DETERMINATION OF THE INFECTIVE STRAIN OF HYDATID CYST IN IRAQI CATTLE BY USING *CO1* GENE

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ABSTRACT

Hydatid Cysts were obtained from liver, lungs, spleen, heart, and peritoneal cavity of 15 cows, from different Iraqi regions between December 2014 and October 2015. Hydatid cysts (protoscoleces) were used for mitochondrial DNA extraction by using mechanical grinder, and the purification of mtDNA was done by (promega kit, USA). "The mitochondrial cytochrome c oxidase subunit 1 (*CO1*) gene" was used as target for "polymerase chain reaction (PCR)" which successfully amplified the targeted this gene with 450 bp. The PCR products were purified and partial sequences were determine. The obtained sequences were aligned with the corresponding region of *co1* gene in the Gene Bank nucleotide database to confirm the infection with hydatid cyst sheep strain (G1) in Iraq. The amplified *CO1* targeted region was analyzed to obtain the phylogenetic tree. G1 genotype was the most common strain and the actual source of infection of Iraqi's cattle. All of 15 samples were G1 strain (sheep strain) according to the partial sequences of (*CO1*) genes.

Keyword : DNA extraction, Hydatid cyst, *CO1*gene, sheep, Sequences.

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تحديد السلالة المسببة للاكياس المائية في الابقار العراقية باستخدام جين *co1*

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المستخلص

تم الحصول على الأكياس العداريه من الكبد، الرئة،الطحال،القلب والتجويف البريتوني ل 15 بقرة من مختلف مناطق العراق ما بين كانون الاول 2014 و تشرين الاول 2015 استخلص دنا DNA المايتوكوندريا من رؤيسات الاكياس العداريه باستخدام طريقة السحق الميكانيكي، وجرى تنقيتة باستخدام العده (بروميكا. امريكي المنشأ). استخدم جين المايتوكوندريا *CO1* كهدف لتفاعل سلسلة البوليمرات PCR والذي نجح في مضاعفة هذا الجين لجميع الاكياس العداريه بحجم 450 زوجاً قاعدياً. جرى تنقية ناتج التضاعف وتحديد تتابع القواعد النايتروجينية له. اجريت مطابقة للتتابعات الناتجه مع تلك الموجوده في بنك الجينات العالمي لأثبات الأصابة بالأكياس العداريه سلالة الاغنام G1 في العراق. حللت التتابعات الوراثية للجين للحصول على الشجرة الوراثية. كان النمط الوراثي G1 اكثر الاصناف انتشاراً و يعد مصدراً لإصابة الأبقار العراقية. كانت جميع ال 15 سلالة تابعه للنمط الوراثي G1 (سلالة الاغنام) اعتماداً على التسلسلات الجزئية ل *CO1*.

كلمات مفتاحيه: استخلاص الدنا،الاكياس العداريه، ألاغنام.تسلسلات الدنا

INTRODUCTION

The larval stage causing Cystic echinococcosis (CE) of tape worm *Echinococcus granulosus* has a worldwide distribution and is one of the most important zoonosis all over the world (1,2) . Cattle acquire the infection by coming in handle with infected dogs harboring mature *E. granulosus* in their intestine resulting in excretion of eggs in the feces thus, one way that humans and other intermediate hosts can be infected is by swallowing ova that contaminate food , water or the environment (3). The extensive difference in *E. granulosus* may influence life cycle, host specificity, rate of development, pathology and consequently, the design plus sensitivity to chemotherapeutic agents and development of vaccines against *E. granulosus* (4). In Iraq , CE constitutes one of the major endemic diseases and has serious influence on animal health or human (5,6,7,8,9,10).To date ten notable genotypes (G1-G10 strains) have been qualified in world based on nucleotide sequences dissection of the (*CO1*) , NADH dehydrogenase 1 (*ND1*) genes and intra transcribed spacer 1 (ITS1), these genotypes have been correlating with prominent, intermediate hosts including: sheep, cattle, goats, horses , pigs, cervides and camels (11,12,13,14,15,16). The G1 genotype was likewise specified as the only strain secluded from sheep, cattle and humans (17). A number of (DNA) correspondence techniques have been used to distinguish *Echinococcus* genotypes and species from various definitive and intermediate hosts and in several geographical settings (18). The aim of this research was to recognize the *E. granulosus* genotypes actually infecting cattle in Iraq. Polymerase chain reaction was used to evaluation the genetic contraindication within the strains by sequencing the "mitochondrial cytochrome c oxidase subunit 1" *CO1* gene.

