云南闭壳龟(*Cuora yunnanensis*)的分子鉴定及进化地位研究

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摘要　云南闭壳龟(*Cuora yunnanensis*, Boulenger, 1906)曾被认为已经灭绝, 在保护生物学上受到广泛的关注. 本文测定了 3 只活的云南闭壳龟线粒体 COI 和 ND4 的部分序列及 His, Ser, Leu tRNA 序列片段, 共 1725 个碱基序列. 结合闭壳龟属其他物种序列, 包括之前测定的一只云南闭壳龟标本(MNHN 1907.10)的 DNA 序列, 进行了分子系统学分析. 与 100 年前的标本比较, 无论是形态上、还是本文的分子系统分析结果都显示, 这种新发现的活龟确实是云南闭壳龟. 同时, 我们的结果确证了标本序列的可信性, 揭示云南闭壳龟不是近期杂交形成的, 而是代表了进化上独立的遗传谱系, 且种内仍存在一定的遗传多样度.

关键词　云南闭壳龟　线粒体**DNA**　保护生物学

闭壳龟属(*Cuora* Gray, 1855)为亚洲特有类群, 广布于东南亚、南亚大陆及其邻近各岛屿地区, 是进行系统地理学研究的理想模型[1)]. 其中, 金头闭壳龟、百色闭壳龟、潘氏闭壳龟、云南闭壳龟和周氏闭壳龟等 5 个种是我国特有的. 近年来, 闭壳龟属系统分类研究的广泛开展增加了我们对这个类群的认识. 闭壳龟类物种间的系统发育关系错综复杂, 加之本属所有物种都是红皮书上的濒危物种, 鲜有野外采集的标本, 取样较为困难. 另外, 中国人几千年来素有繁殖、饲养龟类的传统, 更加剧了系统分类研究的复杂性[1~3]. 目前有关该类群的系统分类与进化研究中仍存在许多疑难问题. 首先, 闭壳龟属(*Cuora*)的适用范围一直存在很大争议. 有的学者支持将本属黄缘闭壳龟(*Cuora flavomarginata*)和黄额闭壳龟(*Cuora galbinifrons*)另立盒龟属(*Cistoclemmys* Gray, 1963)[4~11]; 2002 年, Honda等人[12]应用分子系统发育方法探讨了亚洲闭壳龟(Asian box turtle)的系统发育关系, 支持广义的闭壳龟类群(*Cuora* sensu lato, 包括狭义闭壳龟属和盒龟属)与单种锯缘龟属(*Pyxidea*)聚成一个单系闭壳龟类群(*Cuora* group), 建议闭壳龟属包括原来的狭义闭壳龟属、盒龟属和锯缘龟属. 其次, 其中一些物种的系统地位还有待进一步确证[13,14].

云南闭壳龟是中国特有种, 在中国也仅发现于云南境内. IUCN 2000 年的濒危物种列表(IUCN 2000 Red List of Threatened Species)中将云南闭壳龟列为已灭绝物种, 认为自 1906 年被发现报道以来这个物种已经在地球上消失了. 目前对它的了解仅来自于大约 100 年前采自我国云南省境内的 12 只标本[15]; 而且由于这些标本都是从市场上购得, 缺乏野外观测证据, 同时云南闭壳龟与潘氏闭壳龟形态上十分相似, 与乌龟(*Chinemys reevesii*)共享独特的颈部斑纹, 因此该种也可能由近期杂交形成. 形态比较分析认为, 云南闭壳龟与潘氏、金头、三线闭壳龟(*Cuora pani*, *C. aurocapitata* and *C. trifasciata*)复合类群更近似[11,13,16]. 但由于标本珍贵、不易获得, 上述形态比较分析主要基于前人对标本的描述, 一般研究者没能直接研究标本, 其结果有待检验. 2004 年, Parham 等人[17]测定了一只云南闭壳龟标本(MNHN 1907.10, 保存于巴黎自然历史博物馆中, 这可能是DNA保存情况最好的一个标本[15])的线粒体DNA序列, 结合所有闭壳龟类(*Cuora* group)物种序列, 构建了分子系统

