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New genetic lineages, host associations and circulation pathways of *Neorickettsia* endosymbionts of digeneans

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Abstract

Neorickettsia is a genus of intracellular bacteria endosymbiotic in digeneans that may also invade cells of vertebrates and are known to cause diseases of wildlife and humans. Herein, we report results of screening for *Neorickettsia* of an extensive collection of DNA extracts from adult and larval digeneans obtained from various vertebrates and mollusks in the United States. Seven isolates of *Neorickettsia* were detected by PCR and sequenced targeting a 527 bp long region of 16S rRNA. Sequence comparison and phylogenetic analysis demonstrated that four isolates matched published sequences of *Neorickettsia risticii*. Three other isolates, provisionally named "catfish agents 1 and 2" (obtained from *Megalogonia ictaluri* and *Phyllodistomum lacustri*, both parasitic in catfishes) and *Neorickettsia* sp. (obtained from cercariae of *Diplostomum* sp.), differed from previously known genotypes of *Neorickettsia* and are likely candidates for new species. All 7 isolates of *Neorickettsia* were obtained from digenean species and genera that were not previously reported as hosts of these bacteria. Members of four digenean families (Dicrocoeliidae, Heronimidae, Macroderoididae and Gorgoderidae) are reported as hosts of *Neorickettsia* for the first time. Our study reveals several new pathways of *Neorickettsia* circulation in nature. We have found for the first time a *Neorickettsia* from a digenean (dicrocoeliid *Conspicuum icteridorum*) with an entirely terrestrial life cycle. We found *N. risticii* in digeneans (*Alloglossidium corti* and *Heronimus mollis*) with entirely aquatic life cycles. Previously, this *Neorickettsia* species was known only from digeneans with aquatic/terrestrial life cycles. Our results suggest that our current knowledge of the diversity, host associations and circulation of neorickettsiae is far from satisfactory.

Keywords

Neorickettsia, Anaplasmataceae, 16S rRNA, new genotypes, phylogeny, Digenea, circulation, host associations

Introduction

Neorickettsia is a small genus of bacteria belonging to the order Rickettsiales Gieszczkiewicz, 1939. They are obligate intracellular endosymbionts of digeneans (Platyhelminthes, Digenea), but may also infect cells and tissues of vertebrate hosts of digeneans and are capable of causing diseases of wildlife and humans (Rikihisa 1991, Walker and Dumler 1996, Madigan and Pusterla 2000, Newton *et al*. 2009, Headley *et al*. 2011, Vaughan *et al*. 2012). Neorickettsiae persist through all stages of digenean complex life cycles that always include an aquatic or a terrestrial mollusk as the first intermediate host and may incorporate an invertebrate or a vertebrate second intermediate host. Digeneans almost invariably mature in a vertebrate definitive host. *Neorickettsia* were first recognized as a cause of animal and human diseases in the mid-1950s when these bacteria were identified as the etiological agents of "salmon" dog poisoning disease in the weastern United States (Philip *et al*. 1953, Philip 1955) and Sennetsu fever of humans in Japan (Fukuda *et al*. 1954, Misao and Kobayashi 1954). Diseases caused by neorickettsiae in vertebrates can be severe (e.g., Sennetsu fever in humans) or even fatal (e.g., salmon dog poisoning, Potomac horse fever). Detailed accounts of the history of discovery and pathogenicity of *Neorickettsia* can be found in several published reviews (Mulville 1991, Rikihisa 1991, Palmer 1993, Madigan and Pusterla 2000, Rikihisa *et al*. 2004, Headley *et al*. 2011, Vaughan *et al*. 2012).

Although *Neorickettsia* have now been reported from several countries and all continents other than Antarctica, a majority of the records are based on the serological testing of horses. As discussed by Vaughan *et al*. (2012) the validity of some of these records is doubtful because of potential previous exposure to vaccines and trade/relocation histories. Records resulting from PCR detection of neorickettsial DNA

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are known from North and South America and Asia. However, even those records are mostly based on *Neorickettsia* found in vertebrate hosts (horses, dogs, humans, fish), but very rarely from digeneans. Only 14–15 digenean species, in most cases identified only to genus or family level, have been previously confirmed as hosts of *Neorickettsia* (Vaughan *et al*. 2012).

