



Assessing the Genetic Distinctiveness of *Cambarus (Puncticambarus) acuminatus*

A Recently Discovered Crayfish in Valley Creek within Valley Forge National Historical Park

Natural Resource Technical Report NPS/VAFO/NRTR—2013/752



ON THE COVER

Background image: Valley Creek, Valley Forge National Historical Park, near a roadside parking area, ~0.5 mi S. of SR23 on SR252 (40.09158, -75.45633, WGS-84).

Inset image: *Cambarus (Puncticambarus) cf. acuminatus* collected from the same locality as the background image.

Photographs by: James W. Fetzner Jr.

Assessing the Genetic Distinctiveness of *Cambarus (Puncticambarus) acuminatus*

A Recently Discovered Crayfish in Valley Creek within Valley Forge National Historical Park

Natural Resource Technical Report NPS/VAFO/NRTR—2013/752

James W. Fetzner Jr.

Section of Invertebrate Zoology
Carnegie Museum of Natural History
4400 Forbes Avenue
Pittsburgh, PA 15213-4080
(FetznerJ@CarnegieMNH.Org)

Keith A Crandall

Department of Biology
Brigham Young University
401 Widtsoe Building
Provo, UT 84602

Current Address:
Computational Biology Institute
The George Washington University
Innovation Hall
45085 University Drive
Ashburn, VA 20147

May 2013

U.S. Department of the Interior
National Park Service
Natural Resource Stewardship and Science
Fort Collins, Colorado

The National Park Service, Natural Resource Stewardship and Science office in Fort Collins, Colorado, publishes a range of reports that address natural resource topics. These reports are of interest and applicability to a broad audience in the National Park Service and others in natural resource management, including scientists, conservation and environmental constituencies, and the public.

The Natural Resource Technical Report Series is used to disseminate results of scientific studies in the physical, biological, and social sciences for both the advancement of science and the achievement of the National Park Service mission. The series provides contributors with a forum for displaying comprehensive data that are often deleted from journals because of page limitations.

All manuscripts in the series receive the appropriate level of peer review to ensure that the information is scientifically credible, technically accurate, appropriately written for the intended audience, and designed and published in a professional manner. This report received formal peer review by subject-matter experts who were not directly involved in the collection, analysis, or reporting of the data, and whose background and expertise put them on par technically and scientifically with the authors of the information.

Views, statements, findings, conclusions, recommendations, and data in this report do not necessarily reflect views and policies of the National Park Service, U.S. Department of the Interior. Mention of trade names or commercial products does not constitute endorsement or recommendation for use by the U.S. Government.

This report is available from the Natural Resource Publications Management website (<http://www.nature.nps.gov/publications/nrpm/>).

Please cite this publication as:

Fetzner Jr., J. W., and K. A. Crandall. 2013. Assessing the genetic distinctiveness of *Cambarus (Puncticambarus) acuminatus*: A recently discovered crayfish in Valley Creek within Valley Forge National Historical Park. Natural Resource Technical Report NPS/VAFO/NRTR—2013/752. National Park Service, Fort Collins, Colorado.

Contents

	Page
Figures.....	v
Tables.....	vii
Appendices.....	vii
Acknowledgments.....	vii
Abstract	ix
Introduction.....	1
Background.....	1
Taxonomic Problems	3
Materials and Methods.....	7
Specimen Acquisition.....	7
Molecular Analyses	11
DNA Extraction	11
PCR Amplification.....	11
DNA Sequencing	12
Genetic Data Analysis.....	12
Results.....	13
<i>Cambarus acuminatus</i>	13
Pennsylvania Populations	13
The <i>C. acuminatus</i> Species Complex.....	19
Syntype MCZ #3624.....	20
Relationships Among Members of the Subgenus <i>Puncticambarus</i>	21

Contents

	Page
Discussion	23
Status of Pennsylvania Populations	24
A New Species?	25
Conclusions and Future Directions	27
Literature Cited	29

Figures

	Page
Figure 1. Sampling localities (flag icons) and topography of Valley Creek in Valley Forge National Historical Park (green shaded area).	2
Figure 2. Illustration of female specimen of <i>Cambarus acuminatus</i> from Plate III, Fig. 5, of Faxon (1885) showing the acuminate rostrum (r) and the cervical (=“branchiostegian”) spines (cs).	4
Figure 3. A) Syntype (MCZ 3624) of <i>Cambarus acuminatus</i> from the Museum of Comparative Zoology at Harvard showing the extremely poor condition of the specimen and B) its associated specimen data labels.	5
Figure 4. Gonopod (1 st pleopod) of a Form II (juv) male of <i>Cambarus acuminatus</i> . A) illustrations found in Faxon (1885; Plate VII, Figs. 6a, 6a’), B) photographs of the gonopod housed with MCZ specimen #3624 and C) its associated data labels.	6
Figure 5. Locations where collections were made for A) members of the <i>Cambarus</i> subgenus <i>Puncticambarus</i> and B) for taxa used as outgroups.	8
Figure 6. Map showing locations in Pennsylvania in and around Valley Forge National Historical Park where samples of <i>C. acuminatus</i> were collected for this study (see also Fetzner 2010).	14
Figure 7. Mean estimates of catch per unit effort ($\pm$ SE) for Pennsylvania populations of <i>C. acuminatus</i> based on collections made in 2005 (Valley Creek) and 2009 (Pickering, Darby, Crum creeks) (see Fetzner 2010).	15
Figure 8. Neighbor-joining tree showing relationships among the 170 unique COI haplotypes found in this study.	17
Figure 9. Maps showing the distribution of mtDNA COI gene haplotype clades that include presumed <i>C. acuminatus</i> samples or related species, as depicted in the phylogeny in Figure 8.	18
Figure 10. Image of an agarose gel showing attempts at amplification of COI gene fragments of 50, 100, and 150 base pairs for three samples extracted from the <i>C. acuminatus</i> Syntype (#’s 1-3), a positive control (+) from the South Saluda River population, and a negative control (-).	20

Tables

	Page
Table 1. Species, number of specimens and locality data for samples included in this project.	9
Table 2. Population sizes (Θ) (in bold along diagonal) and symmetric migration rates among populations estimated using the program Migrate-n for A) Clade A, subgroup A1 and B) Clade F, subgroup F2.	15

Appendices

	Page
Appendix A. List of haplotypes by population.	33
Appendix B. Average pairwise divergences (= number of mutations) within and among populations (two pages).	37
Appendix C. Summary of the 43 haplotype networks recovered from the TCS analysis.....	39

Acknowledgments

We kindly thank R. Thoma for his assistance in the field while collecting specimens for this project and M. Leary for laboratory assistance in setting up DNA sequencing reactions. We also thank J. Kilian for his help in collecting the Maryland populations, and staff at Valley Forge National Historical Park for collecting and sending a batch of crayfish from Valley Creek for analysis.

Abstract

This investigation into a newly discovered crayfish from Valley Creek, Valley Forge National Historical Park (Chester/Montgomery counties, Pennsylvania; ~30 km NW of Philadelphia), was undertaken in order to help resolve long-standing taxonomic questions related to the *Cambarus (Puncticambarus) acuminatus* Faxon (1884) species complex, and to help determine the conservation status and geographic origin of Pennsylvania populations assignable to this taxon. The crayfish considered to be a part of this species complex currently range from southeastern Pennsylvania (Valley Creek) in the north, southward to the Saluda River drainage in South Carolina. Morphologically, members of this complex are quite variable across their range, with many populations often lacking the prominent cervical spines Faxon (1884) mentions in his original species description. Taxonomically, the species is difficult to delineate because the type specimens are either lost or degraded to the point where they have no distinguishing characteristics, and is further complicated because the type locality apparently no longer exists; therefore, characterizing which populations of the species complex are most similar to the original type population described by Faxon (1884) cannot currently be determined with available materials, or by recollecting from the type locality. In this report, we attempt to use molecular methods (mitochondrial COI gene sequences) to examine variation from throughout the range of the species complex in order to delimit groups of related populations in the hopes that these data may shed light on the relationships in this group of crayfish and help us to understand their evolutionary history and conservation status. Our results show that the Pennsylvania populations that were sampled (Valley, Pickering and Darby creeks) are closely related to one another, and shared several haplotypes that are unique to Pennsylvania. Interestingly, these Pennsylvania populations appeared to be most closely related to populations sampled from Virginia than they were to the more geographically proximal Maryland populations. DNA sequence divergences averaged 2.4% between PA and VA populations, suggesting that the PA populations share a recent common ancestry with central VA (although some intervening areas were not sampled). The Pennsylvania populations were divergent from what we consider to be the type population from the Saluda River, South Carolina. The magnitude of the divergence suggests they are a different species from “true” *C. acuminatus*, but the current taxonomic confusion in the genus *Cambarus* prevents us from defining which species it might represent. In addition, we also identified several other genetically divergent groups in the *C. acuminatus* complex, indicating that it harbors higher genetic variation than previously thought. Further studies are needed to test if these newly discovered lineages might represent additional undescribed species. We also recommend that additional sampling and analyses should be conducted in order to help sort out the taxonomic issues in this group of crayfish.

Introduction

Background

Valley Forge National Historical Park (VAFO) is a 1,400-ha park located in southeastern Pennsylvania and situated approximately 30 km NW of Philadelphia. The main stream in the park, Valley Creek, flows from southwest to northeast along the western edge of the park boundary and forms part of the border between Chester and Montgomery counties (Figure 1). The Valley Creek watershed encompasses approximately 60 km², with the lower 3.2 km of the stream flowing through VAFO to its confluence with the Schuylkill River. Valley Creek has been designated an “exceptional value” watershed, which garners the highest level of protection from the Pennsylvania Department of Environmental Protection (PA DEP). It has also been classified as a “Class A” wild trout fishery, making it an important stream for local fishermen and prime habitat for aquatic organisms, such as crayfish.

A survey of Valley Creek was conducted during the 2003 field season (see Lieb et al. 2007, Lieb et al. 2008), and it was discovered that the majority of the crayfish specimens collected from the creek were assignable to *Cambarus (Puncticambarus) acuminatus*, which previously had not been recorded from the state of Pennsylvania (Lieb et al. 2007, Lieb et al. 2008). They did note, however, that specimens had been collected three years earlier by Jan Briede (Sciencetech, NES, Inc.) and Jamie Krejsa (Enviroscience, Inc.), and that these specimens had been identified by Roger Thoma (The Ohio State University, Museum of Biological Diversity) as belonging to the *Cambarus acuminatus* complex. Thus, this was the first documented case of *C. acuminatus* occurring north of the Patapsco River drainage (Maryland). Subsequent sampling in streams in the region surrounding VAFO located several other Pennsylvania populations of this species (Lieb et al. 2011a, Lieb et al. 2011b), and Fetzner (2010) reconfirmed the presence of the species at those same sites.

Historically, collections were made in southeastern PA by Arnold Ortmann (1906), who lists specimens of *Cambarus bartonii* as occurring in the vicinity of the town of Valley Forge (the exact location is listed as “Chester County: Valley Forge”, pg. 381). No mention was made of *C. acuminatus* by Ortmann in his 1906 work. On the same page, he also mentions another collection from the region that was made from “Montgomery County: West Manayunk (H. Gera, coll.)” which also contained *C. bartonii*. Carnegie Museum accession records indicate that Hugo Gera donated specimens that were collected from the Schuylkill River at Manayunk (in 1904) and Domino Lane Run (Roxboro, PA; in 1905). Unfortunately, the exact location of most of Ortmann’s collections remain ambiguous (either no specific stream names were given or detailed location information on which stream reaches were visited is lacking), so it is difficult to determine exactly where these collections were made. It appears that most records from this area may have come from larger rivers, and this might explain why *C. acuminatus* may have been missed, if it was indeed present at that time. The species was not detected by Lieb et al. (2005) in the mainstem of the Schuylkill River near Valley Forge during their recent survey, so it is likely that this species prefers smaller streams. As a result, the available historical records cannot really help us determine the past history of the Pennsylvania *C. acuminatus* populations, including those from VAFO.

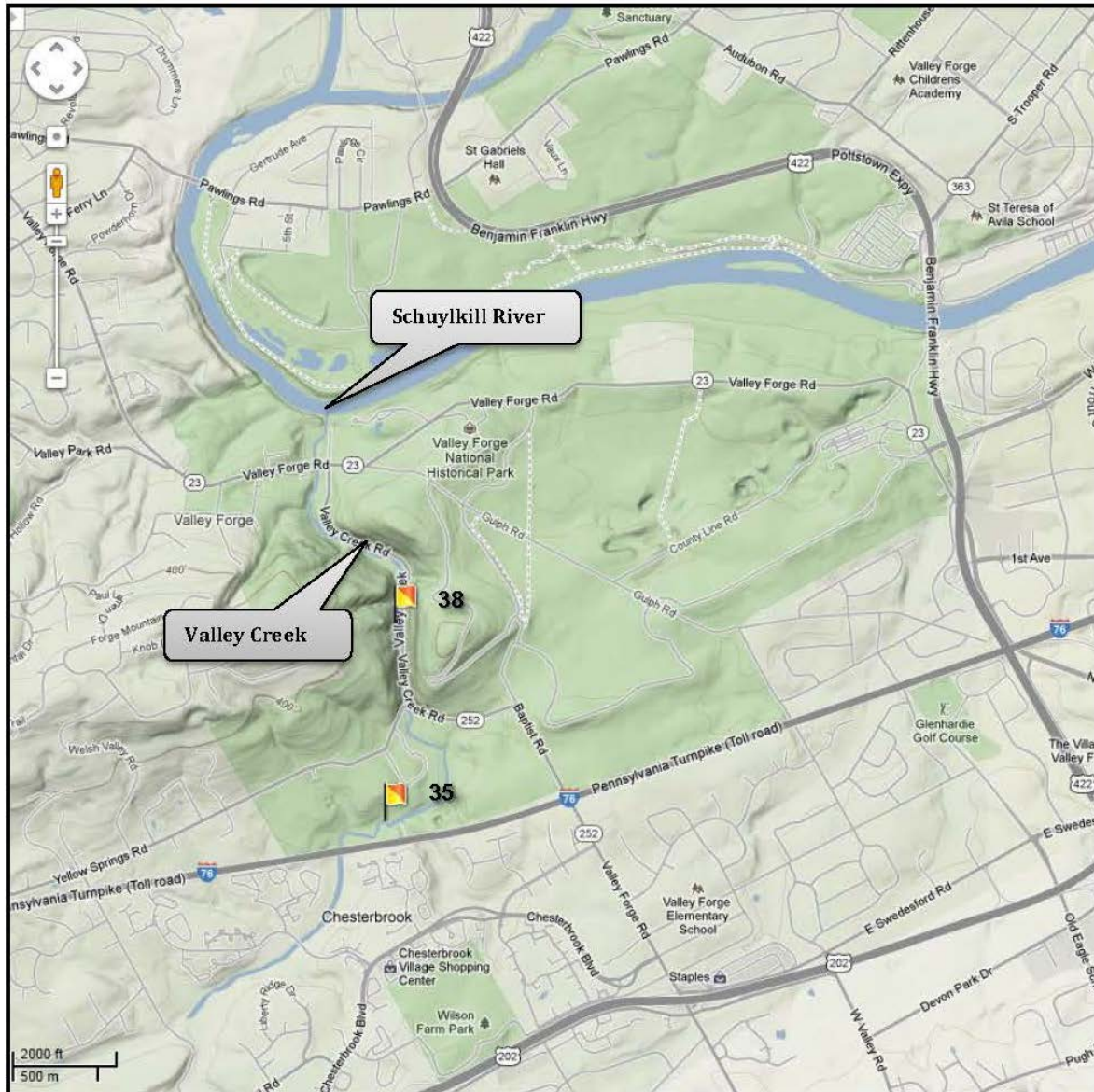


Figure 1. Sampling localities (flag icons) and topography of Valley Creek in Valley Forge National Historical Park (green shaded area). Numbers by flags indicate the population ID number from Table 1

Cambarus (Puncticambarus) acuminatus Faxon 1884 is currently a geographically widespread species that is thought to represent a species complex (Hobbs 1989). There is a significant amount of variation seen in morphological characteristics among populations of this species throughout its range, and indeed, this variation lead Hobbs (1989) to state that “Even in the Santee Cooper Basin (of which the Saluda River is a tributary), South Carolina, more than one rather distinct ‘variant’ is recognizable.” John Cooper of the North Carolina Museum of Natural Sciences is the current authority on this group of crayfish. He has been studying them for the past several decades, and a few years ago he described several of the more distinctive North Carolina morphs as new species (e.g., Cooper 2001, Cooper and Cooper 2003). However, *C. acuminatus* is very difficult to work with for various taxonomic reasons (see below); this has led Dr. Cooper

to list North Carolina populations of the *C. acuminatus* complex as *Cambarus* sp. C. Dr. Cooper has examined specimens from the Valley Creek population, and he is of the opinion that they are morphologically distinct from the southern populations in this complex (John Cooper 2010, personal communication).

This study was initiated because several questions about the Pennsylvania *C. acuminatus* populations were unanswered, including: 1) are Pennsylvania populations of *C. acuminatus* native to the state, or are they the result of a previously undetected introduction from somewhere else (i.e., bait bucket introduction or similar human-aided movement)? 2) If they are an introduction, can the likely source population be identified? 3) If these populations are native, do they represent the same taxonomic entity as that originally described by Faxon (1884), or are they a new undescribed species? and 4) what is the conservation status of these populations?

The purpose of this project was to try to answer these questions by using molecular (= DNA) methods; specifically, by examining sequence variation in the mitochondrial COI gene. This gene has been used previously to help delimit geographic groups in other crayfish species, so it was thought that this gene would be a good choice for an initial assessment of the geographic spread of genetic variation in *C. acuminatus*. The results of this project are made even more urgent because the rusty crayfish (*Orconectes rusticus* [Girard 1852]), an aggressive invasive species, started appearing in Valley Creek in 2008. This alien species has invaded the Susquehanna River drainage and has displaced native crayfish species such as *Orconectes obscurus* and *O. limosus* from the mainstem and many tributaries in this watershed. Therefore, its presence in Valley Creek is a great concern because a potential new species to science may be wiped out before it can be formally named and protected. This invasion has lead VAFO scientists to institute the “Crayfish Corps” which allows volunteers to remove the invasive “rusties” from Valley Creek in order to preserve the native crayfish.

Taxonomic Problems

Walter Faxon (1884) first described *Cambarus acuminatus* from three specimens from the designated type locality of “Saluda River west of Greenville, South Carolina” (he then illustrated specimens in Faxon 1885; Figure 2).

“Rostrum long, tapering, ending in a long, sharp acumen, without lateral spines; upper surface smooth, somewhat hollowed out, margins punctate, ciliate, raised into low sharp crests. Post-orbital ridges with sharp anterior spines. Carapace smooth, punctate, granulated on the sides, cervical groove sulcate, sinuate; a sharp lateral and branchiostegian spine; sub-orbital angle rounded; an irregular indentation on the side of the carapace, below the lateral spine, on the hepatic region and anterior part of the branchial region; areola broad, smooth, punctate, less than one half as long as the distance from the tip of the rostrum to the cervical groove. Telson bispinose on each side. Epistoma triangular, angles rounded. Second and third segment of the antennae with a strong sharp spine; scale of moderate length, rather broad, inner margin rounded, outer margin thick, turned outwards at the tip. Third maxillipeds hairy within. Chela moderate, punctate, serrato-tuberculate on internal border, fingers setose on their inner margins, external border of outer finger submarginate. Carpus armed with a strong internal spine and smaller inferior median and external spines. Meros with well-developed biserial spines below and two obliquely placed near the distal end of the superior border. In some specimens one of the superior pair is obsolete. Third pair of legs hooked. First pair of abdominal appendages as in *C. Bartonii*. Length, 48 mm. Carapace, 23 mm. Rostrum, 6 mm. Areola, 7 mm. Breadth of areola, 2 mm.”

