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Molecular Identification of Three *Argas* Species Using Polymerase Chain Reaction (PCR) Amplification and Restriction Analysis of the Small-Subunit Ribosomal RNA Gene

Mohammed H. Awwad, Sahar M. Abou El kheir and Gehan H. Lashien
Department of Zoology, Faculty of Science, Zagazig University, Benha Branch

Abstract

The polymerase chain reaction (PCR) and restriction fragment length polymorphisms (RFLPs) of SrRNA gene of *Argas* species (~1800 bp) is a very useful technique for differentiation of *Argas persicus, Argas hermanni* and *Argas arboreus* species. The PCR/RFLPs profile of *Eae*I and *Eco*RI restriction endonucleases were highly characteristic of the genetic interspecific levels and low genetic intraspecific levels of the three species. Other enzymes proved that *A. persicus* and *A. hermanni* may be a single or monophyletic species (*Sac*II and *Sst*II restriction endonucleases). *Ava*II restriction enzyme showed that *A. hermanni* and *A. arboreus* could be a monophyletic species. *Ava*I restriction endonuclease was the only restriction enzyme to prove that the three *Argas* species may be polyphyletic species and identified uniquely by this enzyme.

Key words: Identification, *Argas*, RFLPs, SrRNA gene.

Introduction

Ticks are beneficial to humans through the direct effects of their feeding and as vectors for various agents of disease in both man and livestock (Sonenshine, 1991; Jongejan and Uilenberg, 1994; Noda *et al.*, 1997 and Cumming, 1998). Tick-transmitted bacterial pathogens are quite diverse and include organisms belonging to the genera *Borrelia*, *Rickettsia*, *Francisella*, *Ehrlichia*, *Anaplasma*, *Cowdria*, and *Coxiella* (Sonenshine, 1993). Ticks also harbor unidentified nonpathogenic rickettsia- and wolbachia-like bacteria which are possibly mutualistic endosymbionts (Hayes and Burgdorfer, 1981 and Dasch and Weiss, 1992). Numerous reports have been published on the morphological appearance and distribution of symbionts in various tick species (Burgdorfer). Endosymbionts of the soft tick *Argas spp.* (*Persicargas*) *arboreus* (previously thought to be *Argas persicus* and hereafter referred to as *A. arboreus*) and the hard tick *Dermacentor andersoni* have been cultured in the yolk sacs of chicken embryos and found to be pathogenic for guinea pigs (Suitor and Weiss, 1961; Suitor, 1964 and Burgdorfer *et al.*, 1973). The *A. arboreus* microbe has been placed in the genus *Wolbachia* and named *W. persica* (Suitor and Weiss, 1961 and Suitor, 1964).

Ticks have traditionally been viewed as relatively host-specific, and it has frequently been assumed that their geographical distributions can be determined by that of their host(s) (Hoogstraal and Aeschlimann, 1982).
In humans, ticks can cause severe toxic conditions such as paralyses and

Refree : Prof ; *Dr. Eman Helal*

toxicoses, irritation and allergy, and their ability to transmit a great variety of infectious diseases is a major public health concern.

Argas spp. transmit a greater variety of pathogenic micro-organisms than any other arthropod vector group (Kohls and Hoogstraal, 1961; Filippova, 1966; Fedorov, 1968; Grzywacz and Kuzmicki, 1975; Keirans *et al.*, 1979; Miadonna *et al*., 1982; Schwan *et al.*, 1992 and Estrada-Pena and Jongejan, 1999). *Argas* spp. is usually associated with bats, although Hoogstraal (1956) identified it as a human parasite in Egypt.

The importance of ticks in the natural history of West Nile virus is unknown (Hoogstraal, 1985). The isolation of West Nile virus from *A. hermanni* infesting pigeon houses in Egypt during winter suggests that this species could serve as a host for the virus during winter (Schmidt and Said, 1964).

Argas spp. is wide distributed in Egypt and threatened our poultry evolution and human health. Many clinical signs and pathogenicity appe - ared among animals. Infections with *Argas* spp. may cause ruffled feathers, poor appetite (anorexia), diarrhea, emaciation and lowered production. Heavy infections with *Argas* spp. can cause loss of blood leading to anaemia and eventually death (Jongejan, 1999).