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1) Iverson J B. A revised checklist with distribution maps of the turtles of the world. 1992. Privately printed. Richmond

树. 结果显示, 云南闭壳龟隶属闭壳龟类群代表了不同于任何其他已知物种的、独特的遗传谱系. 数据还支持云南闭壳龟和黄缘盒龟(*C. flavomarginata*)的姊妹群关系, 二者再与周氏、潘氏、金头、三线闭壳龟复合类群聚成一枝. 有趣的是, 云南闭壳龟和黄缘盒龟在形态上却差异较大. 但是, 该研究只包括了一个云南闭壳龟的样本, 而且用于测序的这个标本起初曾用福尔马林固定, 而后才转移到乙醇溶液中保存(浸泡长达一个世纪), DNA保存情况不甚理想, 因此其结果招致了很多怀疑[15]. 对更多的标本进行研究是解决云南闭壳龟系统地位的关键.

令人鼓舞的是, 距云南闭壳龟首次被发现近 100 年后, 近期两只活的云南闭壳龟(一雌、一雄)先后被发现报道[18,19], 之后我们又获得了一只雌性个体. 这些标本的发现引起了国内外极大的关注. 在原有形态鉴定的基础上, 通过进一步的分子鉴定, 确定这些标本的可靠性和进化地位具有重要的意义.

我们获得了上述 3 个云南闭壳龟个体及一个周氏闭壳龟(*C. zhoui*)的血样, 用标准酚/氯仿法提取总DNA, 然后进行PCR扩增、测序. 扩增及测序引物参照文献[14]. 我们测定了线粒体基因组上编码部分细胞色素氧化酶第一亚基(COI)831 bp片段的序列及编码部分NADH脱氢酶第四亚基(ND4)、全部组氨酸(His)、丝氨酸(Ser)、部分亮氨酸(Leu)tRNA的 894 bp 片段序列, 总共 1725 bp. 结合闭壳龟属其他物种序列(自测序列和其他DNA序列的GenBank登录号见表 1), 排序采用Clustal-X[20], 使用PAUP 4.0 软件包对合并数据, 分别进行最大简约法(MP)[21]和最大似然法(ML)[22]系统发育分析, 以*Chinemys reevesii*, *Ch. Nigricans*和*Mauremys mutica*为外群. 用MP法构建系统发育关系时, 核苷酸位点被认为是具有 4 种状态的无序位点, 性状具有相同的权重. 参数设置为启发式(heuristic)搜索, 分支交换算法为树二等分再连接(tree-bisection-reconnection, TBR)方式. 用ML法构建系统发育关系时, 我们首先采用了MODELTEST 3.7[23]中的分级似然比检验(hierarchical likelihood ratio tests, hLRT)选择适合目前序列数据的最佳DNA替换模型. 一旦模型和参数选定后, 在PAUP4.0 软件中根据选定的模型和参数构建ML树. 自展重抽样分析(bootstrap analysis)[24]被用来确定树上各分枝的可靠性(MP法 1000 次重复, ML法 100 次重复).

与Parham等人[17]的工作相比较, 我们的分析排除了其中一只黄缘盒龟、一只安布闭壳龟和一只百色闭壳龟的数据(因为缺乏ND4 序列片段), 以确保最多的序列数据用于分子系统发育分析. 简约法及最大似然法分析得到与Parham等人[17]相似的结果(图 1). 新发现、鉴定的 3 只云南闭壳龟各自代表了独特的单倍型类群, 与之前测序的云南闭壳龟标本以较高的支持率聚为单系(BS =100%), 非校正的序列分歧度为 0.2%~1.9%. 云南闭壳龟与黄缘盒龟非校正的序列分歧度为 5.3%~5.8%, 与周氏闭壳龟及潘氏、金头、三线闭壳龟复合类群之间的非校正的序列分歧度为 3.0%~4.4%. 周氏闭壳龟与潘氏、金头、三线闭壳龟复合类群以较高的支持率(BS = 97%~100%)形成姊妹群, 它们与云南闭壳龟、黄缘盒龟三者间形成不明确

