中枢神経系に嚢虫が寄生して重篤な嚢虫症を引き起こす。しかしながら，無鉤条虫とアジア条虫はヒトが虫卵を経口摂取しても嚢虫症を引き起こすことはない。この感染動態の違いは，アジア条虫と無鉤条虫が有鉤条虫とは系統発生学的に起源を異にすることが起因と考えられている[3]。

アジア条虫症による健康被害は，成虫が小腸に寄生することから，持続的に片節が排出されることに伴う精神的な不快感や軽微な下痢である。排出された成虫片節や虫卵の形態に基づいて無鉤条虫や有鉤条虫と鑑別することは困難であり，また中間宿主に寄生する嚢虫も形態による種鑑別が困難であることから，診断には遺伝子同定が不可欠である[8,9]。今回の一連の発生事例は，すべて国立感染症研究所寄生動物部において成虫の遺伝子検査によって確定診断された。しかし，現在までの知見では，アジア条虫は遺伝子の地理的変異に乏しく，国内で発生したアジア条虫の由来の特定など分子疫学的検討は困難である。

治療はプラジカンテル（商品名ビルトリシド），あるいはガストログラフィンによる駆虫が効果的である。

今般，関東地方で連続的に発生した邦人のアジア条虫症患者は，最近の数年間に海外渡航歴が無い，あるいは渡航歴があってもアジア条虫症流行地への渡航歴が無いことから，関東地方のと畜場で食肉処理された豚を感染源とする原発症例であることが強く疑われた。さらに，今回紹介する症例の数例は食品衛生法に準じて，寄生虫による食中毒事例として保健所に届出することが検討されたにもかかわらず，摂取から発病までの期間が長く原因食材の特定が困難であること，また国産豚におけるアジア条虫感染の実態が不明であるなどの理由によって，届出には至っていない。今後，地方自治体関係機関との連携によって上述の問題点を解明しつつ，本症の発生予防に関する早急な対策の立案が強く求められる。

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国立感染症研究所寄生動物部第二室
山﨑　浩　森嶋康之　杉山　広　武藤麻紀

<ミニ特集>

豚あるいは牛レバー刺し摂食によるアジア条虫症の4例

アジア条虫（*Taenia asiatica*）はヒトに寄生する寄生虫で，形態学的には無鉤条虫（*Taenia saginata*）に酷似し，韓国，中国，台湾，タイ，インドネシア，フィリピンなど，豚の内臓を生食，あるいは加熱不十分な状態で食べる習慣を持つ地域に分布している[1]。アジア条虫の生活環は豚（中間宿主）とヒト（終宿主）の間で維持されている。虫卵が含まれる感染者の糞便を豚が経口摂取すると，豚の肝臓で幼虫（＝嚢虫）に発育し，その嚢虫が寄生した豚肝臓をヒトが生食すると2～3カ月後に小腸内で成虫となり，片節が持続的に排出される。日本ではアジア条虫の分布は知られていなかったが，1968年と1996年に出雲市と米子市に住む日本人患者から得られ，無鉤条虫と処理された虫体をretrospective に遺伝子解析したところ，アジア条虫であったことが最近判明した[2]。しかし，それらの症例の詳細は不明であり，その後，アジア条虫症の報告

表1．当院で経験したアジア条虫症4例のまとめ

症例	年齢/性別	症状	食歴	海外渡航歴	検便内虫卵
1	55/M	虫体排出（2010年4月）	牛レバー刺し（2010年2月）	オーストラリア（7年前）	(−)
2	58/M	虫体排出（2010年5月）	牛レバー刺し（2010年2月）	香港（10年前）	(+)
3	41/F	虫体排出（2010年8月）	豚レバー刺し（月に1回）	グアム（2010年7月）	未検査
4	40/M	虫体排出（2010年8月）	なし	シンガポールとマレーシア（5～6年前）	(+)

は無かった。ところが，2010年6～9月の間に当院で4例のアジア条虫症患者を相次いで経験したので報告する。

症例：患者の臨床的特徴は前ページ表1にまとめた。いずれも虫体（片節）排出を主訴に当院を紹介受診している。4例のうち3例に牛または豚レバー刺しの食歴があり，アジア条虫が分布している地域への渡航歴は無かった。検便を行った3例のうち2例で虫卵が検出された。全例ともプラジカンテル＋下剤の投与で治療に成功した。駆虫によって得られた虫体はcytochrome *c* oxidase subunit 1, elongation factor 1-α, ezrin/radixin/moesin-like protein 遺伝子の塩基配列解析[3,4]からアジア条虫と同定された。

考察：今回，当院で経験した4症例の患者はすべて関東在住者であり，アジア条虫症流行地への渡航歴が無いことから日本国内で感染した事例と考えられ，関東地方を中心にアジア条虫の幼虫（＝アジア嚢虫）が感染した食材が流通していることは明らかである。アジア条虫のヒトへの感染経路は豚の肝臓に寄生する嚢虫の経口摂取によることが感染実験や疫学調査から証明されており[5]，当院で経験した症例では，豚レバー刺しの食歴があったのは症例3の1例だけで，症例1と2は牛レバー刺しの食歴があった。感染実験では，アジア条虫は仔牛の肝臓に寄生するが，嚢虫の発育は悪く，感染後1～1.5カ月後にはすでに石灰化しているという報告があることから[5]，牛の肝臓が果たしてアジア条虫の感染源となり得るのか否かについては今後の研究が必要である。

アジア条虫感染によるヒトの健康被害は持続的な虫体排出による不快感であり，腹痛や下痢など消化器症状は特に見られない。治療にはプラジカンテルを用いた駆虫が効果的である。

レバー刺しを好む日本人の嗜好性を考慮すると，今後もアジア条虫症患者の発生が懸念され，豚レバー刺しの摂食を避けるなど，感染予防対策の周知徹底が求められる。

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都立墨東病院感染症科
中村（内山）ふくみ　小林謙一郎
岩渕千太郎　大西健児
国立感染症研究所寄生動物部第二室
山﨑　浩

＜ミニ特集＞

アジア条虫症の1例

症例：患者は埼玉県狭山市在住の31歳男性。生来健康であったが，2010年8月中旬に便の中に白色で長さ約1cm，きしめん様の動くものを発見し，近医を受診した。寄生虫疾患を疑い，精査目的で獨協医科大学越谷病院消化器内科への紹介となった。自覚，他覚症状はなく血液生化学検査においても異常所見はなかった。自然排泄された虫体片節の形態から *Taenia* 属条虫であることは確認できたが，正確な種鑑別のために国立感染症研究所寄生動物部に虫体の遺伝子同定を依頼したところ，アジア条虫（*Taenia asiatica*）であることが判明した。患者の生活環境として実家が焼肉店を経営しており，そこで頻繁に摂食していた豚レバー刺しが感染源と考えられた。

治療に当たっては，駆虫前日は低残渣食とし，入院時クエン酸マグネシウム1包服用，寝前にピコスルファートナトリウム4錠内服，翌日，排便後，プラジカンテル20mg/kg 1回服用，2時間後に硫酸マグネシウム30gを服用したのち，虫体が排出された。駆虫された虫体は破壊されており，頭節を確認することはできなかったが，その後，片節の排出は見られていない。

アジア条虫は豚の肝臓に寄生する幼虫をヒトが経口摂取することによって感染する寄生虫で，中国や東南アジア諸国で見られ，日本には分布しないと考えられていた。今回の患者は海外渡航歴が無いことと，国産豚のレバー刺しの摂取歴から国内において感染した事例と考えられ，わが国でもアジア条虫の生活環が定着，維持されていることが示唆された。アジア条虫感染予防として豚肝臓の生食を避けることが最も効果的な対策として注意喚起しておく。

この報告に当たりPCR検査や多くのご助言をいただきました国立感染症研究所寄生動物部・山﨑浩先生に深謝いたします。

獨協医科大学越谷病院臨床検査部
春木宏介
獨協医科大学越谷病院消化器内科
玉野正也
石心会狭山病院内科
三好洋二
目黒寄生虫館
荒木　潤

皮膚臨床 53(6)；887～890，2011

症例

有棘顎口虫による幼虫移行症の1例

青笹　尚彦*1　常深祐一郎*1　大藤　由佳*1　甲斐　浩通*1　森村　壮志*1
柿沼　　誉*1　玉置　邦彦*1　佐藤　伸一*1　前田　卓哉*2　山﨑　　浩*3

要　約

39歳，男性。生魚を摂食後，有痛性で移動する線状の紅斑や硬結が腹部，大腿に出現した。顎口虫に対する抗体価の上昇がみられたため，顎口虫症と臨床診断した。抗寄生虫薬を内服し症状は軽快した。抗顎口虫抗体価も低下したため，虫体は死滅したものと考えたが，その後皮疹は再燃した。再度抗寄生虫薬を内服の上で皮疹を切除したところ，病理組織標本中に虫体が検出され，その組織学的ならびに遺伝子解析から有棘顎口虫（*Gnathostoma spinigerum*）であることが判明した。その後，腹痛，皮疹等の自覚症状は消失した。血清抗体価や好酸球数が内服治療後に低下しても，治癒と判断するには長期間の経過観察が必要である。

キーワード：有棘顎口虫，幼虫移行症

I．はじめに

幼虫移行症とは，ヒト以外の動物を終宿主とする寄生虫がヒトに感染するものの，成虫になることができず，幼虫のままヒト体内を移行してさまざまな症状を引き起こすものである。寄生部位により，皮膚幼虫移行症と内臓幼虫移行症に大別される。今回我々は，抗寄生虫薬投与後に一時軽快したものの皮疹は再発し，その後皮疹を切除して虫体を摘出することで治癒した有棘顎口虫症を経験した。本症の治療法，経過の追跡についても文献的に考察したのであわせて報告する。

II．症　　例

患　者　39歳，男性

初　診　2008年5月1日

主　訴　右側腹部の移動性の浮腫性紅斑

家族歴・既往歴　特記すべきことなし。

現病歴　初診の2カ月前にカンボジアで詳細不明の生魚を食べた。1カ月前より腹痛が出現した。その後，有痛性で移動する線状の紅斑や硬結が腹部や背部，大腿に出没を繰り返した。

臨床検査成績　白血球数は6800/μl，好酸球数990/μl，IgE 330 IU/ml と軽度上昇していたが，便検査では，虫体や虫卵は検出されなかった。各種寄生虫抗原を用いたmultiple-dot ELISAでは[1]，顎口虫に対して弱陽性，マンソン孤虫に疑陽性であった。