“Saluda River, west of Greenville, S.C. Collected by Prof. D. S. Jordan. Three specimens, one male of the second form, two females. For the opportunity to examine these I am indebted to Prof. O. P. Hay of Butler University, Irvington, Ind. Differs from the other species of the *C. Bartonii* group by its long, gradually tapering rostrum, short metacarapace, strongly developed spines of carapace, antennae, and meros. The acumen of the rostrum is scarcely upturned at the tip.”

“Specimens from North Carolina, Old Fort, McDowell Co., and French Broad River, (in Mus. Comp. Zool. and Acad. Nat. Sci. Phila.), differ from the above in having the rostrum flatter and less attenuated at the tip, a shorter antennal scale, sub-orbital angle produced into a sharp spine. These may prove to be a distinct species from the Saluda River specimens. They approach *C. robustus*, but may be distinguished from that species by the longer-pointed rostrum, shorter metacarapace, better-developed spines, etc.”

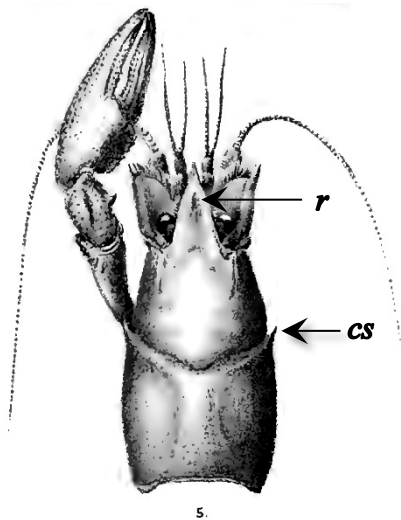


Figure 2. Illustration of female specimen of *Cambarus acuminatus* from Plate III, Fig. 5, of Faxon (1885) showing the acuminate rostrum (*r*) and the cervical (=“branchiostegian”) spines (*cs*).

This was amended by Hobbs (1989) as “Saluda River, at Farr’s Mills, west of Greenville, Greenville County, South Carolina.” A place name called “Farr’s Mills” cannot be located, however, on any current map and does not appear in the Geographic Names Information System, so the exact location of the type locality is unknown, and probably no longer exists, as the Saluda River west of Greenville was dammed in order to form a 331-ac lake (Saluda Lake) back in 1905. We have noted, however, that there is a “Farrs Bridge Road” that crosses the northern portion of Saluda Lake (and the Saluda River), so perhaps this may be related in some way to the “Farrs Mills” mentioned in the type locality description given by Hobbs. Perhaps the mill was located at the intersection of where this road crosses the river, but this is pure speculation.

In addition to ambiguity in the location of the type locality for the species, there are problems with examining the type specimens as well. Hobbs (1989) indicated that the “Cotypes” formerly said to have been at Butler University (Faxon 1914: 424) were no longer there (i.e., have been lost). The remaining Syntype (MCZ 3624), presumably a female based on the labels, housed in the Museum of Comparative Zoology at Harvard collection, has been examined by us, but is completely disintegrated and is essentially unusable for any morphological comparisons (see Figure 3). Also housed with this specimen is a male Form II gonopod that was apparently illustrated in Faxon’s (1885) “Revision of the Astacidae” [Plate VIII, 6a, 6b in that work](see also Figure 4). Unfortunately, it is the male Form I gonopod that is useful in identifying crayfish specimens to species and not the Form II. The annulus ventralis of the female was not described or figured by Faxon (1885). Recent sampling conducted by one of us (JWF) at several sites in the Saluda River west of Greenville only turned up specimens that are assignable to *Cambarus bartonii*.

Given that the type specimens are missing or destroyed (so there are no comparative specimens available that can help us determine what truly represents *Cambarus acuminatus*), and the location of the type locality is ambiguous, we are only left with the original description of Faxon (1884), along with some other details.

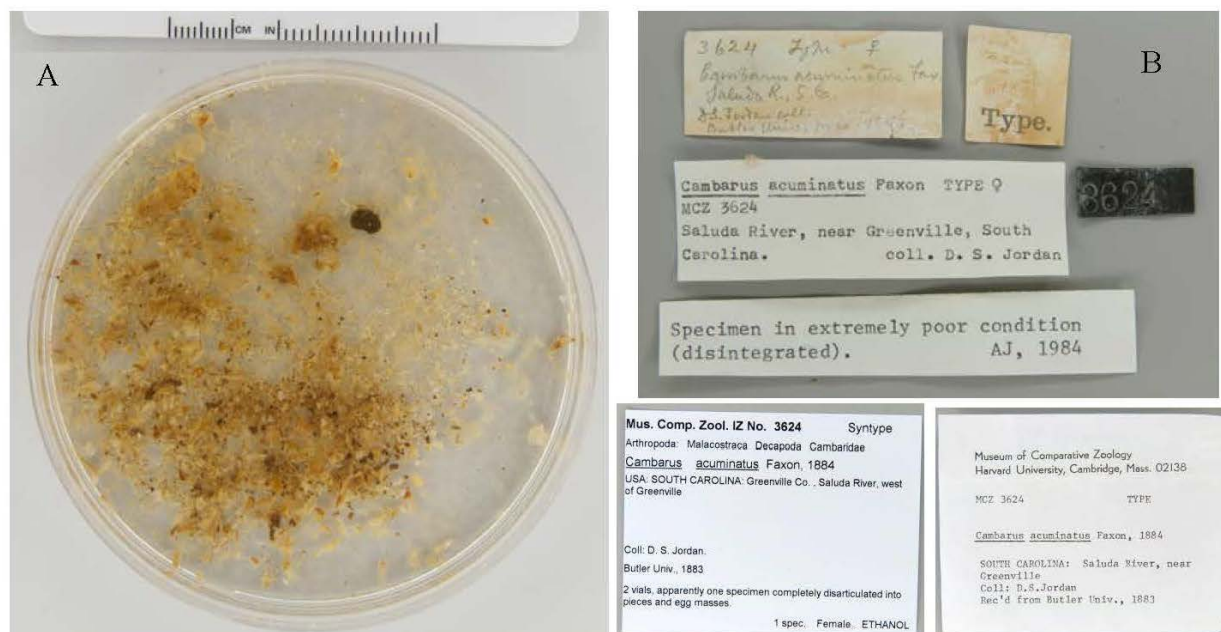


Figure 3. A) Syntype (MCZ 3624) of *Cambarus acuminatus* from the Museum of Comparative Zoology at Harvard showing the extremely poor condition of the specimen and B) its associated specimen data labels.

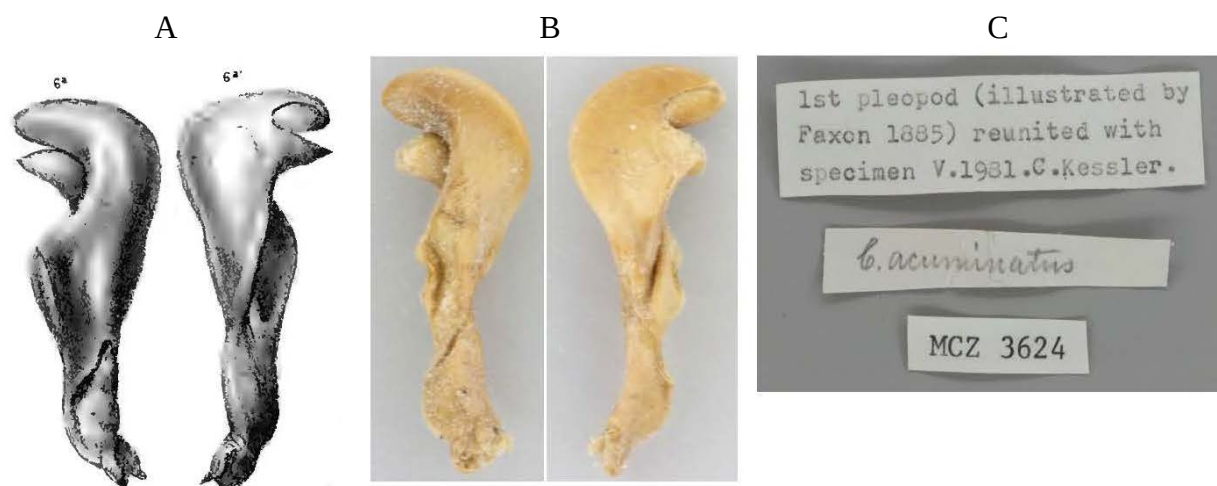


Figure 4. Gonopod (1st pleopod) of a Form II (juv) male of *Cambarus acuminatus*. A) illustrations found in Faxon (1885; Plate VII, Figs. 6a, 6a'), B) photographs of the gonopod housed with MCZ specimen #3624 and C) its associated data labels.

Materials and Methods

Specimen Acquisition

Crayfish specimens were collected from throughout the known range of *Cambarus acuminatus* in order to get the broadest representation of possible genetic variation from the species complex as possible (Figure 5). We felt that it was important to sample broadly from the southern end of the range, as the type locality occurs in this region. However, we also tried to include samples from as many places as we could. The project, as originally proposed, would attempt to examine a total of 600 specimens. In total, we collected 932 specimens (see Table 1) for the project and were able to obtain both an amplified PCR product and an mtDNA COI sequence from 625 of them. A good portion of the failed samples also seemed to amplify to varying degrees, but either did not generate readable sequences (i.e., peaks on top of peaks) or just completely failed. Others produced good readable sequence, but were very divergent from other sequences obtained, and were later found to be bacterial in origin, based on Genbank BLAST searches (although no close bacterial match was found – ~80% match). It could be that additional attempts at PCR and sequencing of the failed, non-bacterial, samples may produce viable sequences in some cases that could be used in future analyses, and this would help to expand the geographic coverage of the dataset before final publication in a peer reviewed journal; however, the timing of this report and a lack of available funds for further laboratory analyses did not allow us to attempt these re-amplifications before submission.

In total, 376 presumed *Cambarus* (*Puncticambarus*) *acuminatus* specimens were sequenced from 41 different populations (Table 1). Specimens were also collected and included in the analysis for 17 of the 21 species that currently comprise the subgenus *Puncticambarus* (212 samples from 27 sites) in order to examine and compare levels of genetic variation within and among populations of these species (i.e., does *C. acuminatus* show similar levels of variation?), as well as variation among the species. This also allows us to generate a molecular phylogeny for the subgenus and look for evolutionary patterns and relationships, and should give a solid framework that can be used later to map morphological variation seen in the group. A few other specimens from other *Cambarus* subgenera, including: *Cambarus* (*Cambarus*) *bartonii* (four sites, n=20), *Cambarus* (*Depressicambarus*) sp. (two sites, n=3), *Cambarus* (*Hiaticambarus*) *longulus* (one site, n=3), *Cambarus* (*Jugicambarus*) *dubius* (n=1), and *Cambarus* (*J.*) sp. (one site, n=8), as well as specimens from different genera, including: *Orconectes* (*Faxonius*) *limosus* (n=1) and *Procambarus* (*Ortmannicus*) *zonangulus* (n=1), were also included as outgroups and to help identify specimens (based on their grouping in the phylogeny) that may have been misidentified in the field.

The majority of collected specimens were sampled for tissues (usually a leg) and then retained as vouchers in 85% ethanol and are currently housed in the CMNH crayfish collection. Since this complex is quite variable, specimens from multiple localities will be needed for later morphological analyses and for descriptions of possible new species that may result from the project.

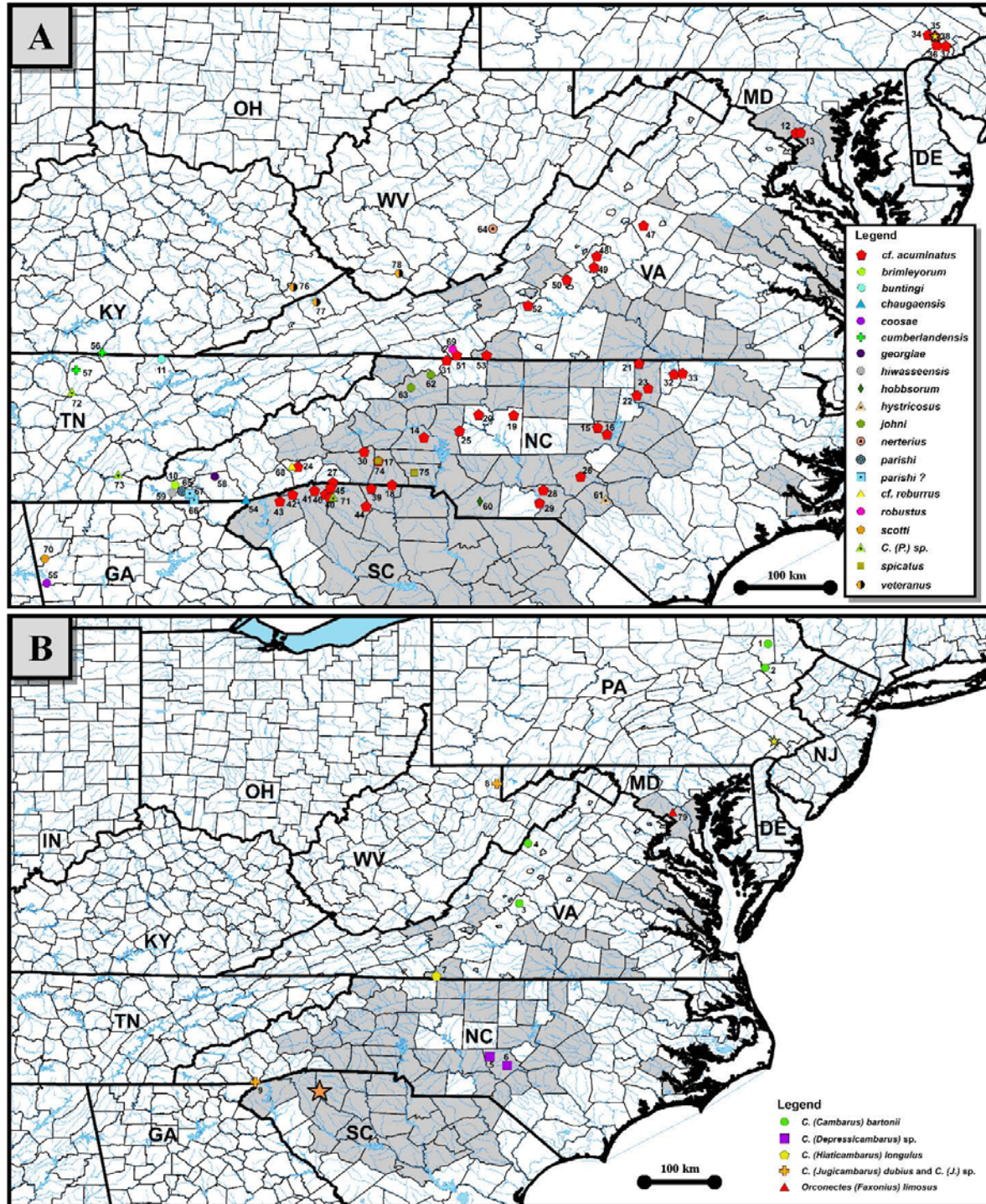


Figure 5. Locations where collections were made for A) members of the *Cambarus* subgenus *Puncticambarus* and B) for taxa used as outgroups. Note that the locality for *P. zonangulus* is not depicted on these maps. The yellow star in SE Pennsylvania indicates the location of Valley Forge NHP (VAFO). Gray shaded counties indicate historical county records based on *C. acuminatus* specimen localities contained in the USNM crayfish database. Note that these records may not accurately reflect the actual distribution of the species, either historically or currently, but are used to depict the general distribution. Numbers by map markers are population ID numbers from Table 1. Orange star indicates approximate location of the *C. acuminatus* type locality.

Table 1. Species, number of specimens and locality data for samples included in this project. “N” indicates the number of specimens that were sampled from the population, while “n” indicates the number of specimens that were successfully PCR amplified and sequenced.