Identification of genotype of ticks would be a key to control it. So, in this research it has been attempted to accomplish two main goals. First, complete detection of the nuclear small ribosomal subunit RNA genes have been used in RFLPS analysis to study phylogenetic relationships among three *Argas* species (*A. persicus, A. hermanni* and *A. arboreus*). The purpose was to discover whether the three species are a valid monophyletic group or whether the three species are more likely polyphyletic. The second goal was to find some specific restriction endonucleases to identify individual species.

Material And Methods

Arthropods. Three species of soft tick *Argas*, family *Argasidae*, were collected from three different birds (*Argas persicus* from chicken; *Argas hermanni* from pigeon and *Argas arboreus* from heron). These ticks were transferred to the laboratory of Faculty of Science, Zagazig University, Benha branch.

DNA preparation and PCR amplification. DNA genome was extracted from the three *Argas* spp. by homogenizing them in UNSET lysis solution (Hugo *et al.*, 1992 and Awwad and Morsy, 2001). One µl of the DNA was checked by 0.8% agarose gel electrophoresis for the presence of DNA, as in Figure 1.

To amplify the complete nuclear SrRNA gene, one µl of whole-cell DNA template was used plus oligonucleotide primers complementary either to the 5` and 3` ends of the gene (ssu1 and ssu2). The standard PCR reaction mixture was used (Kessing *et al.*, 1989). The entire nuclear SrDNA was amplified using the primers SSU1 (5′-CGACTGGTTGATC CTGCCAGTAG-3′) and SSU2 (3′-TCCTGATCCTTCTCAGGTTCAC-5′) anchored respectively in the conserved extremities of the 18S ribosomal gene (Stohard and Rollinson, 1997). The standard polymerase chain reaction program for amplification of nuclear SrRNA was: 30-35 cycles; one minute, at 94^0C; two to three minutes, at 45^0C; and three minutes, at 72^0C.

PCR\RFLPs of rDNA. The enzymes were tested including *Eae*I (Roche Applied Science), *Eco*RI, *Ava*I, *Sst*II (Sigma-Aldrich) and *Sac*II, *Ava*II (Boehringer Mannheim) to distinguish the rDNA gene of the three *Argas*

species. One microlitre (10-12 units) was used for each digestion reaction, together with 1.2 µl of the respective enzyme buffer for a final volume of 12.2 µl. The digestion was performed for ~3.5 h at ~37°C, and the digestion products were evaluated on 2% TBE-agarose gels and stained with ethidium bromide. Bands were detected upon ultraviolet transillumination and photographed (Awwad and Morsy, 2001).

Results

Complete nuclear SrRNA genes (SrDNA) were obtained for the three *Argas* strains from the PCR products. The sizes of the nuclear SrDNA genes were approximately 1800 bp (Figure 2).

*Eae*I and *Eco*RI restriction endonucleases did not differentiate the three *Argas* species (Figures 3 and 4; Tables 1 and 2). *Eae*I restriction enzyme digested the PCR product (SrDNA) of the three *Argas* species (*A. persicus, A. hermanni* and *A. arboreus*) into three restriction patterns (~300, ~500 and ~1000 bp, lanes 1-3; Figure 3; Table 1), whenever *Eco*RI restriction endonuclease fragmented the same gene of the three species into two restriction bands (~250 and ~1550 pb, lanes 1-3; Figure 4; Table 2).

A. persicus and *A. hermanni* were remained undifferentiated when their SrRNA gene digested with *Sac*II and *Sst*II restriction endonucleases, but the same restriction enzymes differentiated *A. arboreus* when compared to the other two species (Figures 5 and 6; Tables 3 and 4). *Sac*II restriction enzyme cut the PCR product of *A. percicus* and *A. hermanni* into two restriction fragments (~550 and ~1250 bp, lanes 1 and 2; Figure 5; Table 3) and digested the gene of *A. arboreus* into three restriction patterns (~200, ~400 and ~1200 bp, lane 3; Figure 5; Table 3). Also, *Sst*II restriction enzyme digested the nuclear gene of *A. persicus* and *A. hermanni* into two restriction bands (~500 and ~1300 bp, lanes 1 and 2; Figure 6; Table 4) and *A. arboreus* into three restriction patterns (~300, ~600 and ~900 bp, lane 3; Figure 6; Table 4).