表 1 用于本工作分子系统发育分析的线粒体DNA序列数据

物种	GenBank 登录号 (COI/ND4 + His + Ser + Leu)
C. yunnanensis B	AY590460/AY572868
*C. yunnanensis*1[18]	EF685038/EF685042
*C. yunnanensis*2[19]	EF685037/EF685041
*C. yunnanensis*3	EF685039/EF685043
C. flavomarginata A	AY357739/AY364610
C. aurocapitata B	AY590463/AY572867
C. aurocapitata A	AY357740/AY364606
C. pani B	AY590457/AY590461
C. pani A	AY357741/AY364607
C. trifasciata A1	AF348270/AF348297
C. trifasciata A2	AF348271/AF348296
C. zhoui B1	AY590458/AY590462
C. zhoui B2	AY593968/AY572865
C. zhoui B3	AY593969/AY572866
C. zhoui	EF685040/EF685044
P. mouhotii A1	AF348272/AF348285
P. mouhotii A2	AF348273/AF348286
C. amboinensis A1	AY357738/AY364609
C. galbinifrons galbinifrons B1	AY357748/AY364615
C. galbinifrons galbinifrons B2	AY357742/AY364612
C. galbinifrons bourreti B1	AY357757/AY364618
C. galbinifrons bourreti B2	AY357751/AY364624
C. galbinifrons picturata B1	AY357760/AY364628
C. galbinifrons picturata B2	AY357745/AY364630
C. mccordi A	AY357737/AY364608
Mauremys mutica A	AF348261/AF348279
Chinemys nigricans A	AF348264/AF348289
Chinemys reevesii A	AF348263/AF348288

