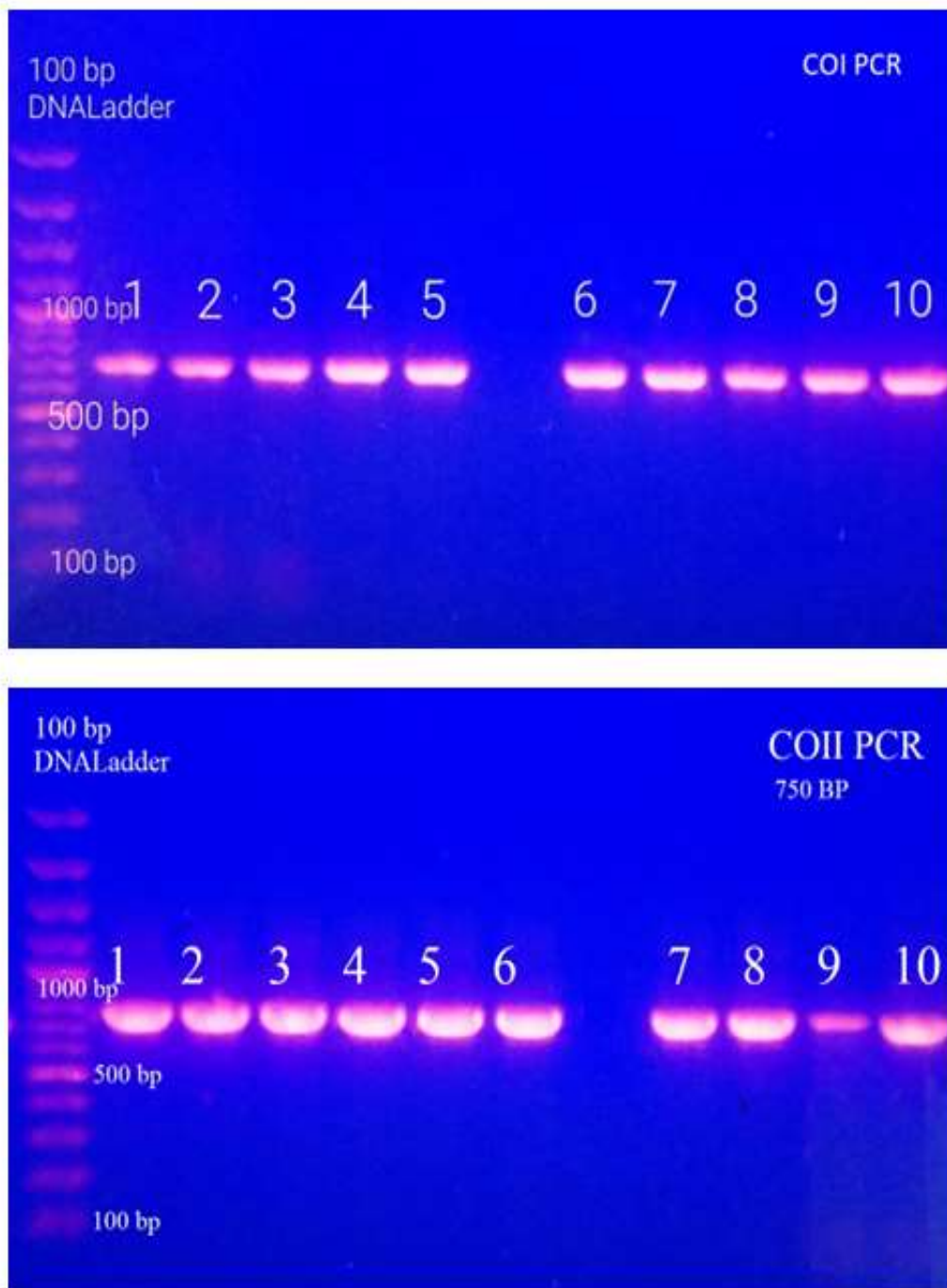


**TABLE 4** Biochemical analytes of the hemolymph for *B. germanica* (n=50) and *P. americana* (n=50)

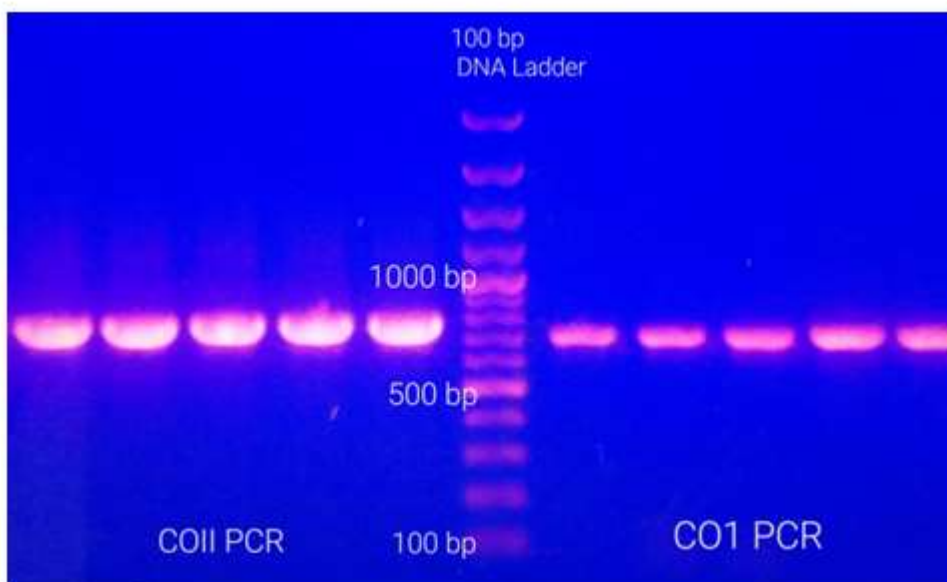
Analytes	<i>B. germanica</i>	<i>P. americana</i>
Total Protein (mg/g b.wt)	11.2±1.0*	9.5±0.7
Total Glucose (mg/g b. wt)	17.2±1.8*	9.8±0.4
Alanine Transaminase (U/L)	102.3±2.4*	99.5±1.8
Aspartate Transaminase (U/L)	205.1±3.4*	188.9±3.0