ID	Genus	Subgenus	Species	N	n	State	County	River	Locality Data	Lat	Lon
1	Cambarus	Cambarus	bartonii	5	5	PA	Lackawanna	Lackawanna River	at bridge on Delaware St., 0.63 km S Jermyn	41.52531	-75.54688
2	Cambarus	Cambarus	bartonii	15	5	PA	Lackawanna	Lehigh River	at bridge on SR-4003 (Locust Ridge Rd.), 1 km SW Thornhurst	41.17239	-75.58888
3	Cambarus	Cambarus	bartonii	4	4	VA	Amhearst	North Fork Buffalo River	at bridge on SR-635 (North Fork Rd.), ~0.1 mi N of junct w/US-60	37.68073	-79.22218
4	Cambarus	Cambarus	bartonii	6	6	VA	Rockingham	Dry River	at bridge along US-33, ~5 mi E of VA and WV state line and 18 mi WNW of Harrisonburg	38.56967	-79.09518
5	Cambarus	Depressicambarus	sp.	2	2	NC	Moore	Cabin Creek	at bridge on Pine Grove Church Rd., 7.52 km WSW Robbins	35.40203	-79.66008
6	Cambarus	Depressicambarus	sp.	1	1	NC	Moore	Little River	at bridge on McCaskill Rd., 2.96 km WNW Whispering Pines	35.26683	-79.40198
7	Cambarus	Hiaticambarus	longulus	3	3	VA	Patrick	Dan River	at bridge on SR-773, 2.79 km WNW Claudville	36.59678	-80.44852
8	Cambarus	Jugicambarus	dubius	1	1	VA	Preston	from burrow	Hillside seep at cemetary at Terra Alta (TYPE LOCALITY), North side of Terra Alta, SR-7	39.45269	-79.55266
9	Cambarus	Jugicambarus	sp.	9	8	NC	Jackson	Scottsman Creek	at bridge on SR-1100 (Bullpen Rd.), 8.9 km SE Highlands	35.02060	-83.11385
10	Cambarus	Puncticambarus	brimleyorum	20	6	NC	Cherokee	Valley River	at roadside pulloff on SR-1515 (Fairview Rd.), 0.9 km E Marble	35.17321	-83.91618
11	Cambarus	Puncticambarus	buntingi	7	6	TN	Campbell	No Business Creek	at US-25W/SR-9	36.55162	-84.06752
12	Cambarus	Puncticambarus	cf. acuminatus	18	18	MD	Montomery	Northwest Branch	at bridge on US-29 (Columbia Pike-Colesville Rd.), 1.7 km SW White Oak	39.03025	-77.00627
13	Cambarus	Puncticambarus	cf. acuminatus	21	21	MD	Prince Georges	Paint Branch	at Powdermill Community Park on SR-212 (Powdermill Rd.), 0.8 km NE Hillandale	39.03235	-76.00627
14	Cambarus	Puncticambarus	cf. acuminatus	20	14	NC	Catawba	McLin Creek	at bridge on SR-1722 (Bethany Church Rd.), 1.9 km SSE Claremont	35.69759	-81.14567
15	Cambarus	Puncticambarus	cf. acuminatus	24	11	NC	Chatham	Dry Creek	at bridge on Old Graham Rd., 7.82 km SE Mandale	35.80342	-79.21136
16	Cambarus	Puncticambarus	cf. acuminatus	2	2	NC	Chatham	Unnamed Trib. to Haw River	at bridge on US-64, at end of parking lot, 6.45 km E Pittsboro	35.73043	-79.10698
17	Cambarus	Puncticambarus	cf. acuminatus	8	1	NC	Cleveland	Hinton Creek	at bridge on SR-226, 2.28 km NNW Polkville	35.43660	-81.65263
18	Cambarus	Puncticambarus	cf. acuminatus	7	5	NC	Cleveland	Lick Branch	Lick Branch at bridge on Rippy Rd., 3.7 km SE Earl	35.17678	-81.50062
19	Cambarus	Puncticambarus	cf. acuminatus	15	1	NC	Davidson	Abbotts Creek	at bridge on SR-1800 (Midway School Rd.), 6.65 km ESE Midway	35.93832	-80.14664
20	Cambarus	Puncticambarus	cf. acuminatus	20	13	NC	Davie	Dutchmans Creek	at bridge on US-158, 6.27 km NNE Mocksville	35.94675	-80.53623
21	Cambarus	Puncticambarus	cf. acuminatus	20	13	NC	Granville	Aarons Creek	at bridge on SR-96, 4.77 km SSE Virgilina, VA	36.50510	-78.75054
22	Cambarus	Puncticambarus	cf. acuminatus	21	6	NC	Granville	Knap of Reeds Creek	at bridge on Range Rd., 3.26 km NNW Butner	36.39884	-78.26655
23	Cambarus	Puncticambarus	cf. acuminatus	15	15	NC	Granville	Tar River	at bridge on Belltown Rd., 10.19 km SSW Oxford	36.23215	-78.64977
24	Cambarus	Puncticambarus	cf. acuminatus	20	12	NC	Henderson	Boylston Creek	at bridge on SR-1314 (Ladson Rd), 2.16 km SE Mills River	35.37545	-82.54896
25	Cambarus	Puncticambarus	cf. acuminatus	20	17	NC	Iredell	Fourth Creek	at bridge on Elmwood Rd., 12.52 km E Statesville	35.76849	-80.74993
26	Cambarus	Puncticambarus	cf. acuminatus	15	15	NC	Moore	Little River	at bridge on McCaskill Rd., 2.96 km WNW Whispering Pines	35.26683	-79.40198
27	Cambarus	Puncticambarus	cf. acuminatus	14	4	NC	Polk	North Pacolet River	North Pacolet River at bridge on SR-1501 (Hunting Country Rd.), 6.2 km SE Columbus	35.20481	-82.15884
28	Cambarus	Puncticambarus	cf. acuminatus	18	16	NC	Richmond	Big Mountain Creek	at bridge on SR-73, 7.27 km NW Ellerbe	35.11958	-79.81627
29	Cambarus	Puncticambarus	cf. acuminatus	8	1	NC	Richmond	Carledges Creek	at bridge on Blewitt Falls Rd., 8.72 km WNW Rockingham	34.97736	-79.85773
30	Cambarus	Puncticambarus	cf. acuminatus	20	1	NC	Rutherford	Little First Broad River	at bridge on SR-1700 (Cane Creek Mountain Rd.), 23.12 km SW Rutherford	35.54016	-81.81084
31	Cambarus	Puncticambarus	cf. acuminatus	5	1	NC	Surry	Gully Creek	off Goodson Rd. (SR-1420)	36.54022	-80.89247
32	Cambarus	Puncticambarus	cf. acuminatus	20	20	NC	Vance	Anderson Creek	at bridge on Mabrey Mill Rd., 3.83 km WSW Middleburg	36.38835	-78.36270
33	Cambarus	Puncticambarus	cf. acuminatus	20	10	NC	Warren	Fishing Creek	at bridge on SR-1100 (Manson-Axtell Rd.), 1.2 km SSE Soul City	36.39884	-78.26655
34	Cambarus	Puncticambarus	cf. acuminatus	20	7	PA	Chester	Pickering Creek	at bridge on SR-29 (State Rd.), 3.7 km SSW Phoenixville	40.10157	-75.53585
35	Cambarus	Puncticambarus	cf. acuminatus	20	9	PA	Chester	Valley Creek	VAFO Section 4 between iron birdge and buffer 45, 2.06 km SSE Valley Forge	40.08116	-75.45701
36	Cambarus	Puncticambarus	cf. acuminatus	4	0	PA	Chester/Delaware	Crum Creek	at bridge and roadside pulloff on Boot Rd. at intersection with Goshen Rd., 0.4 km WSW Echo Valley	39.98972	-75.43630
37	Cambarus	Puncticambarus	cf. acuminatus	8	5	PA	Delaware	Darby Creek	at roadside pulloff on SR-1006 (Darby Creek Rd.), 0.92 km SW Brookthorpe Hills	39.98350	-75.33768
38	Cambarus	Puncticambarus	cf. acuminatus	16	4	PA	Mongomery	Valley Creek	at roadside pulloff, ~0.6 mi S of SR-23 on SR-252 (~0.2 mi N of covered bridge)	40.09158	-75.45633
39	Cambarus	Puncticambarus	cf. acuminatus	20	19	SC	Cherokee	Cherokee Creek	along Mill Gin Rd., at mill parking area, 10.2 km NW Gaffney	35.13655	-81.73161
40	Cambarus	Puncticambarus	cf. acuminatus	15	6	SC	Greenville	Barnes Creek	at bridge on SR-14 (Cockrell Rd.), 9.6 km SW Campobello	35.07448	-82.24356
41	Cambarus	Puncticambarus	cf. acuminatus	10	7	SC	Greenville	Barton Creek	at roadside pulloff on SR-42, 18 km NNE Travlers Rest	35.11435	-82.36269
42	Cambarus	Puncticambarus	cf. acuminatus	20	20	SC	Greenville	South Saluda River	at bridge on SR-11 (Cherokee Foothills Scenic Hwy)(at Greenville/Pickens County Line), 10.7 km NW Slater	35.07099	-82.60734
43	Cambarus	Puncticambarus	cf. acuminatus	13	11	SC	Pickens	Oolenoy River	at bridge on SR-11, 9 km W Pumpkintown	34.99742	-82.74943
44	Cambarus	Puncticambarus	cf. acuminatus	4	2	SC	Spartanburg	Lawsons Fork Creek	at bridge on SR-108 (Goldmine Rd.), 5.4 km NNW Pacolet	34.94327	-81.78862
45	Cambarus	Puncticambarus	cf. acuminatus	20	13	SC	Spartanburg	Middle Tyger River	at bridge on SR-770 (Holly Springs Church Rd.) 12 km NNE Greer	35.03858	-82.19560
46	Cambarus	Puncticambarus	cf. acuminatus	11	6	SC	Spartanburg	Spivey Creek	at bridge on SR-208 (Spivey Creek Rd.), 3.4 km SSE Landrum	35.14435	-82.18353
47	Cambarus	Puncticambarus	cf. acuminatus	18	8	VA	Albemarle	Mechams River	at bridge on CR-635 (Miller School Rd.), 2.73 km NE Batesville	38.01427	-78.70153

ID	Genus	Subgenus	Species	N	n	State	County	River	Locality Data	Lat	Lon
48	Cambarus	Puncticambarus	cf. acuminatus	2	1	VA	Amherst	North Fork Buffalo River	at bridge on SR-635 (North Fork Rd.), ~0.1 mi N of junction with US-60	37.68073	-79.22218
49	Cambarus	Puncticambarus	cf. acuminatus	12	2	VA	Amherst	Pedlar River	at bridge on Road 835 (Buffalo Springs Turnpike), 7.82 km NE Coleman Falls	37.55965	-79.25278
50	Cambarus	Puncticambarus	cf. acuminatus	21	4	VA	Bedford	Stony Creek	at bridge on Road 850, 10.09 km NNW Bedford	37.42161	-79.55504
51	Cambarus	Puncticambarus	cf. acuminatus	20	13	VA	Carrol	Stewarts Creek	Entrance to Stewarts Creek WMA at Lambsburg, off VA SR-795	36.59598	-80.78066
52	Cambarus	Puncticambarus	cf. acuminatus	4	4	VA	Franklin	Maggodee Creek	at bridge on SR-852 (Guthrie Rd.), ~0.1 mi N of junction with SR-613 (Naff Rd.), ~2.5 mi NW Boones Mill	37.14002	-79.98762
53	Cambarus	Puncticambarus	cf. acuminatus	18	18	VA	Patrick	Dan River	at bridge on SR-773, 2.79 km WNW Claudville	36.59678	-80.44852
54	Cambarus	Puncticambarus	chaugaensis	20	17	NC	Jackson/Macon	Chattooga River	at bridge on SR-1603 (Bullpen Rd.), 8.2 km SE Highlands	35.01580	-83.12630
55	Cambarus	Puncticambarus	coosae	1	1	GA	Floyd	Little Cedar Creek	at School for Deaf on SR-100	34.09968	-85.34419
56	Cambarus	Puncticambarus	cumberlandensis	2	2	KY	McCreary	Rock Creek	at picnic area near Great Meadows Campground off Forest Rd. 137 (Rock Creek - Bell Farm Rd.), 25.9 km W Pine Knot	36.62337	-84.72687
57	Cambarus	Puncticambarus	cumberlandensis	1	1	TN	Fentress	Popular Cove Creek	at bridge on Little Crab Rd., 0.2 mi W of SR-52 (TYPE LOCALITY)	36.43355	-85.01504
58	Cambarus	Puncticambarus	georgiae	7	7	NC	Macon	Burningtown Creek	at bridge on SR-1371 (Welch Rd.), 2.3 km SSE Stiles	35.26652	-83.47361
59	Cambarus	Puncticambarus	hiwasseensis	20	18	NC	Cherokee	Peachtree Creek	at bridge on SR-141, 0.4 km S Peachtree	35.08823	-83.94669
60	Cambarus	Puncticambarus	hobbsorum	20	19	NC	Union	Bearskin Creek	at bridge on SR-200 (Morgan Mill Rd.), 2.71 km ENE Monroe	34.99604	-80.52267
61	Cambarus	Puncticambarus	hystricosus	15	15	NC	Hoke	Puppy Creek	at bridge on US-401 (Johnson Mill Rd.), 9.66 km ENE Raeford (TYPE LOCALITY)	35.01984	-79.12921
62	Cambarus	Puncticambarus	johni	20	19	NC	Wilkes	East Prong Roaring River	along SR-1739 (Stone Mountain Rd.), 1.2 km NNW Joynes (TYPE LOCALITY)	36.38151	-81.06831
63	Cambarus	Puncticambarus	johni	20	18	NC	Wilkes	South Fork Reddies River	at small "bridge" on Elder Mountain Rd.	36.23990	-81.28989
64	Cambarus	Puncticambarus	nerterius	1	1	WV	Greenbriar	US-219 Cave	U.S. 219 cave, US-219 just north of Spring Creek	37.98055	-80.37804
65	Cambarus	Puncticambarus	parishi	18	18	NC	Clay	Unnamed Trib to Fires Creek	upstream from bridge on National Forest Rd.	35.10857	-83.83791
66	Cambarus	Puncticambarus	parishi ?	4	1	NC	Clay	Hot House Branch	at "bridge" on service driveway, 3.7 km SW Shooting Creek	35.01176	-83.70360
67	Cambarus	Puncticambarus	parishi ?	20	19	NC	Clay	Tusquitee Creek	at bridge on SR-1326 (Downing Creek Rd.), 1.3 km SE Tusquitee	35.08159	-83.75198
68	Cambarus	Puncticambarus	reburus	16	16	NC	Henderson	South Fork Mills River	along SR-1338 (South Mills River Rd.) at Mills River Baptist Church, 4.4 km W Mills River	35.38149	-82.61474
69	Cambarus	Puncticambarus	robustus	16	12	VA	Carrol	Elkhorn Creek	at bridge on Elkhorn Rd.	36.65997	-80.82774
70	Cambarus	Puncticambarus	scotti	1	1	GA	Cattooga	Clarks Creek	on SR-100 (TYPE LOCALITY)	34.36770	-85.36601
71	Cambarus	Puncticambarus	sp.	2	2	SC	Spartanburg	North Tyger River	at bridge on Hannon Rd., 4.5 km NNE Holly Springs	35.04314	-82.16120
72	Cambarus	Puncticambarus	sp.	1	1	TN	Fentress	Rocky Branch	at mouth (campground road)	36.19490	-85.06831
73	Cambarus	Puncticambarus	sp.	4	3	TN	McMinn	Cane Creek	at bridge at Carlock at CR-804 and 809, near Etowah	35.29192	-84.55298
74	Cambarus	Puncticambarus	spicatus	5	1	NC	Cleveland	Hinton Creek	at bridge on SR-226, 2.28 km NNW Polkville	35.43660	-81.65263
75	Cambarus	Puncticambarus	spicatus	10	5	NC	Gaston	Long Creek	at bridge on SR-1452 (Costner School Rd.), 7.93 km NW Gastonia	35.31084	-81.25068
76	Cambarus	Puncticambarus	veteranus	1	1	KY	Pike	Long Fork	along Long Fork Rd., 10.9 km E Wheelwright	37.33892	-82.59749
77	Cambarus	Puncticambarus	veteranus	1	1	VA	Dickenson	McClure River	at bridge on SR-738, 2.4 km NE Clinchco	37.17683	-82.34172
78	Cambarus	Puncticambarus	veteranus	5	1	WV	Wyoming	Pinnacle Creek	at bridge on CR-18, 7.87 km SSW Northfork	37.49049	-81.42153
79	Orconectes	Faxonius	limosus	1	1	MD	Prince Georges	Paint Branch	at Powdermill Community Park on SR-212 (Powdermill Rd.), 0.8 km NE Hillandale	39.03235	-76.95331
80	Procambarus	Ortmannicus	zonangulus	1	1	LA	E Baton Rouge	LSU Research Ponds	source probably within 60-mi radius of coordinates, and likely from the Atchafalaya River basin	30.37066	-91.18794
TOTALS				932	625						

Molecular Analyses

DNA Extraction

Crayfish tissue samples (typically a leg, but in some cases, chela or whole juvenile abdomens) were snipped and then cut up into ~2-mm pieces directly into 900 µl of cell lysis buffer (CLB: 10 mM Tris, 100 mM EDTA, 2% SDS, pH 8.0 along with 10 µl of Proteinase K (20 mg/ml)) and returned to the laboratory for processing in order to obtain purified total genomic DNA extracts. The DNA was extracted using a high salt precipitation method previously described by Crandall et al. (2000) and Fetzner and Crandall (2003). The tubes containing tissue samples were placed into a heat block and were allowed to incubate overnight at 58°C with periodic mixing for the first few hours. The extraction continued the following day after cooling the samples to room temperature and adding 4 µl of RNase A (10 mg/ml). The samples were then incubated at 37°C for 30 min, after which 300 µl of 7.5 M ammonium acetate was added, the samples were mixed and then placed on ice. After 15 minutes, the samples were centrifuged at high speed (14,000 RPMs) for five minutes and the supernatant was then added to 900 µl of isopropanol. Samples were inverted several times to precipitate the DNA and placed at -20°C overnight. The next day the samples were centrifuged and the pellet washed with 500 µl of 70% ethanol. After drying the pellet completely, 30 to 125 µl of TLE buffer (10 mM Tris, 0.1 mM EDTA, pH 8.0) was added to resuspend the DNA. The quantity of DNA was then checked using a spectrophotometer and dilutions were made to give a final concentration of 100ng/µl for use in the polymerase chain reaction (PCR).

PCR Amplification

PCR amplifications of the mitochondrial DNA (mtDNA) cytochrome *c* oxidase subunit I gene (COI; EC 1.9.3.1) were conducted in a total volume of 25 µl. Each PCR reaction contained the following components: 1X PCR buffer, 3 mM magnesium chloride, 1.25 mM each dNTP, 1 µM each primer, 0.6 units of hotstart *Taq* DNA polymerase (Promega), and 300 ng of sample DNA. PCR cycling conditions included an initial denaturation step of 1:00 minute at 94°C followed by five cycles at a lower initial annealing temperature (48°C) followed by 35 cycles performed at 94°C for 1:00 min, 51°C for 1:30 min, and 72°C for 1:15 min. A final extension at 72°C for 7:00 min was conducted followed by a final soak at 4°C until samples could be processed further (usually overnight). Primers used in the reaction were the standard set of Folmer et al. (1994) primers but a universal primer sequence was added to the 5' end of the Forward and Reverse COI primer (T7 and T3, respectively). These nondegenerate, nonhomologous 5' tails (in bold) are then used to sequence all PCR products. Primers used were: HybLCO 5'-**TAATACGACTCACTATAGGGGGTCA** ACAAATCATAAAGATATTGG-3' and HybHCO 5'-**ATTAACCCTCACTAAAGTAACTT** CAGGGTGACCAAAAATCA-3'. The PCR reactions were checked for amplification products in the correct size range (~700 bp) by electrophoresis through a 1% agarose gel (run at 140 volts for 20 min in TAE buffer). If a sample failed to amplify for the 700 bp fragment, it was typically caused by: 1) loss of integrity of the sample [=degraded DNA]; 2) PCR inhibitors in the sample; or 3) bacterial contamination. PCR products were then cleaned and purified using MultiScreen PCR_{µ96} plates (Millipore) in preparation for DNA sequencing.

In addition, due to the very poor condition of the syntype, we received permission from the MCZ to attempt a DNA extraction from the type material to see if we could use its DNA to match it with recently collected individuals from the same general vicinity of the presumed type locality.

We designed PCR primers for the COI gene (using sequences from specimens collected from the South Saluda River) which would amplify the gene in 50 bp chunks. Initial tests tried to amplify fragments of 50, 100, and 150 base pairs; however, given its condition and age, and the unknown storage history of the specimen, we obviously were skeptical of obtaining any useful DNA.

DNA Sequencing

Sequencing reactions were conducted in a total volume of 10 µl using the Big Dye 3.1 Cycle Sequencing Kit from Applied Biosystems. Each PCR sequencing reaction contained 0.5 µl of Big Dye ready reaction mix, 2.1 µl 5x buffer, 2.0 µl of the purified PCR product, 1 µl of primer (from 10 µM stock), and 6.4 µl water. The cycle sequencing protocol followed the manufacturer's recommendations. Each cycle of the amplification process included denaturation at 96°C for 20 sec, annealing at 50°C for 25 sec, and extension at 60°C for four min. When cycling was completed, samples were held at 4°C until analysis. After the amplification step, the sequencing products were purified using Sephadex G₅₀ Fine columns in a 96 well plate format to remove any unincorporated dye-labeled nucleotides. The samples were then dried down in a vacuum centrifuge for 30 min, resuspended in 35 µl formamide and overlaid with a drop of mineral oil before running on an Applied Biosystems 3730xl DNA Analyzer. Sequences obtained from the automated sequencer were initially corrected and aligned using the program Sequencher, version 5.0.1 (GeneCodes Corp., Inc.) and then adjusted, as necessary, by eye.