In North America, two named species (*Neorickettsia helminthoeca* and *N. risticii*) and two forms known as Elokomin Fluke Fever (EFF) agent and Rainbow trout agent, were described. These infections were invariably connected to lotic habitats and to digeneans having either terrestrial/aquatic (*N. helminthoeca*, *N. risticii*, EFF agent) or fully aquatic (Rainbow trout agent) life cycles. Only 7 digenean species were reported as hosts of *Neorickettsia* in the United States (Philip *et al*. 1953; Farrell *et al*. 1973; Pusterla *et al*. 2000, 2003; Gibson *et al*. 2005; Vaughan *et al*. 2012).

In this study, we used PCR-based detection methods for *Neorickettsia* to screen DNA extracted from an extensive collection of adult digeneans accumulated by the senior author from many groups of hosts and many regions of the United States as well as DNA extracted from digenean cercariae collected in North Dakota and Minnesota. The results of this study (1) reveal the presence of new genetic lineages of *Neorickettsia* that can potentially represent new species; (2) reveal neorickettsial infections in several groups of digeneans not previously reported as hosts of these bacteria; (3) expand the range of circulation pathways and types of habitats known for *Neorickettsia*; and (4) provides new geographic records for these endosymbionts.

Materials and methods

Genomic DNA in the already available collection was extracted from single individuals of well over 100 species of digeneans obtained by Vasyl Tkach from freshwater fishes, amphibians, reptiles, birds and mammals in more than 20 states in the USA between 1999 and 2009.

Snails were collected by hand, or by dip net, and placed into plastic containers for transportation to the lab. We have screened snails belonging to several species, most of our samples were represented by *Stagnicola elodes*, *Lymnaea stagnalis*, *Helisoma trivolvis* and *Physa* sp. The snails were rinsed with tap water and placed into glass jars filled with aged tap water conditioned with commercial aquarium conditioner to remove chlorine/chloramines. Snails were kept for several hours under fluorescent lamps followed by several hours without light. Then the water in jars was examined for cercariae. In cases when snails were found to shed cercariae, some of the cercariae were used for immediate DNA extracts while some of them were fixed in ethanol to provide source for additional extracts if needed.

Genomic DNA was extracted using either the guanidine thiocyanate method as described by Tkach and Pawlowski (1999) or the Qiagen DNeasy kit (Qiagen, Santa Clarita, CA) as per the manufacturer's instructions.

Most of the DNA extracts were tested for the presence of *N. risticii* using a nested PCR protocol described previously by Barlough *et al*. (1997). The nested PCR amplifies a 527-bp portion of the 5' end of the 16s rRNA gene and is designed to amplify this fragment in *N. risticii*, but in our experience it also amplified some other forms such as "catfish agents 1 and 2" (Table I). The primer pairs used were ER-2 (5'-GTTTTAAATGCAGTTCTTGG-3') and ER-3 (5'-ATTTGAGAGTTTGATCCTGG-3') in the first round and ER-2a (5'-CACACCTAACTTACGGG-3') and ER-3a (5'-CTAGCG GTAGGCTTAAC-3') in the nested round. Alternatively, the cercarial samples collected in 2011 were screened using primers newly designed by one of the authors (SEG): n16s-25F (5'-TCAGAACGAACGCTAGCGGT-3') and n16s-610R (5'- GACGTTCCTCTTGATATCTACG-3') for the first round and n16s-70F (5'- GAATCAGGGCTGCTTGCA-3') and ER2-R (5'- GTTTTAAATGCAGTTCTTGG-3') for the nested round.

The PCR reactions were run on a Mastercycler Gradient thermocycler from Eppendorf (Hauppauge, NY) using either Eppendorf MasterTaq polymerase or Quick load OneTaq mastermix from New England Biolabs (Ipswich, MA), according to the manufacturers' instruction. Positive control DNA used in PCR runs was graciously donated by Dr. John Madigan (University of California Davis). It was extracted from the buffy coat of blood taken from a horse showing clinical signs of Potomac horse fever, a disease caused by *Neorickettsia risticii*. Prior to use in our analyses, the positive control was tested by PCR and sequenced. PCR products were visualized on agarose gels (Fig. 1) and PCR-positive samples were sequenced.

