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Molecular phylogenetics of the burrowing crayfish genus *Fallicambarus* (Decapoda: Cambaridae)

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The crayfish genus *Fallicambarus* contains 19 species of primary burrowing freshwater crayfish divided into two distinct subgenera. We test current hypotheses of the phylogenetic relationships among species within the genus as well as the monophyly of the genus. Our study samples all 19 species for five gene regions (both nuclear and mitochondrial) to estimate a robust phylogenetic hypothesis for the genus. We show that the genus is not a monophyletic group. The subgenus *Creaserinus* does fall out as a monophyletic group, but distinct from the subgenus *Fallicambarus*. The subgenus *Fallicambarus* appears to be monophyletic with the exception of the species *Procambarus (Tenuicambarus) tenuis*, which falls in the midst of this subgenus suggesting that it might be better classified as a *Fallicambarus* species. We also show that the species *Fallicambarus fodiens* is a species complex with distinct evolutionary lineages that are regionalized to different geographic areas.

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Introduction

The genus *Fallicambarus* consists entirely of primary burrowers, crayfish that inhabit burrows for all of their lives. Among crayfish there are also secondary burrowers, individuals that inhabit burrows for part of the season, and tertiary burrowers, individuals that seldom inhabit burrows (Hobbs 1981). Because of their burrowing nature, the genus *Fallicambarus* is distinct from the more typical stream-based crayfish species, and this habitat shift may impact migration, speciation and conservation. Their terrestrial habitat also has significant impact on studies of *Fallicambarus* as they are difficult to collect and it is often difficult to identify potential suitable habitat (Welch & Eversole 2006). In addition, primary burrowers are susceptible to having their populations isolated because their habitat is typically patchy.

The range of *Fallicambarus* is also noteworthy because of its breadth, as the genus extends from Ontario Canada to the southern United States, from Florida to Texas (Fig. 1). Some species, *Fallicambarus (Creaserinus) fodiens* in particular, have very large geographic ranges. The crayfish within the genus *Fallicambarus* have great economic importance because of the effect that they can have on the land. As *Fallicambarus* is composed completely of primary burrowers, they all spend their lives creating complex burrows. The burrows can have a negative impact when their habitat overlaps with human land-based activities, such as farmland. One species, *Fallicambarus (Fallicambarus) devastator*, was named for the devastating effect that it had on the farming industry in Texas (Hobbs & Whiteman 1987). The burrows will erode the farmland, kill the crops and create 'pot holes' that ruin the farm equipment. They are also known to burrow in lawns and fields, destroying the aesthetics of developed areas. There is much economic value in controlling these populations without destroying the biological diversity that already exists.

Hobbs (1973) defined the genus *Fallicambarus* by a rostrum in adults that is devoid of spines, pleopods bent caudally at angle of 90° or more, and pleopods that terminate in two or three distinct parts. Hobbs also split the genus

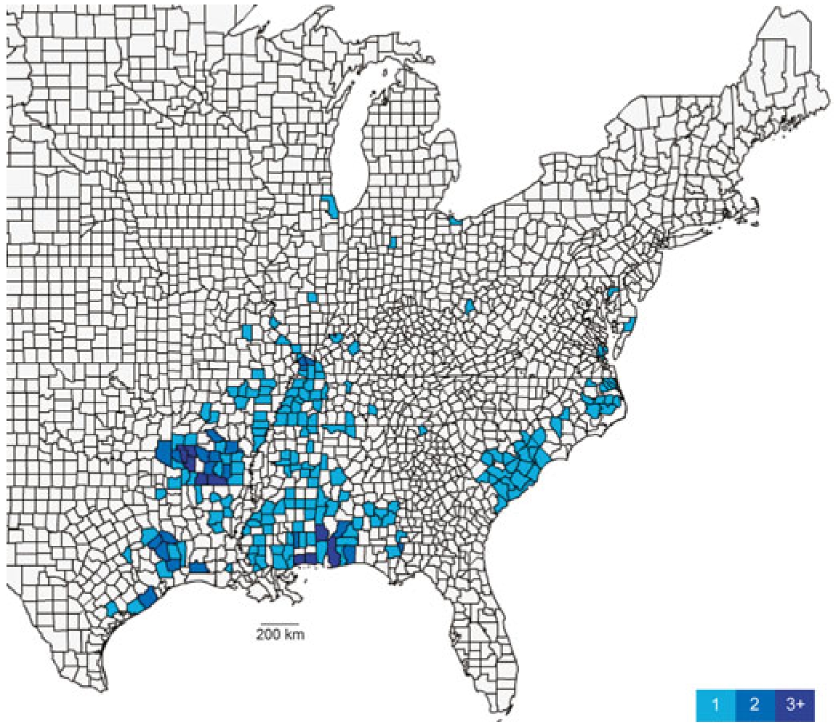

Fig. 1 Choropleth map of the distribution of *Fallicambarus* generated with the open source web tool Openheatmap (http://www.openheatmap.com/) with counts of species in each US county from the SI USNM Invertebrate collection data base (downloaded April, 2012 from http://collections.mnh.si.edu/search/iz/). Each County is coloured according to the number of species listed in the SI USNM records. Counties coloured white have no records and a colour scheme scale shown in the lower right corner of figure for counties with species records.