A表示来自Stuart和Parham [14]的序列, B表示来自Parham等人[17]的序列

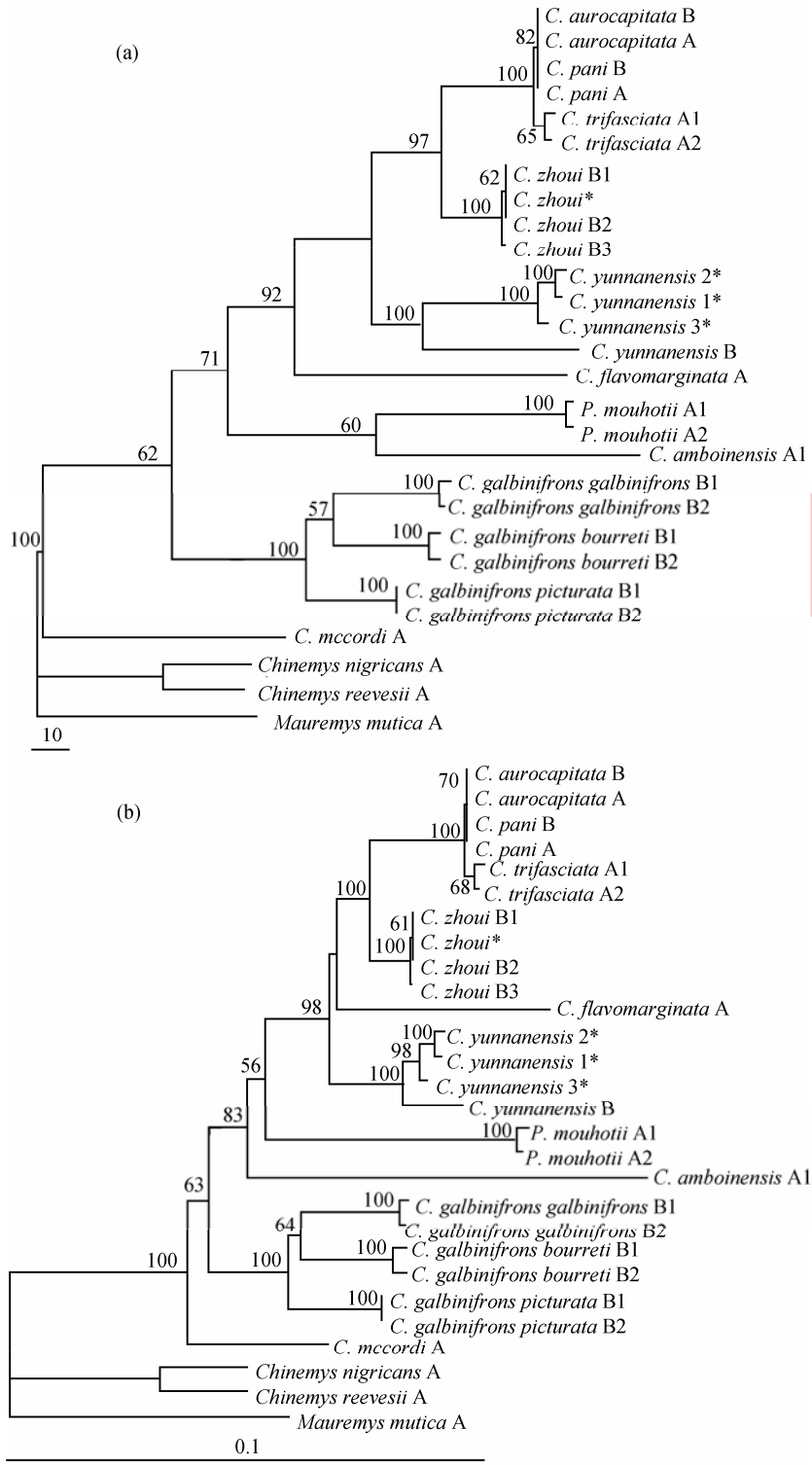

图 1 基于线粒体 DNA 1725 碱基序列构建的闭壳龟属内部系统发育关系

(a) 简约法构建的一致树, (b) 最大似然法系统发育树. 以 *Chinemys reevesii*, *Ch. Nigricans* 和 *Mauremys mutica* 为外群, 各枝上所示数字代表重抽样分析获得的支持率(BS). 本工作新增的样本序列以*标示

的三分叉关系. 对比Parham等人的结果[17], 我们的结果并不支持云南闭壳龟与黄缘盒龟的姊妹群关系.

与 100 年前的标本比较, 无论是形态上还是本文的分子系统学分析结果都显示, 新发现的活龟确实是云南闭壳龟. 同时, 我们的结果也确证了标本序列的可信性. 分子系统发育分析揭示, 云南闭壳龟不是近期杂交形成的, 代表了进化上独立的遗传谱系, 且种内仍存在一定的遗传多样度. 野外调研也显示, 适合云南闭壳龟生存的野外栖息地依然存在[25]. 综上所述, 我们认为云南闭壳龟是在进化上独立的谱系. 我们的结果为推翻云南闭壳龟已经灭绝的观点提供了进一步强有力的分子生物学证据, 但该物种个体数量极其稀少的状况提示其前景不容乐观, 必须尽快采取有力的措施予以重点保护.

在各种传统分类学手段受到诸多限制时, 基于分子系统发育基础的物种界定和鉴别发挥了至关重要的作用. DNA序列具有容易获得和信息量丰富等优点, 尤其是能在不破坏样本的情况下获得来自濒危稀有物种或标本的特异DNA序列信息, 使之在物种界定及鉴别方面发挥着越来越重要的作用[26,27]. 本文是分子系统学在濒危物种保护应用中的成功案例.

参 考 文 献

1 Parham J F, Simison W B, Kozak K H, et al. New Chinese turtles: Endangered or invalid? A reassessment of two species using mitochondrial DNA, allozyme electrophoresis and known-locality specimens. Anim Conserv, 2001, 4: 357—367

2 Wink M, Guicking D, Fritz U. Molecular evidence for hybrid origin of *Mauremys iversoni* Pritchard et McCord, 1991, and *Mauremys pritchardi* McCord, 1997 (Reptilia: Testudines: Bataguridae). Zool Abh Mus Tierkde Dresden, 2001, 51: 41—49

3 Spinks P Q, Shaffer H B, Iverson J B, et al. Phylogenetic hypotheses for the turtle family *Geoemydidae*. Mol Phylogenet Evol, 2004, 31: 164—182

4 Bour R. Position systématique de *Geoclemys palaeannamitica* Bourret, 1941 (Reptilia-Testudines-Emydidae). Amphibia-Reptilia, 1980, 1: 149—159