Adult digeneans were identified based on their morphology using stained whole mounts. *Neorickettsia*-positive cercariae were identified using partial sequence of the nuclear large ribosomal subunit gene (28S). Digenean DNA was amplified by PCR using forward primer digl2 (5-AAGCATATCACTAAGCGG-3') and reverse primer 1500R (5-GCTA TCCTGAGGGAAACTTCG-3'). PCR primers as well as internal forward primers 300F (5-CAAGTACCGTGAGGGA AAGTTG-3), 900F (5-CCGTCTTGAAACACGGACCAAG-3) and internal reverse primers 300R (5-CAACTTTCCCTCACGGTACTTG-3) and ECD2 (5-CTTGGTCCGTGTTT CAAGACGGG-3) were used for sequencing.

PCR amplicons of both *Neorickettsia* and digeneans were purified using Qiaquick™ PCR Purification Kit (Qiagen, Santa Clarita, CA) or ExoSap PCR clean-up enzymatic kit from USB (now Affimetrix, Santa Clara, CA) according to the manufacturer's instructions. The PCR products were then cycle-sequenced using ABI BigDye™ chemistry, alcohol precipitated, and run on an ABI Prism 3100™ automated capillary sequencer. Contiguous sequences of *Neorickettsia* and cercariae were assembled and edited using Sequencher™ ver. 4.2 (GeneCodes Corp., Ann Arbor, MI) and

Table I. *Neorickettsia* species/isolates, their natural hosts, types of life cycles and geographic origin

Neorickettsia species	Digenean family	Digenean genus and species	Digenean life cycle stage	Host	Life cycle	Locality	GenBank numbers
Neorickettsia risticii	Dicrocoeliidae	*Conspicuum icteridorum*	adult	dark-eyed Junco *Junco hyemalis*	terrestrial	Grand Forks Co., North Dakota	JX262948
	Echinostomatidae	*Echinoparyphium rubrum*	cercariae	planorbid snail *Helisoma trivolvis*	aquatic/ terrestrial	Itasca state park Minnesota	JX262946
	Heronimidae	*Heronimus mollis*	adult	painted turtle *Chrysemys picta*	aquatic	Oshkosh, Wisconsin	JX262947
	Macroderoididae	*Alloglossidium corti*	cercariae	planorbid snail *Helisoma trivolvis*	aquatic	Itasca state park Minnesota	JX262949
Neorickettsia sp. 1 (Catfish agent#1)	Gorgoderidae	*Phyllodistomum lacustris*	adult	stonecat catfish *Noturus flavus*	aquatic	Grand Forks Co., North Dakota	JX227941
Neorickettsia sp. 2 (Catfish agent#2)	Allocreadiidae	*Megalogonia ictaluri*	adult	channel catfish *Ictalurus punctatus*	aquatic	Jackson Co., Mississippi	JX227942
Neorickettsia sp. 3	Diplostomidae	*Diplostomum* sp.	cercariae	lymnaeid snail *Lymnaea stagnalis*	aquatic/ terrestrial	Nelson Co., North Dakota	JX227943

submitted to GenBank under accession numbers JX227941-JX227943 and JX262946-JX262949 (*Neorickettsia* spp., see Table I) and JX262943-JX262945 (cercariae of *Alloglossidium corti*, *Echinoparyphium rubrum* and *Diplostomum* sp., see Table I).

Newly obtained sequences (Table I) and sequences of neorickettsiae from the GenBank were used in the phylogenetic analysis. Sequences from GenBank are listed as follows: *N. risticii* (cercariae, CA) (AF036658), *N. risticii* str. Illinois (NC_013009), *Neorickettsia* sp. (SF agent) (U34280), *N. sennetsu* (M73225), *Neorickettsia* sp. (trout isolate) (AF206298), *N. helminthoeca* (NHU12457), *Neorickettsia* sp. (EU780451), Anaplasmataceae sp. (from *Mastacembelus armatus*) (EU780452), and Anaplasmataceae sp. (from *Channa striata*) (EU780453).

The new sequences and sequences obtained from GenBank were aligned initially with the aid of Clustal W as implemented in the BioEdit program, version 7.0.1 (Hall 1999).