経　過　生魚の摂食歴と移動性の紅斑という所見に加え，multiple-dot ELISA陽性所見により，顎口虫による幼虫移行症と臨床診断した。イベルメクチン12 mg単回投与，アルベンダゾール600

*1 Naohiko AOZASA, Yuichiro TSUNEMI, Yuka OTO, Hiromichi KAI, Soushi MORIMURA, Takashi KAKINUMA, Kunihiko TAMAKI & Shinichi SATO，東京大学，皮膚科学教室（前主任：玉置邦彦名誉教授，現主任：佐藤伸一教授）
*2 Takuya MAEDA，東京大学医科学研究所，感染症国際研究センター
*3 Hiroshi YAMAZAKI，国立感染症研究所，寄生動物部第二室
別刷請求先　青笹尚彦：東京大学医学部皮膚科（〒113-8655 東京都文京区本郷7-3-1）

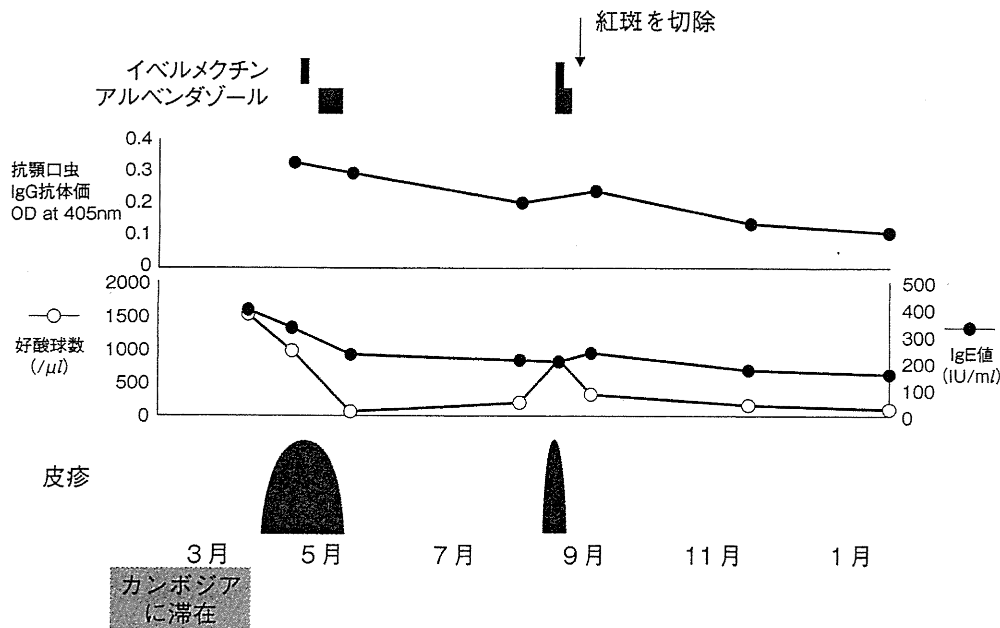

図1　治療および経過

mg/日の3週間連日投与を行った。その後，いったん皮疹は消失したが，腹部の違和感は続いた。なお，カンボジアから帰国後は，顎口虫感染を引き起こしうる食物の摂食歴はない。治療の3カ月後には，好酸球数，IgE値に加えて，ドロレス顎口虫抗原を用いたplate-ELISA法による抗体価も低下傾向となり，虫体が死滅したものと考えた（図1）。しかしながら，さらに2週間後，再度右下腹部に硬結を伴う浮腫性紅斑が出現した（図2）。そのため，虫体の生存を考慮しイベルメクチン12 mg/日を2日間投与し，アルベンダゾール600 mg/日を再投与したが，皮疹は出現と消退を繰り返したため，浸潤を触れる紅斑を切除した。

病理組織学的所見　真皮上層には，食道腺質部と腸管部における2個の虫体断面があり，その周囲には好中球と好酸球主体の炎症細胞が浸潤していた（図3-a）。体腔内に大きく突出した双葉状の側索や，体表面の皮棘が観察された（図3-b）。腸管は円柱状の上皮細胞からなり，その数は20～30個程度，1細胞当たりの核数は1～7個のものまでさまざまであった（図3-b）。以上の所見は，有棘顎口虫の組織学的特徴と最もよく合致した。

さらに，組織学的同定結果を確認するために，虫体の分子同定を行った。無染色のパラフィン切片中の虫体組織より市販のキットを用いてDNAを抽出しPCRによりミトコンドリアDNAのシトクローム *c* オキシダーゼサブユニット1遺伝子を増幅し，その増幅産物の塩基配列解析[2]を行った結果，虫体は有棘顎口虫であることが確認された（DDBJ/GenBank/EMBL データベースアクセッション番号 AB551552）。

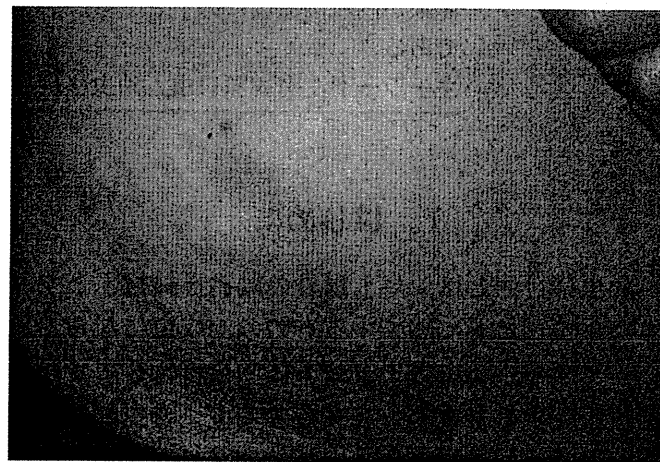

図2　皮疹再燃時の臨床像：右下腹部に浮腫性紅斑がみられ，中央には硬結を触れた。

診　断　臨床経過，病理組織学的所見，免疫血清学的所見に加え，遺伝子解析結果を合わせて，有棘顎口虫による幼虫移行症と確定診断された。

治療および経過　虫体切除後は皮疹の再燃はない。好酸球数，IgE値や顎口虫に対する抗体価は，

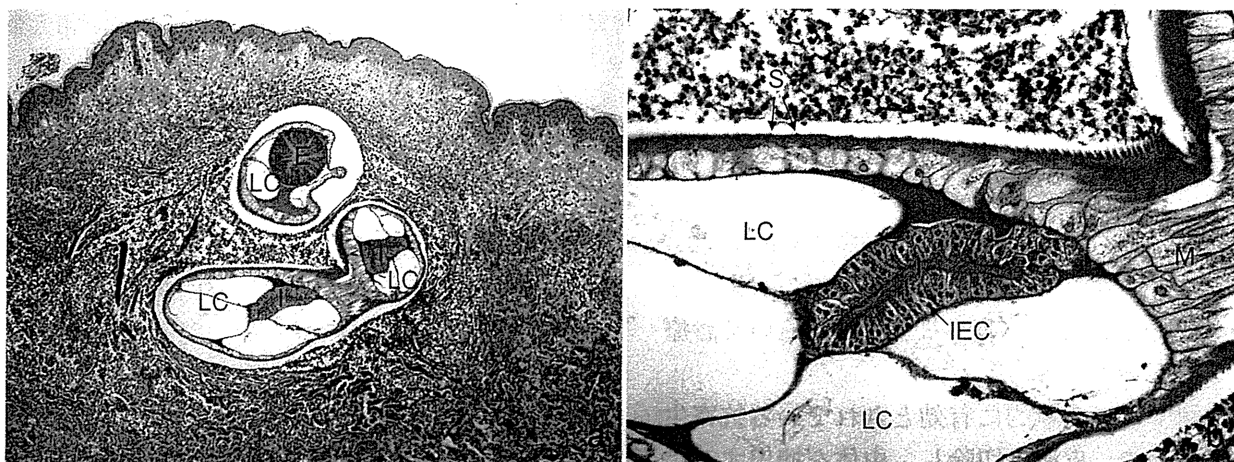

図3　病理組織像

a：弱拡大像；真皮上層に虫体の断面をみる（×100）。E：食道腺質部，I：腸管，LC：側索。

b：強拡大像；虫体表面には皮棘（S）がある。中央に腸管（I）がみられ，円柱状の上皮細胞（IEC）の数は 20〜30 個程度，1 細胞当たりの核数は 1〜7 個程度であった（×400）。LC：側索，M：筋層。

虫体切除直後には軽度上昇したが，その後低下した（図 1）。

Ⅲ. 考　　察

現在，世界には 12 種類の顎口虫が知られており，そのうち人体寄生の報告があるのは，有棘顎口虫，ドロレス顎口虫，日本顎口虫，剛棘顎口虫，二核顎口虫の 5 種類である[3]。自験例の原因となった有棘顎口虫は，東南アジアから東アジアにかけて広く分布しており，これらの地域で淡水産の魚類を生食すると感染する可能性が高く，自験例はカンボジアで喫食した生魚料理が感染源と推定された。

顎口虫症は，ライギョ，ドジョウなど淡水魚の摂食歴と移動性の皮疹という臨床像から疑う。末梢血好酸球数増多や IgE 値の上昇がみられることが多い。診断に有用な検査としては血清反応があり，顎口虫症に対する陽性率は 94％と高いが，各種顎口虫間で互いに強い交叉反応を示すため，種の同定はできない[4]。自験例では，ドロレス顎口虫抗原を用いたが，この抗原は交叉反応により，顎口虫全体を検出できる。

皮疹自体は，虫体が通過した後の遅延型反応によるため，外科的切除による虫体の検出率は 30％程度と低く[5)6]，切除範囲を広範にとるなどの工夫が必要である。虫体断端が得られた場合には，その腸管上皮細胞の形態や細胞内の核数などから種の同定が可能な場合もある[7]。有棘顎口虫は，腸管を構成する細胞数が 20〜30 個であり，形態は円柱状，1 細胞当たりの核数は 0〜7 個とされ，自験例と合致した。しかしながら，形態学的に種の同定が困難である場合には，遺伝子検査が有効である[2]。

顎口虫症の治療としては，抗寄生虫薬の投与と外科的摘出とに大別される。アルベンダゾールとイベルメクチンの有効性が報告されているが[8)〜10]，いったん皮疹が消失してもその後に再燃がみられた症例報告もあり，注意が必要である[11]。自験例も同様の経過をとり，内服終了 3 カ月後に皮疹が再燃した。初回の内服は虫体の活動性を低下させたものの，虫体の死滅までには至らなかったと考えられる。そのため，第一選択は外科的摘出であり，最も確実な治療法と考えられる。なお，アルベンダゾール内服により虫体の移動速度が下がり虫体が表皮近くへ遊走してくる傾向があることが知られており，この時に皮膚切除を行えば，虫体を切除できる可能性が高くなる[12]。自験例もアルベンダゾール内服中であり，虫体は表皮直下に検出された。