MATERIALS AND METHODS

The samples of 15 hydatid cysts were collected from different region in Iraq (Baghdad, Babylon, Nasiriyah, Basrah, Kirkuk) during 2014 to 2015. The cysts were washed more than one times with normal saline to decrease contamination with host tissue, and then they were extensively washed with 70% ethanol. According to McManus (19), each cyst was separated into membrane and intra cystic fluid with protoscoleces. The cyst contents (fluid and protoscoleces) were aspirated aseptically by sterile syringes (10 ml) into a flask. Cysts were opened with longitudinal incision and all the remaining protoscoleces and fluid were aspirated and added to the flask content. The fluid was carefully and gradually decanted into a sterile test tubes with spinning at 3000 rpm for l0 minutes at room temperature collect the protoscoleces' pellet. The germinal membrane was peeled away and washed several times with Hanks saline (pH 2.0) containing 0.2% (w/v) pepsin to release the remaining attached scoleces (20). The suspension was centrifugated at 3000 rpm for 10 minutes, and the pellet of scoleces was collected. Protoscoleces were finally rinsed 3-4 times with sterile normal saline by repeating centrifugation followed by 70% ethanol, and stored in 70% ethanol at 4 ^{o}C for further analysis. Pellet of protoscoleces were rinsed several times with sterile distilled water and Phosphate buffer saline (PBS) to remove ethanol prior to DNA extraction (21). DNA extraction was done by using Wizard ®Genomic DNA Purification Kit.(USA) and following the instructions of the manufacture (22). Twenty nanograms of DNA from protoscoleces pellet were used for all samples.

PCR process

20 ng of mitochondrial DNA specimens were resolved by employing the method described by (22) with minor modifications. The target segment of *CO1* gene was amplified by PCR using Sense *CO1* and Antisense *CO1* primers **(**23) Table [1]. The thermal conditions for the PCR reaction were as follows: denaturation for 5 mins at 94ºC, followed by 35 cycles of 45 sec at 94ºC, 45 sec at 58ºC and 45 sec at 72ºC and a final extension at 72ºC for 7 min(24).

Table 1. The *CO1* gene primer

gene	size	Primer's name	Primer's sequence
CO1	450 bp	Forward	" 5'-TTT TTT GGG CAT CCT GAG GTT TAT -3'"
		Reverse	" 5'-TAA AGA AAG AAC ATA ATG AAA ATG-3'"

Sequences analysis

Following amplification of specific sequences of $_{mt}$DNA *CO1* genes from 15 cows infected with hydatid cyst, that sequences amplicon was compared with a reference *CO1* sequence available on NCBI website (http://www. ncbi. nlm. nih. gov). Furthermore, the amplicon sequence was also aligned with that obtained from previous publications Phylogenetic tree of the *CO1* genotypes was analyzed in this study.

Results Target gene amplification

The target DNA was successfully amplified from 15 hydatid cyst isolates were prepared for PCR process by using specific primers in the PCR technique. The PCR products were confirmed as bands of approximately 450 bp by using agarose gel electrophoresis as shown in (Fig.1). A fractional part of the *CO1* gene was a magnified following a formerly described protocol.