Genetic Data Analysis

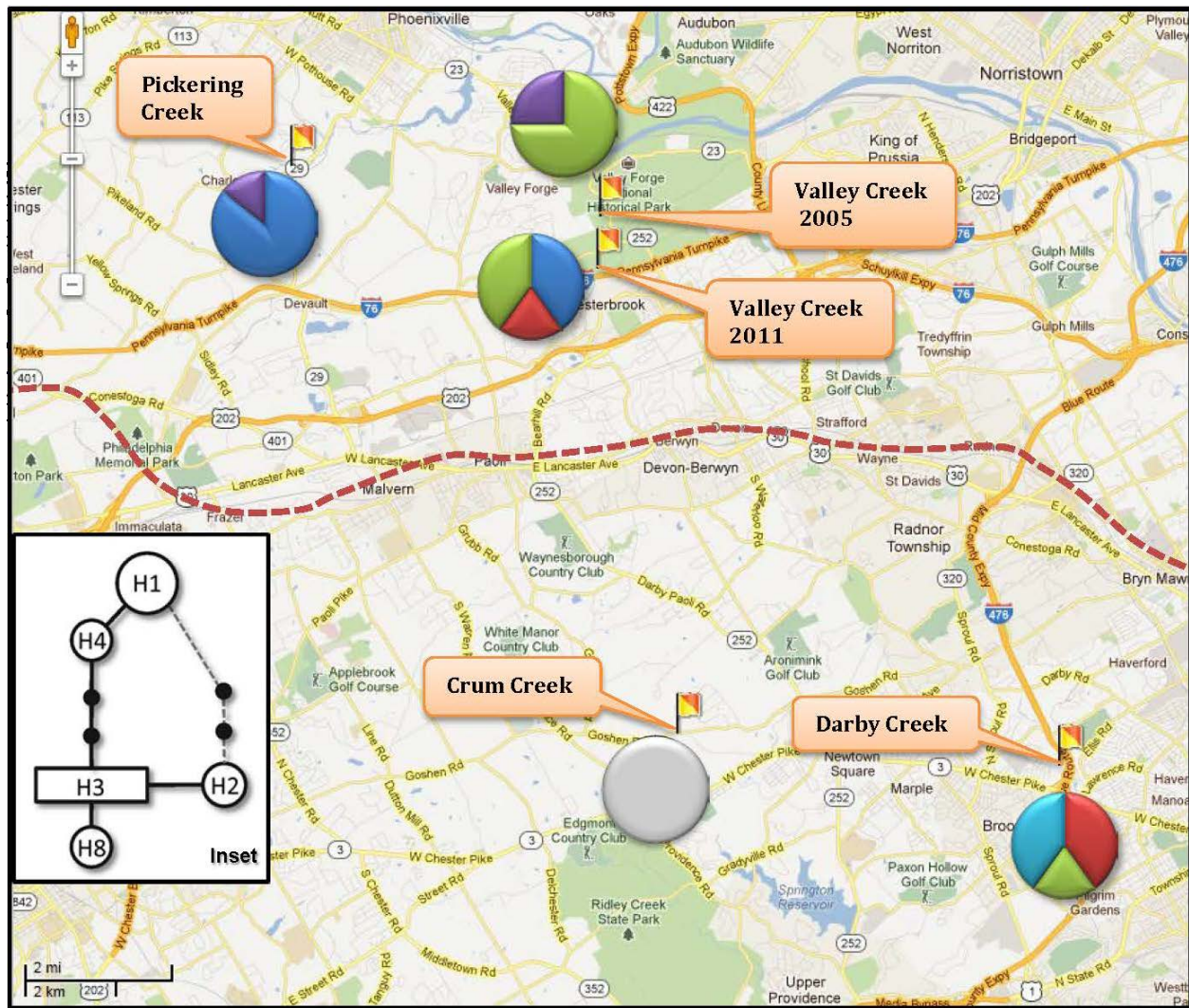
After alignment in Sequencher, the COI barcode sequence data were translated into amino acids using Mesquite (Maddison and Maddison 2011) in order to verify an open reading frame without stop codons and avoid incorporating mtDNA nuclear pseudogenes (=numts) in the analysis (Song et al. 2008). All sequences were found to have open reading frames. The data was then imported and analyzed using PAUP* v4.0b10 (Swofford 2003) via PaupUp v1.0.3.1 (Calendini and Martin 2005). Fifty-six different models of DNA sequence evolution were tested using MODELTEST v3.7 (Posada and Crandall 1998) and pairwise distances were then generated under the best selected model and used to create a neighbor-joining tree to depict relationships among the samples. The DNA sequence models are used to describe the process by which one DNA sequence transitions or changes into another (e.g., the steps necessary to have one haplotype evolve into another). The program TCS v1.23 (Clement et al. 2000) was used to collapse redundant sequences into unique haplotypes and to generate haplotype networks. Arlequin v3.5.1.3 (Excoffier and Lischer 2010) was used to generate summary statistics (such as haplotype and nucleotide diversity), and for creating a matrix of within and among population average pair-wise genetic distances and to test for population structure using AMOVA. GenALEx v6.1 (Peakall and Smouse 2006) was used to test for associations between genetic distance and geographic distance using the Mantel test. GCcalc v1.0 (Fetzner 2007) was used to generate a matrix of geographic distances among populations for use in the Mantel test. Mutation scaled population sizes (Θ) and migration rates among populations were estimated using Bayesian Inference in MIGRATE v3.3.1 (Beerli and Felsenstein 2001, Beerli 2006). These estimates were also scaled according to the geographic distances calculated among populations (in km). Bayesian phylogeographic reconstructions (e.g., Lemey et al. 2009) which incorporated spatial-temporal diffusion were carried out using BEAST v1.7.4 (Drummond et al. 2012) with visualizations for display in Google Earth™ created by SPREAD v1.0.4 (Bielejec et al. 2011).

Results

Cambarus acuminatus **Pennsylvania Populations**

We obtained sequences from 13 specimens from Valley Creek (VAFO), seven from Pickering Creek, and five from Darby Creek (Figure 6). We also collected four specimens from Crum Creek but only one of these amplified well; unfortunately, the sequence obtained was messy and unusable. The two Valley Creek sites were pooled together for analyses. We also found that four of the 13 sequences we obtained from the 2011 collections made in Valley Creek had sufficient variation; suggesting these specimens were not actually *C. acuminatus*. In three cases, sequence divergence was ~8% and the specimens were genetically assignable to *C. bartonii* (a species which also occurs in Valley Creek). This is likely a case of misidentification in the field, possibly of juvenile crayfish, which, in some cases, due to their size, makes it harder to distinguish which species they belong to. The fourth sequence diverged from the “typical” *C. acuminatus* sequences by 4.2%, but was a unique haplotype in the dataset, so it is unknown what this actually might have been. It is possible that it represents a sample mix-up of some sort, as we have not heard of any other *Cambarus* species being found in Valley Creek that this sequence might represent. A search of Genbank produces 94% match to a specimen of *C. bartonii* (accession AY701190), but at this percentage match, this indicates the sequence is likely from a member of the genus *Cambarus*, but not *C. bartonii* per se. There appears to be limited COI sequence data in Genbank for the genus *Cambarus*, which currently makes a positive ID impossible until such time that additional known and verified sequences are added to the database.

Among the three Pennsylvania populations with available COI data, we detected eight unique haplotypes. However, three of these eight were associated with the misidentified *C. bartonii* and one was the “unknown” sample described above, which means there were four haplotypes assignable to *C. acuminatus* in PA. Interestingly, Valley Creek shared haplotypes with both Pickering and Darby creeks, but the latter two sites did not share any haplotypes between them (Figure 6, see also Appendix A). Darby Creek was the only population where we detected haplotype H8; however, this result is probably just an artifact of the low sample sizes examined from these sites (i.e., sampling error). Indeed, even the two sites sampled from Valley Creek (~2 km apart) showed some differences in haplotype frequencies and composition (but they were also sampled six years apart, thus subject to effects of genetic drift). Estimated migration rates can be seen in Tables 2A and B. Alternatively, some differences among populations may be attributable to founding events (i.e., when only a few individuals reach a new habitat and start a new population), so initially only the genetic variation that these migrants carry will be available to future generations at that site (barring new mutations or additional migrants). If their initial numbers are limited and they do not carry the full set of genetic variation from the originating population, there will be differences detected between locations. A different, yet related, cause for such a pattern may be the influence of genetic drift due to low population sizes. When population numbers are low, significant changes in haplotype frequencies can occur from generation to generation and the haplotypes present in the population depend greatly on which individuals (and the haplotypes they carry) are able to breed successfully. Under this low population density scenario, with each new generation, haplotype frequencies can vary widely, and even lead to the loss of haplotypes from a population. This can quickly lead to a noticeable genetic difference between populations (in either frequencies or haplotype composition).



Haplotypes ■ H1 ■ H2 ■ H3 ■ H4 ■ H8

Figure 6. Map showing locations in Pennsylvania in and around Valley Forge National Historical Park where samples of *C. acuminatus* were collected for this study (see also Fetzner 2010). Haplotypes and their frequencies in each population are indicated by the pie graphs. The gray haplotype pie for Crum Creek indicates no haplotype data is available for this site, even though it was sampled. Red dashed line indicates the watershed boundary between the Schuylkill (above) and Lower Delaware (below) drainages. **Inset** shows the inferred relationships among the haplotypes (open circles and rectangle), with sizes proportional to their frequency. The rectangle around H3 indicates that it is the inferred root of the network. Smaller solid black dots indicate un-sampled haplotypes (or mutational steps) that fall between detected haplotypes (so, for example, there are 3 mutational changes between H3 and H4). The dashed line indicates an alternate lower probability connection path. See Appendix A for haplotype distributions by population.

Table 2. Population sizes (Θ) (in bold along diagonal) and symmetric migration rates among populations estimated using the program Migrate-n for **A)** Clade A, subgroup A1 and **B)** Clade F, subgroup F2. Values below diagonal are migration rates for MX→Y while values above the diagonal are for rates MY→X.

A.	Darby	Pickering	Valley 1	Valley 2	Mechums	NF Buffalo	Pedlar
Darby	0.01627	82.6	54.2	65.9	132.9	45.55	165.32
Pickering	163.4	0.00137	92.2	271.2	202.8	135.49	282.19
Valley 1	177.7	122.2	0.00599	158.0	64.83	128.85	68.01
Valley 2	59.4	119.4	42.2	0.00057	176.3	79.01	147.05
Mechums	96.68	75.93	111.45	43.79	0.00268	75.83	79.87
NF Buffalo	259.04	184.62	261.12	61.58	259.21	0.00873	150.85
Pedlar	152.59	277.07	69.84	131.04	51.56	46.11	0.00685

B.	Boylston	S. Saluda	Oolenoy	SF Mills
Boylston	0.00186	86.2	264.3	367.4
South Saluda	86.1	0.01028	64.4	131.0
Oolenoy	155.4	257.2	0.00916	168.4
SF Mills	93.2	99.4	58.7	0.00700

In fact, in sampling the Pennsylvania populations in 2009 (Fetzner 2010), there was a clear difference in catch per unit effort (CPUE), or relative abundance, at these sites (Figure 7). Part of this difference may also be due to variation in habitat quality and the presence of invasive species at some of these sites—Pickering had *O. virilis*, Crum had *P. acutus*, and note that Valley Creek now has *O. rusticus*, but the sampling event analyzed here, from 2005, occurred before

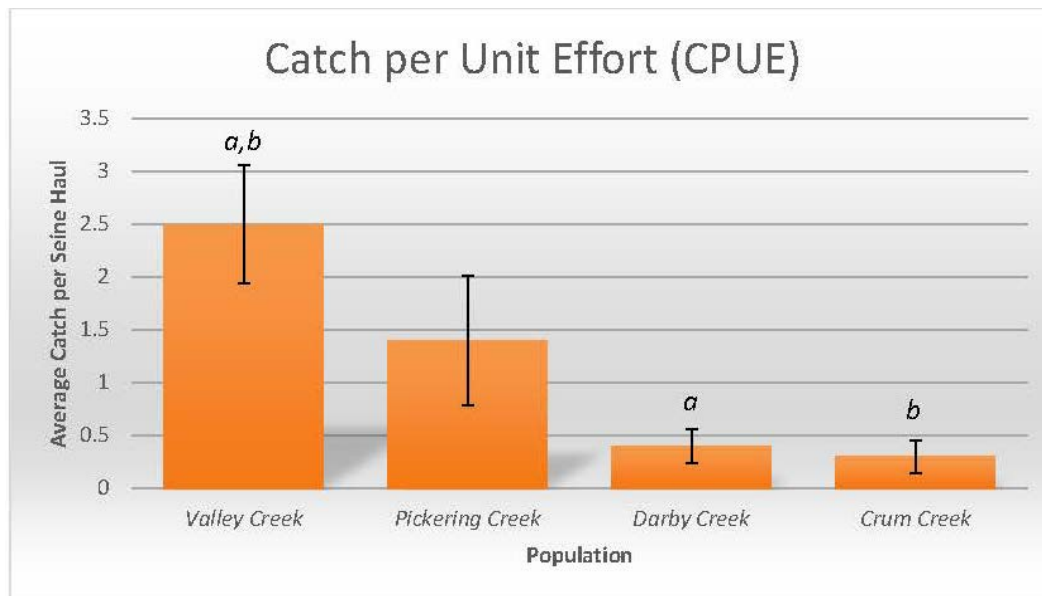


Figure 7. Mean estimates of catch per unit effort ($\pm$ SE) for Pennsylvania populations of *C. acuminatus* based on collections made in 2005 (Valley Creek) and 2009 (Pickering, Darby, Crum creeks) (see Fetzner 2010). CPUE was calculated using number of individuals captured during 10 seine hauls made using a 4'x4' net and sampling an ~1 m² area of stream bottom. Significant differences ($P < 0.05$, t-test) were detected between those samples with the same letter shown above error bars.

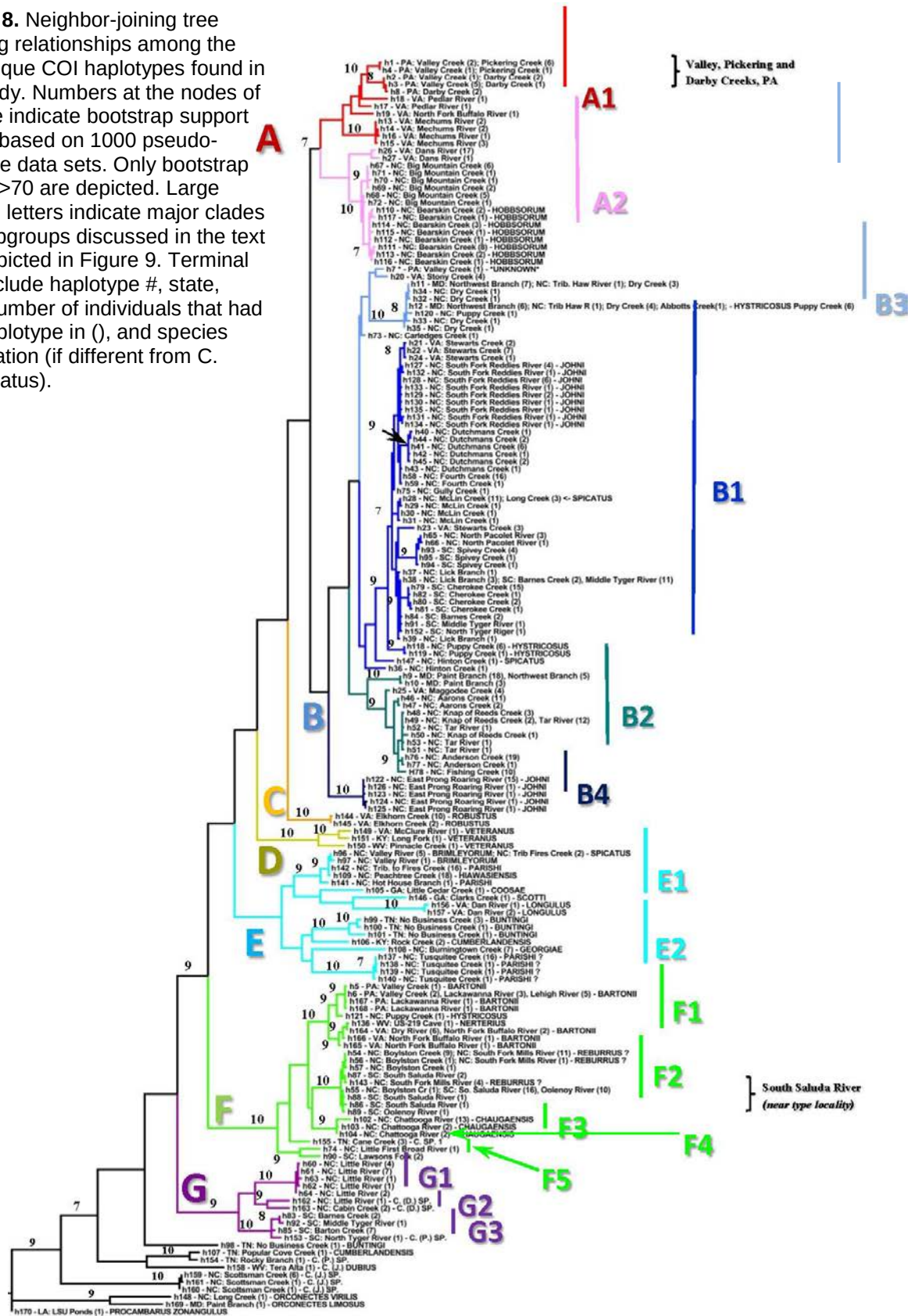
that species invaded the creek. Valley Creek clearly supported the largest population of *C. acuminatus* in the region based on our observations and sampling effort, while specimens were rather sparse at Darby and Crum creeks, and Pickering Creek seemed intermediate. All three populations contained multiple haplotypes, and these haplotypes were closely related to one another, differing by only a few mutational steps (see Figure 6 inset).

We generated a phylogenetic tree that contained the entire dataset (625 taxa); however, the tree (a figure) was too big to display in this report without being confusing, as it broke across multiple pages. Therefore, we also generated a tree that only contained the set of unique haplotypes that were detected. This subset contained 170 terminal taxa, and is displayed in Figure 8. When generating these trees, a model of DNA sequence evolution was selected by the program MODELTEST, which indicated that the best-fit model chosen by the AICc criterion for the 625 taxon tree was the HKY+I+G model (see Hasegawa et al. 1985) with the following parameters: Base= (0.2585 0.1191 0.2015), Nst=2, TRatio=6.7179, Rates=gamma, Shape=0.6421, Pinvar=0.5209. The same model was also selected for the 170 taxon haplotype tree, but with slightly different parameter values [Base= (0.2566 0.1152 0.1988), Nst=2, TRatio=6.7670, Rates=gamma, Shape=0.6519, Pinvar=0.5222]. These values were input into PAUP* and a neighbor-joining tree was generated.

The neighbor-joining haplotype tree indicates that the Pennsylvania populations are most closely related to three populations that were sampled from central Virginia (Pedlar River, North Fork Buffalo River, and Mechums River) (Figure 8). The first two are from Amherst County and the latter from Albemarle County. Pennsylvania populations diverged from the first two Virginia populations by an average of 2.4% (range 1.6 – 2.9%), which is about 16 base pair (bp) differences, and from Mechums River by 3.3% (Appendix B). The next closest group in the tree to these PA populations consisted of Dan River (VA), Big Mountain Creek (NC), and Bearskin Creek (NC), the latter being a population of *Cambarus hobbsorum*!

The two populations of *C. acuminatus* from Maryland were only distantly related to the Pennsylvania crayfish (7.4% sequence divergence or ~49 bp), with their closest relationships being to populations from North Carolina. Northwest Branch also appeared to have two entities, as some of the samples grouped on the tree in two different places. In addition, the Pennsylvania specimens were very different from populations in South Carolina from near the type locality (South Saluda River, etc.), differing on average by 9% (59 bp). These populations clearly belong to different species. If the different groups of taxa in the tree (clades) are plotted on a map, one can get an idea of the geographic distributions of these different groups (Figure 9). Interestingly, many of the subgroups in each of the seven major clades are geographically restricted to a relatively small area, except for a few (e.g., A1, B3, E1, and F1) which tended to have one to several locations that are geographically distant from the main grouping of populations. This result is encouraging, as these subgroups may be useful in the future in defining distributions of the different species in this complex. Results from the Bayesian phylogeographic diffusion analysis are graphically depicted in Figure 9F, and shows the connections among populations. However, additional field work will be necessary in order to delimit ranges, and morphological studies of specimens will be necessary to define characters that might be used to distinguish species. This study gives us a good start along the path of trying to understand variation in this species complex.

Figure 8. Neighbor-joining tree showing relationships among the 170 unique COI haplotypes found in this study. Numbers at the nodes of the tree indicate bootstrap support values based on 1000 pseudo-replicate data sets. Only bootstrap values >70 are depicted. Large colored letters indicate major clades and subgroups discussed in the text and depicted in Figure 9. Terminal taxa include haplotype #, state, river, number of individuals that had that haplotype in (), and species designation (if different from *C. acuminatus*).



