

SCIENCE PRESENTATION SERIES

BY

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**Studies on
the Molecular Diversity
and
Geographical Distribution of
the Invasive Species
*Rhynchophorus ferrugineus***

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REFERENCE

Rabab A.A. El-Mergawy (2011). Studies on the molecular diversity and geographical distribution of the invasive species *Rhynchophorus ferrugineus*. Ph.D. Thesis, Molecular Biology Department, Genetic Engineering and Biotechnology Research Institute (GEBRI), Minoufia University: Egypt p. 126.

*Introduction

→ Invasive species

→ *Rhynchophorus ferrugineus*

✓ Classification

✓ Host palms

✓ Life cycle

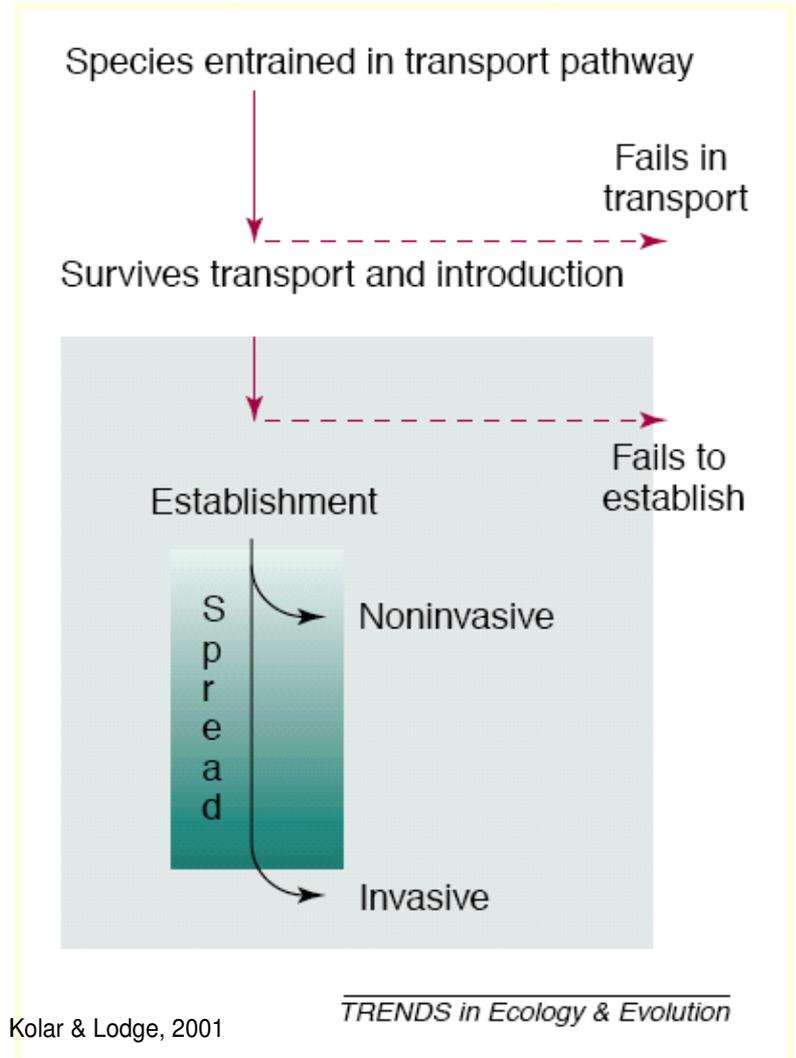
✓ Symptoms of infestation

✓ Geographical distribution

→ Objectives

→ Invasive species

- ✓ **A stimulating topic for ecologists and evolutionary biologists.**
- ✓ **A worldwide ecological, Economical and health problem.**

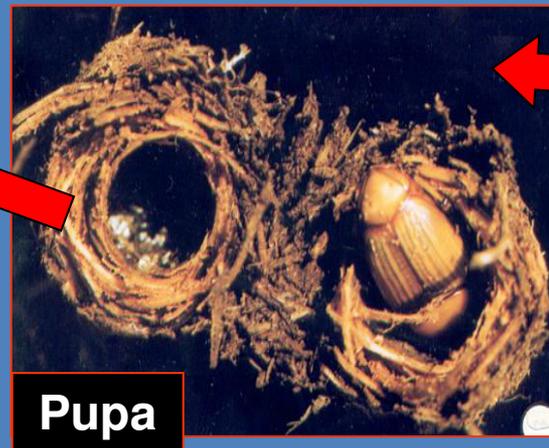
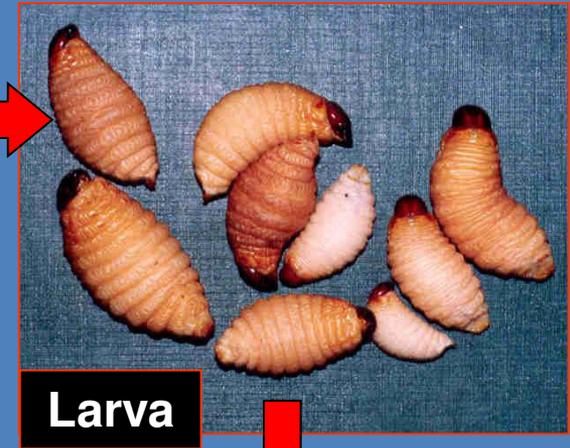
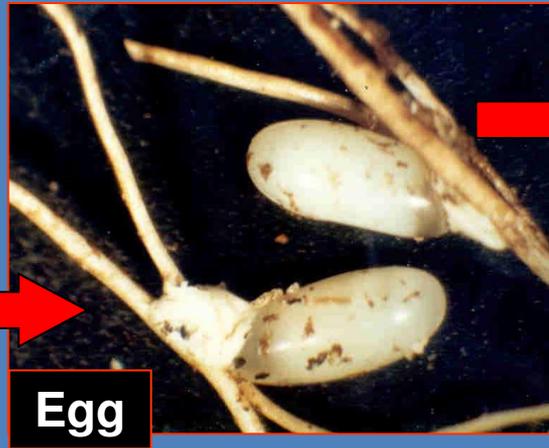
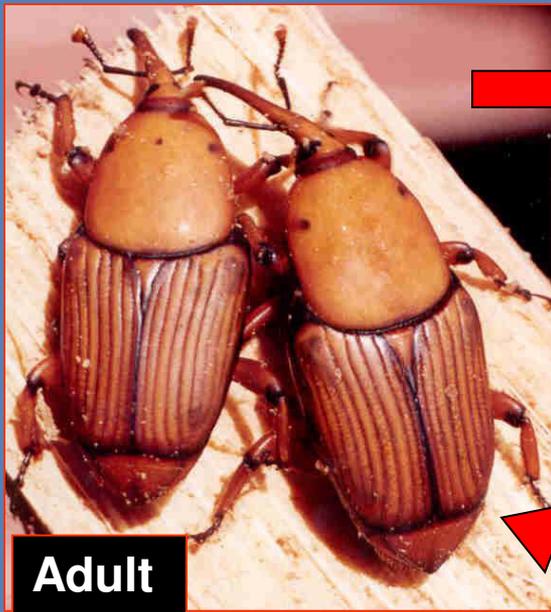


➔ *Rhynchophorus ferrugineus* Classification

- Red Palm Weevil – *Rhynchophorus ferrugineus*
- Order: Coleoptera
- Family: Curculionidae
- Subfamily: Dryophthorinae (Rhynchophorinae)
- Genus: *Rhynchophorus*
- Species: *ferrugineus*

→ *Rhynchophorus ferrugineus*

Life cycle: 45-298 days-4 generations



➔ *Rhynchophorus ferrugineus*

Host palms

➤ Palms species-17 species

- Coconut palm
- Date palm
- Nigbong palm
- Oil palm
- Ornamental palm
- Palmyra palm
- Royal palm
- Sago palm
- Sedang palm
- Sugar palm
- Talipot palm
- Wild date (toddy) palm

➤ Palm age preference

- Infest palms below the age of 20 years.
- Soft, juicy and easily penetrated stem.

➤ Sites of infestation

- The trunk of the palm can be infested in any parts, including the crown.
- Females lay their eggs on any part of the palm specially in:
 - Cracks.
 - Wounds.
 - At the leaf axil.
 - At from where offshoots emerge.

✓ Symptoms of infestation

➤ Brownish viscous liquid oozed out from date palm.

➤ Opening of a tunnel on the base of a leaf petiole.

➤ Breaking of the trunk.

➤ Shewed plant tissues.

➤ Gnawing sound due to feeding.

➤ Fermented odor.

➤ Drying of offshoots.

➤ Fallen empty pupal cases and dead adults around the

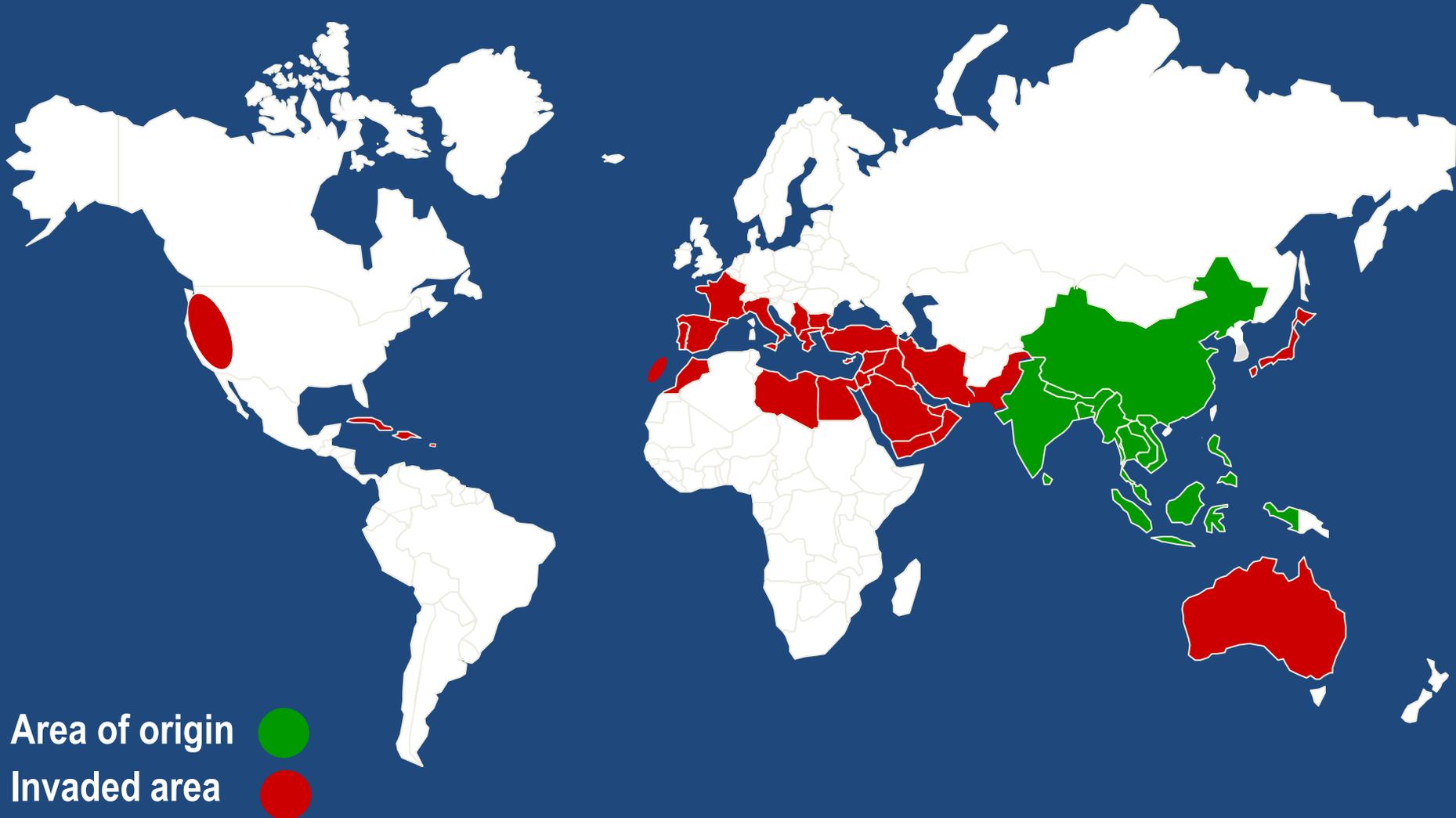
➤ infested palms.