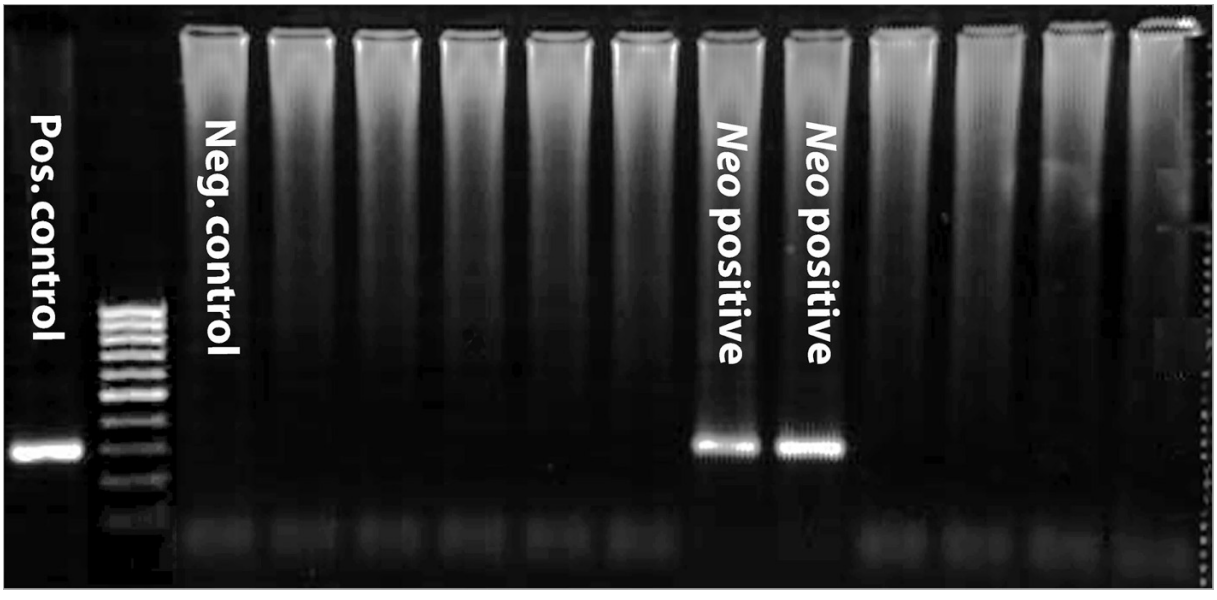

Fig. 1. Agarose gel showing results of digenean DNA screening for *Neorickettsia* by PCR. An approximately 530 bp long fragment of 16S rRNA was amplified. Negative and positive controls were used in each PCR run

The alignments were then manually refined in MacClade, version 4 (Maddison and Maddison 2005).

Two different datasets were analyzed. The first (larger)

dataset included all our newly obtained sequences in addition to sequences of *N. risticii* (strains from Illinois and California), *N. sennetsu*, *N. helminthoeca* and 4 different previously published species-level lineages of *Neorickettsia* spp. (Fig. 2). We did not use an outgroup in this analysis because inclusion of an outgroup would eliminate otherwise unambiguously aligned bases from the analysis. The second (smaller) dataset included only members of a clade that was 100% supported in the first analysis. Sequence of Anaplasmataceae sp. (from *Mastacembelus armatus*) from Seng *et al*. (2009) that appeared basal in the initial tree, was used as an outgroup in the second analysis.

Phylogenetic analyses was carried out using Bayesian inference (BI) as implemented in the MrBayes program, version 2.01 (Huelsenbeck and Ronquist 2001) with the following nucleotide substitution parameters: lset nst = 2, rates = gamma, ngammacat = 4, that correspond to a k80+G model. Posterior probabilities were approximated over 2,000,000 generations for both data-sets, log-likelihood scores plotted and only the final 75% of trees were used to produce the consensus trees by setting the "burnin" parameters at 500,000 generations. The K80+G model was used for both analyses based on the results obtained from jModelTest, version 0.1.1 (Guindon and Gascuel 2003, Posada 2008).

Results

A total of 680 digenean samples were screened for *Neorickettsia*. Screening revealed 4 different forms of *Neorickettsia* in 7 digenean species belonging to 7 different families (Table I). One of the forms discovered in our study corresponded well to *N. risticii*. The three other forms (*Neorickettsia* 1, 2, 3 in Table I) clearly differed from *N. risticii* by their sequences.