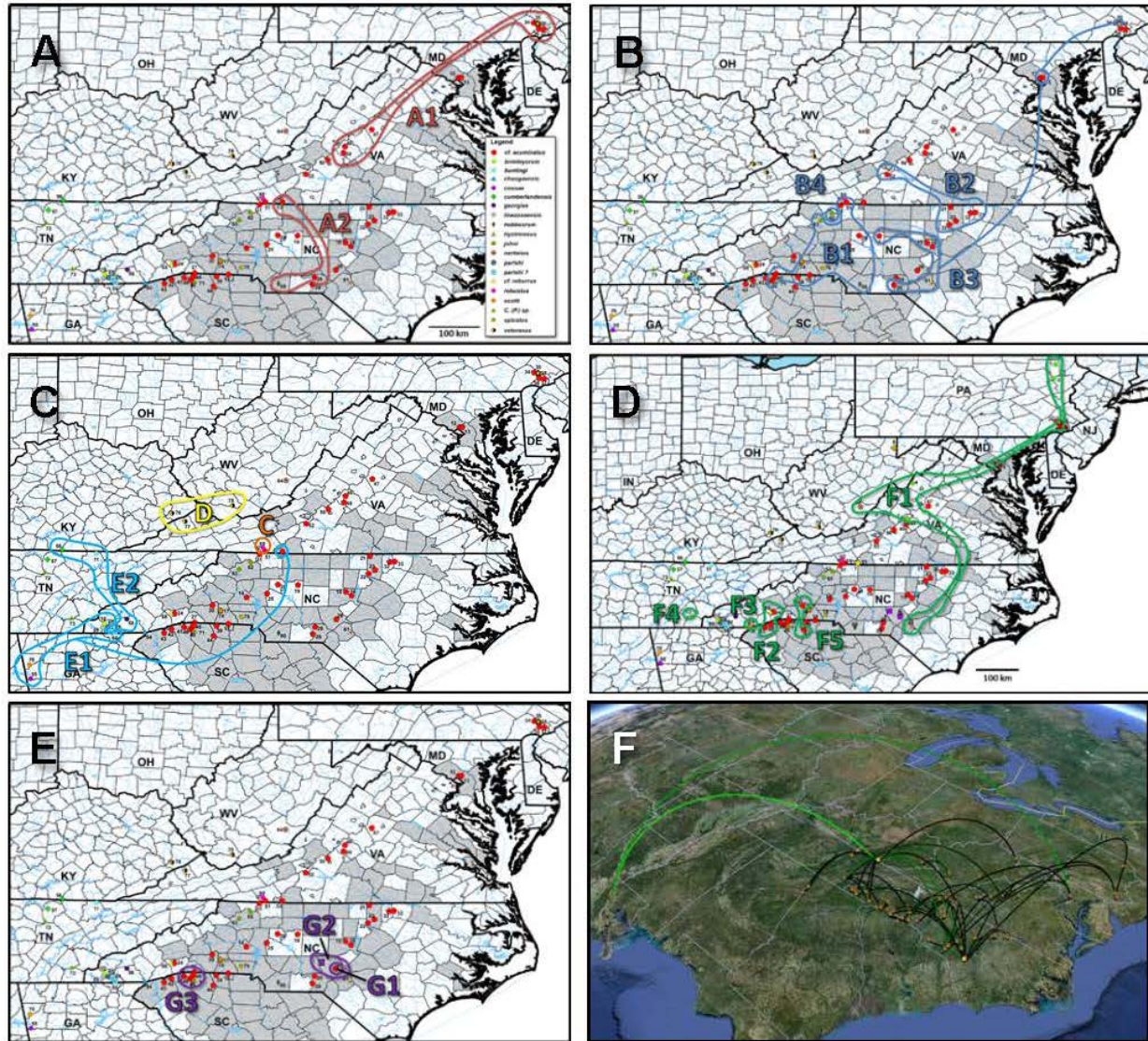


Figure 9. Maps showing the distribution of mtDNA COI gene haplotype clades that include presumed *C. acuminatus* samples or related species, as depicted in the phylogeny in Figure 8. Gray shaded counties indicate county occurrence records for *C. acuminatus* from the USNM crayfish database. A) Clade A and subgroups A1–A2, B) Clade B and subgroups B1–B4, C) Clades C, D and E with subgroups E1–E2, D) Clade F showing subgroups F1–F5, E) Clade G showing subgroups G1–G3 and F) phylogeographic diffusion patterns among the populations analyzed in this study. The green-black line color gradient indicates the relative ages of the transitions (oldest–youngest).

However, in genetic studies, there is no set rule of thumb or arbitrary cutoff level that can be used to define species-level differences when using percent sequence divergence for the COI (or any other) gene, as there are always exceptions to every rule. Part of this uncertainty comes from the fact that different crayfish taxonomists may differ in opinion on what morphological characteristics are important for defining a new species. However, as rough estimates, *within populations* of crayfish, COI divergences among haplotypes are typically less than 1%, often in the range of 0.0% – 0.5%. *Among populations* of a species, these divergences can range widely from say 0% to 5%. *Among species within a genus*, divergences can range anywhere from 2% up to 9+%, with 5% being a rough average. *Among genera*, values of 7% to 20% are not uncommon. Note that there is often overlap in these ranges, which leads to a gray area when trying to define species-level differences using only percent sequence divergence. Also, these values and ranges may be different depending on which species, genera, or genes are being considered, so they cannot be used as a yardstick for comparisons to other studies, especially if other genes are involved.

The C. acuminatus Species Complex

The DNA analysis confirms that the taxon currently considered to be *Cambarus acuminatus* does indeed represent a complex of species. Genetic relationships in this group, similar to previous morphological studies, are quite difficult to interpret. For example, several northern populations appear more closely related to populations further south than they are to neighboring populations. Additional analyses will need to be conducted in order to tease out the necessary information that will allow us to clarify relationships in this group and to delimit species at a finer geographic scale. An in depth examination of the voucher specimens will also be necessary to see if any morphological differences can be correlated with the genetic findings, and to corroborate field identifications for samples that appeared aberrant in the DNA tree. For example, several populations appeared to have more than one taxon present based on the DNA (e.g., Northwest Branch, Puppy Creek). It is clear from the DNA tree that different populations of *C. acuminatus*-like things group with many of the other currently described species in the subgenus *Puncticambarus*, and in one case, the subgenus *Cambarus*. It could be that these populations actually represent members of those species, rather than “true” *C. acuminatus*, or some of these species, as currently delimited, may be no good.

We also generated haplotype networks for this dataset using the TCS program. This analysis combined the 625 sequences into 170 unique haplotypes (see Appendix A), and these haplotypes were grouped into 43 different networks (see summary of networks in Appendix C). However, given the large number of networks, it is not possible to display them in a meaningful manner in this report, so they have been summarized in Appendix C. The maximum connection limit for joining haplotypes together into a network was 11 mutational steps, a value which is directly related to the length of the gene fragment being analyzed (e.g., 658 bp). The largest network (#2) contained 46 haplotypes from 17 locations, mostly from North and South Carolina, and basically represents Clade B, subgroup B1 as shown in Figures 8 and 9. As for the PA populations, they formed their own network (#1 in Appendix C and see Figure 6 inset for actual network), while the closely related Virginia populations formed three different networks (#s 4, 5, and 6 in Appendix C). Due to some of the large sequence divergences seen among populations, there were a lot of networks that were represented by only a single collection locality, which makes it difficult to interpret population relationships using the networks, and is thus better visualized using the phylogeny in Figure 8.

Syntype MCZ #3624

Our attempts to amplify the COI gene from the *C. acuminatus* syntype failed to produce any viable product, even though running the sample through a spectrophotometer to quantify the DNA seemed to indicate that DNA was present (but probably highly degraded). The agarose gel shows that the positive control amplified for all three fragments (50, 100, 150 bp), while the three samples extracted from the syntype did not (Figure 10). Thus, it would seem that any DNA left in the sample is degraded to the point where no fragments are greater than 50 bp, or that mtDNA just is not present. The storage history of the sample is not known, and may have involved formalin fixation. It also seems likely that the specimen dried out at some point in the past and that it was then ground up, possibly by the small lead tag that was with the sample and contained the specimen ID number on it. As a result, we were unable to obtain any DNA sequence from the syntype and thus were not able to determine its genetic affinities to other extant populations of *C. acuminatus*.

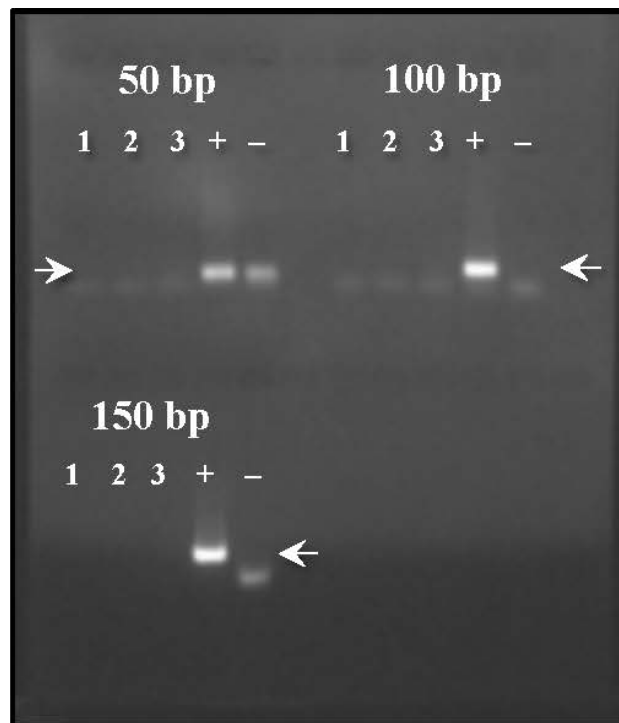


Figure 10. Image of an agarose gel showing attempts at amplification of COI gene fragments of 50, 100, and 150 base pairs for three samples extracted from the *C. acuminatus* Syntype (#'s 1-3), a positive control (+) from the South Saluda River population, and a negative control (-). Arrows show position of expected product length (see positive controls).

Relationships Among Members of the Subgenus *Puncticambarus*

In order to put the levels of genetic variation seen in *C. acuminatus* into perspective, we included representatives of most of the other species (17 of 21) from the *Cambarus* subgenus *Puncticambarus* in the analysis. This also allows us to examine the phylogenetic relationships among members of this group and to explore phylogeographic patterns. As can be seen from the haplotype tree in Figure 8, the relationships in this group are complex. Populations classified as *C. acuminatus* are placed all over the tree (=polyphyletic) and in various combinations with other described species of the subgenus, suggesting that our current understanding of *C. acuminatus* as a taxonomic concept is in need of revision and also indicates that additional undescribed species may be present. Similar results were also found for some of the other species in the subgenus where we had multiple populations sampled, perhaps most notably, *Cambarus johnei* Cooper (2006). We sampled this species from two stream sites in the Upper Yadkin watershed that were listed by Cooper (2006, pg. 76) and were about 25 air km (16 mi) apart. However, in the phylogenetic tree, they were moderately divergent, differing by 4.4% (28 bp), which is a higher divergence than one might expect within a species sampled from sites within the same watershed.

We also found that *Cambarus bartonii* was placed in amongst the ingroup taxa, rather than more basal in the tree, as might be expected by its subgeneric classification, and the group was sister to the clade containing the *C. acuminatus* populations from near the type locality (Figure 8, Clade F). However, it is well known, at least within the genus *Orconectes*, that the subgeneric classification created based on morphological characters does not hold up when compared to results from molecular or biochemical studies. Based on molecular data, these species tend to group based on geographic proximity rather than morphological similarities (Crandall and Fitzpatrick 1996, Fetzner 1996, Taylor and Hardman 2002, Taylor and Knouft 2006).

Recently, Breinholt et al. (2012) tested the phylogenetic validity of the current subgeneric classification of the genus *Cambarus* and found that the majority of the subgenera do not form monophyletic groups. They also suggest that the morphological characters used to define these groups are subject to convergent evolution. The data presented in the current report also seem to support these conclusions, with many species classified in different subgenera being mixed on the COI phylogeny depicted in Figure 8. Clearly, additional field sampling and analysis will be necessary to help sort out the evolutionary relationships of the North American freshwater crayfishes, as basing studies solely on the current subgeneric classifications may not tell the whole story or worse, lead to erroneous conclusions.

Specimens from the three Pennsylvania populations formed a tight cluster in the phylogeny in Figure 8 and had a bootstrap support of 100. Sister to the PA clade was group of samples from Virginia that included specimens from the Pedlar River (n=1), North Fork Buffalo River (n=1) and a grouping of four haplotypes from the Mechums River (n=8). Related to this big group is a clade containing haplotypes from Dans River (VA), Big Mountain Creek (NC), and *C. hystricosus* from Bearskin Creek (NC). All of these samples comprise what we have called Clade A in Figure 8. Clade B is a large group that contains 74 haplotypes, or almost half of the dataset. Within this clade, at least four subgroups could be distinguished based on their relationships, and the branches for these have been color coded in Figure 8 and also displayed graphically on a map in Figure 9B.

The majority of the other described species in the subgenus *Puncticambarus* grouped in clades C, D, E, and F. *Cambarus robustus* (Clade C) and *C. veteranus* (Clade D) seemed to be distinct from many of the other species, although *C. robustus* is itself considered to be another large species complex. Clade E consisted of *C. brimleyorum*, *C. parrishi*, *C. hiwasseeensis*, *C. coosae*, *C. scotti*, *C. buntingi*, *C. cumberlandensis*, *C. georgiae* and, interestingly, *C. (H.) longulus*, the latter being from the subgenus *Hiaticambarus*. Clade F contained several groups, including a set of *C. bartonii* (*Cambarus* subgenus *Cambarus*) from PA and VA, along with a presumed *C. hystricosus* from Puppy Creek (a possible mis-ID?). It also groups *C. nerterius* closely with Virginia *C. bartonii*. Clade F also includes a group containing presumed *C. reburrus*, and the South Carolina populations of *C. acuminatus* from near the inferred type locality (Saluda River). The “true” *C. acuminatus* appears to be restricted to two watersheds, the Saluda River in South Carolina, and the Upper French Broad in North Carolina. Related to this was *C. chaugaensis* and then a little more distantly related were some odds and ends from various populations. Clade G contained the Little River population from North Carolina, an as yet unidentified species from the subgenus *Depressicambarus* and then several samples from 4 populations in South Carolina (Barnes Creek, Barton Creek, Middle Tyger River and North Tyger River). The major clades containing various populations of *C. acuminatus* probably represent a mix of new species or possibly might be assigned to one of the already described species. Further morphological examination of the specimens will be necessary before that determination can be made.

Discussion

Mitochondrial DNA is a circular, double-stranded molecule with a length of nearly 16,600 base pairs that encodes for 37 genes, some of which are proteins required for oxidative phosphorylation. The mtDNA genome is passed down to the offspring only through the maternal line (although a few documented exceptions exist), whereas nuclear DNA comes from both the maternal and paternal lines. The mtDNA also has a higher mutation rate than nuclear genes, which makes it a good genetic marker for studies of within and among population variation. There is also a lack of recombination and it occurs in high copy numbers per cell, making it a good target for PCR amplifications.

In this study, we report on levels of genetic variation gathered from multiple populations of *Cambarus acuminatus* for the mtDNA COI gene. However, these data and analyses are based on partial sequence from a single mitochondrial gene, and they were used to generate a hypothesis about relationships among taxa, as is depicted in the phylogenetic tree presented in Figure 8. However, single gene trees may not accurately reflect the actual species tree (e.g., Pamilo and Nei 1988, Wu 1991, Doyle 1992, Maddison 1997, Ballard and Rand 2005), due to various factors such as stochastic lineage sorting, introgression, mistaken orthology or the influence of selection. A combined multi-gene phylogeny has a better chance of finding the “true” species tree. This is not to say that the results in this report are erroneous, only that these factors, if they occur in this group of crayfish, can skew the inference of evolutionary relationships in the group. In fact, there is currently no evidence that these factors play a role or have influenced the evolutionary history of this group of crayfish, although some cases of hybridization among crayfish species have been reported in the genus *Orconectes* (e.g., Perry et al. 2001), and usually involve the invasive rusty crayfish (*O. rusticus*).

In the past few years, the COI gene has been used successfully to examine population structure in several other crayfish species from the central and eastern U.S. (e.g., Fetzner and DiStefano 2008, Fetzner and Thoma 2011). The haplotypes detected in these studies showed geographic patterning that allowed for the segregation of distinct population groups (typically by river drainage). However, in at least one recent case involving a study of *C. bartonii*/*C. howardi*, another species complex with a similar distribution to that of *C. acuminatus*, the recovered genetic relationships were confusing in a manner similar to what we have seen here in the current study. It is possible that stream drainages in the southern portion of the respective ranges of these species (specifically in North and South Carolina) have had a complex and diverse history that have affected the crayfish fauna in some manner (e.g., stream captures). It may be that the only way to tease out the evolutionary relationships in these widely distributed species complexes will be to examine geographic patterns of variation in a more comparative manner, combining data from multiple species with similar distribution patterns along the east coast, and then seeing if a consistent pattern arises.

Other molecular datasets representing multiple independent loci (microsatellites, single nucleotide polymorphisms [SNPs], or nuclear gene sequences) may also be necessary in order to establish independent estimates of relationships to get around possible gene tree/species tree issues; however, there are currently no microsatellite or SNP primers available for the genus *Cambarus*, so these would require quite an initial undertaking to develop. Little to no data exists on the cross-amplification potential in *Cambarus* of microsatellite loci developed in other

crayfish species, but typically most loci either do not amplify, or if they do, they tend to be non-variable. Nuclear gene sequences can also run into problems due to their diploid nature, and also finding a gene that is variable enough at the population level to give useful information on relationships may be difficult.

Status of Pennsylvania Populations

One of the initial questions raised after populations of *C. acuminatus* were discovered in Pennsylvania back in 2003 was whether they were native (and just previously overlooked), or if they represented a case of an introduced, and thus nonnative, species similar to the rusty crayfish (which today can also be found in the same watershed). The current study found that three of the four known Pennsylvania populations of *C. acuminatus* were very closely related to one another and shared many of the same exact haplotypes (we had no viable sequences from the fourth population). This suggests that these populations have a shared evolutionary history. We also found that these populations, based on our current dataset, were most closely related to populations sampled from central Virginia, differing from them by an average sequence divergence of about 2.4%. Would sampling additional *C. acuminatus* populations from the historical range of the species in northern Virginia help identify a closer match? It is hard to say without actually doing the sampling, and there is no real guarantee of success. Unfortunately, aside from the results given above, we cannot determine with any certainty whether the Pennsylvania populations are native or the result of an introduction, since we did not find any potential source populations among those we sampled that shared the same exact COI haplotypes as those discovered in Pennsylvania. Additional population sampling and perhaps other genetic markers will be necessary to help address this question in the future.

The fact that the PA populations differed quite significantly (7.4%) from the MD populations was a surprise. If anything, we thought that geographically proximal populations would be the source of the PA populations, either through natural (i.e., range expansion or dispersal) or unnatural (i.e., bait-bucket, pet trade, etc.) means. The high degree of genetic variation in this species was not much of a surprise, given the variable habitats it occurs in and the large geographic distances involved between populations from one end of the species distribution to the other. However, we expected the outcome to be a little clearer than it actually turned out to be.

Another result of this project is that we have gained some insight into the genetic relationships of the *C. acuminatus* species complex, as well as among species of the subgenus *Puncticambarus*. The historical difficulty and confusion when trying to delimit and define new species from the complex based on morphology also appears to hold true when examining the genetics. As a result, much more field work still needs to be done on this group, and perhaps the starting point should be to taxonomically re-describe *C. acuminatus* and delimit its distribution. As with many of the historically difficult crayfish species complexes in the eastern U.S., a dense sampling scheme (i.e., multiple samples from each drainage system) is going to be necessary in order to understand how the various populations are related to one another and how we might then group them into coherent species definitions. Whether those species are grouped based on geography (i.e., by drainage systems), or some other means, remains to be seen. Eventually, it will all come down to how many populations can be sampled and examined (the more the better). After that, if we can focus our efforts and include data from multiple sources (morphological and genetic) we may eventually resolve this long-standing species complex.

A New Species?

The other main question this project has tried to address was: Are the Pennsylvania populations of *C. acuminatus* related to the type population from South Carolina, or do they represent a new species? Without a doubt, we can say that the Pennsylvania populations are genetically distinct from South Carolina populations of *C. acuminatus* that were collected from near the presumed type locality (Saluda River drainage). This result was similar to findings made by John Cooper (2010, personal communication), who has indicated that PA specimens he examined from Valley Creek were morphologically distinct from populations he is familiar with from North and South Carolina. However, he has not examined material from Virginia (Cooper 2012, personal communication), and so is not able to verify our results of an apparent close relationship between PA and VA. Since our results indicate that the PA populations are most closely related to populations found in central Virginia (based solely on the limited population sampling for this study), the interpretation of these results are complicated with regard to the intervening populations, both those that were not sampled (i.e., northern VA; how different are they and how do they fit into this picture?), and those that were sampled in relatively close geographic proximity but were more divergent (i.e., Maryland). Should central Virginia and Pennsylvania populations be considered a new species, or are the Pennsylvania populations just the result of a past introduction originating from Virginia, or vice versa?