into two morphologically distinct subgenera *Fallicambarus* and *Creaserinus* (Hobbs 1973). Two of the major morphological features that separate these groups are the presence of a proximomesial spur on the first pleopod of male (present in *Fallicambarus*, absent in *Creaserinus*) and the size of the boss on the coxa of the fourth pereipod (conspicuously large boss in *Fallicambarus*, not conspicuously large boss in *Creaserinus*) (Hobbs 1973).

The subgenus *Fallicambarus* consists of ten species found in Texas, Louisiana and Arkansas USA (Table 1). The subgenus *Creaserinus* has nine species, which together have a much larger distribution than the subgenus *Fallicambarus* (Table 1). *Fallicambarus (C.) fodiens* has the largest distribution of all the *Fallicambarus* species which spans the entire distribution of all other species in the genus and extends into Canada and a disjunct region from New Jersey, USA to South Carolina, USA encompassing the coastal plain and the lower Piedmont provinces. The genus contains one critically endangered species (*Fallicambarus (Creaserinus) hortoni*), one endangered (*Fallicambarus (C.) petilicarpus*), and five near threatened species.

As Hobbs described the subgenera, a number of new species have been described in the genus and assigned to one of the two subgenera, but a formal phylogenetic analysis has never been conducted on the genus to test the evolutionary relationships among species nor the validity of the subgeneric designations. We update the Hobbs (1973) hypothesis by adding species described after this publication following the describing authors' hypotheses of closest relatives (Fig. 2) (Hobbs 1975; Fitzpatrick 1987; Hobbs & Whiteman 1987; Hobbs & Robison 1989; Johnson 2008, 2011). Our study capitalizes on complete species sampling and extensive molecular sampling to estimate phylogenetic relationships among the species of the genus *Fallicambarus*. We then use the resulting phylogeny to test the updated hypothesis of Hobbs (Fig. 2) and the robustness of the subgeneric designations for the genus.

Materials and methods

Sampling, DNA extraction, DNA amplification and sequencing

We targeted each species of the genus *Fallicambarus* to obtain a robust phylogenetic estimate. We obtained samples from multiple individuals of every species except for *Fallicambarus (C.) hortoni* and *Fallicambarus (Fallicambarus) houstonensis* for which one sample was obtained for each species. In a few select species with wider distributions, such as *F. (C.) fodiens*, we collected individuals from multiple localities to test the monophyly of the species and their genetic cohesion (Templeton 2001) across their widespread geographic distributions. Outgroup taxa were selected to represent several of the genera in the superfamily Astacoidea (*Astacus*, *Pacifasticus*, *Cambarellus*, *Procambarus*, *Orconectes*, *Faxonella*, *Cambarus*, *Barbicambarus*). Sequence data for these outgroup taxa were obtained from GenBank (Table 1).

Table 1 Species, collection number, locality and GenBank accession number for specimens used in this study (NA = Not Available)