\*is significant at  $P \leq 0.05$  within the two species of cockroaches

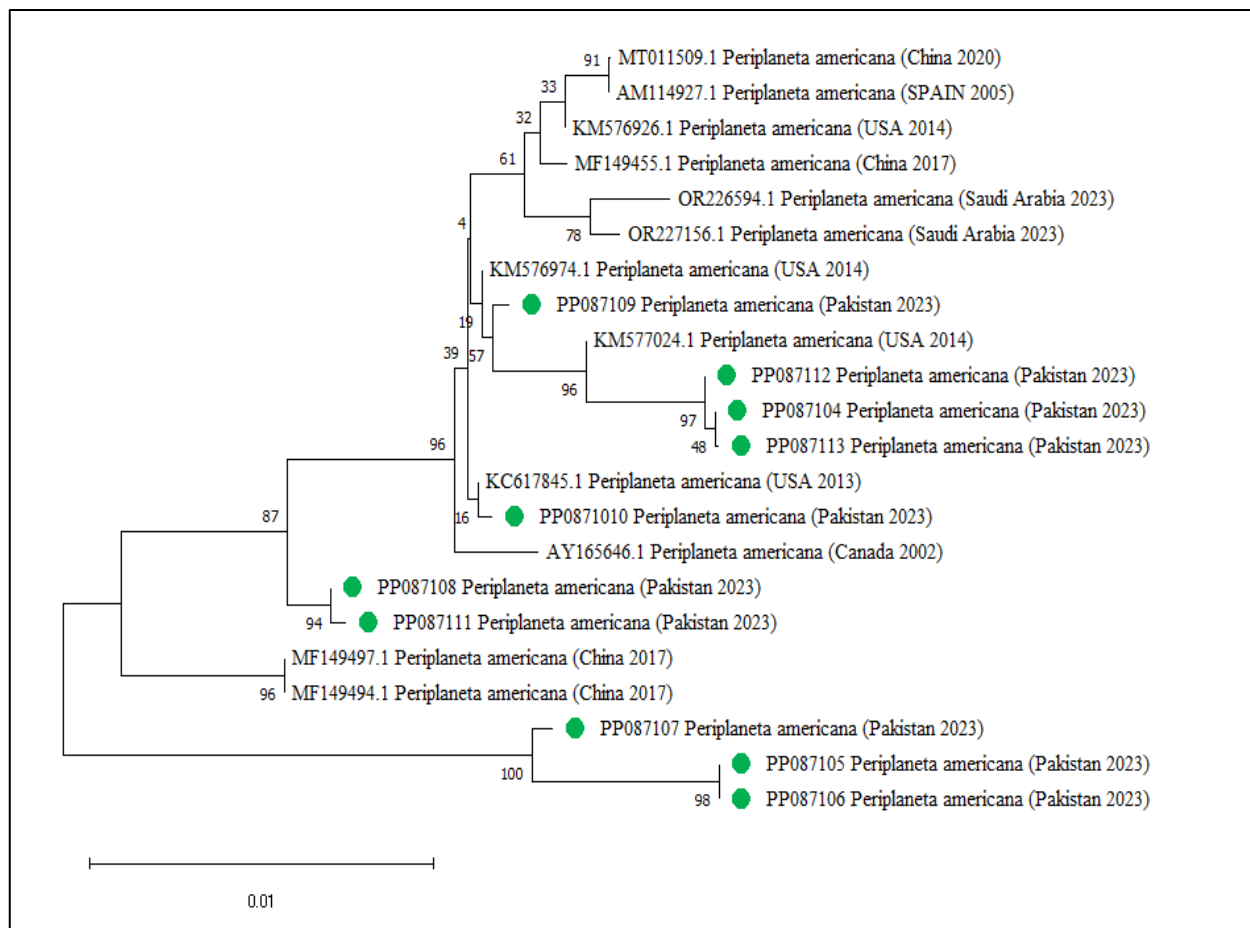
**FIGURES**