5 Hirayama R. Cladistic analysis of batagurine turtles (Batagurinae: Emydidae: Testudinoidea): A preliminary results. In: de Broin F, Jiménez-Fuentes E, eds. Stvdia Geologica Salamanticensia. vol 1, Especial Stvdia Palaeocheloniologica 1, 1984. 141—157

6 Gaffney E S, Meylan P A. A phylogeny of turtles. In: MJ Benton, ed.The Phylogeny and Classification of the Tetrapods Vol 1. New York: Oxford Univ Press, 1988. 157—219

7 King F D, Burke L R. Crocodilian, Tuatara, and Turtle Species of the World: A Taxonomic and Geographic Reference. Washington DC: Assoc Syst Col, 1989

8 Ota H. Systematics and biogeography of terrestrial reptiles of Taiwan. In: Lue KY, Chen TH, ed. Proceeding of the Symposium on the Phylogeny, Biogeography and Conservation of Fauna and Flora of East Asian Region. Taipei: National Taiwan Normal University, 1991. 75—86

9 Zhao E, Adler K. Herpetology of China, Contribution to Herpetology, vol 10. Ohio: Soc Stud Amphibians Reptiles, 1993

10 Yasukawa Y, Ota H. Geographic variation and biogeography of the geoemydine turtles (Testudines: Bataguridae) of the Ryukyu Archipelago, Japan. In: Ota H, ed. Tropical Island Herpetofauna: Origin, Current Diversity and Conservation. Amsterdam: Elsevier Science, 1999. 271—297

11 Yasukawa Y, Hirayama R, Hikida T. Phylogenetic relationships of geoemydine turtles (Reptilia: Bataguridae). Cur Herpetol, 2001, 20: 105—133

12 Honda M, Yasukawa Y, Hirayama R, et al. Phylogenetic relationships of the Asian box turtles of the genus *Cuora sensu lato* (Reptilia: Bataguridae) inferred from mitochondrial DNA sequences. Zoolog Sci, 2002, 19: 1305—1312

13 McCord W P, Iverson J B. A new box turtle of the genus Cuora (Testudines: Emydidae) with taxonomic notes and a key to the species. Herpetologica, 1991, 47: 407—420

14 Stuart B L, Parham J F. Molecular phylogeny of the critically endangered Indochinese box turtle (*Cuora galbinifrons*). Mol Phylogenet Evol, 2004, 31: 164—177

15 Blanck T. *Cuora yunnanensis* (Boulenger, 1906), the Yunnan Box Turtle, rediscovered after one-hundred Years? Radiata, 2005, 14: 10—33

16 Ernst C H. Redescriptions of two Chinese Cuora (Reptilia: Testudines: Emydidae). Proc Biol Soc Wash, 1998, 101: 155—161

17 Parham J F, Stuart B L, Bour R, et al. Evolutionary distinctiveness of the extinct Yunnan box turtle (*Cuora yunnanensis*) revealed by DNA from an old museum specimen. Proc Biol Sci, 2004, 271(Suppl 6): 391—394

18 周婷, 赵尔宓. 58 年来首次发现生活的云南闭壳龟及其描述. 四川动物, 2004, 23: 325—327

19 周婷. 一只生活的雄性云南闭壳龟被发现. 四川动物, 2005, 24: 345—346

20 Thompson J D, Gibson T J, Plewniak F, et al. The Clustal_X windows interface: Flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acids Res, 1997, 25: 4876—4882

21 Fitch W M. Toward defining the course of evolution: Minimum change for a specific tree topology. Syst Zool, 1971, 20: 406—416

22 Felsenstein J. Evolutionary trees from DNA sequences: A maximum likelihood approach. J Mol Evol, 1981, 17: 368—376

23 Posada D, Crandall K A. Modeltest: Testing the model of DNA substitution. Bioinformatics, 1998, 14: 817—818

24 Felsenstein J. Confidence limits on phylogenies: An approach using the bootstrap. Evolution, 1985, 39: 783—791

25 Lau M, Shi H. Conservation and trade of terrestrial and freshwater turtles and tortoises in the People's Republic of China. In: van Dijk P P, Stuart B L,. Rhodin A G. J, eds. Asian Turtle Trade: Proceedings of a Workshop Conservation and Trade of Freshwater. Turtles and Tortoises in Asia. 1999 Dec 1-4, Cambodia. Lunenburg: Chelonian Research Foundation, 2000, vol 2. 30—38