➤ Toppling of the crown.

➤ Larva normally never comes to the surface, since it begins its life inside the palm. Therefore, neither the damage nor the larva can be seen.

➤ Yield has been estimated to have dropped from 10 tonnes to 0.7 tonnes per hectare in infested plantations.

→ *Rhynchophorus ferrugineus*
Geographical distribution



*Objectives

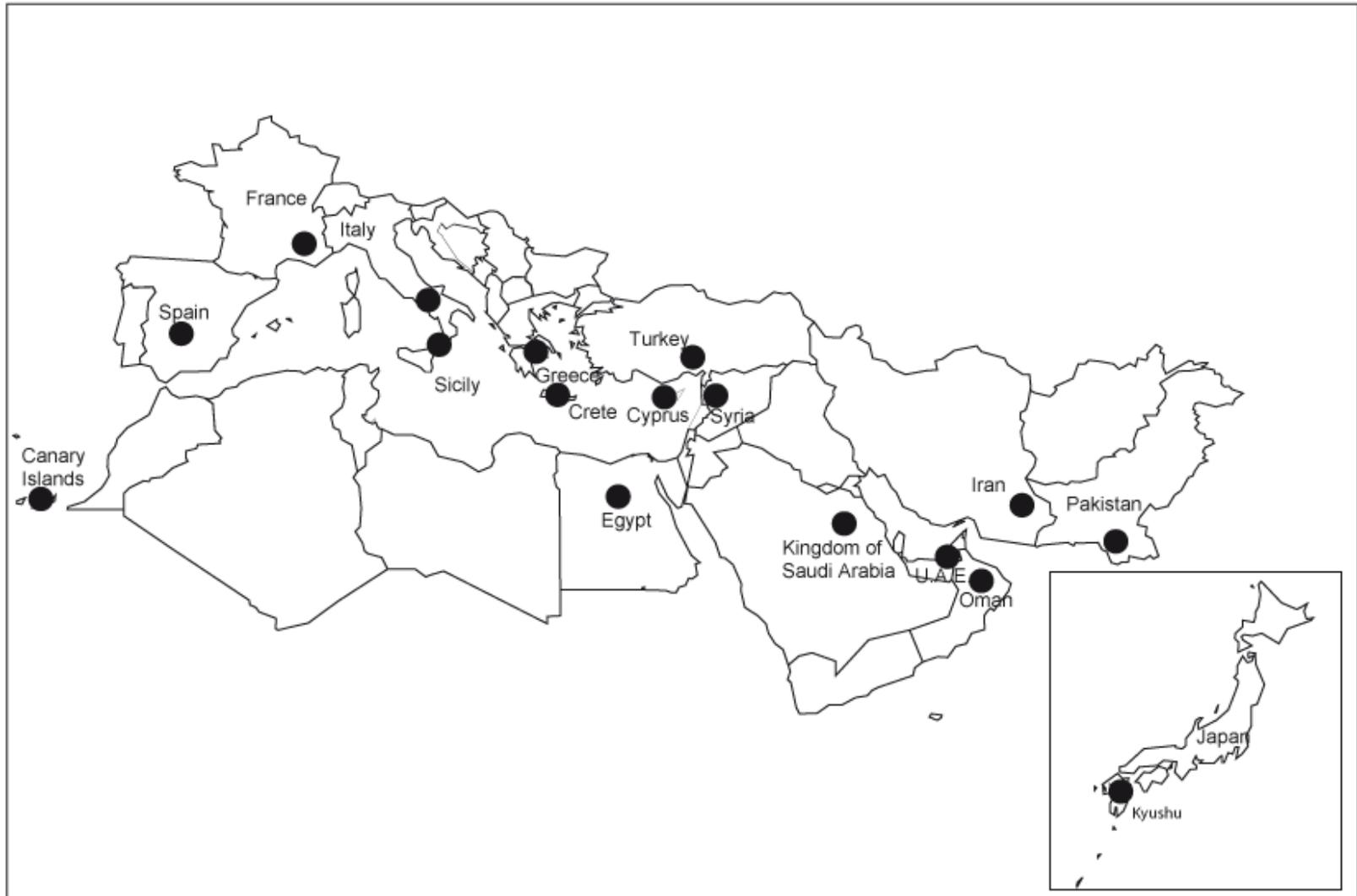
→ The objectives of this study are as follows:

- ✓ 1) investigate whether RPW populations collected from different invaded countries are homogenous or not using different molecular markers.
- ✓ 2) investigate how and why the detected genetic variation of RPW distributed into different regions.
- ✓ 3) determine the systematic position of RPW among the other *Rhynchophorus* spp.
- ✓ 4) determine the systematic position of subfamily Dryophthorinae among the other subfamilies of Curculionidae.
- ✓ 5) determine the systematic position of Curculionidae among the other families of Coleoptera.

*How to proceed

- Sampling.
- Detection of variation.
- Sequence analyses and measurement of variation.
- Demographic analyses.
- Interpretation of the spatial pattern and genetic relationships.

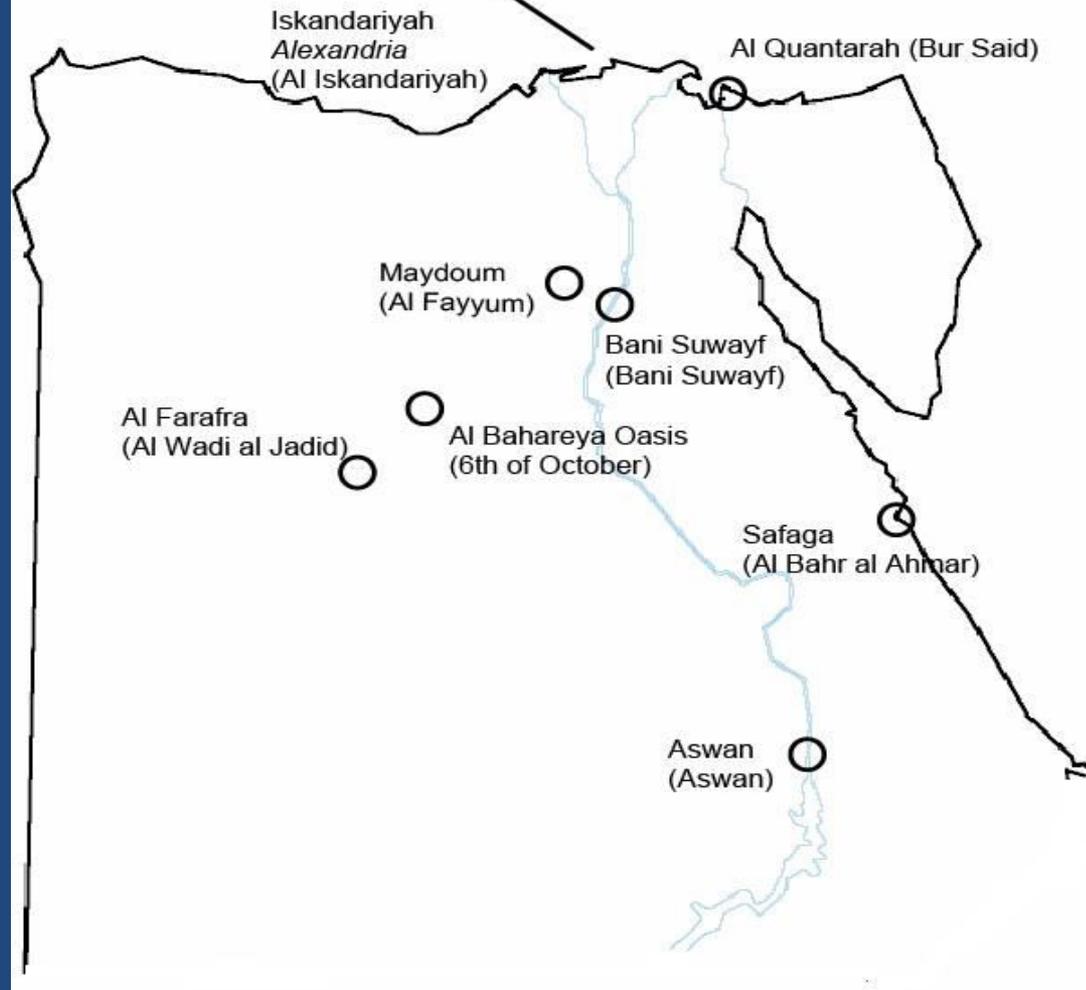
→ Sampling I



→ Sampling II

Nile Delta:

(Al Buhayra, Al Ismailiyah, Al Jizah, 6th of October, Helwan, Al Minufiyah, Al Qalyubiyah, Ash Sharqiyah, Dumyat & Kafr ash Shykh).



→ Sampling III

✓ *Rhynchophorus* spp.

- *R. bilineatus* – Papouasie
- *R. palmarum* - French Guiana
- *R. phoenicis* - Ivory Coast

✓ Sequences from Genbank

- Partial CO1 gene sequences from other Coleopteran species.