Based on the results of this study, the PA populations are different enough genetically from the Saluda River populations to be designated a different species. But would this be described as a new species, or would it be considered part of an existing species, such as *C. hobbsorum*, as might be indicated by the grouping of populations in Clade A in Figure 8? The current uncertainty in how many species we are dealing with, and the unknown extent of their distributions, not to mention the uncertainty in the higher-level (subgeneric) classification, makes it all but impossible at this point to define a new species that would include the PA populations. Again, additional sampling of populations (as many as possible) and adding them to the analysis will greatly improve our ability to resolve these questions.

In a way, this result is unfortunate because one impetus for this study was that the Valley Creek population is threatened by the invasive rusty crayfish (*Orconectes rusticus*), which has invaded many stream systems in the Lower Susquehanna River basin and has caused the decline, displacement, or eradication of the native crayfish species in those streams where it is now found (e.g., see Fetzner 2010). In 2008, rusty crayfish started showing up in Valley Creek, and it is not yet known what effect this invasion has, or will have, on *C. acuminatus*. Given the limited distribution of *C. acuminatus* in Pennsylvania, a factor which often plays a large role in crayfish conservation status assessments (e.g., Taylor et al. 1996, Taylor et al. 2007), and the introduction of a known invasive crayfish into the system, it would seem to be important to conserve the limited number of populations of this unique Pennsylvania crayfish.

Conclusions and Future Directions

As stated above, while we are not able to directly answer some of the initial questions posed at the beginning of the project, especially as they relate to Pennsylvania native status, we have learned some interesting and important information on the *acuminatus* complex. Based on our somewhat limited sampling, we now know that “true” *Cambarus acuminatus* appears to be restricted to the Saluda River drainage in South Carolina and the Upper French Broad drainage in North Carolina (Figure 8, Clade F, subgroup F2; Figure 9D). Additional targeted sampling of other populations from the region should help delimit this species range with even greater accuracy. This process might also be aided by using historical collection records from the USNM crayfish database, and perhaps records from other institutions as well.

We also found that the Pennsylvania populations (three of the four, at least) were genetically very similar to one another, and shared many of the same haplotypes. This suggests that they share a common history, although whether that history is the result of natural dispersal, or human aided introductions, cannot be determined at this point. Based on genetic distances, and limited sampling, the Pennsylvania populations appear to be most closely related to populations sampled from central Virginia, differing on average by 2.4% [range 1.6 – 2.9%]. This result was somewhat of a surprise, as geographic proximity is often an indicator of relationship in crayfish (i.e., populations located at shorter distances apart tend to be more closely related). Thus, we would have predicted that the Maryland populations would have been the most similar to the Pennsylvania populations, but this was clearly not the case, with divergences at roughly 7.3%.

A complete morphological analysis of all specimens collected as part of this study should also be conducted in order to see if morphological characteristics correlate with the genetic findings. Including and examining existing specimens from other museums might also help shed light on this group and help in delimiting the geographic extent of the different forms, which would aid in the description of potential new species. Given the lack of type specimens, an ambiguous type locality, and less than adequate general descriptive information for *C. acuminatus*, a re-description of this species may be necessary.

The genetic data presented in this report give us a glimpse into the evolutionary history of this group, but by no means have we been able to resolve the many taxonomic issues that this group poses. This is clearly a difficult group to work with and is probably why it has had a long standing history of being classified as a species complex. Issues at the subgeneric level will also have to be worked out before we will gain a full understanding of species relationships within the genus *Cambarus*. To this end, future directions for work on this species complex should focus on obtaining population samples from throughout the range, especially where there are gaps in the current sampling (i.e., northern Virginia, etc.). In addition, other molecular methods (microsatellites, SNPs, or nDNA) should also be explored to see if they will be useful in defining population groups and separating the myriad of species that seem to be present.

Literature Cited

- Ballard, J. W. O., and D. M. Rand. 2005. The population biology of mitochondrial DNA and its phylogenetic implications. *Annual Review of Ecology, Evolution and Systematics* **36**:621-642.
- Beerli, P. 2006. Comparison of bayesian and maximum likelihood inference of population genetic parameters. *Bioinformatics* **22**:341-345.
- Beerli, P., and J. Felsenstein. 2001. Maximum likelihood estimation of a migration matrix and effective population sizes in n subpopulations by using a coalescent approach. *Proceedings of the National Academy of Sciences* **98**:4563-4568.
- Bielejec, F., A. Rambaut, M. A. Suchard, and P. Lemey. 2011. SPREAD: Spatial phylogenetic reconstruction of evolutionary dynamics. *Bioinformatics* **27**:2910-2912.
- Breinolt, J. W., M. L. Porter, and K. A. Crandall. 2012. Testing phylogenetic hypotheses of the subgenera of the freshwater crayfish genus *Cambarus* (Decapoda: Cambaridae). *PLoS ONE* **7**:e46105.
- Calendini, F., and J.-F. Martin. 2005. PaupUP v1.0.3.1. A free graphical frontend for Paup* Dos software.
- Clement, M., D. Posada, and K. A. Crandall. 2000. TCS: a computer program to estimate gene genealogies. *Molecular Ecology* **9**:1657-1659.
- Cooper, J. E. 2001. *Cambarus (Puncticambarus) hobbsorum*, a new crayfish (Decapoda: Cambaridae) from North Carolina. *Proceedings of the Biological Society of Washington* **114**:152-161.
- Cooper, J. E., and D. G. Cooper. 2003. A new crayfish of the genus *Cambarus* Erichson, 1846 (Decapoda: Cambaridae) from the Cape Fear River basin in the Sandhills of North Carolina. *Proceedings of the Biological Society of Washington* **116**:920-932.
- Crandall, K. A., J. W. Fetzner, Jr., C. G. Jara, and L. Buckup. 2000. On the phylogenetic positioning of the South American freshwater crayfish genera (Decapoda: Parastacidae). *Journal of Crustacean Biology* **20**:532-542.
- Crandall, K. A., and J. F. Fitzpatrick, Jr. 1996. Crayfish molecular systematics: Inferences using a combination of procedures to estimate phylogeny. *Systematic Biology* **45**:1-26.
- Doyle, J. J. 1992. Gene trees and species trees: molecular systematics as one-character taxonomy. *Systematic Botany* **17**:144-163.
- Drummond, A. J., M. A. Suchard, D. Xie, and A. Rambaut. 2012. Bayesian Phylogenetics with BEAUti and the BEAST 1.7. *Molecular Biology and Evolution* **29**:1969-1973.
- Excoffier, L., and H. E. L. Lischer. 2010. Arlequin suite ver 3.5: A new series of programs to perform population genetics analyses under Linux and Windows. *Molecular Ecology Resources* **10**:564-567.

- Faxon, W. 1884. Descriptions of new species of *Cambarus*, to which is added a synonymical list of the known species of *Cambarus* and *Astacus*. Proceedings of the American Academy of Arts and Sciences **20**:107-158.
- Faxon, W. 1885. A revision of the Astacidae. Part I. The genera *Cambarus* and *Astacus*. Memoirs of the Museum of Comparative Zoology at Harvard College **10**:1-86.
- Fetzner, J. W., Jr. 1996. Biochemical systematics and evolution of the crayfish genus *Orconectes* (Decapoda: Cambaridae). Journal of Crustacean Biology **16**:111-141.
- Fetzner, J. W., Jr. 2007. GCcalc v1.0: A GPS coordinate converter and vector and distance calculator for use among populations based on the great circle distance. Carnegie Museum of Natural History, Pittsburgh, PA. Available from: <http://iz.carnegiemnh.org/fetzner/software.htm>.
- Fetzner, J. W., Jr. 2010. Crayfish of Pennsylvania: Research to assist management, law enforcement, conservation and public awareness of Pennsylvania's unique aquatic resources. Final Project Report to the DCNR Wild Resource Conservation Program, Harrisburg, Pennsylvania. Project Number 07-256.
- Fetzner, J. W., Jr., and K. A. Crandall. 2003. Linear habitats and the Nested Clade Analysis: An empirical evaluation of geographic vs. river distances using an Ozark crayfish (Decapoda: Cambaridae). Evolution **57**:2101-2118.
- Fetzner, J. W., Jr., and R. J. DiStefano. 2008. Population genetics of an imperiled crayfish from the White River drainage of Missouri, USA. Freshwater Crayfish **16**:131-146.
- Fetzner, J. W., Jr., and R. F. Thoma. 2011. A population genetic analysis of two rare crayfish found in southwestern Virginia. Final Project Report Project No. 2008-11221, Final project report submitted to the Virginia Department of Game and Inland Fisheries, Richmond, VA.
- Folmer, O., M. Black, W. Hoeh, R. Lutz, and R. Vriegenhoek. 1994. DNA primers for amplification of mitochondrial cytochrome c oxidase subunit I from diverse metazoan invertebrates. Molecular Marine Biology and Biotechnology **3**:294-299.
- Hasegawa, M., K. Kishino, and T. Yano. 1985. Dating the human-ape splitting by a molecular clock of mitochondrial DNA. Journal of Molecular Evolution **22**:160-174.
- Hobbs, H. H., Jr. 1989. An illustrated checklist of the American crayfishes (Decapoda: Astacidae, Cambaridae, and Parastacidae). Smithsonian Contributions to Zoology **No. 480**:iii + 236 p.
- Lemey, P., A. Rambaut, A. J. Drummond, and M. A. Suchard. 2009. Bayesian Phylogeography Finds Its Roots. PLoS Comput Biol **5**:e1000520.
- Lieb, D. A., R. W. Bouchard, and R. F. Carline. 2011a. Crayfish fauna of southeastern Pennsylvania: Distributions, ecology, and changes over the last century. Journal of Crustacean Biology **31**:166-178.

- Lieb, D. A., R. W. Bouchard, R. F. Carline, T. R. Nuttall, J. R. Wallace, and C. L. Burkholder. 2011b. Conservation and Management of Crayfishes: Lessons from Pennsylvania. *Fisheries* **36**:489-507.
- Lieb, D. A., R. F. Carline, and V. M. Mengel. 2005. Discovery of a member of the *Cambarus acuminatus* complex (Decapoda: Cambaridae) in Pennsylvania with notes on its biology and habitat preferences. Final Report., Valley Forge National Historic Park, Valley Forge, Pennsylvania.
- Lieb, D. A., R. F. Carline, and V. M. Mengel. 2007. Crayfish survey and discovery of a member of the *Cambarus acuminatus* complex (Decapoda: Cambaridae) at Valley Forge National Historical Park in southeastern Pennsylvania. Technical Report NPS/NER/NRTR—2007/084, National Park Service, Philadelphia, PA.
- Lieb, D. A., R. F. Carline, J. L. Rosenberger, and V. M. Mengel. 2008. The discovery and ecology of a member of the *Cambarus acuminatus* complex (Decapoda: Cambaridae) in Valley Creek, southeastern Pennsylvania. *Journal of Crustacean Biology* **28**:439-450.
- Maddison, W. P. 1997. Gene trees in species trees. *Systematic Biology* **46**:523-536.
- Maddison, W. P., and D. R. Maddison. 2011. Mesquite: a modular system for evolutionary analysis. Version 2.75. <http://mesquiteproject.org/>.
- Ortmann, A. E. 1906. The crawfishes of the state of Pennsylvania. *Memoirs of the Carnegie Museum* **2**:iv+343 - 523.
- Pamilo, P., and M. Nei. 1988. Relationships between gene trees and species trees. *Molecular Biology and Evolution* **5**:568-583.
- Peakall, R., and P. E. Smouse. 2006. GENALEX 6: genetic analysis in Excel. Population genetic software for teaching and research. *Molecular Ecology Notes* **6**:288-295.
- Perry, W. L., J. L. Feder, G. Dwyer, and D. M. Lodge. 2001. Hybrid zone dynamics and species replacement between *Orconectes* crayfishes in a northern Wisconsin lake. *Evolution* **55**:1153-1166.
- Posada, D., and K. A. Crandall. 1998. Modeltest: Testing the model of DNA substitution. *Bioinformatics* **14**:817-818.
- Song, H., J. E. Buhay, M. F. Whiting, and K. A. Crandall. 2008. Many species in one: DNA barcoding overestimates the number of species when nuclear mitochondrial pseudogenes are coamplified. *Proceedings of the National Academy of Sciences* **105**:13486-13491.
- Swofford, D. L. 2003. PAUP*. Phylogenetic Analysis Using Parsimony (*and Other Methods). Version 4. Sinauer Associates, Sunderland, Massachusetts.
- Taylor, C. A., and M. Hardman. 2002. Phylogenetics of the crayfish subgenus *Crockerinus*, genus *Orconectes* (Decapoda: Cambaridae), based on cytochrome oxidase I. *Journal of Crustacean Biology* **22**:874-881.

- Taylor, C. A., and J. H. Knouft. 2006. Historical influences on genital morphology among sympatric species: gonopod evolution and reproductive isolation in the crayfish genus *Orconectes* (Cambaridae) *Biological Journal of the Linnean Society* **89**:1-12.
- Taylor, C. A., G. A. Schuster, J. E. Cooper, R. J. DiStefano, A. G. Eversole, P. Hamr, H. H. Hobbs, III, H. W. Robison, C. E. Skelton, and R. F. Thoma. 2007. A reassessment of the conservation status of crayfishes of the United States and Canada after 10+ years of increased awareness. *Fisheries* **32**:372-389.
- Taylor, C. A., M. L. Warren, Jr., J. F. Fitzpatrick, Jr., H. H. Hobbs, III, R. F. Jezerinac, W. L. Pflieger, and H. W. Robison. 1996. Conservation status of crayfishes of the United States and Canada. *Fisheries* **21**:25-38.
- Wu, C.-I. 1991. Inferences of species phylogeny in relation to segregation of ancient polymorphisms. *Genetics* **127**:429-435.

Appendix A. List of haplotypes by population.

The ID column is the same as that shown in Table 1 and used to plot population ID numbers on the maps. “N” is the total number of specimens sampled for the indicated locality. Mean estimates of population size, Theta (Θ), generated by MIGRATE. *H* is the haplotype diversity and π is nucleotide diversity. Haplotypes detected at a locality are indicated by an ‘H’ number in bold (e.g., H10), the number in parentheses after the haplotype number indicates the number of individuals sampled from the population that contained that COI haplotype. * the two Valley Creek populations were pooled for estimated of *H* and π .

ID	Species	Locality	N	Θ	H	$\pi(\pm SD)$	Haplotypes (Count)
35	<i>C. acuminatus</i>	PA: Chester Co., Valley Creek	7	0.00599	0.6944	0.00304 (± 0.00214)	H1(2), H2(1), H3(2), H5(1), H6(1)
38	<i>C. acuminatus</i>	PA:Montgomery Co., Valley Creek	5	0.00057	*	*	H2(3), H4(1), H7(1)
34	<i>C. acuminatus</i>	PA: Chester Co., Pickering Creek	7	0.00137	0.2857	0.000434 (± 0.00059)	H1(6), H4(1)
37	<i>C. acuminatus</i>	PA: Delaware Co., Darby Creek	5	0.01627	0.8000	0.003647 (± 0.00277)	H2(2), H3(1), H8(2)
13	<i>C. acuminatus</i>	MD: Prince Georges Co., Paint Branch	21	0.00325	0.2571	0.003647 (± 0.00051)	H9(18), H10(3)
12	<i>C. acuminatus</i>	MD: Montgomery Co., Northwest Branch	18	0.01524	0.6993	0.026591 (± 0.01386)	H9(5), H11(7), H12(6)
47	<i>C. acuminatus</i>	VA: Albemarle Co., Mechums River	8	0.00268	0.8214	0.001683 (± 0.00139)	H13(2), H14(2), H15(3), H16(1)
49	<i>C. acuminatus</i>	VA: Amherst Co., Pedlar River	2	0.00685	1.0000	0.027356 (± 0.02811)	H17(1), H18(1)
48	<i>C. acuminatus</i>	VA: Amherst Co., North Fork Buffalo River	1	0.00873	1.0000	0.000000 (± 0.00000)	H19(1)
50	<i>C. acuminatus</i>	VA: Bedford Co., Stony Creek	4	0.01571	0.0000	0.000000 (± 0.00000)	H20(4)
51	<i>C. acuminatus</i>	VA: Carrol Co., Stewarts Creek	13	0.00238	0.6795	0.008729 (± 0.00502)	H21(2), H22(7), H23(3), H24(1)
52	<i>C. acuminatus</i>	VA: Franklin Co., Maggoodee Creek	4	0.00099	0.0000	0.000000 (± 0.00000)	H25(4)
53	<i>C. acuminatus</i>	VA: Patrick Co., Dan River	18	0.00387	0.1111	0.001013 (± 0.00091)	H26(7), H27(1)
14	<i>C. acuminatus</i>	NC: Catawba Co., McLin Creek	14	0.00232	0.3956	0.000635 (± 0.00069)	H28(11), H29(1), H30(1), H31(1)
16	<i>C. acuminatus</i>	NC: Chatham Co., Unnamed Trib. To Haw River	2	0.01951	1.0000	0.063830 (± 0.06459)	H11(1), H12(1)
15	<i>C. acuminatus</i>	NC: Chatham Co., Dry Creek	11	0.00713	0.8364	0.028597 (± 0.01555)	H11(3), H12(4), H32(1), H33(1), H34(1), H35(1)
17	<i>C. acuminatus</i>	NC: Cleveland Co., Hinton Creek	1	0.02062	1.0000	0.000000 (± 0.00000)	H36(1)
18	<i>C. acuminatus</i>	NC: Cleveland Co., Lick Branch	5	0.01291	0.7000	0.001426 (± 0.00142)	H37(1), H38(3), H39(1)
19	<i>C. acuminatus</i>	NC: Davidson Co., Abbotts Creek	1	0.00587	1.0000	0.000000 (± 0.00000)	H12(1)
20	<i>C. acuminatus</i>	NC: Davie Co., Dutchmans Creek	13	0.00207	0.7821	0.002650 (± 0.00185)	H40(1), H41(6), H42(1), H43(1), H44(2), H45(2)
21	<i>C. acuminatus</i>	NC: Granville Co., Aarons Creek	13	0.00147	0.2821	0.000429 (± 0.00055)	H46(11), H47(2)
22	<i>C. acuminatus</i>	NC: Granville Co., Knap of Reeds Creek	6	0.02196	0.7333	0.002214 (± 0.00186)	H48(3), H49(2), H50(1)
23	<i>C. acuminatus</i>	NC: Granville Co., Tar River	15	0.00256	0.3714	0.000608 (± 0.00067)	H49(12), H51(1), H52(1), H53(1)
24	<i>C. acuminatus</i>	NC: Henderson Co., Boylston Creek	12	0.00186	0.4545	0.001220 (± 0.00106)	H54(9), H55(1), H56(1), H57(1)
25	<i>C. acuminatus</i>	NC: Iredell Co., Fourth Creek	17	0.01035	0.1176	0.000179 (± 0.00033)	H58(16), H59(1)
26	<i>C. acuminatus</i>	NC: Moore Co., Little River	15	0.00241	0.7333	0.001621 (± 0.00127)	H60(4), H61(7), H62(1), H63(1), H64(2)
27	<i>C. acuminatus</i>	NC: Polk Co., North Pacolet River	4	0.00740	0.5000	0.000760 (± 0.00094)	H65(3), H66(1)