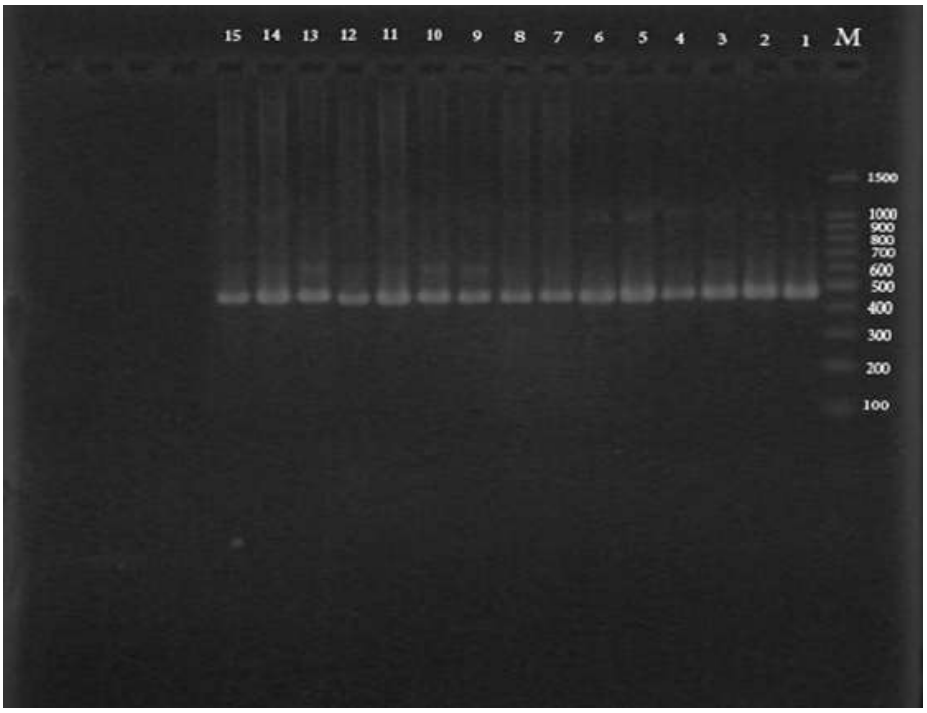

Fig.1 Agarose gel electrophoresis of PCR amplicon of *Co1* gene. Using 2 % agarose , 80 V, 70 Am for 1 hrs, (lanes 1-15: *E. granulosus* isolates; M: 100 bp DNA ladder).

Sequence analysis

To determine the genotypes of 15 isolates of cysts, *CO1* gene was amplified by PCR, then sequenced and resolved by uniformity with reported reference of G1 genotype sequences of *E. granulosus* using Gene bank (Fig.2). The sequence alignment was done by using Bioedit (DNA analysis program) and compared with previously reported references of *E. granulosus* strain. The results revealed 100% compatibility with common sheep strain G1 genotype compared with [ACCESSION KX039951](25).

A phylogenetic tree

A phylogenetic tree of the *CO1* genotypes was organized utilizing the "Maximum Likelihood" calculate with the Tamura-Nei range, as the pattern of development (30) (Fig. 3). The progressive date was conclude by using the ceiling Likelihood style according to the Tamura-Nei technique (30). The tree with the elevated log likelihood (-490.7751) is reveal. Elementary tree for the investigative study were acquired automatically by employing Neighbor-Join and BioNJ method to a template of pairwise stretch evaluated utilizing the Maximum installed Likelihood (MCL) process, and then choosing the topology with officer log likelihood rate. The tree is depicted to gauge, with bough lengths

calibrated in the number of replacement per position. The test participatory 14 nucleotide longitude. Codon location inclusive were 3rd+2nd+ 1st Noncoding. All sites including cavities and absent datum were forsaken. There were a overall of 402 sites in the ultimate dataset. Development dissection were performed in MEGA7 (31).

Score	Expect	Identities	Gaps	Strand
736 bits(398)	0.0	398/398(100%)	0/398(0%)	Plus/Plus

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                                                                    Query  4

GTATAATTAGTCATATTTGTTTGAGTATTAGTGCTAATTTTGATGTGTTTGGGTTCTATG  63
||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  6848  GTATAATTAGTCATATTTGTTTGAGTATTAGTGCTAATTTTGATGTGTTTGGGTTCTATG  6907

Query  64    GGTTGTTGTTTGCTATGTTTTCTATAGTGTGTTTGGGTAGCAGGGTTTGGGGTCATCATA  123
||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  6908  GGTTGTTGTTTGCTATGTTTTCTATAGTGTGTTTGGGTAGCAGGGTTTGGGGTCATCATA  6967