治療後の経過観察としては，臨床症状と好酸球数，IgE 値，顎口虫に対する抗体価の推移が参考になる。自験例では，内服終了後約 2 カ月の時点

ではいずれの指標も改善していたにもかかわらず，皮疹が再燃した。このことは，内服による治療を試みる際には経過観察期間を長めに設定する必要があることを示唆している。Strady らは，内服治療後は少なくとも 6 カ月間は経過をみるべきだと報告している[11]。自験例のように，顎口虫に対する抗体価が低下していても，虫体が排除されていない可能性があることを念頭に置いて診療することが必要である。

自験例では，文献的に有効とされている抗寄生虫薬が無効で，皮疹を切除し，虫体を摘出することで治癒した。血清抗体価や好酸球数が内服治療後に低下しても，治癒と判断するには長期間の経過観察が必要である。

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Comparative Analysis of 18S and 28S rDNA Sequences of *Schistosoma japonicum* from Mainland China, the Philippines and Japan

[1,2]F. Chen, [1]J. Li, [3]H. Sugiyama, [1]Y.B. Weng, [4]F.C. Zou, [1]R.Q. Lin, [1]Z.G. Yuan, [5]H.Q. Song, [4,5]X.Q. Zhu and [2]G.H. Zhao
[1]College of Veterinary Medicine, South China Agricultural University, 510642 Guangzhou, Guangdong Province, China
[2]College of Veterinary Medicine, Northwest A and F University, 712100 Yangling, Shanxi Province, China
[3]Department of Parasitology, National Institute of Infectious Diseases, 113-8421 Tokyo, Japan
[4]College of Animal Science and Technology, Yunnan Agricultural University, 650201 Kunming, Yunnan Province, China
[5]State Key Laboratory of Veterinary Etiological Biology, Key Laboratory of Veterinary Parasitology of Gansu Province, Lanzhou Veterinary Research Institute, CAAS, 730046 Lanzhou, Gansu Province, China

Abstract: In the present study, a portion of the 18S and 28S ribosomal DNA (rDNA) sequences of 35 *Schistosoma japonicum* isolates representing three geographical strains from mainland China, the Philippines and Japan were amplified and compared and phylogenetic relationships were also reconstructed by Unweighted Pair-Group Method with Arithmetic averages (UPGMA) using combined 18S and 28S rDNA sequences as well as the corresponding sequences of other species belonging to the Schistosoma genus available in the public database. The results indicated that the partial 18S and 28S rDNA sequences of all *S. japonicum* isolates were 745 and 618 bp, respectively and displayed low genetic variation among *S. japonicum* strains and isolates. Phylogenetic analysis revealed that the combined 18S and 28S rDNA sequences were not able to distinguish *S. japonicum* isolates from three geographical origins but provided an effective molecular marker for the inter-species phylogenetic analysis and differential identification of different *Schistosoma* species.

Key words: *Schistosoma japonicum*, 18S rDNA, 28S rDNA, phylogenetic analysis, mainland China, Philippines, Japan

INTRODUCTION

Schistosomiasis caused by trematodes of the genus *Schistosoma* is a parasitic disease of medical and veterinary importance in a number of countries with an estimated approximately 200 million people being infected and approximately 200,000 deaths per year (Chitsulo *et al.*, 2004; Taylor, 2008; Huyse *et al.*, 2009). Among six human schistosomes (*Schistosoma japonicum*, *Schistosoma haematobium*, *Schistosoma mansoni* (*Schistosoma intercalatum* = *S. guineensis*), *Schistosoma mekongi* and *Schistosoma malayensis*), three main species (*S. japonicum*, *S. haematobium* and *S. mansoni*) are reported to have significant human health and social-economic impact (Zhao *et al.*, 2009a). *Schistosomiasis japonica*, one of the most serious schistosomiasis caused by *S. japonicum* was mainly distributed in China, Japan, the Philippines and parts of Indonesia and caused various degrees of morbidity and mortality (Zhou *et al.*, 2005; Zhao *et al.*, 2009a, b). In addition to the six human schistosomes, other 13 *Schistosoma* species have been identified as parasites in animals. *S. hippopotami* and *S. edwardiense* are found in hippopotamus (Morgan *et al.*, 2003), *S. mattheei*, *S. bovis*, *S. leiperi*, *S. margrebowiei*, *S. curassoni*, *S. indicum*, *S. spindale* and *S. nasale* found in ruminant animals such as cattle, buffalo, sheep, goat and lechwe (De Bont *et al.*, 1994; Vercruysse *et al.*, 2003; Singh *et al.*, 2004; Vercruysse and Gabriel, 2005; Littlewood *et al.*, 2006; Sato *et al.*, 2008) and *S. rodhaini*, *S. incognitum*, *S. sinensium* mainly inhabited in rodents and carnivores (Bunnag *et al.*, 1983; Walker *et al.*, 1989; Zhang *et al.*, 2001).

Corresponding Author: G.H. Zhao, Department of Veterinary Immunology, College of Veterinary Medicine, Northwest A and F University, 712100 Yangling, Shaanxi Province, The People's Republic of China

Ribosomal DNA (rDNA) forms a tandem array of repeat sequences and each repeat includes 18S, 5.8S and 28S subunits separated by spacers (Zhao *et al.*, 2011). The 18S and 28S rDNA sequences have been identified as good genetic markers for identification and phylogenetic studies of members of the genus *Schistosoma* (Johnston *et al.*, 1993; Barker and Blair, 1996; Attwood *et al.*, 2002; Lockyer *et al.*, 2003; Webster *et al.*, 2006). Recent studies also have demonstrated that different *S. japonicum* strains from mainland China and the Philippines has extensive homology in 18S rDNA sequences and 28S rDNA-D2 domain was also relatively stable among species (Yu *et al.*, 2000; Li *et al.*, 2008). But sequence variation in 18 and 28S rDNA among *S. japonicum* strains from mainland China, the Philippines and Japan have not been investigated comprehensively.

The objectives of the present study were to examine sequence variation in the 18S and 28S rDNA among *S. japonicum* isolates from different endemic regions in mainland China, the Philippines and Japan and to reconstruct the phylogenetic relationships among members of the Schistosoma genus using the combined partial 18S and 28S rDNA sequences.

MATERIALS AND METHODS

Parasites and isolation of genomic DNA: The 35 *S. japonicum* isolates were collected from the endemic areas in mainland China, the Philippines and Japan with sample codes, geographical origin and gender shown in Table 1. The male and female adult parasites were fixed in 70% molecular grade ethanol and stored at -20°C before extraction of genomic DNA. Total genomic DNA was extracted from individual parasites by SDS/proteinase K treatment, column-purified (Wizard® SV Genomic DNA Purification System, Promega) and eluted into 60 μL H_2O according to the manufacturer's recommendations.

Enzymatic amplification and sequencing: A portion of the 18S (p18S) and 28S rDNA were amplified with primers 18Su and 18Sd, 28Su and 28Sd, respectively (Table 2). PCR reactions (25 μL) were performed in 2 mM of $MgCl_2$, 2.5 μM of each primer, 2.5 μL 10×rTaq buffer, 0.2 mM of each dNTPs, 1.25 U of rTaq DNA polymerase (Takara) and 1 μL of DNA sample in a thermocycler (Biometra) under the following conditions: after an initial denaturation at 94°C for 5 min then 94°C for 1 min (denaturation); 45°C for 30 sec (annealing); 72°C for 1 min (extension) for 35 cycles followed by a final extension at 72°C for 10 min. These optimized cycling conditions for the specific and efficient amplification of both rDNA fragments were obtained after adjusting annealing temperatures. Each amplicon (4 μL) was examined by agarose (1%) gel electrophoresis to validate amplification efficiency. The p18S and p28S amplicons of 35 samples were sequenced by BGI-Guangzhou company from both directions using the same primers as used in primary amplification.

Sequences alignment and analysis: Sequences of the p18S and p28S rDNA were separately aligned using the computer program Clustal X 1.81 (Thompson *et al.*, 1997). Meanwhile, Megalign procedure within the DNAStar 5.0 (Burland, 2000) was also used to analyze sequence similarity and to calculate transition and transversion.

Table 1: Information of Schistosoma samples used in the present study

Species/sample codes	Geographical strains	Geographical origin	Gender	GenBank accession number	
				p18S rDNA	p28S rDNA
Schistosoma japonicum/SjYeF55	Mainland China	Yunnan (Eryuan)	Femal	JF721330	JF721363
S. japonicum/SjYeM55	Mainland China	Yunnan (Eryuan)	Male	JF721331	JF721364
S. japonicum/SjHyF54	Mainland China	Hunan (Yueyang)	Femal	JF721328	JF721361
S. japonicum/SjHyM54	Mainland China	Hunan (Yueyang)	Male	JF721329	JF721362
S. japonicum/SjZjF60	Mainland China	Zhejiang	Femal	JF721326	JF721365
S. japonicum/SjZjM60	Mainland China	Zhejiang	Male	JF721327	JF721366
S. japonicum/SjLeF1	The Philippines	Letye	Femal	JF721338	JF721373
S. japonicum/SjLeF2	The Philippines	Letye	Femal	JF721339	JF721374
S. japonicum/SjLeF4	The Philippines	Letye	Femal	JF721340	JF721375
S. japonicum/SjLeM1	The Philippines	Letye	Male	JF721341	JF721376
S. japonicum/SjLeM2	The Philippines	Letye	Male	JF721342	JF721377
S. japonicum/SjLeM4	The Philippines	Letye	Male	JF721343	JF721378
S. japonicum/SjMiF1	The Philippines	Mindoro	Femal	JF721349	JF721384
S. japonicum/SjMiF2	The Philippines	Mindoro	Femal	JF721350	JF721385
S. japonicum/SjMiF4	The Philippines	Mindoro	Femal	JF721351	JF721386
S. japonicum/SjMiM1	The Philippines	Mindoro	Male	JF721352	JF721387
S. japonicum/SjMiM2	The Philippines	Mindoro	Male	JF721353	JF721388
S. japonicum/SjMiM3	The Philippines	Mindoro	Male	JF721354	JF721389