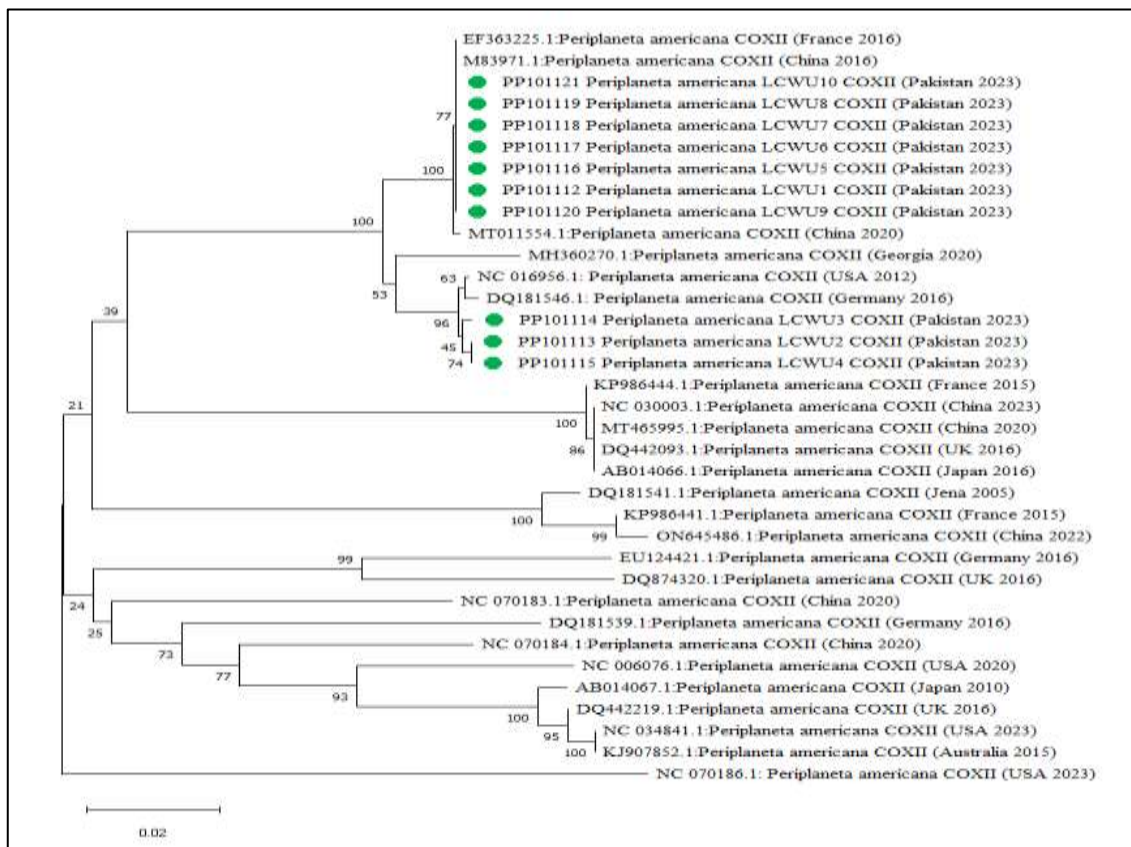
**Fig 1.** Agarose Gel electrophoresis for a) COI and b) COII in *B. germanica* showing 710bp and 750bp DNA, respectively