ID	Species	Locality	N	Θ	H	$\pi(\pm SD)$	Haplotypes (Count)
28	<i>C. acuminatus</i>	NC: Richmond Co., Big Mountain Creek	16	0.00376	0.7833	0.004154 (±0.00260)	H67(6), H68(5), H69(2), H70(1), H71(1), H72(1)
29	<i>C. acuminatus</i>	NC: Richmond Co., Carledges Creek	1	0.02404	1.0000	0.000000 (±0.00000)	H73(1)
30	<i>C. acuminatus</i>	NC: Rutherford Co., Little First Broad River	1	0.00858	1.0000	0.000000 (±0.00000)	H74(1)
31	<i>C. acuminatus</i>	NC: Surry Co., Gully Creek	1	0.01501	1.0000	0.000000 (±0.00000)	H75(1)
32	<i>C. acuminatus</i>	NC: Vance Co., Anderson Creek	20	0.00099	0.1000	0.000152 (±0.00030)	H76(19), H77(1)
33	<i>C. acuminatus</i>	NC: Warren Co., Fishing Creek	10	0.00217	0.0000	0.000000 (±0.00000)	H78(10)
39	<i>C. acuminatus</i>	SC: Cherokee Co., Cherokee Creek	19	0.00196	0.3801	0.000604 (±0.00066)	H79(15), H80(2), H81(1), H82(1)
40	<i>C. acuminatus</i>	SC: Greenville Co., Barnes Creek	6	0.01126	0.8000	0.054189 (±0.03200)	H38(2), H83(2), H84(2)
41	<i>C. acuminatus</i>	SC: Greenville Co., Barton Creek	7	0.03003	0.0000	0.000000 (±0.00000)	H85(7)
42	<i>C. acuminatus</i>	SC: Greenville Co., South Saluda River	20	0.01028	0.3632	0.001320 (±0.00108)	H55(16), H86(1), H87(2), H88(1)
43	<i>C. acuminatus</i>	SC: Pickens Co., Oolenoy River	11	0.00916	0.1818	0.000276 (±0.00044)	H55(10), H89(1)
44	<i>C. acuminatus</i>	SC: Spartanburg Co., Lawsons Fork Creek	2	0.01128	0.0000	0.000000 (±0.00000)	H90(2)
45	<i>C. acuminatus</i>	SC: Spartanburg Co., Middle Tyger River	13	0.00154	0.2949	0.016180 (±0.00896)	H38(11), H91(1), H92(1)
46	<i>C. acuminatus</i>	SC: Spartanburg Co., Spivey Creek	6	0.00178	0.6000	0.000605 (±0.00080)	H93(4), H94(1), H95(1)
10	<i>C. brimleyorum</i>	NC: Cherokee Co., Valley River	6	0.00122	0.3333	0.001272 (±0.00129)	H96(5), H97(1)
11	<i>C. buntingi</i>	TN: Campbell Co., No Business Creek	6	0.00342	0.8000	0.038906 (±0.02307)	H98(1), H99(3), H100(1), H101(1)
54	<i>C. chaugaensis</i>	NC: Jackson/Macon Co., Chattooga River	17	0.01104	0.4118	0.005864 (±0.00362)	H102(13), H103(2), H104(2)
55	<i>C. coosae</i>	GA: Floyd Co., Little Cedar Creek	1	0.01737	1.0000	0.000000 (±0.00000)	H105(1)
56	<i>C. cumberlandensis</i>	KY: McCreary Co., Rock Creek	2	0.00193	0.0000	0.000000 (±0.00000)	H106(2)
57	<i>C. cumberlandensis</i>	TN: Fentress Co., Popular Cove Creek	1	0.01078	1.0000	0.000000 (±0.00000)	H107(1)
58	<i>C. georgiae</i>	NC: Macon Co., Burningtown Creek	7	0.01300	0.0000	0.000000 (±0.00000)	H108(7)
59	<i>C. hiwasseeensis</i>	NC: Cherokee Co., Peachtree Creek	18	0.00091	0.0000	0.000000 (±0.00000)	H109(18)
60	<i>C. hobbsorum</i>	NC: Union Co., Bearskin Creek	19	0.00641	0.8070	0.004142 (±0.00262)	H110(2), H111(8), H112(1), H113(2), H114(3), H115(1), H116(1), H117(1)
61	<i>C. hystricosus</i>	NC: Hoke Co., Puppy Creek	15	0.00267	0.7143	0.039919 (±0.02081)	H12(6), H118(6), H119(1), H120(1), H121(1)
62	<i>C. johni</i>	NC: Wilkes Co., East Prong Roaring River	19	0.00092	0.3860	0.000773 (±0.00076)	H122(15), H123(1), H124(1), H125(1), H126(1)
63	<i>C. johni</i>	NC: Wilkes Co., South Fork Reddies River	18	0.01117	0.8562	0.002592 (±0.00188)	H127(4), H128(6), H129(2), H130(1), H131(1), H132(1), H133(1), H134(1), H135(1)
64	<i>C. nerterius</i>	WV: Greenbriar Co., US-219 Cave	1	0.01010	1.0000	0.000000 (±0.00000)	H136(1)
67	<i>C. parishii ?</i>	NC: Clay Co., Tusquitee Creek	19	0.00547	0.2982	0.000640 (±0.00068)	H137(16), H138(1), H139(1), H140(1)
66	<i>C. parishii ?</i>	NC: Clay Co., Hot House Branch	1	0.01097	1.0000	0.000000 (±0.00000)	H141(1)
65	<i>C. parishii</i>	NC: Clay Co., Unnamed Trib to Fires Creek	18	0.00175	0.2092	0.000434 (±0.00062)	H96(2), H142(16)

ID	Species	Locality	N	Θ	H	$\pi(\pm SD)$	Haplotypes (Count)
68	<i>C. reburrus</i> ?	NC: Henderson Co., South Fork Mills River	16	0.00695	0.4917	0.001912 (± 0.00142)	H54(11), H56(1), H143(4)
69	<i>C. robustus</i>	VA: Carrol Co., Elkhorn Creek	12	0.00381	0.3030	0.000461 (± 0.00058)	H144(10), H145(2)
70	<i>C. scotti</i>	GA: Chattooga Co., Clarks Creek	1	0.00749	1.0000	0.000000 (± 0.00000)	H146(1)
74	<i>C. spicatus</i>	NC: Cleveland Co., Hinton Creek	1	0.00845	1.0000	0.000000 (± 0.00000)	H147(1)
75	<i>C. spicatus</i>	NC: Gaston Co., Long Creek	5	0.04049	0.4000	0.057751 (± 0.03559)	H28(4), H148(1)
77	<i>C. veteranus</i>	VA: Dickenson Co., McClure River	1	0.03935	1.0000	0.000000 (± 0.00000)	H149(1)
78	<i>C. veteranus</i>	WV: Wyoming Co., Pinnacle Creek	1	0.02348	1.0000	0.000000 (± 0.00000)	H150(1)
76	<i>C. veteranus</i>	KY: Pike Co., Long Fork	1	0.00883	1.0000	0.000000 (± 0.00000)	H151(1)
71	<i>C. (P.) sp.</i>	SC: Spartanburg Co., North Tyger River	2	0.01138	1.0000	0.106383 (± 0.10714)	H152(1), H153(1)
72	<i>C. (P.) sp.</i>	TN: Fentress Co., Rocky Branch	1	0.02300	1.0000	0.000000 (± 0.00000)	H154(1)
73	<i>C. (P.) sp.</i>	TN: McMinn Co., Cane Creek	3	0.01398	0.0000	0.000000 (± 0.00000)	H155(3)
8	<i>C. (J.) dubius</i>	WV: Preston Co., from burrow	1	—	1.0000	0.000000 (± 0.00000)	H158(1)
9	<i>C. (J.) sp.</i>	NC: Jackson Co., Scottsman Creek	8	—	0.4643	0.000760 (± 0.00082)	H159(6), H160(1), H161(1)
6	<i>C. (D.) sp.</i>	NC: Moore Co., Little River	1	—	1.0000	0.000000 (± 0.00000)	H162(1)
5	<i>C. (D.) sp.</i>	NC: Moore Co., Cabin Creek	2	—	0.0000	0.000000 (± 0.00000)	H163(2)
4	<i>C. bartonii</i>	VA: Rockingham Co., Dry River	6	—	0.0000	0.000000 (± 0.00000)	H164(6)
3	<i>C. bartonii</i>	VA: Amhearst Co., North Fork Buffalo River	4	—	0.8333	0.003503 (± 0.00294)	H164(2), H165(1), H166(1)
1	<i>C. bartonii</i>	PA: Lackawanna Co., Lackawanna River	5	—	0.7000	0.001216 (± 0.00121)	H6(3), H167(1), H168(1)
2	<i>C. bartonii</i>	PA: Lackawanna Co., Lehigh River	5	—	0.0000	0.000000 (± 0.00000)	H6(5)
79	<i>O. limosus</i>	MD: Prince Georges Co., Paint Branch	1	—	1.0000	0.000000 (± 0.00000)	H169(1)
80	<i>P. zonangulus</i>	LA: East Baton Rouge Co., LSU Research Ponds	1	—	1.0000	0.000000 (± 0.00000)	H170(1)

Appendix B. Average pairwise divergences (= number of mutations) within and among populations (two pages).

Population	ID	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39
Cambarus acuminatus PA: Chester Co., ValleyCr	1	2.0	2.6	1.9	52.8	46.3	22.2	16.0	16.0	28.3	40.0	40.3	25.7	37.3	49.0	45.5	31.7	30.8	43.3	42.4	35.2	37.7	39.4	59.8	40.2	67.4	43.0	29.1	32.7	60.3	39.7	39.4	41.3	42.7	48.0	74.3	59.3	58.9	73.3	33.5
Cambarus acuminatus PA: Chester Co., PickeringCr	2	1.5	0.3	2.4	50.3	44.8	21.0	14.0	14.0	27.1	38.3	39.1	23.1	34.8	47.1	44.0	29.1	28.8	42.1	40.9	34.0	36.7	38.2	59.7	38.7	67.9	41.0	27.6	30.1	59.1	37.1	38.2	40.1	40.1	46.0	73.0	59.1	58.8	72.1	31.5
Cambarus acuminatus PA: Delaware Co., DarbyCr	3	-0.3	1.0	2.4	52.5	46.1	22.1	15.8	15.8	28.2	39.9	40.2	25.4	37.0	48.8	45.4	31.4	30.6	43.2	42.3	35.0	37.6	39.3	59.8	40.0	67.4	42.8	29.0	32.4	60.2	39.4	39.3	41.2	42.4	47.8	74.2	59.3	58.9	73.2	33.3
Cambarus acuminatus MD: Prince Georges Co., Paint Branch	4	51.7	50.0	51.2	0.3	29.4	50.0	44.6	36.1	31.1	39.4	27.1	42.5	35.9	21.0	29.0	41.1	29.5	40.1	41.1	29.3	32.1	33.1	70.9	38.2	77.9	43.1	48.1	32.1	69.1	36.1	31.2	31.1	38.4	46.5	78.1	70.4	70.1	67.1	30.3
Cambarus acuminatus MD: Montgomery Co., Northwest Branch	5	36.6	35.9	36.2	20.5	17.5	43.8	41.6	32.8	28.5	39.2	34.6	41.2	35.4	21.0	16.7	34.2	28.5	11.5	41.4	33.2	32.9	35.6	68.4	39.1	68.8	45.6	40.5	26.6	65.8	37.1	35.1	36.4	39.9	44.8	72.6	69.0	68.9	66.7	29.9
Cambarus acuminatus VA: Albemarle Co., Mechums River	6	20.7	20.3	20.3	49.3	34.5	1.1	20.9	19.9	25.9	38.5	36.9	25.9	33.7	46.4	42.4	29.9	29.3	40.9	40.2	32.7	35.5	36.9	63.7	37.8	71.9	37.9	31.8	30.9	67.9	36.9	36.9	37.9	38.9	46.9	76.9	63.1	62.8	73.9	31.5
Cambarus acuminatus VA: Amherst Co., Pedlar River	7	6.0	4.9	5.6	35.5	23.9	11.3	18.0	11.5	27.0	37.9	36.5	22.6	34.6	43.3	40.9	29.0	28.7	40.0	40.0	32.3	35.1	36.6	60.9	37.9	66.1	40.5	27.3	28.0	63.5	37.0	36.6	37.5	39.5	45.5	72.0	60.3	59.9	69.0	30.9
Cambarus acuminatus VA: Amherst Co., North Fork Buffalo River	8	15.0	13.9	14.6	36.0	24.0	19.3	2.5	0.0	22.0	29.9	30.0	21.2	27.1	34.5	32.1	23.0	26.4	31.0	31.0	26.8	31.3	31.1	60.0	29.1	61.5	34.0	26.4	20.0	58.0	28.0	31.1	31.0	31.1	38.7	64.0	59.2	58.9	61.0	27.8
Cambarus acuminatus VA: Bedford Co., Stony Creek	9	27.3	27.0	27.0	31.0	19.8	25.3	18.0	22.0	0.0	24.7	19.0	25.2	22.5	30.0	28.0	22.0	15.2	27.0	27.0	19.2	20.2	23.1	56.0	24.8	65.1	32.0	30.4	12.0	60.0	23.0	25.1	25.0	28.0	35.3	69.0	57.1	56.9	62.0	18.2
Cambarus acuminatus VA: Carrol Co., Stewarts Creek	10	36.2	35.3	35.8	36.4	27.6	35.1	26.1	27.1	21.8	5.7	32.3	38.9	9.6	40.0	38.4	26.7	6.7	38.7	8.6	30.9	32.9	35.0	61.0	5.1	67.9	17.5	40.1	20.2	57.1	6.4	35.6	35.1	13.9	29.1	72.4	61.8	61.6	68.8	10.6
Cambarus acuminatus VA: Franklin Co., Maggodee Creek	11	39.3	39.0	39.0	27.0	25.9	36.3	27.5	30.0	19.0	29.4	0.0	35.3	29.8	33.0	34.0	34.0	26.0	37.0	33.1	8.2	10.0	10.1	66.8	31.9	70.7	37.0	40.4	26.0	69.0	30.0	10.1	10.0	35.2	43.3	76.0	66.3	65.9	64.0	27.2
Cambarus acuminatus VA: Patrick Co., Dan River	12	24.3	22.7	23.9	42.0	32.1	25.0	13.2	20.8	24.9	35.7	35.0	0.7	33.9	42.3	40.7	28.1	27.2	40.2	40.2	32.1	34.8	36.3	62.1	38.0	69.2	38.2	21.4	24.2	59.1	36.2	36.3	38.2	40.2	42.8	68.1	63.0	63.1	73.2	28.5
Cambarus acuminatus NC: Catawba Co., McLin Creek	13	36.1	34.4	35.6	35.5	26.4	33.0	25.4	26.9	22.3	6.5	29.6	33.3	0.4	36.2	34.9	22.8	4.5	34.7	10.0	25.7	29.0	29.8	59.4	6.1	69.1	13.0	34.9	16.8	52.8	4.9	29.9	29.9	10.3	26.6	70.5	60.4	60.3	66.4	8.5
Cambarus acuminatus NC: Chatham Co., Unnamed Trib to Haw River	14	27.0	26.0	26.6	-0.1	-8.7	24.8	13.3	13.5	9.0	16.1	12.0	20.9	15.0	42.0	20.7	32.0	29.5	21.0	42.0	32.7	33.3	35.5	69.9	39.5	72.3	45.5	43.6	29.9	67.5	37.5	34.6	35.5	40.1	46.0	75.0	70.1	69.3	67.5	30.7
Cambarus acuminatus NC: Chatham Co., Dry Creek	15	35.4	34.7	35.0	19.7	-1.2	32.7	22.7	22.9	18.8	26.4	24.8	31.2	25.6	-9.5	18.3	34.1	28.6	12.0	40.5	32.5	32.2	34.7	68.0	38.3	67.7	45.0	40.3	26.4	65.3	36.5	34.7	35.8	39.3	44.4	71.6	68.6	68.5	66.4	29.9
Cambarus acuminatus NC: Cleveland Co., Hinton Creek	16	30.7	29.0	30.2	41.0	25.4	29.3	20.0	23.0	22.0	23.8	34.0	27.7	22.6	16.0	24.9	0.0	16.6	31.0	26.3	31.8	33.5	36.1	58.0	25.0	70.6	34.0	29.3	16.0	53.0	22.0	34.1	36.0	26.1	35.7	71.0	59.0	59.1	72.0	18.8
Cambarus acuminatus NC: Cleveland Co., Lick Branch	17	29.4	28.3	29.0	29.0	19.3	28.3	19.3	26.0	14.8	3.4	25.6	26.4	3.9	8.1	19.1	16.8	0.8	27.6	8.4	23.4	25.6	24.9	49.0	4.5	57.9	9.4	27.9	11.6	47.6	3.4	24.5	25.0	4.7	19.8	60.2	49.8	49.7	56.0	4.9
Cambarus acuminatus NC: Davidson Co., Abbotts Creek	18	42.3	42.0	42.0	40.0	2.8	40.3	31.0	31.0	27.0	35.8	37.0	39.9	34.5	0.0	2.8	31.0	27.2	0.0	41.0	34.2	32.7	36.1	67.0	39.0	64.8	46.0	37.1	24.0	64.0	37.0	36.1	38.0	39.9	43.7	70.0	68.0	67.9	66.0	29.2
Cambarus acuminatus NC: Davie Co., Dutchmans Creek	19	40.5	39.9	40.2	40.1	31.7	38.7	30.1	30.1	26.1	4.9	32.2	39.0	9.0	20.1	30.4	25.4	7.1	40.1	1.7	31.2	32.3	35.2	59.5	4.4	73.1	18.3	39.5	22.8	59.0	7.1	37.2	35.3	14.4	30.0	70.9	60.5	60.4	69.2	12.1
Cambarus acuminatus NC: Davidson Co., Aarons Creek	20	34.0	33.7	33.7	29.0	24.3	32.0	23.2	26.7	19.0	27.9	8.0	31.6	25.4	11.5	23.2	31.7	22.9	34.0	30.1	0.3	6.7	6.4	62.7	29.8	68.6	32.8	35.8	24.8	67.8	27.8	6.2	6.2	33.1	42.2	76.8	62.1	61.8	65.8	25.4
Cambarus acuminatus NC: Granville Co., Knap of Reeds Creek	21	36.0	35.9	35.7	31.3	23.4	34.3	25.4	30.6	19.5	29.4	9.3	33.7	28.0	11.6	22.3	32.8	24.5	32.0	30.8	5.8	1.4	1.2	60.6	31.9	61.9	34.0	35.9	25.5	68.0	30.5	5.1	7.0	33.6	40.7	69.2	59.9	59.6	61.2	27.0
Cambarus acuminatus NC: Granville Co., Tar River	22	38.2	37.9	37.9	32.7	26.6	36.2	27.4	30.9	22.9	32.0	9.9	35.8	29.4	14.3	25.3	35.9	24.2	6.0	0.3	0.4	62.9	33.9	66.6	34.9	37.6	29.1	72.1	31.9	4.3	6.2	35.1	42.6	74.9	62.3	62.0	66.1	26.2		
Cambarus acuminatus NC: Henderson Co., Boylston Creek	23	58.4	59.1	58.2	70.4	59.3	62.8	51.5	59.6	55.6	57.8	66.3	61.3	58.8	48.5	58.5	57.6	48.2	66.6	58.2	62.1	59.5	62.3	0.8	60.8	73.5	61.8	61.5	59.0	37.8	60.9	63.9	63.8	62.0	59.0	70.8	2.8	2.8	68.7	50.2
Cambarus acuminatus NC: Iredell Co., Fourth Creek	24	39.1	38.5	38.8	38.0	30.3	37.1	28.9	29.0	24.7	2.2	31.9	37.7	5.9	18.5	29.1	24.9	4.0	38.9	3.4	29.6	31.2	33.6	60.4	0.1	69.3	14.1	37.2	18.9	57.8	3.1	34.0	34.1	10.3	27.6	73.8	61.9	61.8	68.7	8.6
Cambarus acuminatus NC: Moore Co., Little River	25	65.8	67.2	65.7	77.3	59.5	70.8	56.5	61.0	64.6	64.5	70.1	68.3	68.3	50.7	58.0	70.1	56.9	64.3	71.7	68.0	60.6	65.9	72.6	68.7	1.1	73.6	66.5	69.7	73.6	71.5	65.8	69.7	73.5	57.3	46.6	72.8	72.6	58.6	55.8
Cambarus acuminatus NC: Polk Co., North Pacolet River	26	41.8	40.6	41.4	42.8	36.6	37.1	31.3	33.8	31.8	14.3	36.8	37.6	12.5	24.3	35.6	33.8	8.8	45.8	17.2	32.5	33.1	34.5	61.2	13.8	72.8	0.5	40.3	28.0	62.0	14.0	35.1	35.0	17.2	31.1	72.3	61.2	60.9	73.0	13.2
Cambarus acuminatus NC: Richmond Co., Big Mountain Creek	27	26.7	26.1	26.4	46.6	30.4	29.8	16.9	25.0	29.0	35.8	39.0	19.7	33.3	21.2	29.8	27.9	26.1	35.8	37.2	34.3	33.8	36.0	59.7	35.8	64.6	38.6	2.7	28.1	66.4	37.3	35.7	40.4	41.3	44.2	70.1	62.4	62.5	74.3	29.7
Cambarus acuminatus NC: Richmond Co., Carldoges Creek	28	31.7	30.0	31.2	32.0	17.9	30.3	19.0	20.0	12.0	17.3	26.0	23.9	16.6	8.0	17.2	16.0	11.2	24.0	22.0	24.7	24.8	28.9	58.6	18.9	69.2	27.8	26.8	0.0	58.0	17.0	29.1	29.0	22.1	33.3	73.0	60.0	59.9	68.0	14.3
Cambarus acuminatus NC: Rutherford Co., Little First Broad River	29	59.3	59.0	59.0	69.0	57.0	67.3	54.5	58.0	60.0	54.2	69.0	58.7	52.6	46.5	56.1	53.0	47.2	64.0	58.1	67.7	67.3	71.9	37.4	57.8	73.1	61.8	65.0	58.0	0.0	55.0	71.1	73.0	59.2	57.3	73.0	37.0	37.1	70.0	48.1
Cambarus acuminatus NC: Surry Co., Gully Creek	30	38.7	37.0	38.2	36.0	28.4	36.3	28.0	28.0	23.0	3.5	30.0	35.8	4.7	16.5	27.4	22.0	3.0	37.0	6.2	27.7	29.8	31.7	60.5	3.0	71.0	13.8	35.9	17.0	55.0	0.0	32.1	32.0	9.2	27.0	74.0	62.0	61.9	69.0	7.5
Cambarus acuminatus NC: Vance Co., Anderson Creek	31	38.3	38.0	38.0	31.0	26.3	36.3	27.5	31.0	25.0	32.7	10.0	35.9	29.6	13.5	25.5	34.0	24.0	36.0	36.3	6.0	4.3	4.0	63.4	33.9	65.3	34.8	34.3	29.0	71.0	32.0	0.1	6.1	35.3	42.4	75.1	63.3	63.0	66.1	25.6
Cambarus acuminatus NC: Warren Co., Fishing Creek	32	40.3	40.0	40.0	31.0	27.7	37.3	28.5	31.0	25.0	32.2	10.0	37.9	29.6	14.5	26.7	36.0	24.6	38.0	34.4	6.0	6.3	6.0	63.4	34.0	69.														