Table 1: Continue

				GenBank accession number	
Species/sample codes	Geographical strains	Geographical origin	Gender	p18S rDNA	p28S rDNA
S. japonicum/SjSoF2	The Philippines	Sorsogor	Femal	JF721344	JF721379
S. japonicum/SjSoF3	The Philippines	Sorsogor	Femal	JF721345	JF721380
S. japonicum/SjSoM1	The Philippines	Sorsogor	Male	JF721346	JF721381
S. japonicum/SjSoM3	The Philippines	Sorsogor	Male	JF721347	JF721382
S. japonicum/SjSoM4	The Philippines	Sorsogor	Male	JF721348	JF721383
S. japonicum/SjAsF1	The Philippines	Asuncium	Femal	JF721332	JF721367
S. japonicum/SjAsF3	The Philippines	Asuncium	Femal	JF721333	JF721368
S. japonicum/SjAsF4	The Philippines	Asuncium	Femal	JF721334	JF721369
S. japonicum/SjAsM1	The Philippines	Asuncium	Male	JF721335	JF721370
S. japonicum/SjAsM2	The Philippines	Asuncium	Male	JF721336	JF721371
S. japonicum/SjAsM4	The Philippines	Asuncium	Male	JF721337	JF721372
S. japonicum/SjYYF1	Japan	Yamanashi	Femal	JF721355	JF721390
S. japonicum/SjYYF2	Japan	Yamanashi	Femal	JF721356	JF721391
S. japonicum/SjYYF4	Japan	Yamanashi	Femal	JF721357	JF721392
S. japonicum/SjYYM2	Japan	Yamanashi	Male	JF721358	JF721393
S. japonicum/SjYYM3	Japan	Yamanashi	Male	JF721359	JF721394
S. japonicum/SjYYM4	Japan	Yamanashi	Male	JF721360	JF721395
S. intercalatum	-	-	-	AY157235	AY157262
S. rodhaini	-	-	-	AY157230	AY157256
S. spindale	-	-	-	Z11979	Z46505
S. bovis -	-	-	AY157238	AY157266	
S. edwardiense	-	-	-	AY197344	AY197344
S. haematobium	-	-	-	Z11976	Z46521
S. hippopotam	-	-	-	AY197343	AY197343
S. incognitum	-	-	-	AY157229	AY157255
S. japonicum	-	-	-	AY157226	Z46504
S. leiperi -	-	-	AY157234	AY157261	
S. malayensis	-	-	-	AY157227	AY157252
S. mattheei	-	-	-	AY157237	AY157265
S. mekongi -	-	-	AY157228	AY157253	
S. nasale -	-	-	AY157232	AY157259	
S. sinensium	-	-	-	AY157225	AY157251
S. mansoni	-	-	-	M62652	Z46503
S. indicum -	-	-	AY157231	AY157258	

Table 2: Sequences of primers used to amplify a portion of the 18S and 28S ribosomal DNA from *Schistosoma japonicum* isolates from mainland China, the Philippines and Japan

Name of primer	Sequence (5'-3')	Product length
For 18S rDNA	-	745 bp
18Su (forward)	CTTATGCTGTGCCTGTTACATT	-
18Sd (reverse)	TTACTTCGGATCCGAAAACCAAC	-
For 28S rDNA	-	618 bp
28Su1 (forward)	GGGTATGTGTAGACGTTCTTAT	-
28Sd (reverse)	AACACAAGGTCGCATGTCTACGT	-

These sequences were used for phylogenetic analyses. The sequences of the two *rRNA* genes were concatenated into single alignments. The Unweighted Pair-Group Method based on Arithmetic averages (UPGMA) (Sneath and Sokal, 1973) in MEGA v. 4.0 (Tamura *et al.*, 2007) was carried out to examine the genetic relationship, starting from a distance matrix based on the Kimura 2-parameter index (Kimura, 1980). The consensus tree was obtained after bootstrap analysis with 1, 000 replications. The pairwise comparisons were made of the level of sequence differences (Chilton *et al.*, 1995). To study the phylogenetic relationships of Schistosoma, the corresponding sequences of other *Schistosoma* species/isolates namely; *S. intercalatum*, *S. rodhaini*, *S. spindale*, *S. bovis*, *S. edwardiense*, *S. haematobium*, *S. hippopotam*, *S. incognitum*, *S. indicum*, *S. japonicum*, *S. leiperi*, *S. malayensis*, *S. mattheei*, *S. mekongi*, *S. nasale*, *S. sinensium* and *S. mansoni* obtained from GenBank were also used for phylogenetic analyses. The phylograms were drawn using the Tree View program version 1.65 (Page, 1996).

RESULTS AND DISCUSSION

Genomic DNA was prepared from 35 individual adult trematodes (including male and female *S. japonicum*) representing three geographical strains from mainland China, the Philippines and Japan. Amplicons of p18S and p28S rDNA (~750 and 620 bp, respectively) were amplified. For each rDNA fragment, no size variation was detected on 1% agarose gel among any of the amplicons examined in dicating that the PCR primers and reactions of this study had good specificity (Fig. 1).

To examine sequence variations in the two rDNA fragments among three geographical strains, amplicons of p18S and p28S rDNA were subjected to direct sequencing. The sequences of p18S and p28S rDNA were 745 and 618 bp in length, respectively. One variable nucleotide position was identified in the sequences of p18S and three in p28S rDNA with intra-specific variation of 0.14% (1/704) and 0.51% (3/586) for p18S and p28S

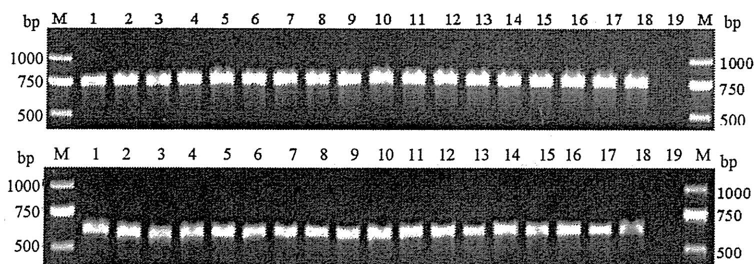

Fig. 1: Representative PCR products for a portion of the 18S (upper) and 28S (bottom) ribosomal DNA of *Schistosoma japonicum* isolates in mainland China, the Philippines and Japan. Lanes 1-18 represent samples SjYeM55, SjHyM54, SjZjF60, SjLeM1, SjLeM4, SjLeF1, SjMiM1, SjMiF1, SjMiF4, SjSoM3, SjSoM4, SjSoF2, SjAsM1, SjAsM2, SjAsF3, SjYYM2, SjYYM3, SjYYF1, respectively (cf. Table 1). Lane 19 represents no-DNA control. M represents a DNA size marker (ordinate values in bp)

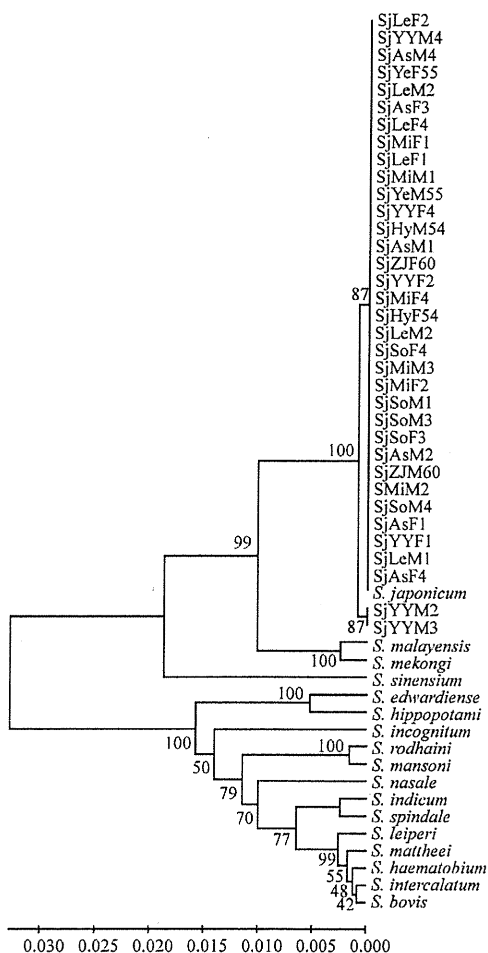

Fig. 2: Phylogenetic relationships of *Schistosoma* species/isolates inferred by UPGMA analysis using the combined sequences of partial 18S and p28S ribosomal DNA. Numbers at nodes indicate bootstrap values (%) resulting from UPGMA tree

rDNA, respectively. There were one transitions (A<->G) with intra-specific variation for p18S and two (A<->G) and one (C<->T) transitions for p28S rDNA. It appeared that the main transition was A<->G and there was no transversion within these two fragments. The low variations between the p18S and p28S rDNA were consistent with previous reports (Yu *et al*., 2000; Li *et al*., 2008).

The combined sequences of p18S and p28S rDNA were aligned over a consensus length of 1,290 bp. The phylogenetic relationships among the individual *S. japonicum* isolates were constructed by UPGMA analyses (Fig. 2). The phylogenetic tree consisted of two large clades.

All of the *S. japonicum* isolates from mainland China, the Philippines and Japan clustered in the *S. japonicum* clade, grouped with *S. malayensis* and *S. mekongi* and sistered to *S. sinensium*. All of the other *Schistosoma* species grouped in the other large clade with high bootstrap values.

The combined sequences of p18S and p28S rDNA allowed the unequivocal differentiation of all the *Schistosoma* species examined in the present study which is consistent with previous studies (Johnston *et al*., 1993; Littlewood and Johnston, 1995; Attwood *et al*., 2002; Morgan *et al*., 2003; Webster *et al*., 2006).

CONCLUSION

The present study revealed low level variation in p18S and p28S rDNA sequences among 35 *S. japonicum* isolates from mainland China, the Philippines and Japan. The combined p18S and p28S rDNA sequences were highly conserved and could not distinguish *S. japonicum* isolates from three geographical origins by phylogenetic analysis but could differentiate species in the *Schistosoma* genus suggesting that the p18S and p28S rDNA sequences were not suitable markers for studying population relationships among *S. japonicum* isolates but

is an effective genetic marker for inter-species phylogenetic analysis of Schistosoma and identification of schistosomes.