	Collection no.	State	County	Latitude	Longitude	16S	CO1	12S	28S	H3
Ingroup Taxa										
Fallicambarus (Creaserinus) burrisi (Fitzpatrick, 1987)	KC7371	MS	Green	31.296	−88.484	KC163510	KC163706	KC163418	KC163606	KC163786
F. (C.) burrisi	KC7372	MS	Green	31.403	−88.452	KC163511	No data	KC163419	KC163607	KC163787
F. (C.) burrisi	KC7373	MS	Green	31.403	−88.452	KC163512	KC163707	KC163420	KC163608	KC163788
F. (C.) byersi (Hobbs, 1941)	KC3844	FL	Escambia	30.773	−87.339	KC163489	KC163665	KC163396	KC163583	No data
F. (C.) byersi	KC4168	FL	Escambia	30.694	−87.435	JX127863	JX128002	JX127720	JX127594	JX127455
F. (C.) byersi	KC6057	FL	Escambia	30.434	−87.324	KC163505	KC163712	KC163413	KC163601	KC163781
F. (C.) byersi	KC6127	FL	Okaloosa	30.716	−86.516	KC163506	KC163682	KC163414	KC163602	KC163782
F. (C.) caesius Hobbs, 1975	JC2197	AR	Hempstead	33.872	−93.570	KC163445	KC163699	KC163350	KC163539	KC163729
F. (C.) caesius	JC2199	AR	Hempstead	33.506	−93.487	KC163446	KC163711	KC163351	KC163540	KC163730
F. (C.) caesius	JC2204	AR	Hempstead	33.506	−93.487	JX127867	JX128007	JX127724	JX127598	JX127459
F. (C.) caesius	KC5649	AR	Hempstead	33.869	−93.488	KC163500	KC163678	KC163409	KC163597	KC163778
F. (C.) caesius	KC5650	AR	Hempstead	33.869	−93.488	KC163501	KC163679	No data	KC163598	KC163779
F. (C.) caesius	KC5651	AR	Hempstead	33.869	−93.488	KC163502	KC163680	KC163410	KC163599	KC163780
F. (C.) danielae Hobbs, 1975	KC7397	MS	Jackson	30.596	−88.864	KC163513	KC163685	KC163421	KC163609	KC163789
F. (C.) danielae	KC7555	MS	Harrison	30.633	−88.962	KC163516	KC163688	KC163424	KC163612	KC163792
F. (C.) danielae	KC7556	MS	Harrison	30.633	−88.962	KC163517	KC163689	KC163425	KC163613	KC163793
F. (C.) danielae	KC7557	MS	Harrison	30.633	−88.962	KC163518	KC163690	KC163426	KC163614	KC163794
F. (C.) fodiens (Cottle, 1863)	DJ130	TX	Hardin	30.297	−94.459	No data	KC163619	No data	KC163525	KC163716
F. (C.) fodiens	DJ60	TX	Calhoun	28.582	−96.833	KC163437	No data	KC163341	KC163531	KC163721
F. (C.) fodiens	DJ61	TX	Newton	30.426	−93.805	KC163438	KC163625	KC163342	KC163532	KC163722
F. (C.) fodiens	DJ71	TX	Refugio	28.471	−96.944	KC163441	KC163630	KC163345	KC163535	KC163725
F. (C.) fodiens	DJ75	TX	Galveson	29.439	−95.111	KC163442	KC163632	KC163346	KC163536	KC163726
F. (C.) fodiens	DJ76	TX	Harris	29.608	−95.474	KC163443	KC163633	KC163347	KC163537	KC163727
F. (C.) fodiens	JC2377	AR	Hempstead	33.872	−93.570	KC163451	KC163702	KC163356	KC163545	KC163735
F. (C.) fodiens	JC2733	AR	Little River	33.882	−94.419	KC163459	KC163636	KC163364	KC163553	KC163743