→ Detection of variation

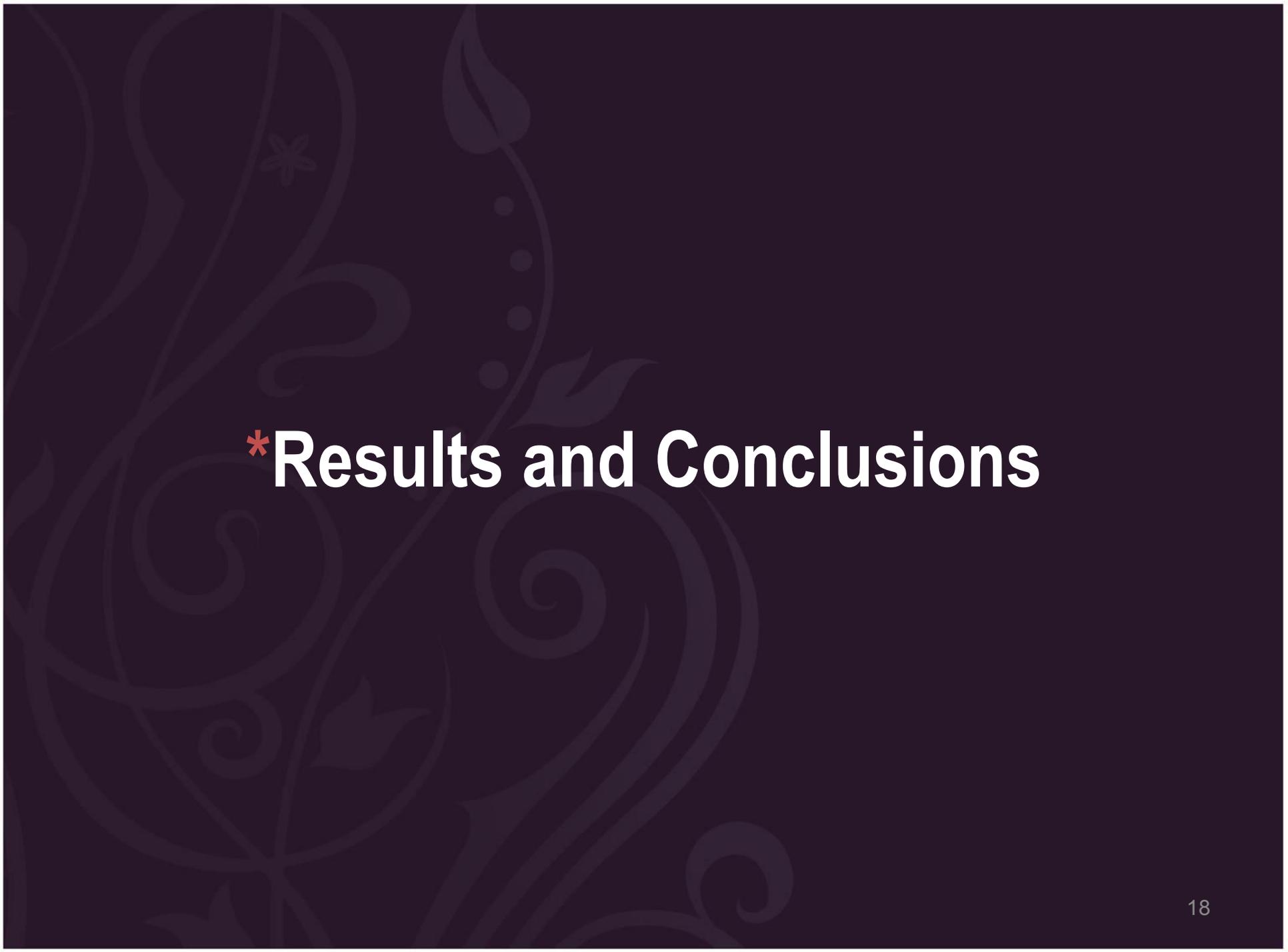
- ✓ DNA based markers
 - Sequence based markers
 - Mitochondrial DNA markers: CO1 and Cytb genes.
 - ITS2.
 - Electrophoresis based markers
 - Random amplified polymorphic DNA (RAPD).
- ✓ PCR amplifications, purification of the resulted products and Gel electrophoresis.
- ✓ Sequencing.
- ✓ Sequences alignment.
- ✓ Depositing the obtained sequences in GenBank.

→ Sequence analyses and measurement of variation

- ✓ Different parameters were estimated.
- ✓ Phylogenetic analyses.
- ✓ Parsimony network

→ Demographic analyses

- ✓ Tajimas'D test.
- ✓ F_{st} - genetic differentiation.
- ✓ N_m – gene flow.



*Results and Conclusions

****PART I**

→ Molecular Diversity and Geographic Distribution of the Invasive Species RPW

- ✓ **Mitochondrial genetic variation and invasion history of RPW in Middle-East and Mediterranean basin.**
- ✓ **Genetic variation of of RPW in Middle-East and Mediterranean basin using ITS2.**
- ✓ **Determination of Different Geographical Populations of RPW Using Random Amplified Polymorphic DNA (RAPD).**

***Mitochondrial genetic variation and invasion history of RPW in Middle-East and Mediterranean basin**

Characteristics of sequences and variation

	CO1	Cytb
Previous studies	No	
Geographic populations	14	12
Amplified region	600 bp	440 bp
Studied sites	546 bp	400 bp
AT %	61.7-62.4	66-66.5
GC %	37.6-38.3	33.5-34
Number of haplotypes	8 (H1 to H8)	3 (HB1 to HB3)
Inter-population variation	Yes	Yes
Intra-population variation	UAE (H1,H3,H4 & H5) OMAN (H1,H5&H6)	No
Type of variation	Nucleotide substitutions	
Variable sites	23 (4.2%)	16 (4.2%)
*Transitional substitutions	A to G	
Transversional substitutions	T to A and T to G	
R	26	8

DNA polymorphism and divergence

Over the entire set of populations

Total no. Of mutations	24
Overall haplotype diversity	0.556
Average pairwise nucleotide diversity (k)	6.759
Nucleotide diversity (Pi)	0.01238

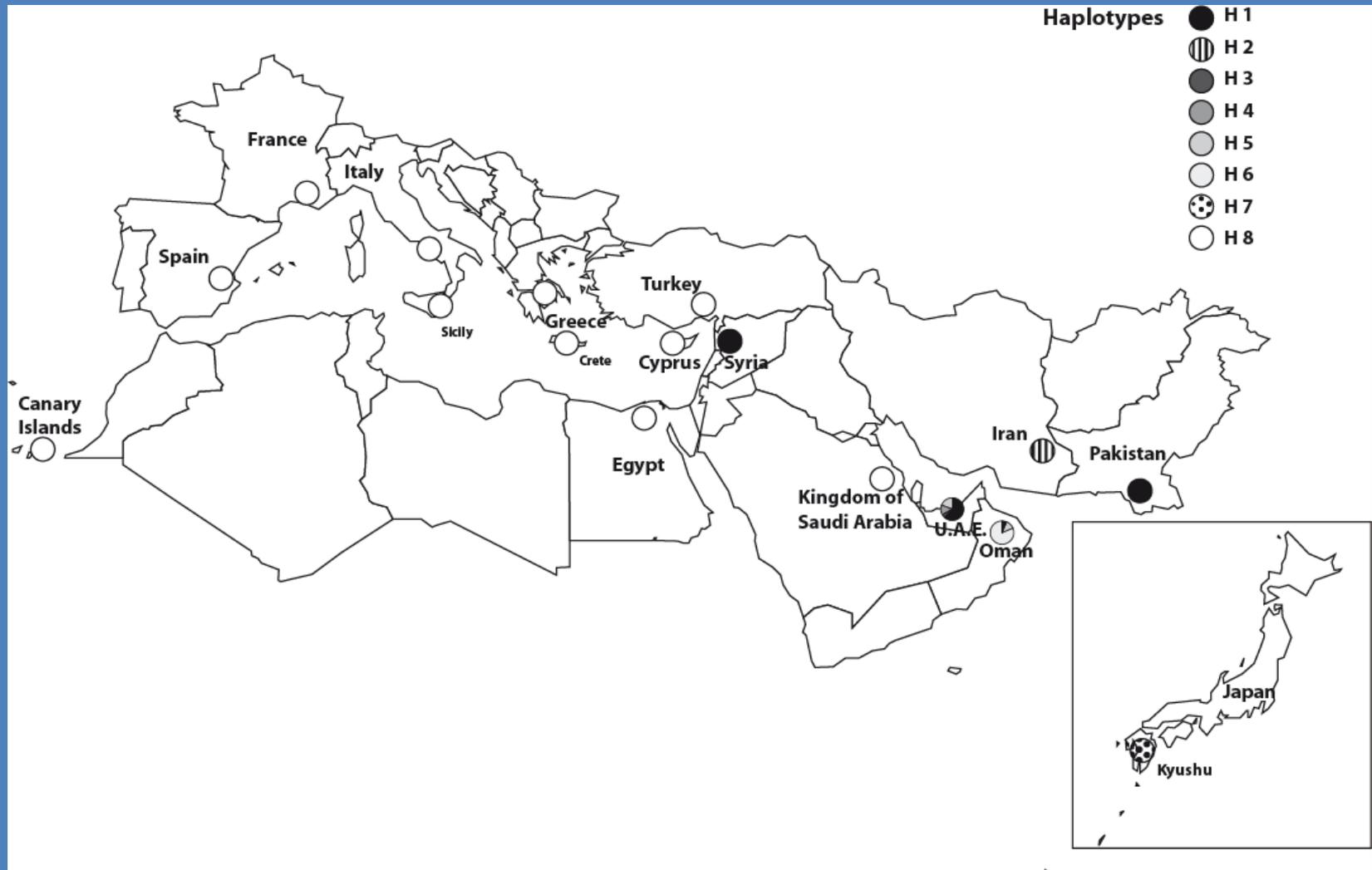
DNA divergence at inter-population level