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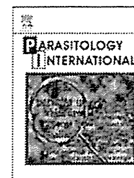

Variability in intron sequences of housekeeping and antigen-coding genes among *Schistosoma japonicum* isolates in mainland China

G.H. Zhao [a,b,c], J. Li [b,c], F. Chen [b,c], F.C. Zou [d], J.F. Yang [d], H. Sugiyama [e], M.J. Xu [b,c], Q. Lin [a], R.Q. Lin [b,c,**], X.Q. Zhu [b,d,*]

[a] College of Veterinary Medicine, Northwest A & F University, Yangling, Shaanxi Province 712100, PR China
[b] State Key Laboratory of Veterinary Etiological Biology, Key Laboratory of Veterinary Parasitology of Gansu Province, Lanzhou Veterinary Research Institute, CAAS, Lanzhou, Gansu Province 730046, PR China
[c] College of Veterinary Medicine, South China Agricultural University, 483 Wushan Street, Tianhe District, Guangzhou, Guangdong Province 510642, PR China
[d] College of Animal Science and Technology, Yunnan Agricultural University, Kunming, Yunnan Province 650201, PR China
[e] Department of Parasitology, National Institute of Infectious Diseases, Shinjuku Ward, Tokyo 162-8640, Japan

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ABSTRACT

The accurate characterization of *Schistosoma japonicum* has important implications for analyzing genetic variation and would provide basic data for disease control. Previous studies using proteins, coding sequences, and especially antigen-coding genes showed lower genetic variation among *S. japonicum* isolates from mainland China. Therefore, the present study focused on variations in intron sequences of housekeeping and antigen-coding genes, which may be more informative for genetic analysis. We compared sequence variation between introns of two housekeeping genes and two antigen-coding genes. All 4 genes were polymorphic among all the *S. japonicum* isolates in mainland China, with 103, 158, 47, and 19 polymorphic (segregating) sites per kilobase in intron sequences of Actin, FBPA, 22.6 kDa antigen and GST-26, respectively. Introns of housekeeping genes were slightly more polymorphic than coding and non-coding regions of antigen-coding genes examined in the present study within or among lake/marshland and mountainous types. Phylogenetic analysis based on sequences of single gene or combined sequences of multiple genes showed no specific clustering comprising parasites from single geographical or endemic regions. These results demonstrated that introns of housekeeping and antigen-coding genes were polymorphic, but the intron sequences examined in the present study were not suitable markers for examining genetic relationship among different isolates from endemic regions in mainland China.
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1. Introduction

Schistosomiasis japonica has been documented for more than two millennia and most probably was known as a specific disease in historic times in China [1]. After approximately 50 years of continued control efforts, schistosomiasis has been eliminated in five of the 12 previously endemic provinces, and the prevalence of schistosomiasis also dropped significantly in the remaining seven endemic provinces [1–3], which were divided into two main endemic types (mountainous and lake/marshland types) based on geographical, water-snail distribution, epidemiological information and some molecular markers [3–6].

The accurate characterization of parasites at different taxonomic levels has important implications for analyzing genetic variation and would provide basic data for disease control. Previous studies using some portions of mitochondrial DNA (mtDNA) [3,6,7] and coding regions of antigen-coding genes [8] showed limited genetic variation among *Schistosoma japonicum* isolates from different countries and between or within two endemic types.

Many eukaryotic genes have their coding regions interrupted by intervening sequences or introns [9]. The presence, absence, and mutation of these introns present us with an opportunity to examine intron transfer events, and the evolutionary history of introns and species or subspecies within the same or different genera. Intron sequences have been successfully used to study the genetic variation and phylogenetic relationships of *Toxoplasma gondii* [10,11], *Metarhizium* [12], chicken and turkey [13], non-human primates [14], the family Cyprinidae [15], and some plants [9,16].

In the present study, we compared sequence variability in introns of two housekeeping genes (Actin and fructose-1,6-bisphosphate aldolase genes) and in non-coding regions of two antigen-coding genes (22.6-kDa tegument membrane-associated antigen and 26 kDa glutathiones-

* Correspondence to: X.Q. Zhu, Department of Parasitology, State Key Laboratory of Veterinary Etiological Biology, Key Laboratory of Veterinary Parasitology of Gansu Province, 1 Xujiaping, Yanchangbu, Lanzhou Veterinary Research Institute, CAAS, Lanzhou, Gansu Province 730046, PR China. Tel.: +86 931 8342837; fax: +86 931 8340977.
** Corresponding author. Tel.: +86 20 85285954; fax: +86 20 85283730.
E-mail addresses: rqlin@scau.edu.cn (R.Q. Lin), xingquanzhu1@hotmail.com (X.Q. Zhu).

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transferase genes) of *S. japonicum* isolates from different geographical origins in mainland China. The results showed that all the intron sequences of four selected representative genes showed variability, and introns of housekeeping genes were slightly more polymorphic than non-coding and coding regions of the examined antigen genes, but the variations were neither associated with geographical origins, nor related to the two classical types of endemicity.

2. Materials and methods

2.1. Parasites and isolation of genomic DNA

A total of 32 *S. japonicum* samples (including male and female parasites), collected from 10 geographical origins in eight endemic provinces in mainland China (Table 1), was used. All the *S. japonicum* samples were prepared as previously described [3,5,6,17], except for *S. japonicum* samples from Hunan and Yunnan provinces. Briefly, *Oncomelania hupensis* snails infected with *S. japonicum* were collected from one village per province, and transported to the laboratory. For each isolate, 50 infected snails were shed under light for 3 h, cercariae were pooled, and each of two male adult rabbits was infected percutaneously with 1,000 cercariae and housed in separate cages. After 45 days, adult schistosomes were perfused from the mesenteric veins of infected rabbits using 0.15 mM NaCl and 25 mM sodium citrate, washed extensively in physiological saline, fixed in 90% molecular grade ethanol, and stored at −20 °C. For isolates from Yunnan (Eryuan) province and Hunan (Changsha city, Junshan county, and Yueyanglou district) province, three infected snails were collected, and two male adult rabbits were infected as mentioned above. After 60 days, adult schistosomes were obtained and fixed in 70% molecular grade ethanol and stored at −20 °C.

Total genomic DNA was extracted from a single adult parasite (male or female representing each of the *S. japonicum* isolates (Table 1) by SDS/proteinase K treatment. Then the genomic DNA was column-purified by the Wizard® SV Genomic DNA Purification System (Promega) and eluted into 60 μl H_2O according to the manufacturer's recommendations [3].

2.2. Genetic markers

Four single-copy genes were chosen for sequencing (Tables 1 and 2; Fig. 1), including two housekeeping genes and two antigen-coding genes. The group named "housekeeping genes" encodes proteins that are unlikely to play a role in protective immunity [11] and were selected on the basis of the presence of an intron at least 250 bp long. It includes actin [18], and fructose-1, 6-bisphosphate aldolase (FBPA) [18,19] genes. FBPA is a ubiquitous enzyme essential for glycolysis, gluconeogenesis and the Calvin cycle, which has been demonstrated to induce immune responses and to be useful in the immunodiagnosis of schistosomiasis japanica of water buffaloes [19] and humans [20]. The group named "antigen-coding genes" encodes proteins that probably plays a role in protective immunity, which includes 22.6 kDa tegumental membrane-associated antigen (22.6 kDa antigen) [21] and 26 kDa glutathione S-transferase (GST-26) [22], whose intron at least 400 bp long was selected. All unique sequences were submitted to GenBank (accession numbers: GU567821–GU567948).

2.3. Enzymatic amplification and sequencing

External and internal sets of primers (Table 2) were designed for each gene, except Actin gene, on the basis of the published sequences and *S. japonicum* genome contigs. PCR reactions (25 μl) were performed in 2 mM of $MgCl_2$, 0.4 μM of each primer, 2.5 μl 10 × r*Taq* buffer, 0.2 mM of each dNTPs, 1.25 U of r*Taq* DNA polymerase (TAKARA), and 1 μl of DNA sample in a thermocycler (Biometra) under the following conditions: after an initial denaturation at 94 °C for 5 min, then 94 °C

Table 1
Schistosoma japonicum samples used in the present study.

Endemic type	Province	Geographical origin	Sample codes	Gender	GenBank accession number			
					Actin	FBPA	22.6 kDa	GST-26
Lake/marshland type	Hunan	Changsha city	SJHCM4	Male	GU567917	GU567885	GU567821	GU567853
			SJHCM33		GU567918	GU567886	GU567822	GU567854
			SJHCM34		GU567919	GU567887	GU567823	GU567855
			SJHCF25	Female	GU567920	GU567888	GU567824	GU567856
		Junshan county	SJHYM23	Male	GU567921	GU567889	GU567825	GU567857
			SJHYM24		GU567922	GU567890	GU567826	GU567858
			SJHYF23	Female	GU567923	GU567891	GU567827	GU567859
		Yueyanglou district	SJHLM23	Male	GU567924	GU567892	GU567828	GU567860
			SJHLM25		GU567925	GU567893	GU567829	GU567861
			SJHLF2	Female	GU567926	GU567894	GU567830	GU567862
			SJHLF3		GU567927	GU567895	GU567831	GU567863
	Hubei	Wuhan city	SJHWM31	Male	GU567928	GU567896	GU567832	GU567864
			SJHWF5	Female	GU567929	GU567897	GU567833	GU567865
			SJHWF7		GU567930	GU567898	GU567834	GU567866
	Anhui	Guichi county	SJAGM1	Male	GU567931	GU567899	GU567835	GU567867
			SJAGF24	Female	GU567932	GU567900	GU567836	GU567868
	Jiangxi	Yongxiu county	SJJYM5	Male	GU567933	GU567901	GU567837	GU567869
			SJJYM24		GU567934	GU567902	GU567838	GU567870
			SJJYF21	Female	GU567935	GU567903	GU567839	GU567871
			SJJYF43		GU567936	GU567904	GU567840	GU567872
	Jiangsu	Wuxi city	SJJWM25	Male	GU567937	GU567905	GU567841	GU567873
			SJJWM35		GU567938	GU567906	GU567842	GU567874
	Zhejiang	Jiashan county	SJZJM21	Male	GU567946	GU567914	GU567850	GU567882
			SJZJM25		GU567947	GU567915	GU567851	GU567883
			SJZJF1	Female	GU567948	GU567916	GU567852	GU567884
Mountainous type	Sichuan	Tianquan county	SJSTM7	Male	GU567939	GU567907	GU567843	GU567875
			SJSTM10		GU567940	GU567908	GU567844	GU567876
			SJSTM11		GU567941	GU567909	GU567845	GU567877
	Yunnan	Eryuan county	SJYEIIM57	Male	GU567942	GU567910	GU567846	GU567878
			SJYEIIM58		GU567943	GU567911	GU567847	GU567879
			SJYEIIM59		GU567944	GU567912	GU567848	GU567880
			SJYEIIF57	Female	GU567945	GU567913	GU567849	GU567881

Table 2
Primers used for each gene and corresponding PCR annealing temperature.