*Ava*II restriction enzyme did not differentiate between *A. hermanni* and *A. arboreus*, but differentiated between these two species and *A. persicus* (Figure 7 and Table 5). The restriction enzyme, *Ava*II digested the SrRNA gene of *A. persicus* into five restriction fragments (~50, ~150, ~300, ~600 and ~700 bp, lane 1; Figure 7 and Table 5). Simultaneously, the same restriction enzyme digested the gene of *A. hermanni* and *A. arboreus* into five restriction fragments but different than the RFLP profile of *A. persicus* in sizes (~150, ~200, ~250, ~450 and ~750 bp, lanes 2 and 3; Figure 7 and Table 5).

*Ava*I restriction endonuclease differentiated between the three *Argas* species (Figure 8 and Table 6). *Ava*I restriction enzyme did not react with the gene of *A. persicus* (lane 1; Figure 8 and Table 6). The same restriction enzyme digested the SrDNA of *A. hermanni* into two restriction bands (~200 and ~1600 bp, lane 2; Figure 8 and Table 6) and gave three restriction patterns with *A. arboreus* (~200, ~300 and ~1300 bp, lane 3; Figure 8 and Table 6).

Table 1: Shows the length of SrRNA genes fragments, resulted from digestion with *Eae*I enzyme in the three *Argas* species.

Argas strain	Band 1	Band 2	Band 3	Band 4	Band 5
A. persicus	~300	~500	~1000	------	------
A. hermanni	~300	~500	~1000	------	------
A. arboreus	~300	~500	~1000	------	------

Table 2: Shows the length of SrRNA genes fragments, resulted from digestion with *Eco*RI enzyme in the three *Argas* species.

Argas strain	Band 1	Band 2	Band 3	Band 4	Band 5
A. persicus	~250	~1550	------	------	------
A. hermanni	~250	~1550	------	------	------
A. arboreus	~250	~1550	------	------	------

Table 3: Shows the length of SrRNA genes fragments, resulted from digestion with *Sac*II enzyme in the three *Argas* species.

Argas strain	Band 1	Band 2	Band 3	Band 4	Band 5
A. persicus	~550	~1250	------	------	------
A. hermanni	~550	~1250	------	------	------
A. arboreus	~200	~400	~1200	------	------

Table 4: Shows the length of SrRNA genes fragments, resulted from digestion with *Sst*II enzyme in the three *Argas* species.

Argas strain	Band 1	Band 2	Band 3	Band 4	Band 5
A. persicus	~500	~1300	------	------	------
A. hermanni	~500	~1300	------	------	------
A. arboreus	~300	~600	~900	------	------

Table 5: Shows the length of SrRNA genes fragments, resulted from digestion with *Ava*II enzyme in the three *Argas* species.

Argas strain	Band 1	Band 2	Band 3	Band 4	Band 5
A. persicus	~50	~150	~300	~600	~700
A. hermanni	~150	~200	~250	~450	~750
A. arboreus	~150	~200	~250	~450	~750

Table 6: Shows the length of SrRNA genes fragments, resulted from digestion with *Ava*I enzyme in the three *Argas* species.

Argas strain	Band 1	Band 2	Band 3	Band 4	Band 5
A. persicus	~1800	------	------	------	------
A. hermanni	~200	~1600	------	------	------
A. arboreus	~200	~300	~1300	------	------

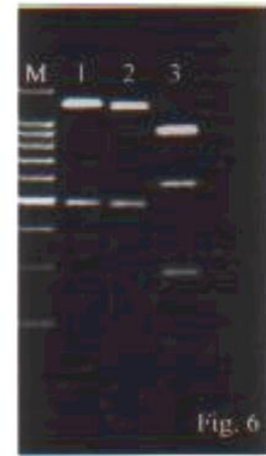
M 1 2 3
Fig. 1

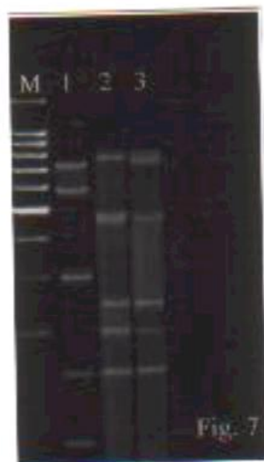
M 1 2 3
Fig. 2

M 1 2 3
Fig. 3

M 1 2 3
Fig. 4

M 1 2 3
Fig. 5


M 1 2 3
Fig. 6


M 1 2 3
Fig. 7

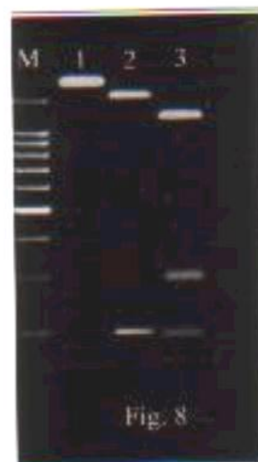

M 1 2 3
Fig. 8

Figure 1: Total DNA genome from *Argas* sp. Lane M is DNA marker. Lanes 1, 2 and 3 represent the chromosomal DNA of *Argas persicus, Argas hermanni* and *Argas arboreus* respectively.