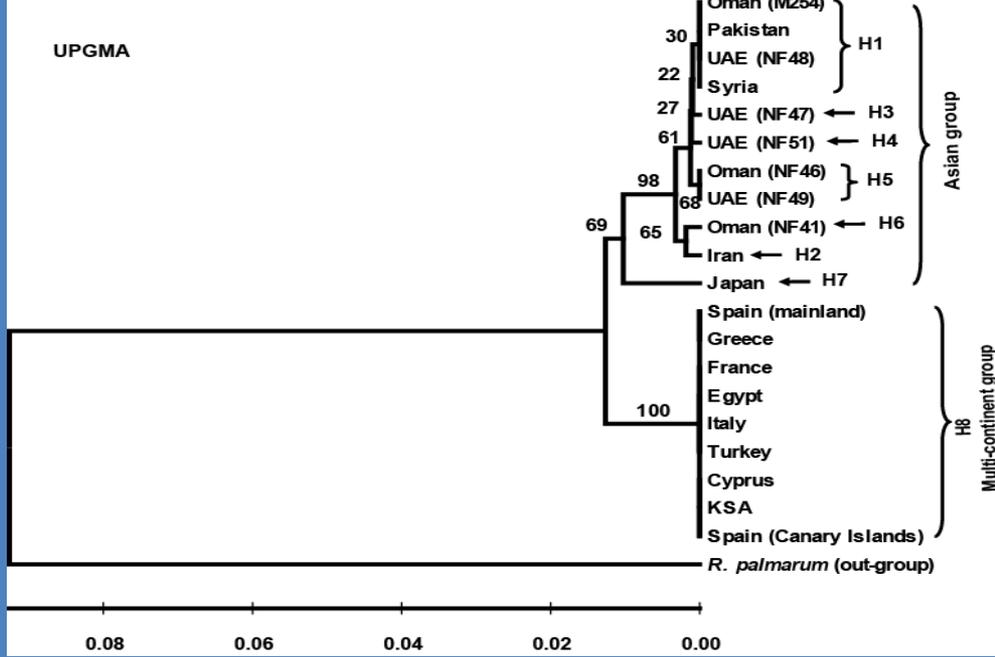
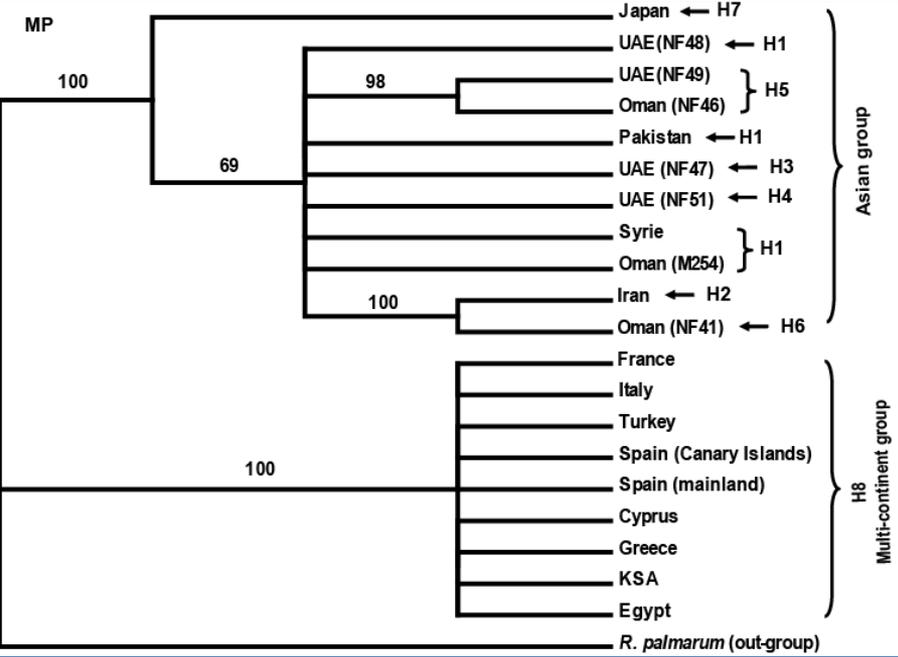
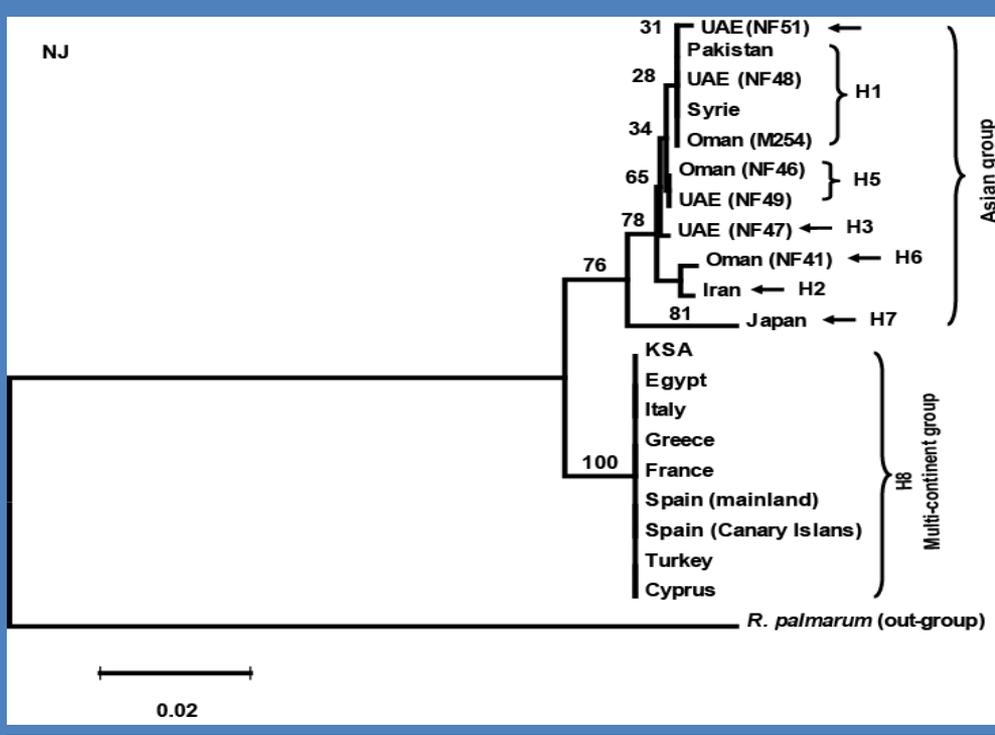
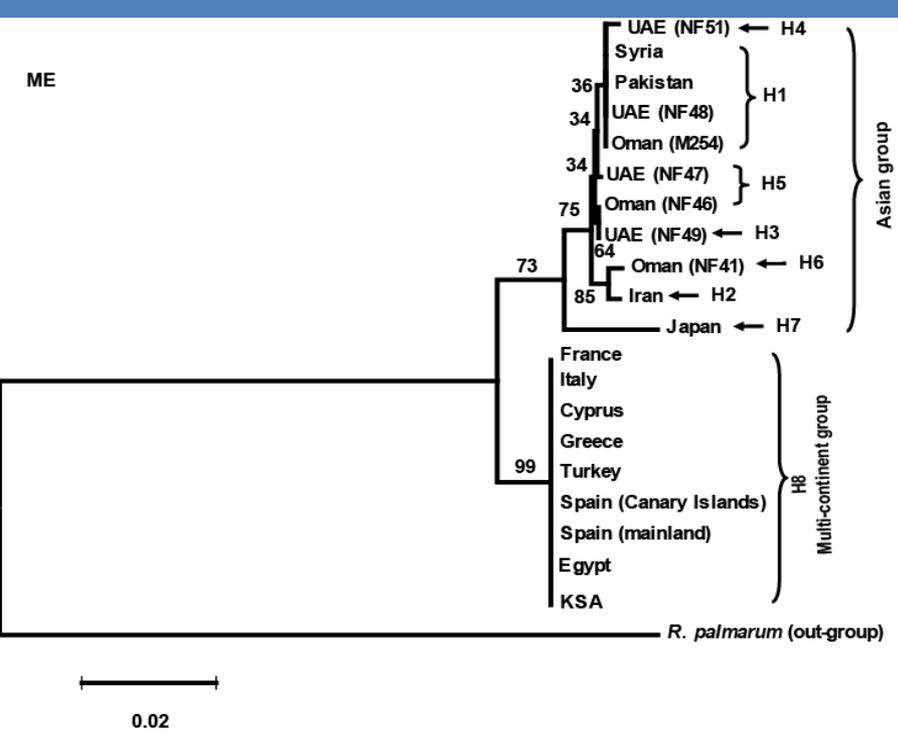
No. fixed differences	1-17
Average pairwise nucleotide diversity (k)	0.377-5.005
Nucleotide diversity (Pi)	0.00069-0.01193

DNA divergence at intra-population level (CO1)

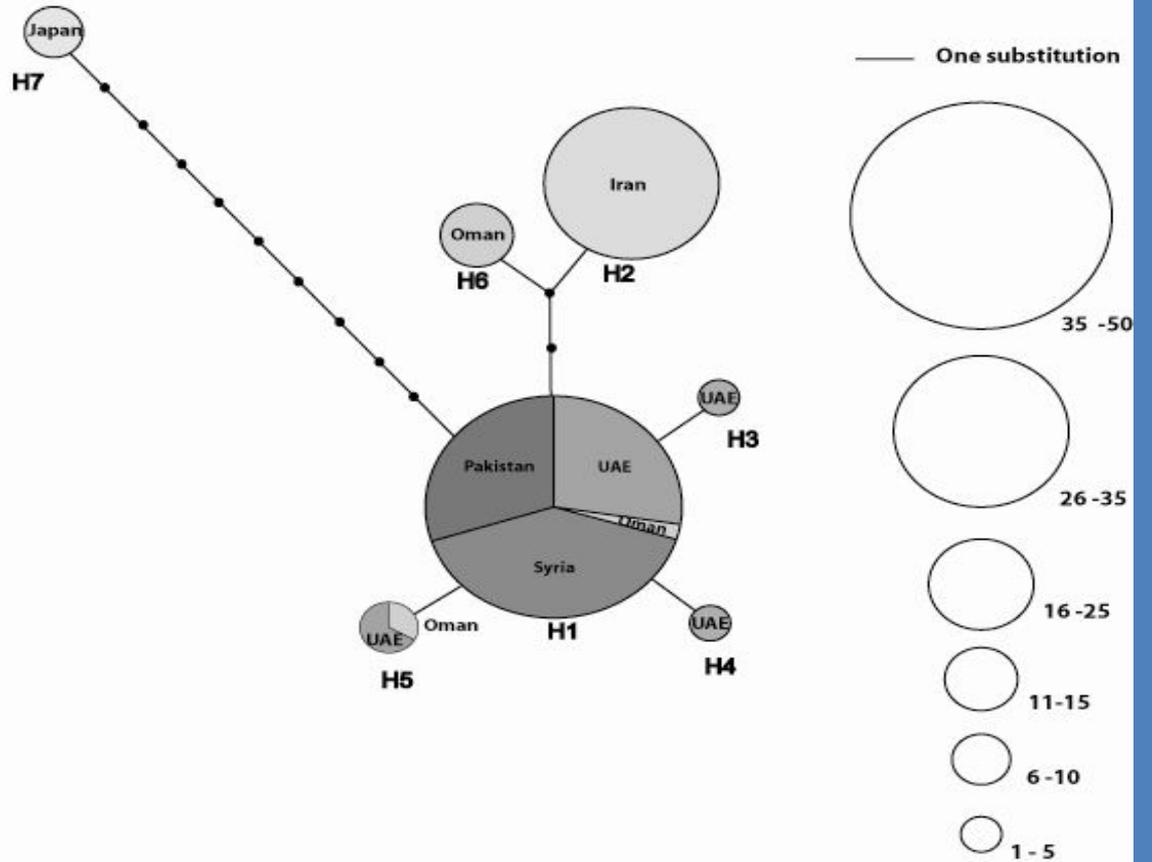
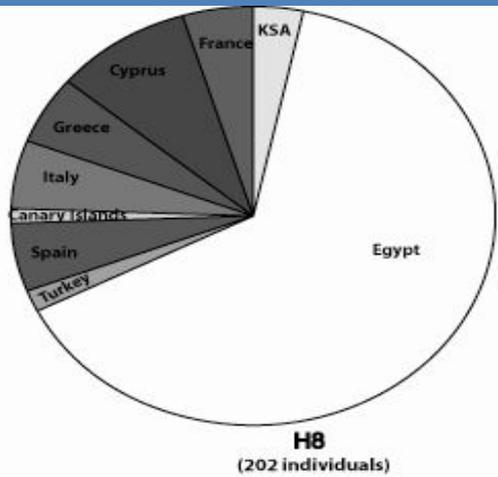
Number of segregating (S) sites	3 & 4 within UAE and Oman populations respectively
Haplotype diversity (h)	0.58571 & 0.34167
Nucleotide diversity (Pi)	0.001240 & 0.00221