The alignment of partial 16S sequences of neorickettsiae included a total of 436 sites, all of which could be aligned unambiguously and used in the analyses of both larger and

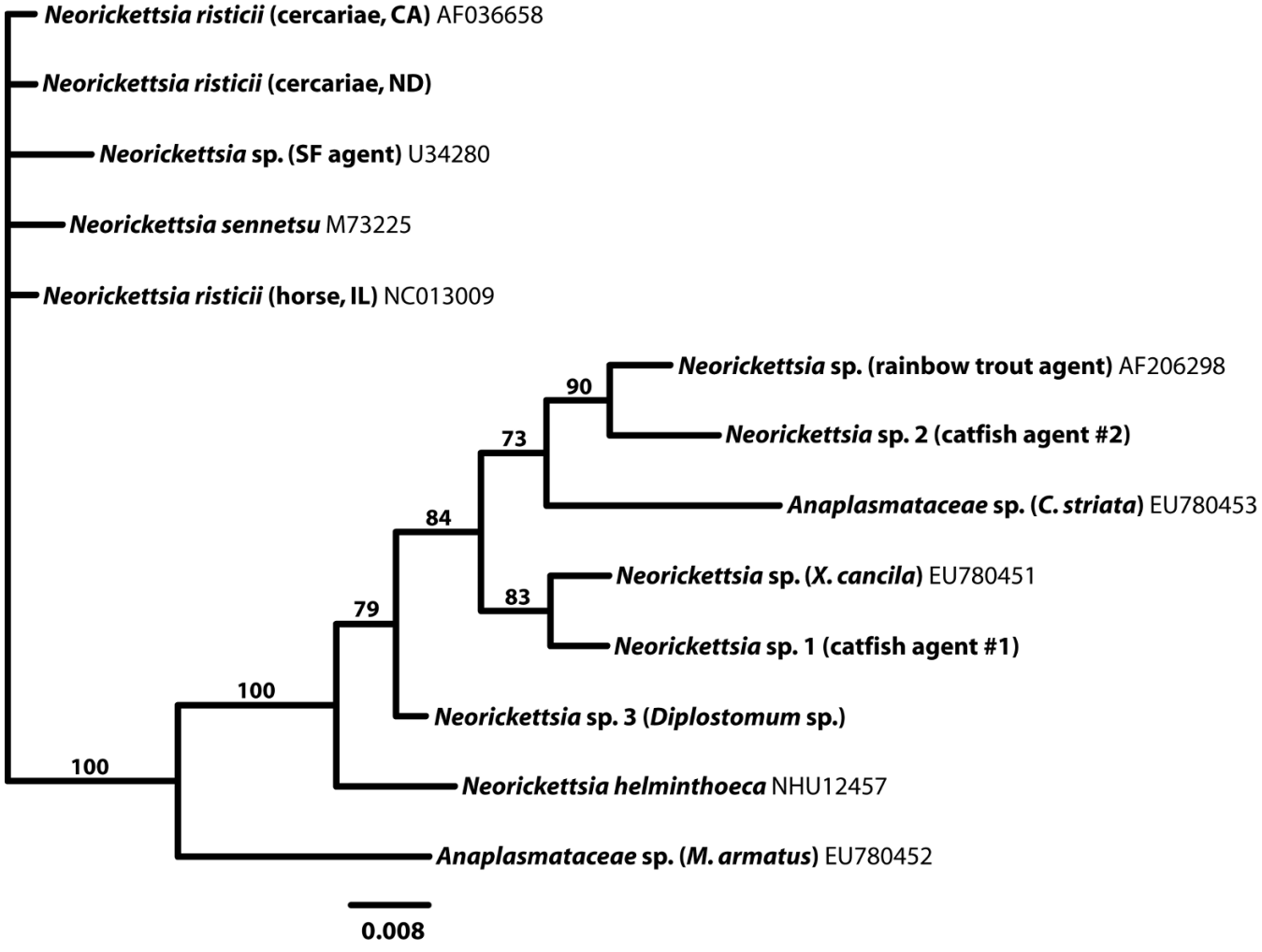

Fig. 2. Unrooted phylogenetic tree showing relationships among *Neorickettsia* spp. based on partial sequences of 16S rRNA gene. Numbers above nodes show posterior probabilities from Bayesian analysis (2,000,000 generations)