Figure 2: Shows full-segment SrRNA gene (~1800 bp) of *Argas* sp. Lane M is the DNA marker. Lanes 1, 2 and 3 represent SrDNA of *Argas persicus, Argas hermanni* and *Argas arboreus* respectively.

Figure 3: Shows the representative SrDNA PCR/RFLPs bands from *Argas persicus* (lane 1), *Argas hermanni* (lane 2) and *Argas arboreus* (lane 3) with *Eae*I restriction endonuclease, which produced roughly the same fragments (three bands: ~300, ~500 and ~1000 bp, for all). Lane M is the DNA marker.

Figure 4: Shows the representative SrDNA PCR/RFLPs patterns from *Argas persicus* (lane1), *Argas hermanni* (lane 2) and *Argas arboreus* (lane 3) with *Eco*RI restriction endonuclease, which produced roughly the same fragments (two bands: ~250 and ~1550 bp, for all). Lane M is the DNA marker.

Figure 5: Shows *Sac*II restriction enzyme digested the SrRNA gene of *Argas persicus* and *Argas hemanni* roughly the same fragments (two bands: ~550 and ~1250 bp; lanes 1 and 2), and the SrRNA gene of *Argas arboreus* into three fragments (~200, ~400 and ~1200 bp; lane 3). M lane is the DNA marker.

Figure 6: Shows *Sst*II restriction enzyme digested the SrRNA gene of *Argas persicus* and *Argas hemanni* roughly the same fragments (two bands: ~500 and ~1300 bp; lanes 1 and 2), and the SrRNA gene of *Argas arboreus* into three fragments (~300, ~600 and ~900 bp; lane 3). M lane is the DNA marker.

Figure 7: Shows the representative SrDNA PCR/RFLPs patterns of *Ava*II enzyme of *Argas hermanni* (lane 2) and *Argas arboreus* (lane 3) producing roughly the same patterns (five bands: ~150, ~200, ~250, ~450 and ~750 bp) and the same gene of *Argas persicus* (lane 1) into five different sized bands (~50, ~150, ~300, ~600 and ~700 bp). M lane is the DNA marker.

Figure 8: Shows *Ava*I restriction enzyme did not react with the gene of *Argas persicus* (lane 1), digested the SrDNA of *Argas hermanni* into two restriction bands (~200 and ~1600 bp) and gave three restriction patterns with *Argas arboreus* (~200, ~300 and ~1300 bp). M lane is the DNA marker.

Discussion

In this study it has been attempted to achieve a target, to resolve whether *A. persicus, A. hermanni* and *A. arboreus* are a single species (monophyletic relationship) or the three species are not related to each other (polyphyletic relationship) and/or if any of them are related to another each other. The objective is very valuable to determine because *A. persicus, A. hermanni* and *A. arboreus* transmit a greater variety of pathogenic micro-organisms than any other arthropod vector group to animals and humans and there have been many complexities with classification of pathogenic carrier *Argas* species in the past. (Kohls and Hoogstraal, 1961; Filippova, 1966; Fedorov, 1968; Grzywacz and Kuzmicki, 1975; Keirans *et al.*, 1979; Miadonna *et al.*, 1982; Schwan *et al.*, 1992 and Estrada-Pena and Jongejan, 1999). Polymerase chain reaction/re-striction fragment length polymor-phisms(PCR/RFLPs) of small-subunit ribosomal RNA gene is a modern molecular biological technique by which, the problem of unclear relationship between *A. persicus, A. hermanni* and *A. arboreus* could be resolved.

A. persicus, A. hermanni and *A. arboreus* remained undifferentiated when their SrRNA gene digested with

*Eae*I and *Eco*RI restriction endonucleases and gave the same PCR/RFLPs profiles (Figures 3 and 4). This indicated that *A. persicus, A. hermanni* and *A. arboreus* species may be a single, or monophyletic species.