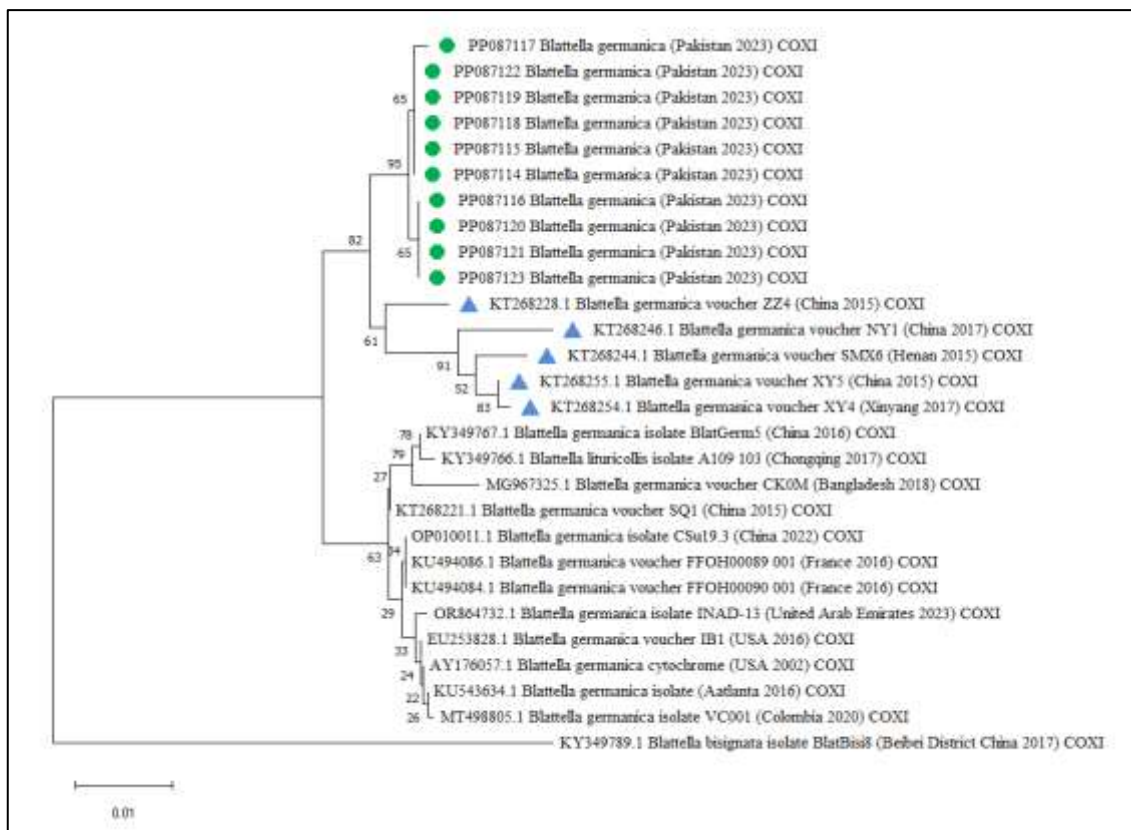
**Fig. 2.** Agarose Gel electrophoresis for COI and COII in *P. americana* showing 710bp and 750bp DNA, respectively