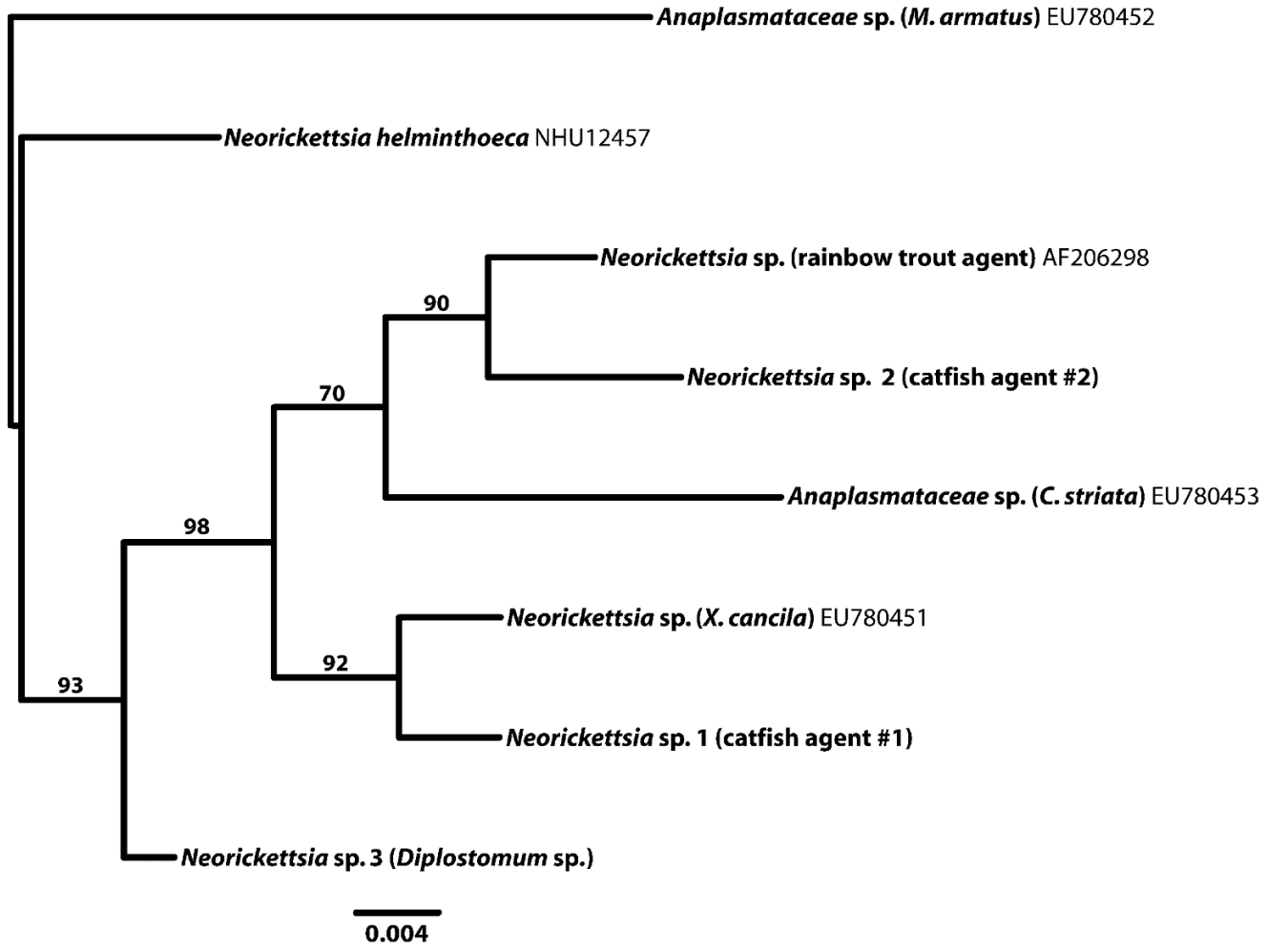

Fig. 3. Phylogenetic tree resulted from the analysis of the smaller dataset (the well supported clade from Fig. 1). Anaplasmataceae sp. from *M. armatus* from Seng *et al*. (2009) was used as an outgroup. Numbers above nodes show posterior probabilities from Bayesian analysis (2,000,000 generations)