F. (C.) fodiens	JC2738	AR	Little River	33.882	−94.419	KC163460	KC163637	KC163365	KC163554	KC163744
F. (C.) fodiens	JC2776	AR	Sevier	34.060	−94.472	KC163461	KC163638	KC163366	KC163555	KC163745
F. (C.) fodiens	JC2983	AR	Ashley	33.114	−92.321	KC163466	KC163643	KC163371	KC163558	KC163750
F. (C.) fodiens	JC3097	AR	Bradley	33.368	−92.024	KC163470	KC163698	KC163376	KC163561	No data
F. (C.) fodiens	KC3843	FL	Santa Rosa	30.912	−87.275	KC163488	KC163664	KC163395	KC163582	No data
F. (C.) fodiens	KC4150	FL	Escambia	30.857	−87.311	KC163490	KC163666	KC163397	KC163584	KC163769
F. (C.) fodiens	KC4153	FL	Escambia	30.857	−87.311	KC163491	KC163667	KC163398	KC163585	KC163770
F. (C.) fodiens	KC4163	FL	Escambia	30.857	−87.311	KC163492	KC163668	KC163399	KC163586	KC163771
F. (C.) fodiens	KC5452	AR	Hot Springs	34.054	−93.245	KC163494	KC163670	KC163401	KC163588	No data
F. (C.) fodiens	KC5453	AR	Hot Springs	34.054	−93.245	KC163495	KC163671	KC163402	KC163589	No data
F. (C.) fodiens	KC5454	AR	Hot Springs	34.054	−93.245	KC163496	No data	KC163403	KC163590	No data
F. (C.) fodiens	KC5659	AR	Hempstead	33.872	−93.570	KC163503	No data	KC163411	KC163600	No data
F. (C.) fodiens (Cottle, 1863)	KC5661	AR	Hempstead	33.872	−93.570	KC163504	KC163681	KC163412	No data	No data
F. (C.) fodiens	KC7428	MS	Lauderdale	32.240	−88.772	KC163514	KC163686	KC163422	KC163610	KC163790
F. (C.) fodiens	KC7513	MS	Winston	33.015	−88.969	KC163515	KC163687	KC163423	KC163611	KC163791
F. (C.) gilpini Hobbs & Robison, 1989	JC2222	AR	Jefferson	34.084	−91.996	KC163448	KC163715	KC163353	KC163542	KC163732
F. (C.) gilpini	JC2223	AR	Jefferson	34.084	−91.996	KC163449	KC163629	KC163354	KC163543	KC163733
F. (C.) gilpini	JC2224	AR	Jefferson	34.084	−91.996	KC163450	KC163628	KC163355	KC163544	KC163734
F. (C.) gordoni (Fitzpatrick, 1987)	JF10166	MS	Perry	No data	No data	KC163471	KC163713	KC163377	KC163562	KC163753
F. (C.) gordoni	SW1203	MS	Perry	No data	No data	KC163520	KC163714	KC163428	KC163616	KC163798
F. (C.) gordoni	SW1206	MS	Perry	No data	No data	KC163521	KC163692	KC163429	No data	KC163799
F. (C.) gordoni	SW2203	MS	Perry	No data	No data	KC163522	KC163710	KC163430	No data	No data
F. (C.) gordoni	SW2205	MS	Perry	No data	No data	KC163523	KC163693	KC163431	KC163617	KC163800
F. (C.) gordoni	SW5002	MS	Perry	No data	No data	KC163524	KC163694	No data	KC163618	KC163801
F. (C.) hortoni Hobbs and Fitzpatrick, 1970	JF8770	TN	Chester	No data	No data	KC163472	KC163646	KC163378	KC163563	No data
F. (C.) oryktes Penn and Marlow, 1959	KC1130	MS	Harrison	30.471	−88.970	KC163475	KC163649	KC163381	KC163566	KC163756