Gene	Primer location	GenBank™ accession no. and Reference sequence	Expected PCR product size (bp)	Annealing temperature (°C)	Primer name	Sequence (5′–3′)
Actin	External	AF223400, genome sequences [18]	797	56	ActinU1 ActinD1	AAGAAGTCCAAGCCCTTGTTG GGCAGAGCATAACCTTCGTAG
FBPA	External	DQ497791, genome sequences [18]	1487	52	FBPAu1 FBPAd1	GAGACAACGACTCAGGGTCTC TCCTTCCAAGTAAACATGGTG
	Internal		1293	52	N-FBPAu1 N-FBPAd1	CGTGCTTGCACGCTACGCCTC AACTTCCTCTGTAACTCTCTG
22.6 kDa	External	U75510, genome sequences [18]	651	52	22.6u1 22.6d1	ATATGTTTTCCAGCCATGGATTG TATTGCTTAGCTTTTGACATGG
	Internal		521	52	N-22.6u1 N-22.6d1	TTTTGTCGTGGATTCGGTCT TTGCCTTCCTTGTCTCTCTT
GST-26	External	AF044411, genome sequences [18]	1018	51	sj26u1 sj26d1	GGTAACTTGGTCATGTCCCCT TTTTTGTTTCGCCATTTATCACCTT
	Internal		956	47	N1-Sj26u N1-Sj26d	CTATACTAGGTTATTGGAAAATT GCTCTTCATATTTTTCTTCAAGA

for 1.5 min (denaturation); 52 °C (or as specified in Table 2) for 30 s (annealing); 72 °C for 1.5 min (extension) for 35 cycles, followed by a final extension at 72 °C for 10 min. These optimized cycling conditions for the specific and efficient amplification of individual gene fragments were obtained after varying annealing temperatures. Samples without genomic DNA (no-DNA controls) were included in each amplification run, and in no case were amplicons detected in the no-DNA controls (not shown). Each amplicon (5 μl) was examined by agarose gel electrophoresis to validate amplification efficiency.

Positive amplicons were selected, purified and sequenced using an ABI 377 automated DNA sequencer (using BigDye Terminator Chemistry) employing the same primers (individually) as used in the PCR. The intron sequences of each gene are available from DDBJ, EMBL, and GenBank™ under the accession numbers shown in Table 1.

2.4. Molecular genetic analysis

Intron sequences of four genes were separately aligned using the computer program Clustal X 1.81 [23] using default settings. Alignments were checked by eye and modifications made as required. Measures of polymorphism including the number of segregating sites, nucleotide diversity (π), and haplotype diversity were computed for intron regions of the genes using DNASP 3.0 [24]. Because isolates belong to a single species, the p-distance (the proportion of polymorphic sites between 2 sequences) was used to calculate genetic distance and all substitutions were assigned the same weight [25]. Meanwhile, the pairwise comparisons of open reading frame (ORF) of housekeeping and antigen-coding gene sequences for *S. japonicum*, *Schistosoma bovis*, and *Schistosoma mansoni* were made of the level of sequence differences using Megalign procedure within the DNAStar 5.0 software (DNASTAR Inc.) using sequences published previously (GenBank™ Accession numbers EU595760, DQ497790, DQ497791, XM_002575518, EU595757, AF223400, and U19945).

Four methods, namely unweighted pair-group method with arithmetic averages (UPGMA), neighbor joining (NJ), maximum likelihood (ML) and maximum parsimony (MP), were used for phylogenetic re-constructions using the combined dataset (the intron sequences of Actin + FBPA + 22.6 kDa + GST-26) [17]. UPGMA, NJ and MP analysis were carried out using PAUP 4.0 Beta 10 program [26], PHYLIP package3.64 [27], and MEGA 4.0 [28], and ML analyses were performed using PUZZLE 4.1 [29] and MrBayes 3.1 [30] under the default setting. Phylograms were drawn using the Tree View program version 1.65 [31].

3. Results and discussion

Genomic DNA was prepared from 32 individual adult trematodes (including male and female *S. japonicum*) representing two epidemic types (mountainous and lake/marshland types) from eight endemic provinces in mainland China (Table 1). Amplicons of introns in housekeeping genes (Actin, FBPA) and antigen genes (22.6 kDa antigen, GST-26) were amplified individually and subjected to agarose gel electrophoresis. The PCR products for introns in housekeeping genes were variable in length, but for antigen genes, no size variation was detected on agarose gel among any of the amplicons examined (Fig. 2).

Sequencing of housekeeping and antigen genes showed that intron sequences of Actin, FBPA, 22.6 kDa antigen and GST-26 were 263–301 bp, 1157–1190 bp, 426 bp and 907 bp in length, respectively. All 4 genes were polymorphic (Table 3) among all the isolates of *S. japonicum* in mainland China, with 103, 158, 47, and 19 polymorphic (segregating) sites per kilobase in intron sequences of Actin, FBPA, 22.6 kDa antigen and GST-26, respectively. In addition to 256 polymorphic sites, a total of

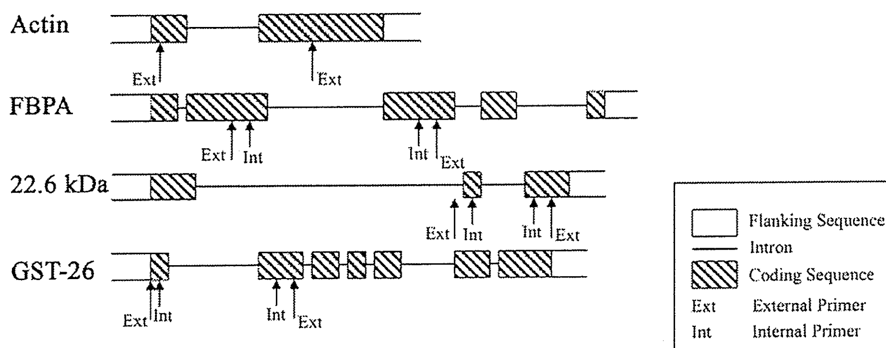

Fig. 1. Diagrams showing organization of each gene and placement of the primers. Sequences are not drawn to scale.

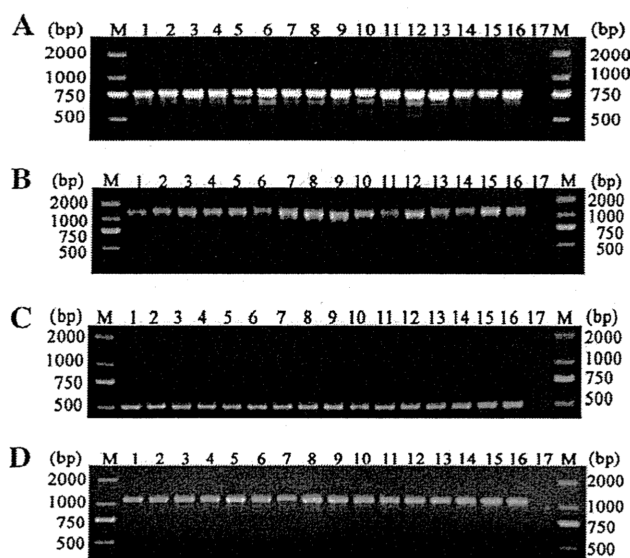

Fig. 2. Representative PCR products for a portion of Actin (A), FBPA (B), 22.6 kDa antigen (C), and GST-26 genes (D) of *Schistosoma japonicum* isolates in mainland China. Lanes 1–16 represent samples SJJYM5, SJJYM25, SJJWM35, SJZJM21, SJZTM10, SJYEIIM59, SJYEIIM57, SJHCM34, SJHCF25, SJHYM23, SJHYF3, SJHLM23, SJHLF2, SJHWF5, SJAGF24, SJAGM1, respectively. Lane 17 represents no-DNA control. M represents a DNA size marker (ordinate values in bp). Refer to Table 1 for sample information.

105 gaps (insertions/deletions) in housekeeping genes were found. The overall levels of polymorphism (π) were 0.02743 and 0.00881 for housekeeping and antigen genes, respectively. And introns of the housekeeping genes appeared to be only slightly more polymorphic than non-coding regions of the antigen-coding genes within or among 2 endemic types (lake/marshland and mountainous types) (Table 3).

Gene trees and bootstrap tests among different *S. japonicum* samples, re-constructed by NJ, MP, ML, and UPGMA analyses based on single gene or combined gene sequences using different methods and models, showed no specific clustering comprising parasites from single geographical or endemic regions. As shown in Fig. 3, the phylogenetic tree was consisted of two large clades. Isolates from Sichuan, Hunan, Hubei and Jiangsu provinces were located in both clusters, and some samples from different geographical locations (except Yunnan province) were clustered together. As a consequence, parasites from the same endemic regions were scattered in different clusters.

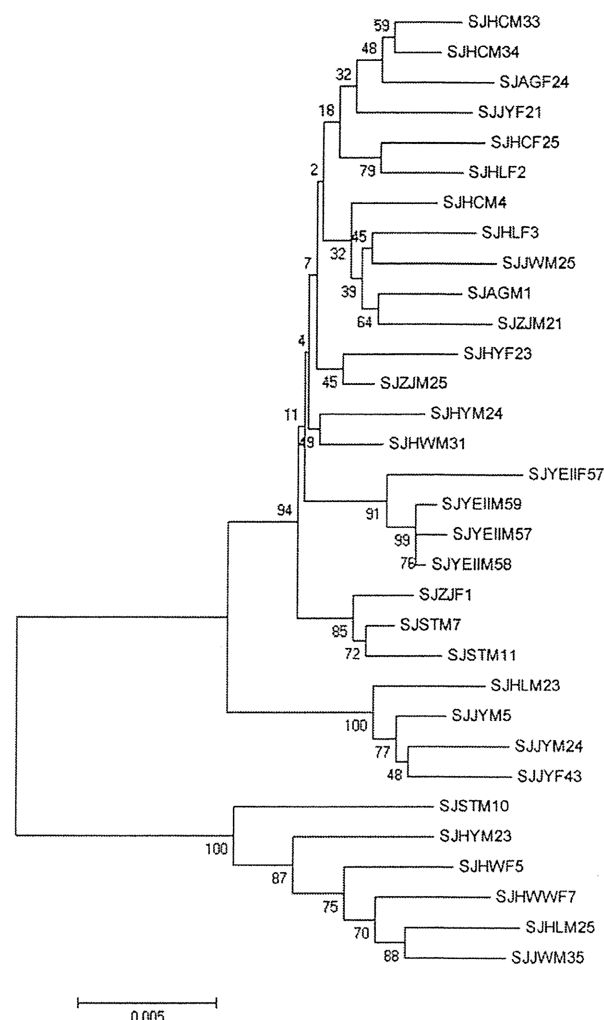

Fig. 3. Phylogenetic relationships of *Schistosoma japonicum* isolates from different provinces in mainland China inferred by neighbor-joining analysis of the combined dataset (the intron sequences of Actin + FBPA + 22.6 kDa + GST-26). Bootstrap values (in percentage) from 1000 pseudo-replicates are shown. Scale bar indicates an evolutionary distance of *p* distance.