Geographic distribution of mtDNA gene haplotypes



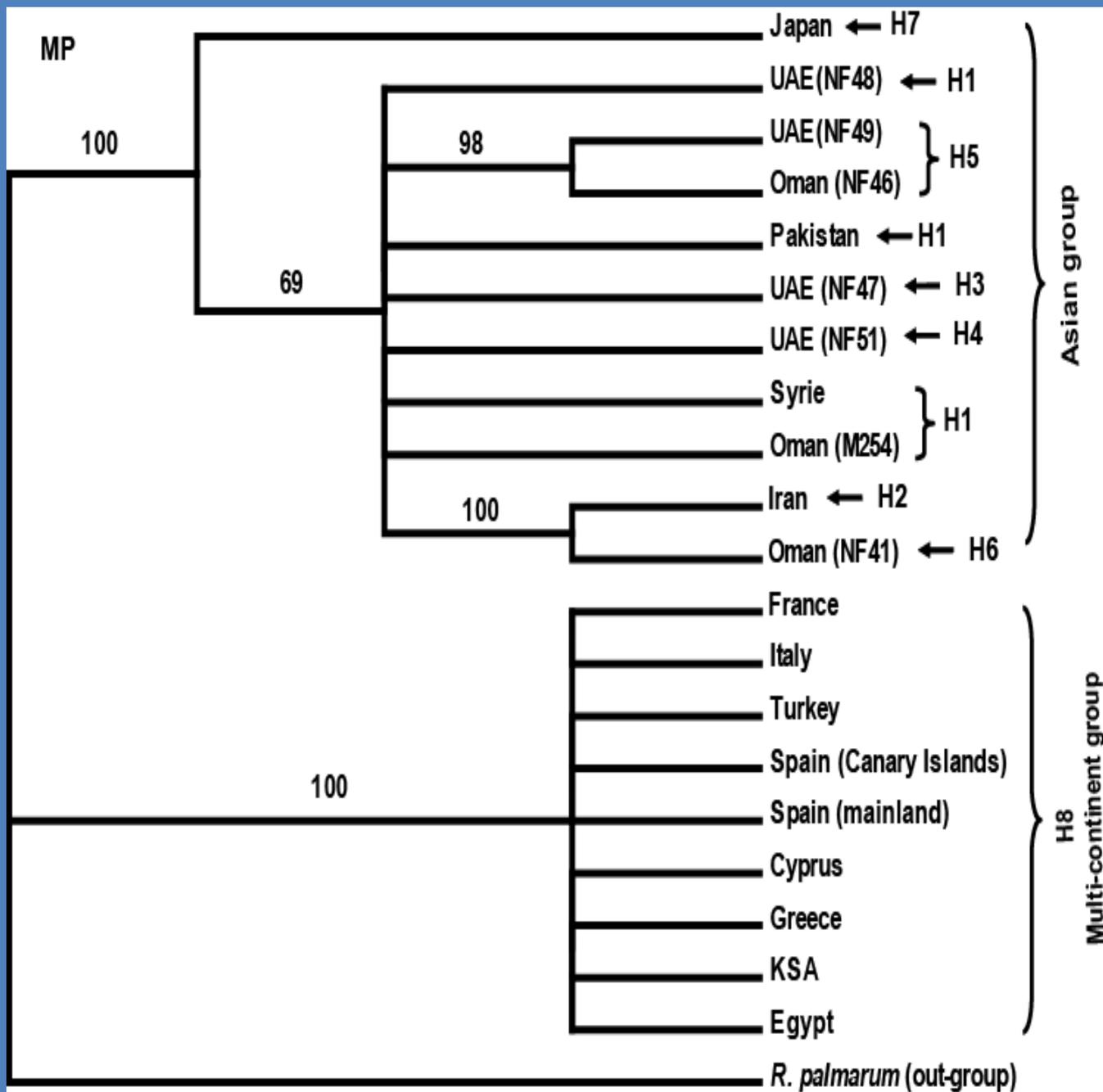


Haplotype Network



→ Genetic distance

- ✓ **Between the 8 haplotypes:**
 - **GD range: 0.002 to 0.033.**
 - **The lowest: H1 and H3, H4 & H5.**
 - **The highest: H8 and H7.**
 - **H8 haplotype more related to H1 and distantly related to H7.**
- ✓ **Within the Asian group: H1, H2, H3, H4, H5 , H6 & H7**
 - **H7 & H6: the lowest GD.....The most close (0.019).**
 - **H7 & H1: The highest GD.....The most distantly relationship (0.025).**
 - **H1 to H6: more related to each other (0.002-0.007).**



→ Demographic analyses

- ✓ Tajimas'D test: 2.05248
- ✓ *Fst* - genetic differentiation: 0.98406
- ✓ *Nm* – gene flow: 0

***Discussion & CONCLUSIONS**

- ➔ **High diversity of RPW in its area of origin.**
- ➔ **Number of haplotypes and introduction events**
 - ✓ **Multiple haplotypes**
 - **Different source populations**
 - **The same source**
 - **Different introduction events**
 - **A single one containing more than one haplotype**
 - ✓ **Fixed haplotypes**
 - **A unique introduction event**
 - **A single successful one**
 - **Multiple introductions of the same haplotype**
- ✓ **A series of secondary invasion events –Egypt governorates**
- ✓ **Egyptian haplotype-not similar to any of the UAE haplotypes**
 - **The offered offshoot of the UAE was not infected by RPW and in the same time infected offshoot have been introduced from another country**
 - **RPW may have been introduced to Egypt through the offshoot offered by the UAE but we have not been able until now to find H8 or HB1 haplotype among UAE samples due to limited sampling effort in that country.**

- ➔ **Wide geographic distribution pattern of the invasive haplotype- a very high invasive potential.**

- ➔ **Paradoxes**
 - ✓ **KSA & Syria –not the same haplotype as the neighboring countries**
 - ✓ **Pakistan-only one haplotype-no natural barriers can prevent the distribution of RPW between Pakistan and India.**

- ➔ **The phylogenetic relationships among the tested populations of RPW were sufficiently resolved and supported by moderately to high bootstrap values.**

- ➔ **According to the GD:**
 - ✓ **The 8 haplotypes can be ranked as follows:
H8/H1/H3/H4/H5/H6/H7.**

- The haplotypes were likely genetically differentiated before being transferred to the new invaded countries.
- Tajima's parameter D : +..... a recent founder event .
- F_{ST} : 0.98a major genetic differentiation among the fourteen populations with each fixed for a different haplotype.
- N_m : 0..... signified the absence of gene flow..... Support the bottleneck event. Nevertheless, we have only access here to the mitochondrial diversity (maternal lineage), consecutively gene flow at the nuclear level cannot be excluded.

RPW populations are subdivided into different sub-populations; this differentiation resulted from a series of founder and bottleneck events when the insect was introduced into the different invaded areas and countries.

- RPW populations diverged genetically under the influence of genetic drift likely through multiple founder events.

- ➔ **RPW may followed three different routes of invasion during the last 30 years, one towards the East of the area of origin that gave rise to the population of RPW in Japan, and two routes towards the West.**
- ➔ **The invaded area was divided in the West between Middle East, where several haplotypes were found and the Mediterranean basin where the invasive haplotype was detected.**
- ➔ **According to the level of sequence divergence between the haplotypes, it can be hypothesize that these different invasion roads are corresponding to three different genetic lineages of RPW populations. These three lineages had independent evolutionary histories for a relatively long period of time.**
- ➔ **Consequences of this situation may be strong. Differences in behaviour between the three kind of RPW populations may be significant.**

✓ **Genetic variation of of RPW in
Middle-East and Mediterranean
basin using ITS2**



**No polymorphism was observed
after the alignment and
comparison among the different
sequences of the amplified ITS2
region.**



**A strong concerted evolution that
has been exhibited.**

✓ **Determination of Different
Geographical Populations of RPW
Using
Random Amplified Polymorphic DNA
(RAPD)**

Previous studies 1) Different morphological forms of RPW from Egypt (Salama & Saker, 2002) and KSA (Al-Ayied <i>et al.</i> , 2006). 2) Individuals of RPW from UAE (Gadelhak & Enan, 2005). 3) Individuals of RPW from Egypt, KSA and Indonesia (Abulyazid <i>et al.</i> , 2002).	PCR products	200-1400 bp
	No. of tested PCR products	776 bands
	Polymorphism	100%
	Unique RAPD markers	Egypt: 17

Individual level

Genetic distance	Between Countries	lowest	Port Said 1 (Egypt) & Turkey 2
		highest	Minoufia 2 (Egypt) & Japan 1
	Same Country	lowest	Cyprus
		highest	Japan
	Egypt	lowest	Giza 2 & Ismailia 2
		highest	Fayoum 2 & Damitta 1

Population level

Average genetic distance	13 geographic populations	lowest	KSA & UAE
		highest	KSA & Japan
	Egypt (15 local populations)	lowest	Bani suef & Aswan
		highest	AlBuhayra & (Bani suef & Aswan)

Cluster Analyses-UPGMA dendrogram

➤ 12 clusters .

➔ Egypt

6 clusters

(clusters 1 to 5 & 9).

Clusters 1 to 5.....