Table 1 Continued

	Collection no.	State	County	Latitude	Longitude	16S	CO1	12S	28S	H3
F. (C.) oryktes	KC7338	MS	Stone	38.846	−86.060	KC163508	KC163684	KC163416	KC163604	KC163784
F. (C.) oryktes	KC7339	MS	Stone	38.846	−86.060	KC163509	KC163695	KC163417	KC163605	KC163785
F. (C.) oryktes	KC7562	MS	Stone	38.846	−86.060	KC163519	KC163691	KC163427	KC163615	KC163795
Fallicambarus sp. nov. 1	JC2627	AR	Ouachita	No data	No data	KC163452	KC163703	KC163357	KC163546	KC163736
Fallicambarus sp. nov. 1	JC2629	AR	Ouachita	No data	No data	KC163453	KC163704	KC163358	KC163547	KC163737
Fallicambarus sp. nov. 1	JC2630	AR	Ouachita	No data	No data	KC163454	KC163700	KC163359	KC163548	KC163738
Fallicambarus sp. nov. 1	JC2631	AR	Ouachita	No data	No data	KC163455	KC163701	KC163360	KC163549	KC163739
Fallicambarus sp. nov. 2	JC2642	LA	Morehouse	No data	No data	KC163456	KC163705	KC163361	KC163550	KC163740
Fallicambarus sp. nov. 2	JC2643	LA	Morehouse	No data	No data	KC163457	KC163709	KC163362	KC163551	KC163741
Fallicambarus sp. nov. 2	KC2929	LA	Morehouse	No data	No data	KC163481	KC163658	KC163388	KC163575	KC163763
Fallicambarus sp. nov. 2	KC2930	LA	Morehouse	No data	No data	KC163482	KC163659	KC163389	KC163576	KC163764
Fallicambarus (Fallicambarus) devastator Hobbs & Whiteman, 1987	KC1019	TX	Angelina	No data	No data	KC163473	KC163647	KC163379	KC163564	KC163754
F. (F.) devastator	KC1020	TX	Angelina	No data	No data	KC163474	KC163648	KC163380	KC163565	KC163755
F. (F.) dissitus (Penn, 1955)	JC2681	AR	Columbia	No data	No data	KC163458	KC163635	KC163363	KC163552	KC163742
F. (F.) dissitus	JC2872	AR	Union	33.226	−92.953	KC163464	KC163641	KC163369	No data	KC163748
F. (F.) dissitus	JC2870	AR	Union	33.226	−92.953	KC163463	KC163640	KC163368	KC163557	KC163747
F. (F.) dissitus	JC2869	AR	Union	33.226	−92.953	KC163462	KC163639	KC163367	KC163556	KC163746
F. (F.) dissitus	JC2874	AR	Union	33.226	−92.953	KC163465	KC163642	KC163370	No data	KC163749
F. (F.) harpi Hobbs and Robison, 1985	KC2900	AR	Pike	34.333	−92.475	KC163477	KC163654	KC163385	KC163571	KC163759
F. (F.) harpi	KC2903	AR	Pike	34.333	−92.475	KC163478	KC163655	KC163386	KC163572	KC163760
F. (F.) harpi	KC2910	AR	Pike	34.333	−92.475	KC163479	KC163656	No data	KC163573	KC163761
F. (F.) houstonensis Johnson, 2008	DJ242	TX	Brazoria	29.430	−95.224	KC163432	KC163620	KC163336	KC163526	No data
F. (F.) jeanae Hobbs, 1973	KC2847	AR	Pike	No data	No data	KC163476	KC163650	KC163382	KC163567	KC163757
F. (F.) jeanae	KC2919	AR	Pike	No data	No data	KC163480	KC163657	KC163387	KC163574	KC163762
F. (F.) jeanae	KC2951	AR	Pike	34.236	−93.756	KC163483	KC163660	KC163390	KC163577	KC163765
F. (F.) jeanae	KC2955	AR	Hot Springs	No data	No data	KC163484	KC163661	KC163391	KC163578	KC163766
F. (F.) jeanae	KC2977	AR	Pike	34.244	−93.642	KC163487	KC163708	KC163394	KC163581	KC163768
F. (F.) jeanae	KC5557	AR	Clark	34.265	−93.469	KC163497	KC163672	KC163404	KC163591	KC163773
F. (F.) jeanae	KC5558	AR	Clark	34.265	−93.469	No data	KC163673	KC163405	KC163592	KC163774
F. (F.) jeanae	KC5559	AR	Clark	34.265	−93.469	No data	KC163674	KC163406	KC163593	KC163775
F. (F.) jeanae	KC5580	AR	Hot Springs	34.328	−93.274	No data	KC163675	No data	KC163594	KC163776
F. (F.) jeanae	KC5582	AR	Hot Springs	34.328	−93.274	KC163498	KC163676	KC163407	KC163595	KC163777
F. (F.) jeanae	KC5583	AR	Hot Springs	34.328	−93.274	KC163499	KC163677	KC163408	KC163596	No data
F. (F.) kountzeae Johnson, 2008	DJ62	TX	Hardin	30.221	−94.378	KC163439	KC163626	KC163343	KC163533	KC163723
F. (F.) kountzeae	DJ63	TX	Hardin	30.331	−94.420	KC163440	KC163627	KC163344	KC163534	KC163724
F. (F.) macneesei (Black, 1967)	DJ310	TX	Brazoria	29.294	−95.276	KC163433	KC163621	KC163337	KC163527	KC163717
F. (F.) macneesei	DJ79	TX	Newton	30.488	−93.807	KC163444	KC163634	KC163348	KC163538	KC163728
F. (F.) macneesei	KC7297	LA	Acadia	No data	No data	KC163507	KC163683	KC163415	KC163603	KC163783
F. (F.) petilicarpus Hobbs & Robison, 1989	JC2986	AR	Union	33.131	−92.480	KC163467	KC163644	KC163372	No data	No data
F. (F.) petilicarpus	JC3034	AR	Union	33.319	−92.978	KC163468	KC163645	KC163373	KC163559	KC163751
F. (F.) petilicarpus	JC3036	AR	Union	33.319	−92.978	KC163469	KC163696	KC163374	KC163560	KC163752
F. (F.) petilicarpus	JC3038	AR	Union	33.319	−92.978	No data	KC163697	KC163375	No data	No data
F. (F.) strawni (Reimer, 1966)	KC2963	AR	Howard	34.277	−93.947	KC163485	KC163662	KC163392	KC163579	KC163767
F. (F.) strawni	KC2966	AR	Howard	34.277	−93.947	KC163486	KC163663	KC163393	KC163580	No data
F. (F.) wallsi Johnson, 2011	DJ326	TX	Sabine	31.325	−93.977	KC163435	KC163623	KC163339	KC163529	KC163719
F. (F.) wallsi	DJ313	TX	San Augustine	31.261	−94.070	KC163434	KC163622	KC163338	KC163528	KC163718
F. (F.) wallsi	DJ327	TX	San Augustine	31.264	−94.068	KC163436	KC163624	KC163340	KC163530	KC163720
Outgroup Taxa										
Astacus astacus (Linnaeus, 1758)	GenBank	NA	NA	NA	NA	AF235983	AF517104	EU920881	DQ079773	DQ079660