Previous studies showed that the genetic differences in the open reading frame (ORF) of 22.6 kDa antigen cDNA for *S. japonicum* were lower than 1%, through comparing parasite samples from Japan, China and Philippine [8]. In the present study, we also analyzed the genetic differences in ORF of Actin and FBPA sequences for *S. japonicum*, *S. bovis*, and *S. mansoni*. The genetic differences in ORF sequences of Actin genes were lower than 8% among *S. japonicum* and *S. bovis* isolates, and in that of FBPA genes were lower than 1% between *S. japonicum* samples from mainland and Formosan, and lower than 16% among *S. japonicum*, *S. bovis*, and *S. mansoni* (data not shown). But the genetic differences in intron sequences were higher than 1.5%, and 10% for antigen genes and housekeeping genes for *S. japonicum* isolates in mainland China, respectively (Table 3). These results showed that the introns were more variable than coding regions in

Table 3
Polymorphism in intron region for different genes of *Schistosoma japonicum* isolates.

Gene	Lake/marshland type					Mountainous type					Among populations				
	P	S/L	π	Gap	H/M	P	S/L	π	Gap	H/M	P	S/L	π	Gap	H/M
Actin	103	31/301	287.9	40	91/16	46	13/283	164.8	23	90.5/5	103	31/301	250.2	56	93.3/19
FBPA	144	172/1191	318.9	37	100/25	68	81/1185	202.1	28	100/7	158	188/1191	298.4	49	100/32
HK mean	136	203/1492	303.4	39	95.5/21	64	94/1468	183.5	26	95.3/6	147	219/1492	274.3	53	96.7/26
22.6 kDa	35	15/426	99.2	0	91.7/16	31	13/426	174.4	0	57.1/2	47	20/426	149.6	0	92.5/17
GST-26	18	16/907	28.9	0	94.3/17	2	2/907	11.6	0	66.7/3	19	17/907	26.6	0	92.3/18
ANC mean	23	31/1333	64.1	0	93/17	11	15/1333	93	0	61.9/3	28	37/1333	88.1	0	92.4/18

P, Number of polymorphic (segregating) sites per kilobase (based on S and L). S, Number of polymorphic (segregating) sites. L, Total sequence length. π, Nucleotide diversity (expected heterozygosity per sites, calculated on the basis of Nei [35], equation 10.5), $\times 10^{-4}$. Gap, Number of gaps. HK, Mean value of housekeeping genes (total values of S and L). ANC, Mean value of antigen genes (total values of S and L). H, Haplotype diversity (expected haplotype heterozygosity, calculated based on Nei [35], equation 8.4), %. M, Number of unique haplotypes.

housekeeping and antigen genes. Introns of housekeeping genes were slightly more polymorphic than coding and non-coding regions of antigen-coding genes examined in this study, which was consistent with similar studies of *T. gondii* [11].

Introns are assumed to be one of the most important genetic factors to modify the pace of genetic evolution, despite uncertainties concerning their early (exon shuffling) or late (intron insertion) evolutionary origin [32,33]. In addition to RNA splicing, introns are implicated in functions as diverse as genetic recombination and gene network multitasking [33,34]. Hence, the evolutionary impact of variations in intron number, length, and base composition may provide a high-resolution classification tool to define the genetic diversity of *S. japonicum* isolates in mainland China. Our study determined the intron sequences of 4 housekeeping and antigen genes for different isolates of *S. japonicum*, and analyzed the genetic variability and phylogenetic relationships based on these sequences. The results showed that the intron sequences of all 4 genes were polymorphic, but there was no relation between geography and genetic diversity.

In conclusion, the intron sequences of 4 housekeeping and antigen genes of *S. japonicum* examined in the present study were polymorphic among different isolates from different endemic provinces in China. Introns of the housekeeping genes appeared to be only slightly more polymorphic than non-coding and coding regions of the antigen genes within or among the 2 endemic types, but the genetic variability was not related to geographical distribution of *S. japonicum* isolates from endemic regions in mainland China. Considering the phylogenetic relationships based on single gene or combined genes may not reflect the geophylogenetic status of *S. japonicum* isolates, further studies of long and more introns of other housekeeping and antigen-coding genes are warranted.

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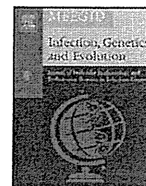

The ribosomal intergenic spacer (IGS) region in *Schistosoma japonicum*: Structure and comparisons with related species

Guang-Hui Zhao [a,b,c,1], David Blair [d,*], Xiao-Yan Li [a,e,1], Juan Li [a,c], Rui-Qing Lin [c], Feng-Cai Zou [f], Hiromu Sugiyama [g], Xi-Hao Mo [c], Zi-Guo Yuan [c], Hui-Qun Song [a,c], Xing-Quan Zhu [a,f,**]

[a] *State Key Laboratory of Veterinary Etiological Biology, Key Laboratory of Veterinary Parasitology of Gansu Province, Lanzhou Veterinary Research Institute, CAAS, Lanzhou, Gansu Province 730046, PR China*
[b] *College of Veterinary Medicine, Northwest A & F University, Yangling, Shaanxi Province 712100, PR China*
[c] *College of Veterinary Medicine, South China Agricultural University, Guangzhou, Guangdong Province 510642, PR China*
[d] *School of Marine and Tropical Biology, James Cook University, Townsville, Queensland 4811, Australia*
[e] *College of Agronomy, Northwest A & F University, Yangling, Shaanxi Province 712100, PR China*
[f] *College of Animal Science and Technology, Yunnan Agricultural University, Kunming, Yunnan Province 650201, PR China*
[g] *Department of Parasitology, National Institute of Infectious Diseases, Shinjuku Ward, Tokyo 113-8421, Japan*

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ABSTRACT

The intergenic spacer (IGS) between the 28S and 18S ribosomal RNA genes was PCR-amplified, sequenced and characterized for *Schistosoma japonicum* from mainland China, and compared with those of other *Schistosoma* species. Excluding flanking portions of the 28S and 18S genes, the IGS in the longest sequenced amplicon from *S. japonicum* IGS was 1457 bp in length. However, intra-specific and intra-individual variation was noted. The IGS region of *S. japonicum* is strikingly different in structure from those of African *Schistosoma* species for which data are available. *S. japonicum* has a shorter IGS and largely lacks a long region of complex repeats seen in the African species. However, careful comparisons with African species highlighted the presence of a few shared repeat motifs that were not apparent from study of African species only. Such motifs presumably have functional significance. Discovery of such motifs may in general be aided by comparisons of relatively distant taxa rather than of sibling taxa.
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1. Introduction

Schistosomiasis caused by *Schistosoma japonicum* is a very important parasitic zoonosis occurring in China and the Philippines (McGarvey et al., 1999). Human schistosomiasis has been listed as one of China's three most important communicable diseases, together with HIV/AIDS and tuberculosis (Li et al., 2000; Zhou et al., 2005, 2008). Many avenues are being explored to further our understanding of this disease and its causative organism. Here, we report the structure of the nuclear ribosomal intergenic spacer (IGS) region.

Nuclear ribosomal genes code for ribosomal RNA, a central component of ribosomes and hence essential for an ancient cellular process that is probably fundamentally similar in all living organisms. Ribosomal RNA typically accounts for about 40% of all transcription within a cell, and ribosomal RNA makes up as much as 80% of cellular RNA (Moss and Stefanovsky, 1995). In schistosomes, as in many eukaryotes, the ribosomal genes form a tandem array with each operon containing the genes for the 18S, 5.8S and 28S subunits respectively, separated by spacers (Simpson et al., 1984). There are about 185 copies of the repeat in *S. japonicum* (*Schistosoma japonicum* Genome Sequencing and Functional Analysis Consortium et al., 2009). By processes such as unequal crossing-over, arrays of tandem operons such as the ribosomal genes, tend towards sequence homogeneity. Thus within a species, all copies tend to be very similar (Hillis and Dixon, 1991). Ribosomal RNA genes and most spacers are transcribed in a single full-length rRNA precursor transcript by RNA polymerase I. The spacers are subsequently excised from this transcript to yield the mature subunits (Capowski and Tracy, 2003). The region separating the 3′ end of the 28S rRNA gene and the 5′ end of the 18S rRNA gene is the intergenic spacer (IGS),

* Corresponding author. Tel.: +61 7 4781 4322; fax: +61 7 4725 1570.
** Corresponding author at: Department of Parasitology, State Key Laboratory of Veterinary Etiological Biology, Key Laboratory of Veterinary Parasitology of Gansu Province, Lanzhou Veterinary Research Institute, CAAS, Lanzhou, Gansu Province 730046, PR China. Tel.: +86 931 8342837; fax: +86 931 8340977.
E-mail addresses: david.blair@jcu.edu.au (D. Blair), xingquanzhu1@hotmail.com (X.-Q. Zhu).
[1] These authors contributed equally to this work.

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which can be subdivided into the external transcribed spacers (ETSs) and the non-transcribed spacer (NTS). The IGS regions in eukaryotes often exhibit an extraordinary variety of repeats and other features including promoters and enhancers for RNA polymerase I (Moss and Stefanovsky, 1995). The IGS and its embedded promoters can evolve more rapidly than other parts of the ribosomal repeat unit, leading to considerable sequence difference, even between related species. Indeed, sequences of promoters tend to be highly species-specific. They can also exhibit considerable intra-individual variation in length, largely as a consequence of numbers of repeats differing between copies (reviewed for parasitic flatworms by Blair, 2006).