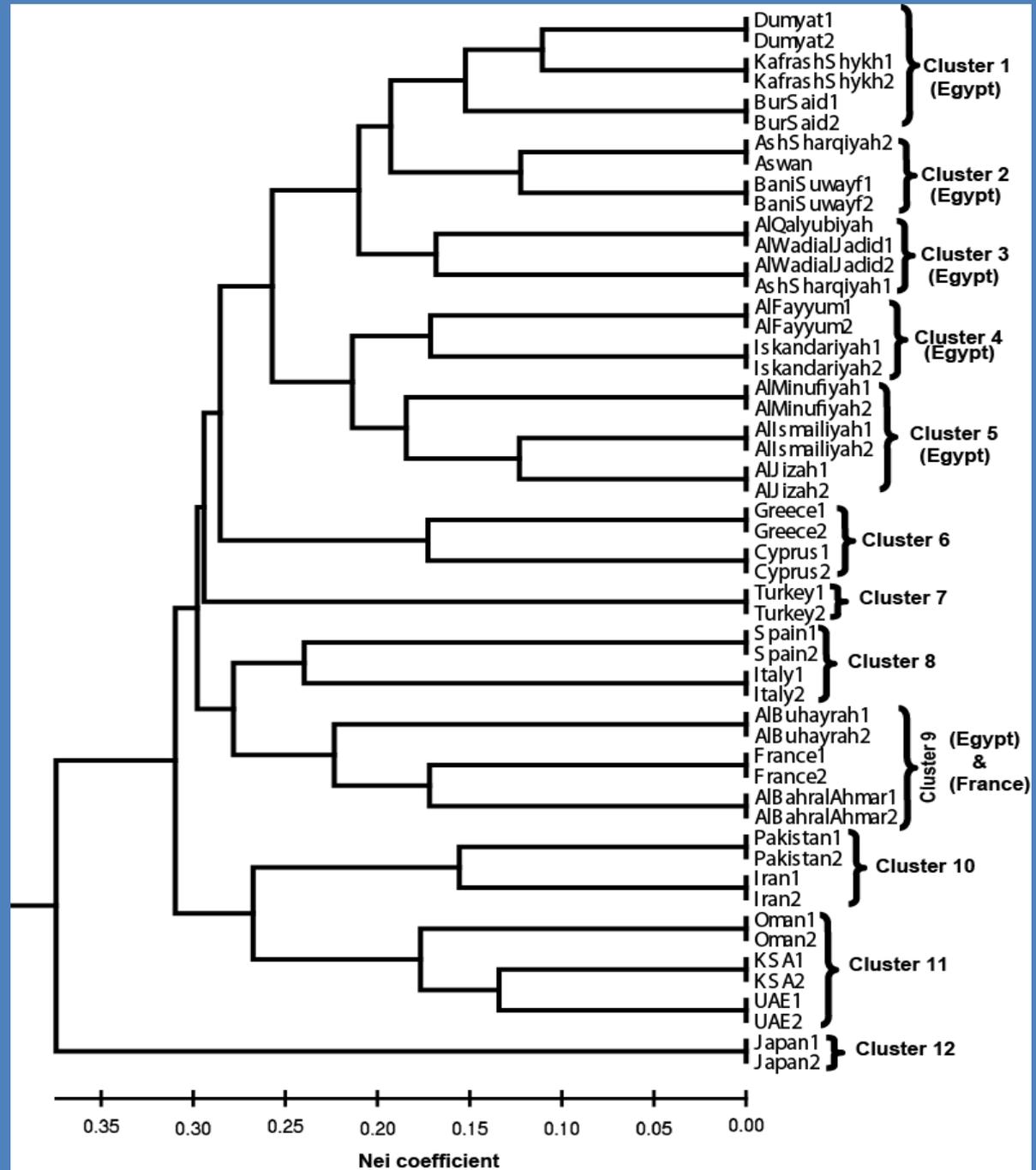
only Egyptian individuals.

Cluster 9.....

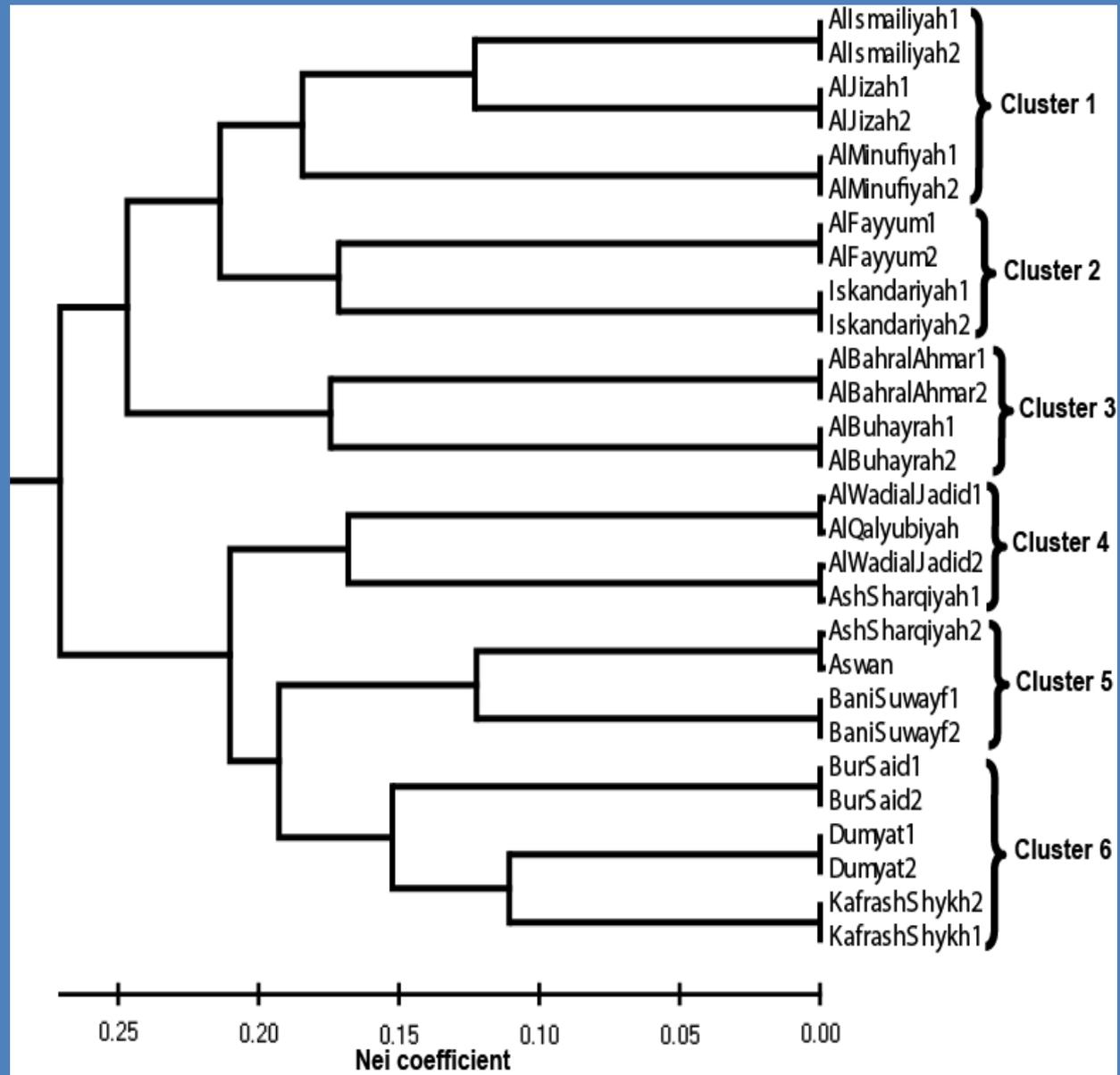
individuals from

Egypt

(Al Bahr al Ahmar & Al Buhayrah) & France .



Cluster Analyses-UPGMA dendrogram



***CONCLUSIONS**

- ✓ **RAPD has the ability to distinguish among populations of RPW.**
- ✓ **Similarity among the analyzed populations ranged from 20% to 70%.**
- ✓ **100% polymorphism reflects the absence of genetic homogeneity.**
- ✓ **17 unique PCR products: can be used as genetic markers that can distinguish the geographic populations of RPW from each other.**
- ✓ **There is a positive correlation between genetic distances and geographic distances.**
- ✓ **Not all the Egyptian individuals have direct relationships with local geographic pattern as some individuals from distant localities were clustered together: Cluster 2) Ash Sharqiyah, Aswan and Bani Suwayf; Cluster 3) Al Qalyubiyah, Al Wadi al Jadid and Ash Sharqiyah; Cluster 4) Al Fayyum and Iskandariyah.**
- ✓ **RPW populations that invaded the Middle East and the Mediterranean area likely came from two different geographical origins:**
 - **One is the source of the Egyptian and related Mediterranean populations.**
 - **The other is the source of the Arabic Peninsula and Asian populations.**

PART II

- ✓ **Phylogenetic relationships between *Rhynchophorus ferrugineus* and the other *Rhynchophorus* spp.**
- ✓ **Systematic position of *Rhynchophorinae* among the Other Coleopteran**

- ✓ **Phylogenetic relationships between *Rhynchophorus ferrugineus* and the other *Rhynchophorus* spp.**

✓ **Rhynchophorus genus**

✓ **10 species**

✓ **1 Africa**

➤ *R. phoenicis* (Fabricius)

✓ **5 Asia**

➤ *R. ferrugineus* (Olivier)

➤ *R. bilineatus* (Montrouzier)

➤ *R. distinctus* (Wattanapongsiri)

➤ *R. lobatus* (Ritsema)

➤ *R. vulneratus* (Panzer)

✓ **4 New World**

➤ *R. cruentatus* (Fabricius)

➤ *R. palmarum* (Linnaeus)

➤ *R. ritcheri* (Wattanapongsiri)

➤ *R. quadrangulus* (Queden)

Previous studies

✓ **Uncertain taxonomic status-Murphy & Briscoe (1999)**

✓ **RAPD-Abulyazid *et al.* (2002)**

➤ Genetic variation was detected among *R. ferrugineus* (Egypt, KSA & Indonesia), *R. cruentatus* (Florida-USA) and *R. palmarum* (Costa Rica).

➤ No similarity was found between *R. ferrugineus* and *R. vulneratus*.

✓ **RAPD & CO1- Hallett *et al.* (2004)**

➤ *R. ferrugineus* and *R. vulneratus* were considered as colour morphs of the same species and they are synonymies under the name *Rhynchophorus ferrugineus* with the common name Asian palm weevil.