26 Hebert P D, Cywinska A, Ball S L, et al. Biological identifications through DNA barcodes. Proc Biol Sci, 2003, 270: 313—321

27 Tautz D, Arctander P, Minelli A, et al. A plea for DNA taxonomy. Trends Ecol Evol, 2003, 18: 70—74

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The molecular identification of *Cuora yunnanensis* and the investigation to the species' phylogeny

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(Partially translated by WEN Cheng)

Abstract: *Cuora yunnanensis* (Boulenger,1906) had been thought to be extinct for decades. It draws great attention from conservational biologists. In this work, 1725bp altogether from the sequences of mitochondria COI and ND4, as well as the sequences of His, Ser, Leu tRNA from 3 *Cuora spp.* individuals were investigated. The molecular phylogeny analysis is based on the comparison between the results and the homologues sequences from other *Cuora* species, as well as the identical sequences from the MNHN1907.10 *Cuora yunnanensis* specimen. By comparing the morphological traits and the molecular sequence of the individuals involved in the work to those of the MNHN1907.10 *Cuora yunnanensis* specimen, it is confirmed that those 3 recently discovered spevimens are *Cuora yunnanensis*. Meanwhile, the work also reveals that *Cuora yunnanensis* is a valid species with genetic diversity and independent phylogenetic origin.

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Encouragingly, a pair of *C. yunnanensis* were found in recent year[18] [19]. Later, another female was obtained. These 3 live individuals arouse great attention. It is important to clarify the validity of these specimens and the species' evolutionary history through further molecular investigation, with morphological investigation preceding. **[The only description in the paper on the origin of specimens. But so far as I know, at least 2 of the known 3 C. yunnanensis are kept by RAO Dingqi. No captive breeding progress has been heard of.]**

Blood samples from those 3 *C.yunnanensis* and 1 *C.zhoui* were collected. DNA was extracted by standard phenol-chloroform method. The original DNA samples were then PCR amplified and sequenced respectively. The primers for PCR amplification and sequencing are referred to the reference [14]. The 831 bp of COI fragment and 894 bp of ND4 fragment, His, Ser, Leu tRNA, 1725 bp in all are sequenced. These sequences were applied to Clustal-X[20] with PAUP 4.0 package, adopting Maximum Parsimony Method(MP)[21] and Maximum Likelihood Method(ML)[22] respectively, in phylogenetic analysis with other *Cuora* species with *Chinemys reevesii, Ch. nigricans* and *Mauremys mutica* as outgroup. (All other sequences were obtained from GenBank.) When MP was adopted, heuristic search mode was chosen, with branch-exchange algorithm as tree-bisection-reconnection method. When ML was adopted, hierarchical likelihood ratio tests (hLRT) in MODELTEST 3.7[23] was applied to choose the most optimal DNA substitution model for those from extracted samples. The Bootstrap Analysis (BA)[24] was used to determine the reliability of each branch in the phylogenetic tree.(Repeated for 1000 times in MP while 100 times in ML.) **[Methodology]**

Compared with the works of Parham et al., the data from 1 *C. flavomarginata,* 1 *C. amboinensis* and 1 *C. mccordi* are excluded in our work for the absence of ND4 sequence….. . **[Methodology]** Each of the 3 recently identified *C. yunnanensis*

individuals represents a unique haplotype. ….. Non-corrected divergence degree between *C. yunnanensis* and *C. flavamarginata* is 5.3%~5.8% while that between *C. yunnanensis* and the sister group of *C. zhoui*, *C. pani, C. trifasciata*, *C. aurocapitata* averaging 3.0%~ 4.4%. The result is not coincident with the work of Parham et. al., in which *C. yunnanensis* and *C. flavamarginata* fall into a sister group. **[Discussion]**