Query  124   TGTTTACTGTTGGGTTGGATGTGAAGACGGCTGTTTTTTTTAGCTCTGTTACTATGATTA  183
||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  6968  TGTTTACTGTTGGGTTGGATGTGAAGACGGCTGTTTTTTTTAGCTCTGTTACTATGATTA  7027

Query  184   TAGGGGTTCCTACTGGTATAAAGGTGTTTACTTGGTTATATATGTTGTTGAATTCGAGTG  243
||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  7028  TAGGGGTTCCTACTGGTATAAAGGTGTTTACTTGGTTATATATGTTGTTGAATTCGAGTG  7087

Query  244   TTAATGTTAGTGATCCGGTTTTGTGATGGGTTGTTTCTTTTATAGTGTTGTTTACGTTTG  303
||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  7088  TTAATGTTAGTGATCCGGTTTTGTGATGGGTTGTTTCTTTTATAGTGTTGTTTACGTTTG  7147

Query  304   GGGGAGTTACGGGTATAGTTTTGTCTGCTTGTGTGTTAGATAATATTTTGCATGATACTT  363
||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  7148  GGGGAGTTACGGGTATAGTTTTGTCTGCTTGTGTGTTAGATAATATTTTGCATGATACTT  7207

Query  364   GGTTTGTGGTGGCTCATTTTCATTATGTTATGTCGTTA  401
|||||||||||||||||||||||||||||||||||||
Sbjct  7208  GGTTTGTGGTGGCTCATTTTCATTATGTTATGTCGTTA  7245
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Fig.2 Alignment of *CO1* gene of *E. granulosus* by using Gene bank

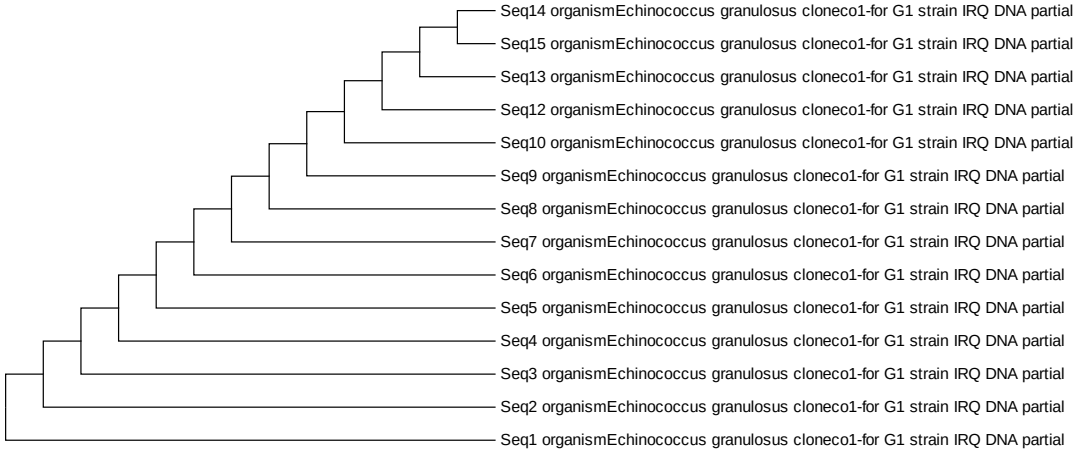

Fig. 3 Molecular phylogenetic analysis by Maximum Likelihood process.

DISCUSSION

The *CO1* gene was amplified by utilizing a primer specific prepared for this study, the PCR dilatation of *CO1* gene was felicitous in all samples and created outputs of nearly 450 bp on agarose gel, this result agreed with previous results done by (23,17,24) where showed the size of *CO1* gene was 450 bp. Genotype G1 was the most common sheep contagious *E. granulosus* strain in the universe with a broad domain of hosts (26). In these area, dogs are predominating feed with domestic animal bowels that probably infected with the hydatid cyst (16). This vigor could be adequate to increase the endemic situation. From the outcome above, it can be indicated that the notable spreading

of the prevalent sheep strain (G1) was prevalent in hyper-endemic regions of Iraq. This finding is consistent with results of several researchers (16), (24) and (26) possibly because it is the most common one and is widespread among intermediate hosts (17, 27). The determination of the strain of *E. granulosus* existent in domestic animals and untamed animals within districts endemic for this parasite is epidemiologically significant. The G1 genotype as well as pathogenic for other intermediate hosts like goats (28, 29). The G1 genotype was the generality popular taxon and the effective provenance of infection of Iraqi's cattle. All of 15 specimens were G1 strain (sheep strain) according to mitochondrial *CO1* gene.