*Sac*II and *Sst*II restriction endonucleases proved that *A. persicus* and *A. hermanni* may be one species or monophyletic and *A. arboreus* is polyphyletic when related to the other two species or different species. SrRNA gene PCR/RFLPs of *Sac*II and *Sst*II restriction enzymes of *A. persicus* and *A. hermanni* gave the same profiles and gave a different profile with *A. arboreus* (Figure 5 and 6).

On the other hand, *Ava*II restriction enzyme showed similarity between *A. hermanni* and *A. arboreus* species and dissimilarity between these two species and *A. persicus* species. SrDNA PCR/RFLPs of *Ava*II restriction endonuclease gave same profile with *A. hermanni* and *A. arboreus* species and different profile with *A. persicus* species when compared to the others. This indicated that *A. persicus* species may be different or polyphyletic species when compared to the single or monophyletic *A. hermanni* and *A. arboreus* species.

*Ava*I restriction endonuclease was a different and more specific enzyme than the other restriction enzymes used in this work. This enzyme gave a different and specific PCR/RFLPs profile with each one of the three *Argas* species. *Ava*I restriction enzyme did not digest the gene of *A. persicus*, digested the gene of *A. hermanni* into two bands and digested SrDNA of *A. arboreus* into three restriction fragments. *Ava*I restriction endonuclease proved that *A. persicus, A. hermanni* and *A. arboreus* may be all polyphyletic or in a different manner they are different species.

From examination of restriction enzyme analysis of SrRNA gene, it is believed that *A. persicus, A. hermanni* and *A. arboreus* may be monophyletic species and related to the same species with some minor differences. These differences could extend to differentiate the three species as a different and polyphyletic species with some exception. *A. persicus* and *A. hermanni* are related to each other, while, *A. hermanni* and *A. arboreus* are close enough to each other, *A. persicus* and *A. arboreus* are not related. It appears also that PCR/ RFLPs of nuclear SrDNA generated by restriction endonuclease could be used for preliminary characterization of this species complex.

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التعرف الجزيئى لثلاثة أنواع من قراد الأرجاس باستخدام تحليل التفاعلات المتتابعة لإنزيم البلمرة و التغاير فى طول القطع المحدد للجين الريبوزومى تحت الصغير للحمض النووى الاريبوزى

محمد حسين عواد, سحر محمد أبو الخير وجيهان حسين لاشين
قسم علم الحيوان – كلية العلوم – جامعة الزقازيق – فرع بنها

إن القراد تعتبر الدراسة عليه من الدراسات المهمة بصفة عامة. حيث أنه يصيب الإنسان والحيوان على حد سواء. علاوة على أن القراد يتغذى على دم الإنسان والحيوان فإنه ينقل لهما الفيروسات والبكتريا والطفيليات التى تسبب الأمراض. وعلى سبيل المثال لا الحصر فإن القراد ينقل بعض الكائنات الحية من فصائل *اللاريليا*, *الريكيتشيا*, *الفرانسيسيلا*, *الإيرليشيا*, *الأنابلازما*, *الكوودريا والكوكسيلا*.

فى هذه الدراسة تم عزل ثلاثة سلالات القراد من ثلاثة طيور وهم *الأرجاس بيريسكاس* من الدجاج, *الأرجاس هيرمانى* من الحمام *والأرجاس أربوريس* من أبو قردان. إن هذه السلالات الثلاث تعتبر من أنواع القراد الناعمة أو الرخوة وكذلك يصعب التعرف عليها شكلا ومن المعروف أن هذه السلالات تعيش على دم الحيوان والإنسان فكان يجب دراستها وتصنيفها بدقة. . وعليه استخدمت تقنية التغاير فى طول القطع المحددة لجين القطعة الصغيرة الريبوزومية للتفرقة بين السلالات الثلاث *للأرجاس*, والذى يختلف حسب الطراز الذى ينتمى اليه الكائن الحى.

وقد تم عن طريق الفرد أو الفصل الكهربى استخلاص جينات الجسم الريبوزى من النواه بطريقة التفاعلات المتتابعة لإنزيم البلمرة. وقد وجد أن مورث القطعة الصغيرة للجسم الريبوزى فى النواه لجميع العزلات عبارة عن حوالى 1800 من أزواج القواعد النيتروجينية.