smaller datasets. Bayesian analysis of the larger dataset produced a tree with *N. risticii* (three genotypes), *N. sennetsu* and SF agent appearing as independent branches in a polytomy while the remaining taxa (Fig. 2) formed a 100% supported clade with Anaplasmataceae sp. (from *M. armatus*) published by Seng *et al*. (2009) as the basal taxon. This clade also included *N. helminthoeca*, the "Rainbow trout agent", two additional forms of *Neorickettsia* reported by Seng *et al*. (2009) and 3 genotypes (*Neorickettsia* 1, 2, 3) obtained by us (Fig. 2). While this clade as a whole was well supported by the posterior probabilities values, the internal topologies received much less support with the exception of close relationship between the "Rainbow trout agent" and *Neorickettsia* sp. 2 ("catfish agent 2") sequenced in our study.

Analysis of the smaller dataset using Anaplasmataceae sp. (*M. armatus*) as an outgroup yielded a tree with stronger support for some of the internal branches, including the already mentioned clade "Rainbow trout agent"+"catfish agent 2" and *Neorickettsia* sp. (*X. canclia*)+"catfish agent 1".

Discussion

We are not providing the complete list of screened species because a negative result from screening individual specimens does not necessarily mean that other specimens of the same species cannot host neorickettsiae. Digeneans do not have any known co-dependency with their *Neorickettsia* endosymbionts and thus different individuals of the same species may or may not be infected.

Development of PCR-based diagnostic and sequencing techniques has made possible reliable detection of *Neorickettsia* at every step of their circulation, whether in the digeneans, or in their hosts. The same technology also allows for reliable identification of the digeneans, regardless of the stage of their development. We used this approach in our study to detect *Neorickettsia* and identify their cercarial hosts by matching their DNA sequences with those of adult forms.

Despite the small number of *Neorickettsia* isolates obtained in our study, 4 different genotypes were found. One of

these genotypes (4 isolates) corresponded to *N. risticii* while three others were different.

This level of diversity was somewhat unexpected because so far only *N.risticii* was known from the continental United States, with the notable exception of the Pacific Northwest where *N. helminthoeca*, Elokomin Fluke Fever and "Rainbow trout agent" occur (Philip *et al*. 1953, Philip 1955; Millemann *et al*. 1964; Farrell *et al*. 1973; Sakawa *et al*. 1973; Pusterla *et al*. 2000; Headley *et al*. 2011; Vaughan *et al*. 2012). One of our sequences (*Neorickettsia* sp. 3 from *Diplostomum* sp.) seems to be closest to *N. helminthoeca* (Fig. 3). This form as well as the "catfish agent 1" and the "catfish agent 2" may be considered candidates for potential new species. Unfortunately, the taxonomy and principles of differentiation among *Neorickettsia* species are currently far from being stable and are not universally accepted. Some formally named species of *Neorickettsia* show lower levels of divergence in the 16S ribosomal RNA sequence than do genotypes that are not named yet. For instance, a few lineages of *N. risticii*, *N. sennetsu* and SF agent differed by 2–3 bases only. At the same time, these species have been proven to possess different biological/immunological properties, pathogenicity and host specificity and their taxonomic separation is well justified. Future phylogenetic analyses and comparisons using longer sequences along with new data on the host specificity, distribution and pathogenicity will provide a more definite answer to the question of the taxonomic status of the new genotypes reported herein.

Our phylogenetic analysis has produced only one strongly supported cluster of forms/species containing Anaplasmataceae sp. (*M. armatus*) from Seng *et al*. (2009) as the basal taxon (Fig. 1), *N. helminthoeca*, and several yet unnamed lineages. Our analysis suggests that the two Anaplasmataceae that were considered by Seng *et al*. (2009) as likely new genera, probably belong to *Neorickettsia* and may deserve a status of new *Neorickettsia* species. The analysis of the smaller dataset provided somewhat better support for most branches and showed with some confidence that two "catfish agents" found in our study are related to two previously published lineages, namely the "Rainbow trout agent" from the United States and *Neorickettsia* sp. (*X. cancila*) from Cambodia. At the same time, our isolate from *Diplostomum* sp. does not appear to be very closely related to any of the currently published genotypes.