Ribosomal RNA (rRNA) repeats in parasitic flatworms have been the subject of many studies on phylogeny/molecular taxonomy (e.g. Nolan and Cribb, 2005). The advantages of this region for such studies are that concerted evolution has led to homogenisation of sequence among repeats (Hillis and Dixon, 1991), and that there are often hundreds of copies of the operon in each cell, constituting an abundant target for PCR. Virtually all studies using rDNA sequences for phylogeny and taxonomy have focused on the main coding regions (18S and 28S, e.g.) or the internal transcribed spacers (Nolan and Cribb, 2005; Blair, 2006). In contrast, the IGS region has been poorly studied in this taxon. IGS sequence data for parasitic flatworms are few but are available for the cestode *Echinococcus granulosus* (Picón et al., 1996) and the monogenean *Gyrodactylus salaris* and *Gyrodactylus thymalli* (Collins and Cunningham, 2000). Kane and Rollinson (1998) characterized the IGS for three *Schistosoma* species from Africa and found many similarities between them as well as some striking differences. In the present study, we sequenced the IGS of *S. japonicum* from several localities for comparison with those of the phylogenetically distant African species and to evaluate its utility as a population-level marker. We discovered that the IGS in *S. japonicum* is very different in many respects from those of the African *Schistosoma* species and identified some features, conserved across a considerable phylogenetic distance, which might be of functional significance.

2. Materials and methods

2.1. Parasite samples

In a search for geographical variation within China, samples were obtained from six provinces: Zhejiang (1 locality, 4 worms), Anhui (1 locality, 2 worms), Jiangxi (1 locality, 3 worms), Hunan (2 localities, 3 worms), Sichuan (3 localities, 6 worms) and Yunnan (1 locality, 4 worms). All the samples were cercariae collected from infected snails, *Oncomelania hupensis*, except one from Yunnan, which was an adult worm collected from infected cattle. Cercariae from a single locality were used to infect a rabbit from which worms were later harvested. The adult parasites were stored in 70% molecular grade ethanol, and stored at −20 °C before extraction of genomic DNA.

2.2. DNA isolation and PCR amplification

Genomic DNA (gDNA) was extracted from individual adult trematodes by sodium dodecyl-sulphate/proteinase K treatment (Zhu et al., 2002), column-purified using Wizard® SV Genomic DNA Purification System (Promega) and eluted into 60 μl H_2O according to the manufacturer's recommendations (Zhao et al., 2009a,b,c). DNA samples were stored at −20 °C until use.

Sequences of primers used are listed in Table 1. The 28S–18S IGS rDNA of *S. japonicum* was amplified by nested-PCR. For the primary PCR amplification, primers used were described by Kane and Rollinson (1998): 28aa (forward) and ET10 (reverse). For the secondary PCR amplification, the following primers were designed and used: ZghIGSu (forward; 151 bp downstream of 28aa) and ZghSJIGSd (reverse; upstream of ET10 and overlapping it by 6 bases). Both pairs are within the 28S or 18S coding regions.

Table 1
Sequences of the primers used in this study. Refer to Fig. 1 for location of each primer.

Forward primers	Sequence (5′–3′)
28aa	AGGTTAGTTTTACCCTACT
ZghIGSu	ACCATCTGAGGGATTAAGACTGAACG
ZghIGSu1	AAGTCTGAATCCCGTCCAAA
IGSF1	GAGGACTTGATCCTCATTCATTTCCCTACTG
IGSF2	ACGGCATGTGAAGCCCTATAGAACGAGTTGC
IGSd1RC	GAATTTTATGCGCTACACAATGG
Reverse primers	
ET10	CTGAGACAAGCATATGACTAC
ZghSJIGSd	GACTACTGGCAGGATCAACCAGTTT
ZghIGSd1	CCATTGTGTAGCGCATAAAATTC
IGSR2	GCAACTCGTTCTATAGGGCTTCACATGCCGT
IGSR1	CAGTAGGGAAATGAATGAGGATCAAGTCCTC

PCR reactions were performed in 2 mM MgCl2, 2.5 μM of each primer, 2.5 μl Ex Taq buffer, 0.2 mM of each dNTP, 1.25 U of Ex Taq DNA polymerase (TAKARA), 1 μl of DNA sample and double-distilled water up to a total volume of 25 μl. The conditions for the secondary PCR were the same as for the primary PCR, except for the use of a different pair of primers and 0.5 μl primary PCR products as templates.

Both procedures were performed in a thermocycler (Biometra) under the following conditions: 94 °C for 5 min (initial denaturation), followed by 35 cycles of 94 °C, 1 min (denaturation), 51 °C, 1 min (annealing), 72 °C, 2.5 min (extension) and a final extension of 72 °C for 10 min. Reactions with and without host (cattle and rabbit) gDNA were included in each amplification run as controls. An aliquot (4 μl) of each amplicon was examined on 1.0% agarose–TBE (65 mM Tris–HCl, 22.5 mM boric acid and 1.25 mM EDTA, pH 9.0) gels, stained with ethidium bromide and photographed using a gel documentation system (UVItec). The DL2000 marker (Takara) was used to estimate the sizes of the amplicons.

2.3. Purification, cloning, and sequencing of the S. japonicum IGS+ PCR product

PCR products were purified using spin columns (Wizard™ PCR-Preps DNA Purification System, Promega) and ligated with pGEM-T easy plasmid vector (Promega) according to the manufacturer's recommendations. The recombinant plasmid was then transformed into *Escherichia coli* JM109 competent cells (Promega). Transformants containing recombinant plasmids were selected by PCR amplification. Cell cultures with confirmed recombinant plasmid were sent to Shanghai Songon Biological Engineering Biotechnology Company for sequencing using an ABI 377 automated DNA sequencer (BigDye Terminator Chemistry). Three clones from a single worm from each geographical location were sequenced in both directions.

2.4. Additional PCRs to investigate length variation

Sequencing of cloned amplicons suggested that length variation occurred between populations of *S. japonicum* in the IGS region and appeared to be due to large indels in the 5′ half of the region. Suspecting possible PCR and/or cloning artifacts, we designed primers to amplify across shorter portions of the IGS, and especially those where variation seemed to occur. We also used this approach to determine the extent to which such variation occurred within a single worm. A reverse primer (ZghIGSd1) was designed to anneal just 3′ of the region where most variation

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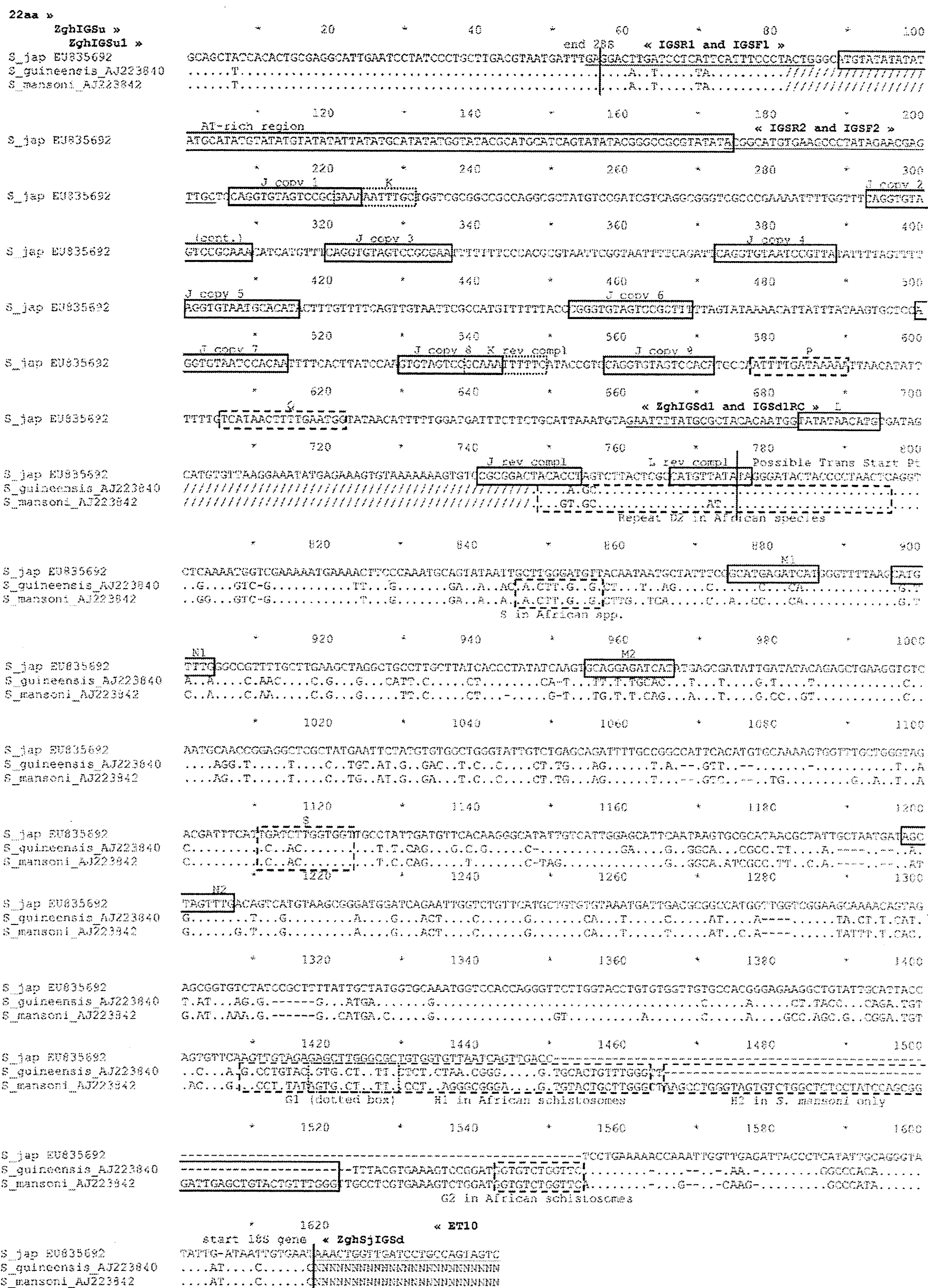

Fig. 1. Alignment of the longest IGS amplicon sequence from *S. japonicum* (EU835692). Portions of the flanking 28S and 18S genes are shown. The sequence in the top line is that of EU835692. Sequences from two African species are shown only where they can be aligned with *S. japonicum* with confidence. Identical nucleotides in other sequences are indicated by a dot (·) and alignment gaps by a dash (–). Primer sequences located within the IGS are underlined and the name and orientation of each indicated. See the text for additional details. Motifs in *S. japonicum* are boxed with a solid line. Motifs also occurring in, or only in, African schistosomes are boxed with a broken line.

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