Genetic comparison among *R. ferrugineus* and four *Rhynchophorus* species



R. phoenicis



R. ferrugineus



R. cruentatus



R. bilineatus



R. palmarum

Amplified and sequenced regions and types of variation

Studied sites	735 bp	
Number of haplotypes	<i>R. ferrugineus</i>	6: H1*-H4* , H6* & H8*
Type of variation	Nucleotide substitutions	
Variable sites	26.1%	
Nucleotide contents	AT: 66 to 69.7%	GC: 30.2 to 34%
overall transition/transversion ratio	R: 32.488	

R. ferrugineus

Characters	<i>R. bilineatus</i>	<i>R. phoenicis</i>	<i>R. Cruentatus</i>	<i>R. Palmarum</i>
Polymorphic sites	88 (0.12%)	104 (0.14%)	109 (0.15%)	111 (0.15%)
Mutations	91	107	112	115

✓ Genetic distances (GD)

- Highest GD: 0.095 - between *R. palmarum* and *R. bilineatus*.
- Lowest GD: 0.058-0.062- between *R. ferrugineus* and *R. bilineatus*.
- *R. ferrugineus* and the other four species:
 - Highest GD: 0.158-0.171- between *R. ferrugineus* and *R. palmarum*.
 - H8* haplotype of *R. ferrugineus* -The most closely related to *R. palmarum* (0.081).
 - H2* and H3* haplotypes-The most distantly related to *R. palmarum* (0.085-0.086).

3 MP tree; 109 parsimony informative site.

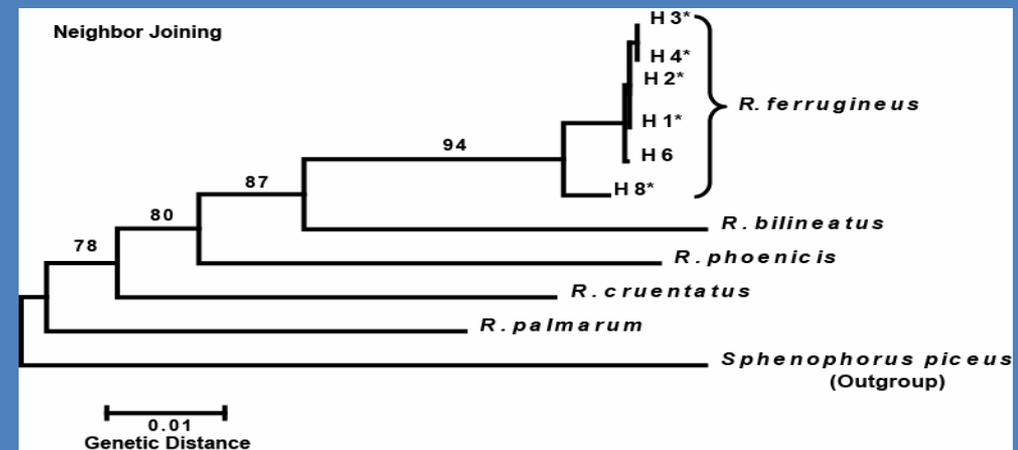
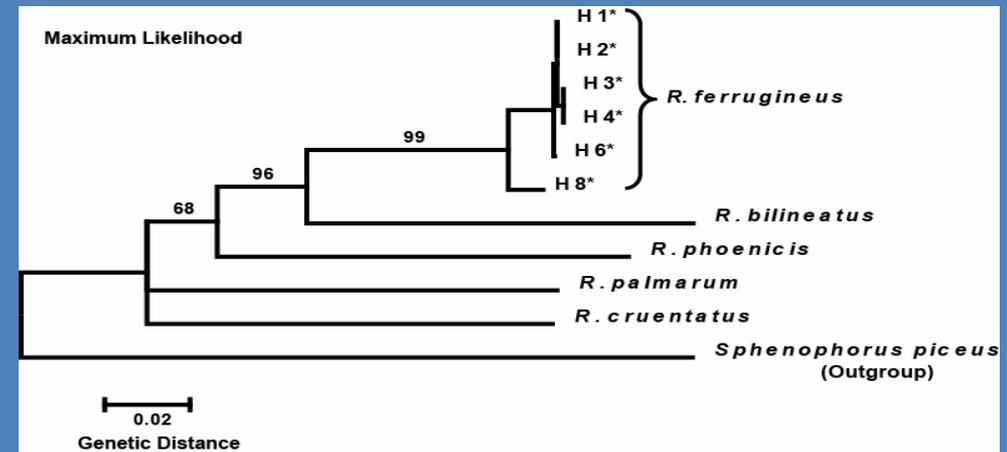
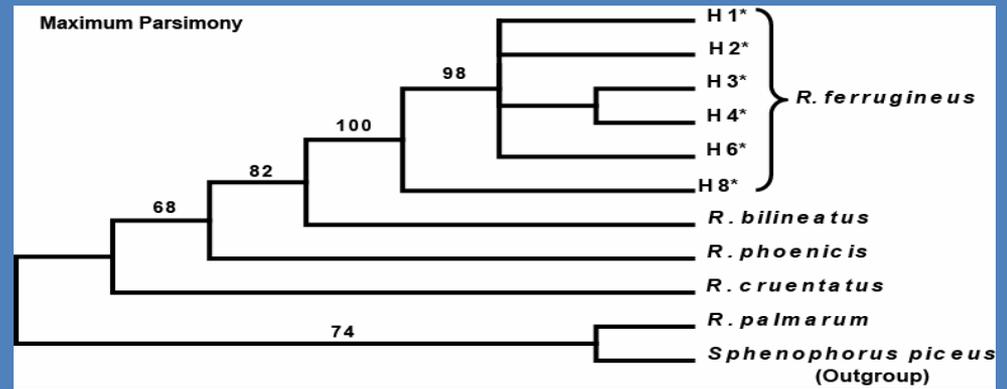
1) The six CO1 haplotypes of *R. ferrugineus* were grouped together in one clade.

2) *R. ferrugineus* was close to *R. bilineatus*, the two Asian species were grouped in the same clade and descended from the same ancestor.

3) *R. phoenicis* was the sister species of *R. ferrugineus* and *R. bilineatus* clade.

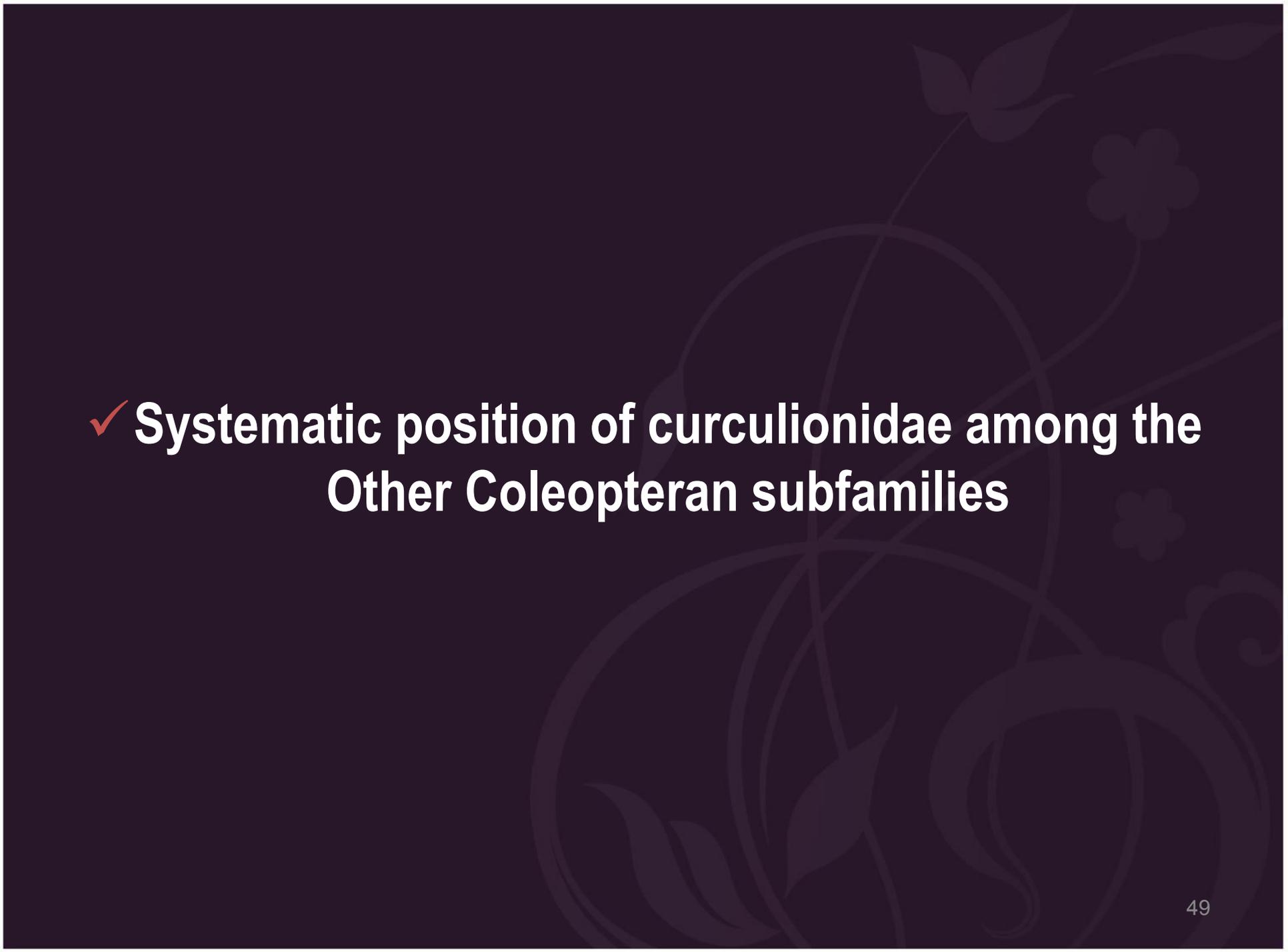
4) *R. cruentatus* was the sister species of the clade formed by the previous species.

5) *R. palmarum* was the most distantly related species of the currently analyzed species.



*CONCLUSIONS

- ✓ *R. palmarum* and *R. bilineatus* were the most distantly related species.
- ✓ *R. ferrugineus* was more closely related to *R. bilineatus*, while it was more distantly related to *R. palmarum*.
- ✓ The six CO1 haplotypes of *R. ferrugineus* were grouped together in one clade.
- ✓ H8* haplotype of *R. ferrugineus* was the most closely related to *R. palmarum*.
- ✓ H2* and H3* haplotypes were the most distantly related to *R. palmarum*.
- ✓ *R. phoenicis* was the sister species of *R. ferrugineus* and *R. bilineatus* clade.
- ✓ *R. cruentatus* was the sister species of the clade formed by the previous species.
- ✓ *R. palmarum*, appeared as the most distantly related species of the currently analyzed species.
- ✓ According to these findings it is possible to rank the tested *Rhynchophorus* species as follow: *R. ferrugineus* H1*/ H2*,H3*,H4*,H6* / H8* / *R. bilineatus* / *R. phoenicis* / *R. cruentatus* / *R. palmarum*.
- ✓ This study was the first step to analyze molecular phylogenetic relationships among *Rhynchophorus* species. Further analysis should be performed using other *Rhynchophorus* species as well try other molecular markers.