Table 1 Continued

	Collection no.	State	County	Latitude	Longitude	16S	CO1	12S	28S	H3
Barbicambarus cornutus (Faxon, 1884)	GenBank	NA	NA	NA	NA	EU920913	DQ113440	EU920883	EU920993	EU921045
Cambarellus shufeldtii Fitzpatrick, 1983	GenBank	NA	NA	NA	NA	AF235986	EU921149	EU921117	DQ079778	DQ079665
Cambarus maculatus Hobbs, 1988	KC64	NA	NA	NA	NA	AF235988	JF737746	EU921119	DQ079780	DQ079667
Cambarus scotti Hobbs, 1981	KC1266	NA	NA	NA	NA	JX514559	JX514500	JX514632	JX514688	No data
Cambarus aculabrum Hobbs and Brown, 1987	KC574	NA	NA	NA	NA	JX514559	JX514500	JX514632	JX514688	No data
Cambarus setosus Faxon, 1889	KC593	NA	NA	NA	NA	JX514539	JX514464	JX514611	JX514674	No data
Cambarus gentryi Hobbs, 1970	JF2508	NA	NA	NA	NA	AY853664	DQ411785	DQ411731	No data	DQ411804
Cambarus friaufi Hobbs, 1954	JF2543	NA	NA	NA	NA	DQ411733	DQ411784	DQ411730	No data	DQ411803
Cambarus brachydactylus Hobbs, 1953	JF2579	NA	NA	NA	NA	DQ411732	DQ411783	DQ411729	No data	DQ411802
Faxonella clypeata (Hay, 1899)	KC4655	NA	NA	NA	NA	JX514563	JX514453	JX514636	JX514692	No data
Orconectes luteus (Creaser, 1933)	KC278	NA	NA	NA	NA	AF376495	JX514454	JX514637	No data	No data
Orconectes negelectus (Faxon, 1885)	KC240	NA	NA	NA	NA	JX514564	JX514455	JX514638	JX514693	No data
Orconectes ronaldi Taylor, 2000	JC1424	NA	NA	NA	NA	JX127865	JX514456	JX127722	JX127596	JX127457
Orconectes virilis Hagen, 1840	GenBank	NA	NA	NA	NA	AF235989	AF474365	EU920900	DQ079804	DQ079693
Pacifastacus leniusculus leniusculus (Dana, 1852)	GenBank	NA	NA	NA	NA	AF235985	EU921148	EU921116	DQ079806	DQ079695
Procambarus clarki (Girard, 1852)	GenBank	NA	NA	NA	NA	AF235990	AY701195	EU920901	EU920970	EU921067
Procambarus geminus Hobbs, 1975	KC5624	NA	NA	NA	NA	JX514566	JX514457	JX514640	JX514695	No data
Procambarus liberorum Fitzpatrick, 1978	JC2668	NA	NA	NA	NA	JX514567	JX514458	JX514641	JX514696	KC163797
Procambarus tenuis Hobbs, 1950	KC2852	OK	Le Flore	34.646	−93.463	EF012346	KC163651	No data	KC163568	KC163758
Procambarus tenuis	KC2854	OK	Le Flore	34.646	−93.463	EF012347	KC163652	KC163383	KC163569	No data
Procambarus tenuis	KC2867	OK	Le Flore	34.646	−93.463	EF012348	KC163653	KC163384	KC163570	No data

DNA extraction, amplification and sequencing protocols were followed as outlined by Porter *et al.* (2005) and Crandall & Fitzpatrick (1996). Polymerase chain reactions (PCRs) were performed for three mitochondrial gene regions, 16S [~ 460 bp; using the primer 16sf-cray (Buhay & Crandall 2005) and 16s-1472r (Crandall & Fitzpatrick 1996)], COI [~659 bp; with primers LCO1-1490 and HCO1-2198 (Folmer *et al.* 1994)] and 12S (~390 bp; using the primers 12sf and 12sr (Mokady *et al.* 1999)), as well as two nuclear gene regions 28S [~800–1000 bp; with primers 28s-rd3a and 28s-rD5b (Whiting *et al.* 2000, 1997) or with 28sF-cray and 28sR-cray (Breinholt *et al.* 2012)] and H3 [~328; with H3AF and H3AR (Colgan *et al.* 1998)]. These genes were chosen because they show the appropriate amount of variation within other crayfish studies (Sinclair *et al.* 2004; Buhay *et al.* 2007; Toon *et al.* 2009).

Sequencing was performed on an ABI Prism 3730XL capillary autosequencer using the ABI Big Dye Ready-Reaction kit following standard protocols with the exception of reducing the standard reaction volume to 1/16th of the recommended volume. To avoid COI nuclear mitochondrial pseudogenes, we followed the procedures laid out by Song *et al.* (2008) and Buhay (2009).

Sequence processing

We used Sequencher 4.9 (GeneCodes, Ann Arbor, MI, USA) to clean and assemble raw chromatograms of the sequence data and screen the protein coding genes for stop codons. The clean sequence data were aligned individually by gene in MAFFT (Katoh *et al.* 2005), using the G-INS-I alignment algorithm. The best-fit model of evolution was then estimated using MODELTEST 3.7 (Posada & Crandall 1998) for each individual gene using the Bayesian information criterion (BIC) (Schwarz 1978) to choose the best-fit model.