REFERENCES

1.Thompson RC. 2008. The taxonomy, phylogeny and transmission of *Echinococcus*. Exp Parasitol. 119: 439-446.

2.Altintas N, M. Oztatlici, N. Altintas, A. Unver and A. Sakarya. 2013. Molecular analysis of cattle isolates of *Echinococcus granulosus* in manisa province of Turkey. Kafkas Univ Vet Fak Derg.19 (3): 455-459.

3. Fasihi HM, Hobbs RP, Adams PJ, Mobedi I., Morgan-Ryan UM, Thompson RCA. 2002. Molecular and morphological characterization of *Echinococcus granulosus* of human and animal origin in Iran. Parasitolo. 125: 367-373.

4. McManus D.P. and R.C.A. Thompson. 2003. Molecular epidemiology of cystic echinococcosis. Parasitol.127:S37-S51.

5. Al-jeboori T.I. 1976. Hydatid disease : A study of the records of the medical city hospital. J. Fact . Med. Baghdad. 18:65-75.

6. Mahmoud S.S. 1980.Studies on Hydatid Disease in Mousl .M.Sc. Thesis, College of Medicine, University of Mosul.PP:12-20.

7. Eckert J, P.M. Schantz, R.B. Gasser, P.R. Torgerson, A.S. Bessonov and S.O.2001. Movsessian. Geographic distribution and prevalence. In: Eckert J, Gemmell MA, Meslin FX, Pawlowski ZS (eds) WHO/OIE Manual on Echinococcosis in Human and animals: A public health problem of global concern, Paris: World Organisation for Animal Health. 100-142.

8. Dinkel A, E.M. Njorage, A. Zimmermann, M. Walz, E, Zeyhle, I.E. Elhamdi, U. and T. Mackenstedt, Roming.2004. A PCR systems for detection of species and genotypes of the *Echinococcus granulosus* complex, with reference to the epidemiological situation in eastern Africa. Int. J. Parasitol. 34(5): 645-653.

9. Sarıözkan S and C. Yalçın. 2009. Estimating the production losses due to cystic echinococcosis in ruminants in Turkey. Vet. Parasitol. 163:330-334.

10. Snabel V, N.Altınta, S. D'Amelio, M. Nakao, T. Romig, A. Yolasımaz, K. Güne, M. Türk, M. Busi, M. Hüttner, D. Sevcova, A. Ito and P. Dubinsky. 2009. Cystic echinococcosis in Turkey: genetic variability and first record of the pig strain (G7) in the country. Parasitol. Res. 105: 145-154.

11. Breyer I, D. Georgieva , R. Kurdova and R. Gottstein.2004. *Echinococcus granulosus* strain typing in Bulgaria: the G1 genotype is predominant in intermediate and definitive wild hosts. Parasitol. Res. 93: 127-130.

12. Romig T and A. Dinkel. 2006. Mackenstedt U . The present situation of echinococcosis in Europe. Parasitol. Int. 55: 187-191.

13. Varcasia A, S. Canu, A. Kogkos, A.P. Pipia, A. Scala, G. Garippa and A. Seimenis .2007. Preliminary data on diffusion and molecular characterization of cystic echinococcosis in small ruminants in Peloponnesus, Greece. Parasitol. Res. 101: 1135-1139.

14. Busi M, V. Snabel, A. Vercasia, G. Garippa, V. Perrone,C. De Liberato and S. D'Amelio . 2007. Genetic variation within and between G1 and G3 genotypes of *Echinococcus granulosus* in Italy revealed by multilocus DNA sequencing. Vet. Parasitol. 150: 75-83.

15. Nakao M, D.P. Mc Manus,P.M. Schantz, P.S. Craig and A. Ito. 2007. A molecular phylogeny of the genus *Echinococcus* inferred from complete mitochondrial genomes. Parasitol. 134: 713-722.