All 7 digenean species and all 7 genera detected as hosts of *Neorickettsia* in our study, represent new host associations for *Neorickettsia*. All these species and genera belong to different families (Table I). Members of the Dicrocoeliidae, Heronimidae, Macroderoididae and Gorgoderidae are reported here as hosts of *Neorickettsia* for the first time. Until now, members of only 10 digenean families have been detected as hosts of *Neorickettsia* worldwide and members of only 5 families were reported as hosts of these bacteria in the U.S. Thus, our study increased the number of the digenean families harboring *Neorickettsia* worldwide by 40% and almost doubled the number of digenean families playing this role in the U.S. This suggests that many more digenean taxa will be found to harbor *Neorickettsia* as a result of future surveys. All vertebrate host species listed in the Table I represent new vertebrate host associations for *Neorickettsia*-positive digeneans. Noteworthy, our finding of *Neorickettsia* in *Heronimus mollis* is the first record of these bacteria in digeneans parasitic in reptiles.

Our findings have revealed new pathways of *Neorickettsia* circulation in natural environment. Until now, most of the authors, at least in North America, reported *Neorickettsia* in association with lotic ecosystems. Report of *N. risticii* from lymnaeid snail *Stagnicola* (typically living in lentic habitats) in Oregon by Barlough *et al*. (1998) represents an exception to the "rule". Contrary to this tendency, 4 of our 7 isolates representing two different genotypes (*N. risticii* and *Neorickettsia* sp. 3, Table I) were obtained from ponds or small lakes. This suggests that *Neorickettsia* can be encountered in most types of freshwater ecosystems providing suitable habitat for the mollusks and definitive hosts of digeneans.

So far, *Neorickettsia* were reported from digeneans characterized by either fully aquatic (Rainbow trout agent) or aquatic/terrestrial (*N. helminthoeca*, *N. risticii*) life cycles. These particular life cycles involve an aquatic mollusk as the first intermediate host and an aquatic invertebrate or vertebrate as a second intermediate host. The definitive host would be either fish (Rainbow trout agent) or mammals and birds (*N. helminthoeca*, *N. risticii*). *Neorickettsia risticii* was previously reported only from digeneans with aquatic/terrestrial life cycles (Vaughan *et al*. 2012). However, we found *N. risticii* for the first time in two digenean species that possess fully aquatic life cycles. Definitive hosts of *Alloglossidium corti* are fish (typically some type of catfish) and the definitive hosts of *Heronimus mollis* are aquatic turtles. Moreover, for the first time for any *Neorickettsia* species anywhere we have found these endosymbionts in a dicrocoeliid digenean, *Conspicuum icteridorum*, that has a completely terrestrial life cycle and uses terrestrial gastropod mollusks and terrestrial arthropods as the first and second intermediate hosts (Patten 1952, Yamaguti 1975). Thus, our data suggest that the circulation of *Neorickettsia* is not restricted to aquatic ecosystems. This finding has implications on the epizootiology of Potomac horse fever and other neorickettsial diseases.

Although we do not know anything about the ability of new *Neorickettsia* genotypes discovered in our study to infect vertebrates, our data clearly indicate that *Neorickettsia* are more genetically divergent and taxonomically diverse than previously thought. This could be one of the potential reasons for Potomac horse fever vaccine failures (e.g., Vemulapalli *et al*. 1995, Dutta *et al*. 1998) and supports previously published conclusions regarding the immunological and genetic heterogeneity of what is considered a single species, *N. risticii* (Chaichanasiriwithaya *et al*. 1994, Gibson *et al*. 2011).

We have found two *Neorickettsia* genotypes in two digenean species infecting catfishes from different regions of the United States (Table I). These genotypes belong to different

clades within the genus (Figs 2 and 3) and are tentatively called catfish agents 1 and 2. These findings, in addition to already known "Elokomin fluke fever agent" and "Rainbow trout agent" suggest that digeneans of North American freshwater fish may harbor a more diverse *Neorickettsia s*pecies/genotype complex than currently appreciated. The "catfish agents" require additional studies in order to clarify their taxonomic rank. They also warrant attention in view of the fact that the channel catfish is a commercially important species consumed throughout the United States and elsewhere. Although the only human disease caused by *Neorickettsia* is currently known to occur only in Southeast Asia, our findings indicate that even in the United States neorickettsiae occur close to the human food chain and thus deserve further study.

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