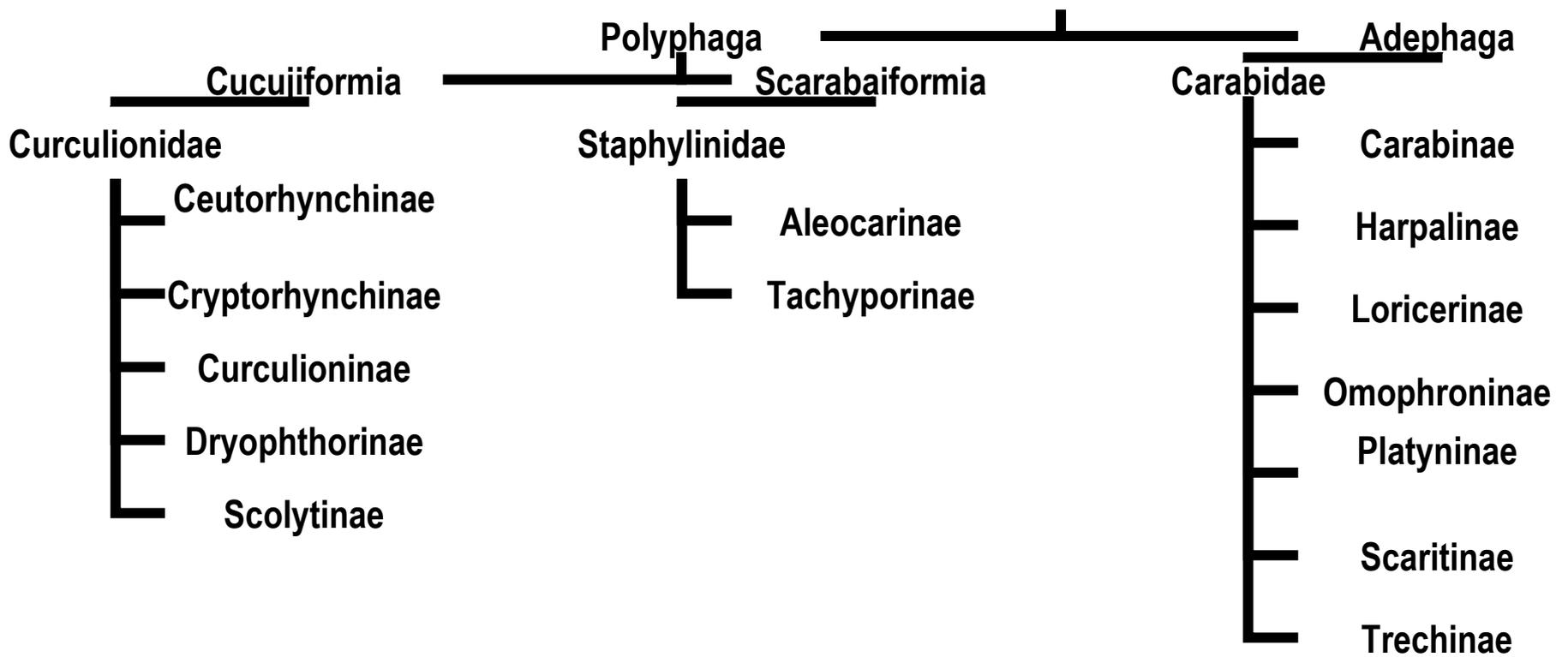
- ✓ **Systematic position of curculionidae among the Other Coleopteran subfamilies**

Coleoptera

- Kingdom: Animalia
- Phylum: Arthropodes.....540 MYA
- Class: Insecta...390 MYA...1/2 animal spp.
- Subclass: Pterogota
- Infraclass: Neoptera
- Suborder: Endopterogota
- Order: coleoptera

✓ Coleoptera

- 25% of all life forms
- 40% of described insect species (40000 species)
- Originated 319-299 MYA
- The largest taxonomic family-Curculionidae
- Phylogeny: 4 suborders:
 - ✓ Polyphaga-170 families, 300000 species
 - ✓ Adephaga-10 families, predators
 - ✓ Archostemata-4 families, wood-eating beetles
 - ✓ Mexophaga-4 families-100 described species
- The phylogenetic relationships is not certain
- Only one study using 18S.
- present an overall picture of the diversity present in the mitochondrial DNA.



Suborders	3	
Families	3	
No. subfamilies	14	
No. genera	65	
No. species	146	
Studied sites	458 bp	
Type of variation	Nucleotide substitutions	
Variable sites	447 (98%)	
Nucleotide contents	AT	60 to 70.3%
	GC	40 to 29.7%

Curculionidae

→ Subfamily:

Genetic distances

Highest GD (4.946): Scolytinae and Cryptorhynchinae

Lowest GD (0.190): Scolytinae and Ceutorhynchinae

→ Family:

Genetic distances

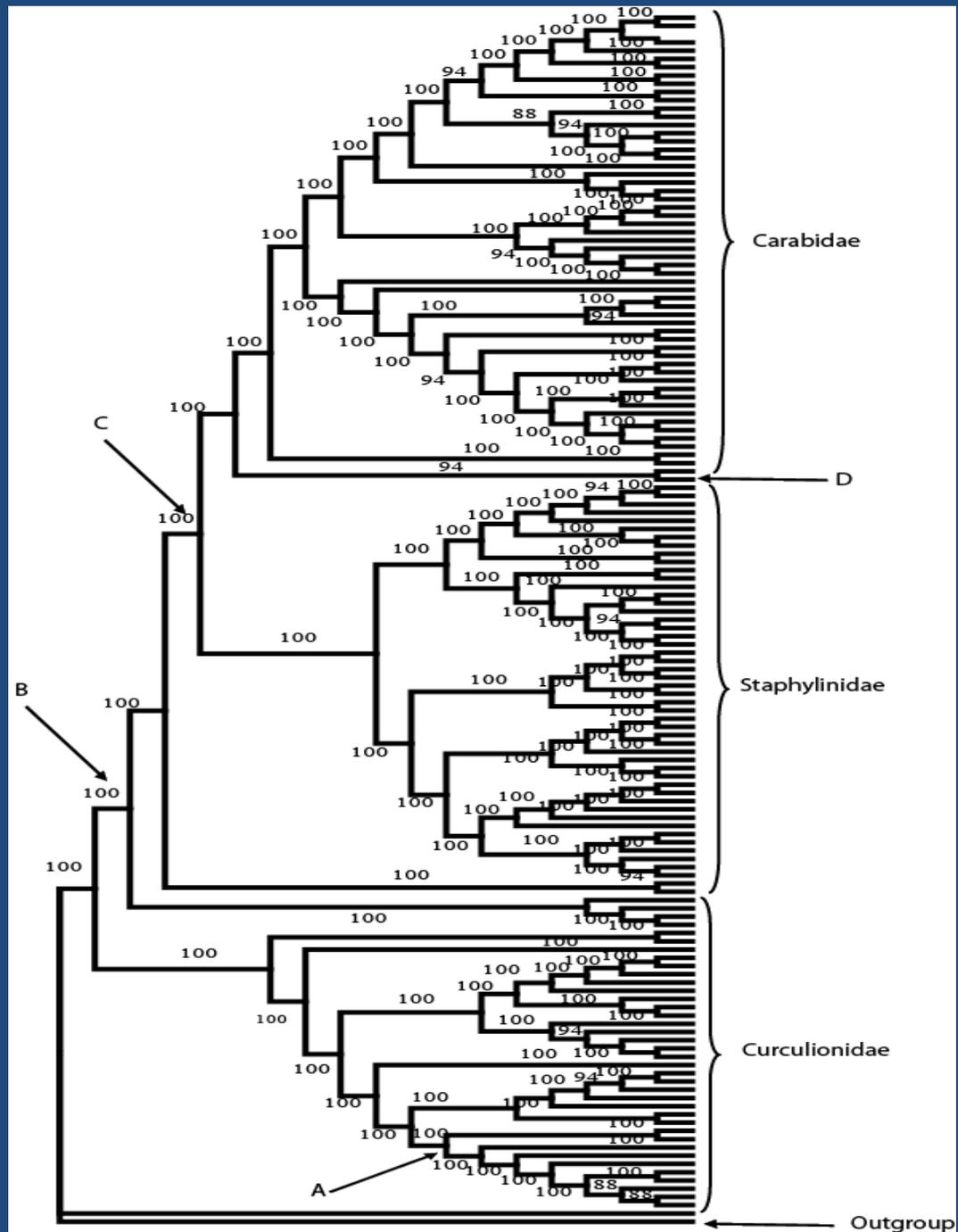
Curculionidae and Staphylinidae: 2.647-5.539

Curculionidae and Carabidae: 2.587-4.665

Staphylinidae and Carabidae: 0.144-0.294

Phylogenetic tree of COI sequences by MP methods

- * Bootstrap values were shown either above or down the branches.
- A) The most common ancestor to the species of Dryophthorinae.
- B) The most common ancestor for the three families: Curculionidae, Staphylinidae and Carabidae.
- C) The most common ancestor for the two families: Staphylinidae and Carabidae.
- D) A clade contained one species of Staphylinidae and another from Carabidae.



*Conclusions

- ✓ The monophyletic of the polyphaga and Adephaga was not supported in our study using CO1 sequences.
- ✓ family Carabidae (Adephaga) was grouped with family Staphylidae (Polyphaga) with Staphylinidae paraphyletic.
- ✓ The subfamily Scolytinae is the most common ancestor for the subfamilies: Ceutorhynchinae, Curculioninae and Dryophthorinae and hence the oldest. The subfamily Cryptorhynchinae is the oldest among the five tested Curculionidae families.
- ✓ At the family level the genetic distances and phylogenetic analysis obtained in this study showed that the family Carabidae was more related to family Staphylinidae than to family Curculionidae with the topology Staphylinida-Carabidae-Curculionidae.
- ✓ The topology was the same when *Micromus igorotus* from order Neuroptera was used as an outgroup taxa as it was Staphylinida, Carabidae, Curculionidae/Neuroptera.
- ✓ An alternative topology was obtained when *Acytolepis puspa* from order Lepidoptera was used as an outgroup that was Carabidae, Staphylinida, Curculionidae-Neuroptera/Lepidoptera, where the species of order Neuroptera placed within family Curculionida.



* perspectives

- * Obtain RPW microsatellite markers in order to analyse the present days RPW genetic structure in Egypt and neighbouring countries and to estimate gene flows among populations. Such markers will also be useful to better understand local scale genetic structure and single palm tree colonization.
- * A simple and cheap molecular test is needed to help plant protection officers to detect new haplotypes that could be introduced in Egypt in the future.

Thank you ! 😊