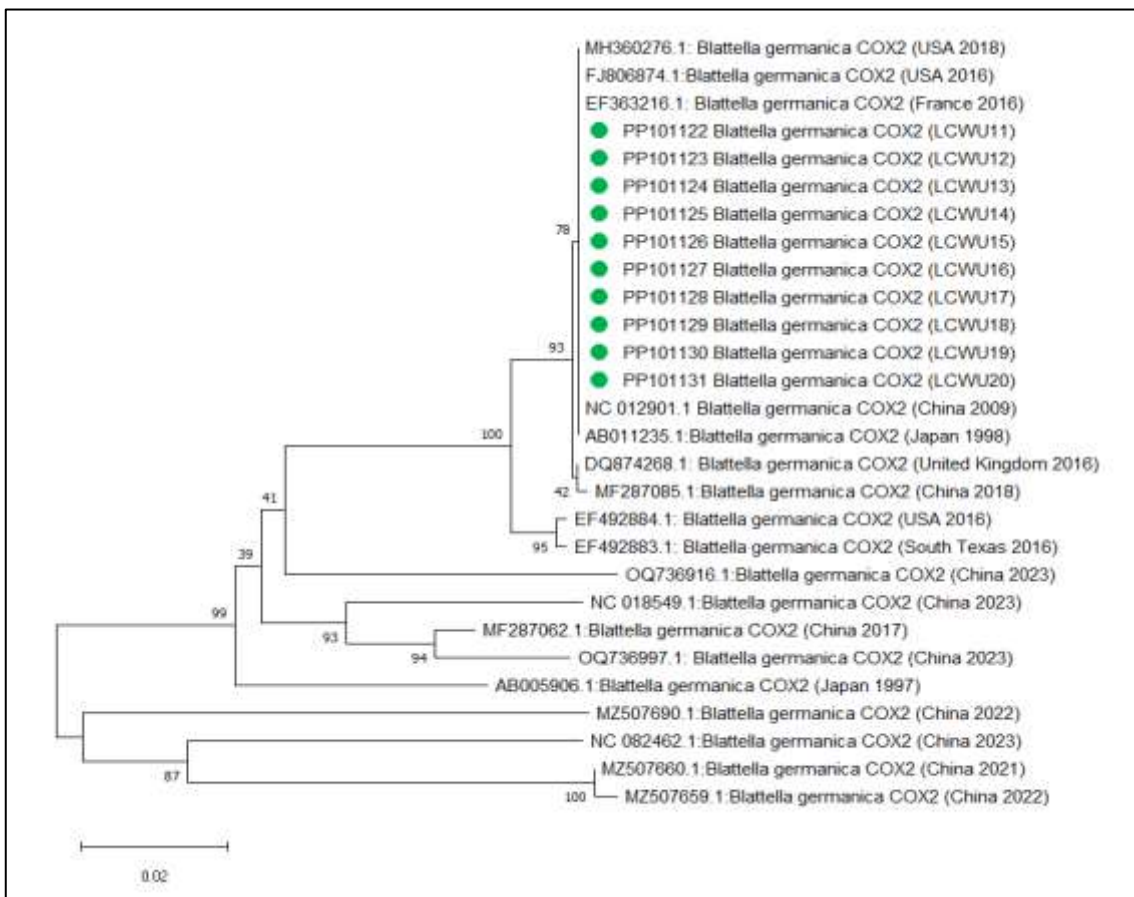
**Fig. 3.** Phylogenetic tree from Bayesian inference and maximum likelihood analysis for COI DNA in *P. americana*



**Fig. 4.** Phylogenetic tree from Bayesian inference and maximum likelihood analysis for COII DNA in *P. americana*



**Fig. 5.** Phylogenetic tree from Bayesian inference and maximum likelihood analysis for COI DNA in *B. germanica*



**Fig. 6.** Phylogenetic tree from Bayesian inference and maximum likelihood analysis for COII DNA in *B. germanica*