16. Sanchez E, O. Caceres, C. Naguira, D. Garcia and G. Patifio. 2010. Molecular characterization of *Echinococcus granulosus* from Peru by sequencing of the mitochondrial cytochrome C oxidase subunit 1 gene. Oswaldo Cruz, Rio de Janeiro. 105 (6): 221-

232.
17. Sánchez E, O. Cáceres , A.C. Náquir, E. Miranda, F. Samudio and O. Fernandes. 2012. *Echinococcus granulosus* genotypes circulating in alpacas (*Lama pacos*) and pigs (*Sus scrofa*) from an endemic region in Peru. Mem Inst Oswaldo Cruz, Rio de Janeiro. 107(2):275-278.
18. Eryıldız C. and N. Şakru.2012. Molecular Characterization of Human and Animal Isolates of *Echinococcus granulosus* in the Thrace Region,Turkey. Balkan Med J. 29: 261-7.
19. McManus DP and S.D. Smyth. 1978. Differences in the chemical composition and carbondrate metabolisim of *Echinococcus granulosus* (horse and sheep strains) and E. multilocularis. Parasitol. 77: 103-109.
20. Rishi A.K and D. McManus. 1987. Genomic cloning of human *Echinococcus granulosus* DNA: isolation of recombinant plasmids and their use as genetic markers in strain characterization . Parasitol. 94: 369–383.
21. Al- Azawiy A.K.2003. Immunization of Experimental Mice by DNA of Protoscoleces of Hydatid Cyst: Immunological and Histopathological Studies. Ph.D. Dissertation. College of Veterinary Medicine, University of Baghdad. Iraq.PP:5-15.
22. Miller S.A, D.D. Dykes and H.F. Polesky.1988. A simple salting out procedure for extracting DNA from human nucleated cells. Nucl. Acids Res. 16: 1215.
23. Ergin S., S. Saribas and P. Yuksel. 2010. Genotypic characterisation of Echinococcus granulosus isolated from human in Turkey. Afr. J. Microbiol. Res. 4 (7):551-555.
24. Baraak M.J. 2014. Molecular Study on Cystic Echinococcosis in Some Iraqi Patients. Ph.D. Dissertation, College of Science, University of Baghdad. Iraq. PP:10-19.
25.Laurimae,T., L. Kinkar, V. Andresiuk, K.L. Haag, F. Ponce-Gordo, G. Acosta-Jamett, T. Garate, L.M. Gonzalez, and U. Saarma.2016. Genetic diversity and phylogeography of highly zoonotic Echinococcus granulosus genotype G1 in the Americas(Argentina,Brazil, Chile and Mexico) based on 8279bp of mtDNA. Infect. Genet. Evol., 45: 290-296.
26. Craig P.S. and M.T. Rogan.2003. Campos-Ponce M. Echinococcosis: disease, detection and transmission. Parasitol. 127 :5-20.
27. Rinaldi L, M.P. Maurelli, F. Capuano, A.G. Perugini, V. Veneziano and S. Cringoli.2008. "Molecular update and cystic echinococcosis in cattle and water buffaloes of southern Italy", J. Blackwell verlag. Zoonosis, Pub. Heal. 55:119-123.
28. Eckert J. and R.C. Thompson. 1997. Intraspesific variation of *E.granulosus* and related species with emphasis on their infectivity to humans. Acta. Trop. 64: 19-34.
29. Mwambete K.D., F. Ponce-Gordo and C. Cuesta-Bandera. 2004. Genetic identification and host range of the Spanish strains of *Echinococcus granulosus*. Acta. Trop. 91: 87-93.
30. Tamura K and M. Nei .1993. Estimation of the number of nucleotide sub- stitutions in the control region of the mitochondrial DNA in hu- mans and chimpanzees. Mol. Biol. Evol. 10: 512-526.
31. Kumar S, G. Stecher and K. Tamura. 2016. MEGA7: Molecular Evolutionary Genetics Analysis version 7.0 for bigger datasets.Molecular Biology and Evolution. 33:1870-1874.