Phylogenetic analyses

RAxML (Randomized Axelerated Maximum Likelihood) (Stamatakis 2006) and MrBayes (Ronquist & Huelsenbeck

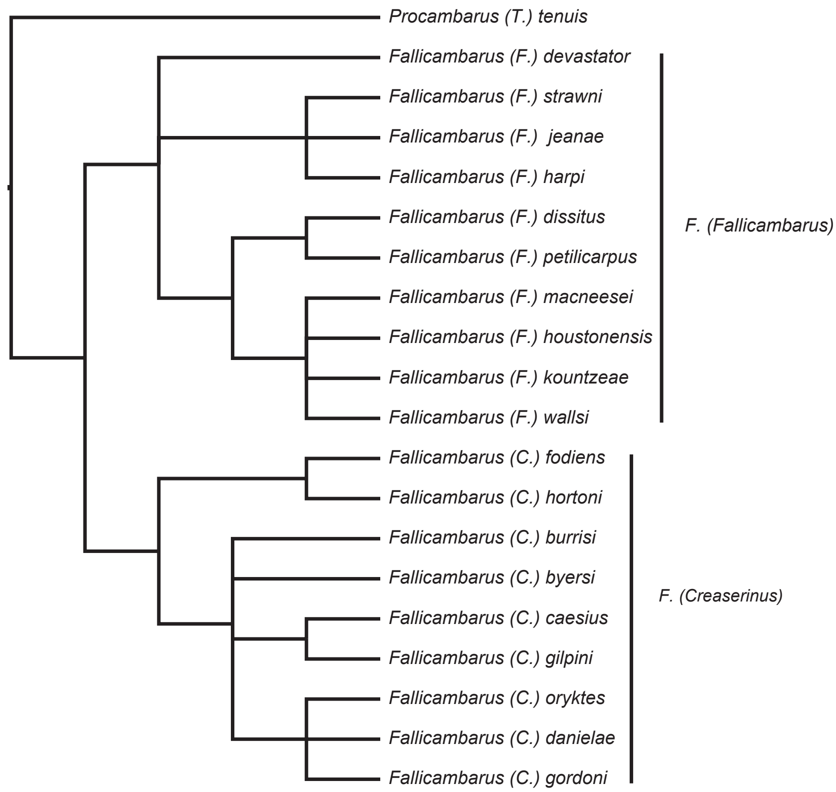

Fig. 2 Hypothesized phylogenetic relationships of species within the genus *Fallicambarus* based on an updated phylogeny of Hobbs classifications (Hobbs 1973, 1989). Taxa described after Hobbs (1973) were added according to hypothesized closest relatives listed by describing authors (Hobbs 1975; Fitzpatrick 1987; Hobbs & Whiteman 1987; Hobbs & Robison 1989; Johnson 2008, 2011).

2003) were used to estimate the phylogenies using the Bayesian optimality criteria (Huelsenbeck *et al.* 2002) and the Maximum likelihood (ML) methods (Felsenstein 1981). All computations were performed on Marylou5, the BYU Fulton Supercomputing Lab's supercomputer. We estimated gene trees for each gene and for a combined mitochondrial data set in RAxML using the GTR+G following the author's recommendation that the alternative, GTR+I+G, may cause problems in model parameter optimization. The ML gene trees were estimated using the RAxML 'f - a' option for a 1000 bootstrap search followed by a search for the best ML gene tree using every fifth bootstrap topology as a starting trees. The gene tree topologies were compared, and highly supported nodes in conflict between gene trees were identified as possible COI nuclear mitochondrial pseudogenes or contaminated/mislabelled sequences and if found were removed from the data set.

After concatenating the five genes we used this data set to estimate phylogenies using RAxML and MrBayes. We partitioned our data set by gene in both RAxML and MrBayes applying independent models to each gene to account for gene specific rates and nucleotide heterogeneity. In MrBayes models of evolution were set following the ModelTest results for the number of parameters and rate heterogeneity for each gene. We unlinked the variables statefreq, revmat, shape and pinvar for all gene models and for all MrBayes runs. We used two independent runs with one cold chain and seven hot chains from random starting trees using the default flat priors for 5×10^8 generations sampling every 5000 generations. To determine the size of burn-in and evaluate convergence, we used split frequencies below 0.01 as well as visually examining the negative log likelihood distribution for convergence and in the program TRACER v1.4 (Rambaut & Drummond 2007). The two MrBayes runs were combined after the deletion of burn-in, and a majority-rule consensus tree was created with nodal confidence for the trees assessed using posterior probabilities of contained nodes. To find the best ML tree, we executed 200 tree searches starting from random as well as 200 ML searches using every fifth bootstrap pseudoreplication of 1000 as a starting topology. The tree with the best ML score from these searches was selected, and we assessed confidence in nodal support through 1000 bootstrap pseudoreplications (Felsenstein 1985) estimated in RAxML.