Population	ID	40	41	42	43	44	45	46	47	48	49	50	51	52	53	54	55	56	57	58	59	60	61	62	63	64	65	66	67	68	69	70	71	72	73	74	75	76	77	78
Cambarus acuminatus PA: Chester Co., ValleyCr	1	40.3	49.0	49.8	58.0	61.0	61.3	70.7	64.3	44.7	27.5	43.1	39.3	37.2	54.7	69.4	36.0	38.3	59.8	34.8	69.3	33.0	49.2	45.0	50.0	48.0	56.2	71.7	58.0	71.7	87.0	85.8	65.3	63.3	45.3	49.0	56.7	56.7	97.7	90.7
Cambarus acuminatus PA: Chester Co., PickeringCr	2	38.3	48.3	48.6	57.7	61.0	59.9	70.0	63.1	44.0	25.8	41.9	37.9	35.7	55.9	68.2	35.9	38.1	59.7	34.7	68.1	31.0	46.9	44.9	49.9	47.9	54.6	71.0	57.9	71.5	87.1	85.3	65.9	63.9	46.0	49.9	57.9	57.9	97.1	90.1
Cambarus acuminatus PA: Delaware Co., DarbyCr	3	40.1	48.9	49.7	58.0	61.0	61.2	70.6	64.2	44.6	27.3	43.0	39.2	37.0	54.8	69.2	36.0	38.3	59.8	34.8	69.2	32.0	48.0	45.0	50.0	48.0	56.0	71.6	58.0	71.7	87.0	85.7	65.4	63.4	45.4	49.1	56.8	56.8	97.6	90.6
Cambarus acuminatus MD: Prince Georges Co., Paint Branch	4	40.3	40.1	54.8	61.7	68.1	53.1	68.1	70.1	37.1	49.7	40.5	40.9	37.4	68.1	62.2	39.1	41.0	71.2	38.0	68.1	31.1	47.5	60.1	58.1	58.1	55.6	69.1	62.1	66.1	84.1	86.1	77.1	71.1	58.1	61.6	68.5	68.1	100.1	90.1
Cambarus acuminatus MD: Montgomery Co., Northwest Branch	5	42.5	38.5	53.4	60.5	60.4	53.4	66.9	61.7	35.2	40.7	27.6	45.5	38.7	64.8	64.7	35.4	38.1	68.4	36.1	65.5	29.2	47.9	60.4	59.8	58.4	53.0	70.8	65.3	68.6	83.7	85.6	68.0	65.6	54.8	58.5	66.6	66.2	98.9	89.7
Cambarus acuminatus VA: Albemarle Co., Mechums River	6	35.5	44.2	55.4	59.5	56.9	64.9	66.9	66.9	39.9	30.8	41.9	39.8	36.0	58.9	64.9	38.0	41.3	63.7	31.7	67.9	29.9	46.5	49.9	51.9	50.9	56.4	66.1	60.9	74.5	83.9	84.0	65.9	67.9	47.9	52.1	62.5	62.9	101.9	93.9
Cambarus acuminatus VA: Amherst Co., Pedlar River	7	38.1	45.8	49.7	57.3	61.0	59.5	65.5	64.0	41.5	27.4	40.4	36.4	35.0	56.5	65.6	34.5	37.1	60.8	32.8	67.5	30.5	46.5	43.5	47.0	44.0	54.0	66.5	60.0	69.2	87.0	84.6	68.0	63.0	48.0	51.1	60.7	60.5	99.5	91.0
Cambarus acuminatus VA: Amherst Co., North Fork Buffalo River	8	32.7	46.3	45.2	52.7	51.0	50.0	57.0	57.0	42.0	28.4	33.1	30.9	26.9	48.0	57.1	29.0	34.4	59.8	30.8	60.0	26.0	38.4	36.0	41.0	35.0	45.5	58.0	55.0	57.3	74.0	75.1	63.0	54.0	46.0	47.0	51.2	51.0	96.0	87.0
Cambarus acuminatus VA: Bedford Co., Stony Creek	9	28.8	39.0	44.3	53.1	56.0	51.0	60.0	59.0	36.0	30.6	27.9	33.0	25.1	59.0	59.1	31.0	35.4	55.8	25.8	63.0	15.0	37.2	47.0	48.0	45.0	45.0	63.0	54.0	63.7	81.0	77.9	57.0	62.0	50.0	52.8	59.0	59.0	91.0	89.0
Cambarus acuminatus VA: Carrol Co., Stewarts Creek	10	15.6	38.5	51.3	55.8	64.9	52.1	64.2	65.6	34.8	40.1	27.8	38.2	5.0	65.2	62.7	34.3	38.1	61.0	38.1	64.1	19.8	27.1	52.6	55.8	50.8	39.5	67.0	51.8	64.1	85.3	86.4	65.3	59.5	52.9	57.3	60.7	60.4	101.6	92.8
Cambarus acuminatus VA: Franklin Co., Maggodee Creek	11	34.7	41.0	51.3	60.6	65.0	60.0	65.0	66.0	38.0	40.1	35.2	38.1	31.4	66.0	63.1	40.0	42.8	67.1	33.8	70.0	28.0	43.2	63.0	61.0	61.0	52.0	66.0	62.0	61.7	84.0	86.9	69.0	68.0	54.0	58.5	68.4	68.0	92.0	84.0
Cambarus acuminatus VA: Patrick Co., Dan River	12	35.5	46.4	52.2	54.9	62.9	53.3	66.2	62.2	42.0	24.0	39.3	34.1	35.7	58.2	64.3	39.0	42.4	61.8	36.0	70.1	30.2	46.0	52.1	49.1	52.1	52.1	67.1	55.2	67.9	83.2	83.3	63.2	66.2	49.1	52.1	60.5	60.1	99.1	87.3
Cambarus acuminatus NC: Catawba Co., McLin Creek	13	11.8	35.9	48.7	54.5	59.3	50.3	66.6	64.5	32.9	36.0	26.2	32.3	7.8	64.4	60.4	32.4	37.1	59.2	33.6	58.5	16.1	19.1	50.4	57.3	51.4	36.8	68.6	50.5	62.5	82.4	84.2	64.5	59.6	52.1	56.5	60.7	60.4	99.4	87.7
Cambarus acuminatus NC: Chatham Co., Unnamed Trib to Haw River	14	42.5	39.7	54.5	61.6	63.5	54.0	68.0	65.0	36.5	44.2	32.2	44.8	38.9	66.5	64.6	37.0	39.5	69.9	37.3	67.0	30.5	48.5	61.0	60.0	59.0	54.5	71.0	65.0	68.5	84.5	86.4	71.5	68.0	56.5	60.1	67.9	67.5	100.0	90.5
Cambarus acuminatus NC: Chatham Co., Dry Creek	15	42.1	37.8	52.6	60.2	58.5	52.6	66.0	60.9	34.5	40.5	27.5	44.6	38.1	63.7	62.9	35.1	37.9	68.0	35.4	64.0	29.3	47.3	59.5	59.1	57.5	52.4	69.4	64.9	67.8	82.0	84.1	67.5	65.0	54.1	57.6	65.7	65.4	98.1	89.3
Cambarus acuminatus NC: Cleveland Co., Hinton Creek	16	31.2	38.0	49.3	53.1	55.0	53.0	66.0	61.0	36.0	28.6	30.1	35.8	24.2	58.0	62.1	37.0	40.7	58.1	34.8	59.0	22.0	37.4	48.0	55.0	48.0	46.5	70.0	54.0	63.0	86.0	83.1	64.0	63.0	46.0	50.8	59.4	59.0	102.0	94.0
Cambarus acuminatus NC: Cleveland Co., Lick Branch	17	8.6	38.4	41.6	43.8	52.0	40.8	55.2	52.4	35.4	31.4	20.7	25.7	5.3	51.2	52.1	24.8	30.4	48.6	28.4	52.2	16.0	20.2	39.6	46.6	40.6	29.5	56.2	40.6	53.3	70.0	74.1	56.0	51.6	49.8	49.5	46.2	46.0	87.4	81.8
Cambarus acuminatus NC: Davidson Co., Abbotts Creek	18	42.8	37.3	52.3	59.6	57.0	53.0	66.0	58.0	34.0	36.8	22.1	46.8	39.1	63.0	65.1	34.0	37.0	66.8	34.8	64.0	28.0	47.6	60.0	60.0	58.0	51.5	71.0	66.0	69.0	83.0	84.9	64.0	63.0	53.0	56.8	65.4	65.0	98.0	89.0
Cambarus acuminatus NC: Davie Co., Dutchmans Creek	19	16.8	35.2	53.5	56.8	65.0	52.8	68.0	64.9	33.1	40.4	29.3	38.7	5.9	65.3	62.1	33.3	34.7	59.8	36.4	63.2	18.2	27.9	55.9	59.7	54.7	39.1	72.0	52.4	66.1	86.1	88.9	69.0	64.0	52.5	57.6	59.8	59.4	105.9	94.9
Cambarus acuminatus NC: Richmond Co., Aarons Creek	20	31.0	41.8	49.3	57.1	60.8	58.8	62.8	63.8	38.8	36.5	33.1	34.0	28.9	63.8	60.0	37.8	41.0	62.9	30.0	67.8	24.8	39.4	58.8	59.8	59.8	51.3	61.8	60.8	66.5	80.8	86.7	69.2	67.8	52.8	56.8	65.2	64.8	90.8	83.8
Cambarus acuminatus NC: Granville Co., Knap of Reeds Creek	21	32.3	41.8	48.5	54.3	57.3	57.0	59.2	58.5	38.8	37.1	33.3	32.7	30.6	59.5	58.7	35.2	38.1	60.7	32.0	65.2	29.3	41.2	58.2	59.7	59.2	47.8	58.2	60.0	63.8	76.2	85.0	63.7	61.7	52.8	55.6	61.2	61.0	86.5	81.7
Cambarus acuminatus NC: Granville Co., Tar River	22	32.9	42.0	52.9	56.9	63.1	62.9	62.9	64.1	39.0	37.8	35.9	36.0	32.4	64.1	63.1	38.9	41.9	63.1	33.8	69.9	28.9	43.1	62.8	63.9	63.9	51.4	61.9	63.1	68.9	82.9	90.8	66.9	65.9	52.1	56.6	65.5	65.1	92.9	85.9
Cambarus acuminatus NC: Henderson Co., Boylston Creek	23	59.1	48.5	64.0	28.2	68.8	68.0	77.6	76.0	46.2	59.9	61.0	64.5	55.8	36.7	74.0	41.3	37.6	1.1	57.6	72.7	52.2	68.4	66.7	73.6	64.7	64.4	78.6	36.8	68.3	89.0	93.7	67.8	68.6	30.7	32.2	34.3	33.9	103.0	103.7
Cambarus acuminatus NC: Iredell Co., Fourth Creek	24	12.6	37.1	52.5	56.0	64.8	50.8	65.8	64.9	34.1	38.1	27.5	36.4	2.4	66.9	61.0	31.8	36.8	60.7	35.7	64.8	18.1	24.2	51.8	55.6	50.8	38.4	67.8	51.9	64.9	84.6	86.6	66.7	61.9	54.1	58.7	61.3	60.9	102.7	91.9
Cambarus acuminatus NC: Moore Co., Little River	25	70.0	51.7	66.3	66.4	76.1	72.8	57.3	82.9	45.5	65.4	66.2	65.8	64.6	61.3	75.5	45.9	46.5	72.9	68.8	79.6	65.2	74.3	69.9	71.9	67.1	60.1	58.1	66.1	79.7	67.7	80.5	37.3	36.5	53.7	56.4	62.3	62.3	86.9	87.1
Cambarus acuminatus NC: Polk Co., North Pacolet River	26	1.5	42.0	54.3	57.4	66.8	55.0	65.8	66.0	39.0	41.6	35.1	40.6	14.7	64.0	60.1	36.0	41.1	61.8	37.8	64.0	23.0	29.5	56.0	62.0	57.0	41.1	65.8	51.0	67.7	86.0	86.9	68.0	61.0	52.0	55.8	61.4	61.0	102.0	88.0
Cambarus acuminatus NC: Richmond Co., Big Mountain Creek	27	37.9	44.0	53.9	55.0	62.5	58.0	66.7	66.0	40.6	7.9	37.8	36.2	36.4	59.1	68.7	35.3	38.9	61.2	32.3	63.9	32.3	46.5	50.6	53.8	51.5	53.7	65.9	58.3	70.5	92.6	84.8	59.4	63.1	50.3	53.2	61.9	61.5	99.3	91.8
Cambarus acuminatus NC: Richmond Co., Carldoges Creek	28	25.0	34.0	47.0	53.4	57.0	49.0	65.0	58.0	31.0	30.3	25.1	32.8	20.0	60.0	60.1	32.0	36.7	58.8	30.8	63.0	14.0	32.8	49.0	50.0	47.0	44.0	68.0	56.0	64.0	86.0	82.9	65.0	62.0	48.0	52.8	62.4	62.0	96.0	89.0
Cambarus acuminatus NC: Rutherford Co., Little First Broad River	29	59.0	50.3	62.3	36.4	68.0	71.0	79.0	78.0	47.0	62.1	58.1	66.4	53.2	50.0	76.1	46.0	46.4	37.4	62.8	70.0	55.0	62.2	66.0	75.0	67.0	65.0	81.0	36.0	69.0	88.0	85.3	67.0	61.0	37.0	42.3	46.4	46.0	108.0	102.0
Cambarus acuminatus NC: Surry Co., Gully Creek	30	12.3	38.0	51.2	55.9	63.0	51.0	67.0	65.0	35.0	37.8	26.5	36.7	3.8	67.0	60.1	34.0	38.9	60.8	36.8	62.0	17.0	23.6	51.0	56.0	51.0	38.0	69.0	51.0	65.0	84.0	84.9	67.0	62.0	53.0	58.3	60.4	60.0	102.0	90.0
Cambarus acuminatus NC: Vance Co., Anderson Creek	31	32.9	41.1	52.4	55.9	63.1	63.1	61.1	64.1	38.1	35.9	35.9	38.1	33.6	65.1	62.2	41.0	43.7	64.1	35.9	69.1	31.1	42.9	61.1	64.1	62.1	51.6	60.1	62.1	66.7	85.1	88.9	65.1	66.1	53.1	57.6	66.5	66.1	92.1	84.1
Cambarus acuminatus NC: Warren Co., Fishing Creek	32	33.0	44.0	55.0	57.8	65.0	63.0	66.0	66.0	41.0	41.9	37.8	38.2	33.1	65.0	62.1	42.0	44.9	64.1	35.8	72.0	29.0	43.4	63.0	66.0															

Appendix C. Summary of the 43 haplotype networks recovered from the TCS analysis.

Network	Number of Haplotypes	Number of Specimens	Number of Locations	Number of Species	States Represented
1	5	21	3	1	PA
2	46	144	17	3	PA, VA, NC, SC
3	2	26	2	1	MD
4	4	8	1	1	VA
5	1	1	1	1	VA
6	2	2	2	1	VA
7	2	18	1	1	VA
8	1	1	1	1	NC
9	9	59	4	2	NC, SC
10	5	15	1	1	NC
11	14	35	2	2	NC
12	1	1	1	1	NC
13	12	68	6	1	NC, VA
14	1	2	1	1	SC
15	1	1	1	1	TN
16	2	4	1	1	TN
17	1	1	1	1	TN
18	3	17	1	1	NC
19	1	1	1	1	GA
20	1	1	1	1	KY
21	2	2	2	1	TN
22	1	7	1	1	NC
23	7	34	6	2	MD, NC
24	5	19	1	1	NC
25	4	19	1	1	NC
26	5	43	4	3	NC
27	2	12	1	1	VA
28	1	1	1	1	GA
29	1	1	1	1	NC
30	1	1	1	1	NC
31	2	2	2	1	VA, KY
32	1	1	1	1	WV
33	4	11	4	1	SC
34	1	3	1	1	TN
35	2	3	1	1	VA
36	1	1	1	1	WV
37	3	8	1	1	NC
38	1	1	1	1	NC
39	1	1	1	1	NC
40	4	11	3	2	VA, WV
41	5	14	7	2	PA, NC
42	1	1	1	1	MD
43	1	1	1	1	LA

The Department of the Interior protects and manages the nation's natural resources and cultural heritage; provides scientific and other information about those resources; and honors its special responsibilities to American Indians, Alaska Natives, and affiliated Island Communities.

NPS 464/120889, May 2013

National Park Service
U.S. Department of the Interior



Natural Resource Stewardship and Science

1201 Oakridge Drive, Suite 150
Fort Collins, CO 80525

www.nature.nps.gov

EXPERIENCE YOUR AMERICA TM