وقد وجد أنه عند هضم الجين بإنزيمى *Eae*I و *Eco*RI أن سلالات جنس *الأرجاس* الثلاث سابقة الذكر جمعت فى مجموعة واحدة كسلالات متشابهة وربما يكونوا ذات أصل واحد. فعند هضم الجين (أو المورث) بإنزيم *Eae*I أعطت ثلاث قطع(حوالى 300 500 و 1000 من أزواج القواعد) بنفس الحجم فى الأنواع الثلاث سابقة الذكر. وكذلك عند هضم المورث بإنزيم *Eco*RI نتج عن ذلك قطعتين (حوالى 250 و 1550 من أزواج القواعد) مع نفس السلالات.

إن سلالاتى *الأرجاس بيريسكاس* و *الأرجاس هيرمانى* ربما تكونا متشابهتان وراثيا أو ذات أصل واحد حيث أنها قد جمعت فى مجموعة واحدة عند هضم موروثها بإنزيمى القطع *Sac*II و*Sst*II وكذلك فإن سلالة *الأرجاس أربوريس* قد كونت مجموعة أخرى مختلفة عند هضم جينها بنفس إنزيمى القطع *Sac*II و *Sst*II. فعند هضم جين الوحيدة الصغيرة الريبوزومية للحمض النووى الريبوزى لسلالاتى *الأرجاس بيريسكاس و الأرجاس هيرمانى* بإنزيم القطع *Sac*II أعطى قطعتين (حوالى 550 و 1250 من أزواج القواعد) وعند هضم المورث لسلالة *الأرجاس أربوريس* بنفس الإنزيم أعطى ثلاث قطع (حوالى 200 و 400 و 1200 من أزواج القواعد). وكذلك عند هضم المورث بإنزيم

*Eco*RI نتج عن ذلك قطعتين (حوالى 500 و 1300 من أزواج القواعد) مع سلالاتى *الأرجاس بيريسكاس* و *الأرجاس هيرمانى*, وأعطى ثلاث قطع مع سلالة *الأرجاس أربوريس* وكان حجم (حوالى 300و 600 و 900 من أزواج القواعد).

إن سلالاتى *الأرجاس هيرمانى* و *الأرجاس أربوريس* ربما تكونا متشابهة وراثيا أو ذات أصل واحد حيث أنها قد جمعت فى مجموعة واحدة عند هضم موروثها بإنزيم *Ava*II وكذلك فإن سلالة *الأرجاس بيريسكاس* قد كونت مجموعة أخرى مختلفة عند هضم جينها بنفس الإنزيم. . فعند هضم جين الوحيدة الصغيرة الريبوزومية للحمض النووى الريبوزى لسلالاتى *الأرجاس هيرمانى* و *الأرجاس أربوريس* بإنزيم *Ava*II أعطى خمسة قطع (حوالى 50 و 150 و 300 و 600 و 700 من أزواج القواعد) وعند هضم المورث لسلالة *الأرجاس بيريسكاس* بنفس الإنزيم أعطى خمسة قطع ولكن لهم حجم مختلف (حوالى 150 و 200 و 250 و 450 و 750 من أزواج القواعد).

إن جميع سلالات *الأرجاس* الثلاث قد تميزت إلى ثلاثة مجموعات حينما تم هضم مورث الوحيدة الصغيرة الريبوزومية للحمض النووى الريبوزى لهذه السلالات بإنزيم . *Eva*Iفقد قطع إنزيم *EvaI* مورث سلالة *الأرجاس هيرمانى* إلى قطعتين (حوالى 200و 1600 من أزواج القواعد) وسلالة *الأرجاس أربوريس* إلى ثلاث قطع (حوالى 200و 300و 1300 من أزواج القواعد) ولم يهضم هذا الإنزيم جين *الأرجاس بيريسكاس*.

وعلى هذا فإن سلالات *الأرجاس* ربما تكون واحدة الأصل ومتعددة الصفات الجينيه أو قد يكون قد حدث طفرة وراثية لهذا الأصل الواحد نتيجة لبيئته أو الحيوان الذى يستخدمه كعائل. كما يتضح أن التغاير فى طول القطعة المحددة يمكن استخدامه كمدخل للتعرف على الخصائص المعقدة لهذا النوع.

أيضا يمكن القول أنه من المعتقد أن استخدام طرق البيولوجيا الجزيئية يعطى صورة تصنيفية أكثر دقة عن استخدام الشكل التركيبى فى هذه الدراسات. وبالرغم من أن الدراسة على جين واحد ليست كافية إلا انه يمكن استخدام بعض الإنزيمات التى فصلت فى هذه الدراسة للعمل كمجسات للتعرف على سلالات *الأرجاس*.