To account for individual gene history and control for possible error associated with incomplete lineage sorting ignored by the concatenation method, we used *BEAST (Heled & Drummond 2010) to estimate a species tree for each subgenus. The species tree analysis co-estimates the gene trees and the species tree and has been shown to outperform concatenated analysis (Heled & Drummond 2010). The model implemented by *BEAST assumes species are definable groups that, after a period of divergence, have no history of interbreeding outside the designated group (Heled & Drummond 2010). We used highly supported clades from our Bayesian and ML analyses for our OTUs in *BEAST, assuming the branch lengths and highly supported clades represent independent lineages. Several species were split into different OTUs in the *BEAST analysis, these groups are labelled either by the species name and a group number (e.g., *F. jeanea* – 1) or in the case of *F. (C.) fodiens* by state samples were collected in and a group number (e.g., AR -1). We estimated the rate of each gene by giving an uninformative uniform prior for the ucld.mean (0–100) and further used uninformative default priors for the remaining parameters. For each subgenus we ran two *BEAST runs starting from random trees for 5×10^7 generations collecting samples every 5000 generations as well as a single run that excluded data and sampled the prior only. Convergence of the independent runs and ESS values were checked, and burn-in was estimated using Tracer V1.4. The postburn-in trees from each run were combined, and a maximum clade credibility tree was estimated.

Phylogenetic hypothesis testing

To test the hypotheses of taxonomic relationships, we compared the best resulting ML topology to topologies constrained to fit taxonomy using the approximately unbiased test (AU) (Shimodaira 2002) in the program CONSEL (Shimodaira & Hasegawa 2001). Constraint topologies were estimated from 200 ML searches starting from random tree topologies to find the best topology given the provided constraint. In addition to the ML topology test, we used Bayesian topological tests (Huelsenbeck *et al.* 2002).

Results

Examination of translated COI sequences yielded no stop codons, and the topology of the COI gene tree was similar to relationships in the 16S gene tree. We found no highly supported nodes in conflict among gene trees and kept all generated sequences in the concatenated data sets. The mitochondrial maximum likelihood tree (Appendix S1), the 28S maximum likelihood tree (Appendix S2) and the H3 maximum likelihood tree (Appendix S3) had no highly supported (>70 bootstrap) nodes in conflict. The best model of evolution for 16S and 12S was a two-parameter model with rates = invgamma and a six parameter model for COI, H3, and 28S with rate = invgamma for COI and rates = propinv for H3 and 28S. The two independent MrBayes runs converged and burin was set at 2.5×10^7 where split frequencies were below 0.01 as well as convergence of negative log likelihood values. Both Bayesian and ML analyses resulted in very similar topologies with the exception of *Fallicambarus (Creaserinus) byersi* and *Fallicambarus (C.) burrisi*. In the ML analysis *F. (C.) byersi* falls out sister to *F. (C.) burrisi* with low support; however, in the BAY this relationship is below 50% of posterior distribution, and therefore, this relationship was collapsed. We chose to present our Bayesian topology (Fig. 3) as it is the same as the ML estimation with a single collapsed node. The genus *Fallicambarus* is paraphyletic with outgroups from the genera *Procambarus*, *Orconectes* and *Barbicambarus* falling out in between the *Fallicambarus* subgenera. The AU test for the monophyly of the genus rejected the group as being monophyletic (P value < 4e−51), and a monophyletic *Fallicambarus* is not found in the set of Bayesian posterior trees (*P*p = 0%).

The subgenus *Creaserinus* results in a monophyletic group with high posterior probability and bootstrap support. The following relationships within the *Creaserinus* are noteworthy. The *Fallicambarus* sp. nov. 2 samples (from LA) form a monophyletic clade and are a poorly supported sister clade to the *Fallicambarus (Creaserinus) caesius* samples (*P*p = 0.76, BS = 62). *Fallicambarus* sp. nov. 1 (from AR) is placed as a highly supported (*P*p = 1, BS = 95) and basal clade to *Fallicambarus (Creaserinus) gilpini*, *F. (C.) caesius*, and *Fallicambarus* sp. nov. 2. The species *F. (C.) fodiens* shows a high degree of diversity. This species is paraphyletic because the group from Florida does not fall out with the rest of the group. Individuals from *F. (C.) fodiens* from both Arkansas and Texas appear in multiple clades within the group, which may suggest multiple species within the fodiens complex. The AU test for a monophyletic *F. (C.) fodiens* fails to reject monophyly as significantly different for our best ML estimation (AU P-value = 0.125); however, Bayesian topology tests give very little support to a monophyletic *F. (C.) fodiens* (*P*p = 0.3%). While *Fallicambarus (C.) oryktes* all group together they are not monophyletic. The identification of *F. (C.) oryktes* is questionable as we were only able to collect form II males; however, the sample KC1130 was identified by Dr. Joseph F. Fitzpatrick, Jr. a notable expert on the taxa in the southern United States. *Fallicambarus (C.) byersi* has surprisingly deep lineages for a species, which we only sampled from Florida. *F. (C.) caesius* formed two clades for which the sample localities are from the same county, yet they are separated by ~40 km.

The subgenus *Fallicambarus* is estimated as being non-monophyletic as *Procambarus (Tenuicambarus) tenuis* falls out in the middle of the subgenus and is sister to *Fallicambarus (Fallicambarus) strawni*. The AU test fails to reject the monophyly of the subgenus (P value = 0.107),

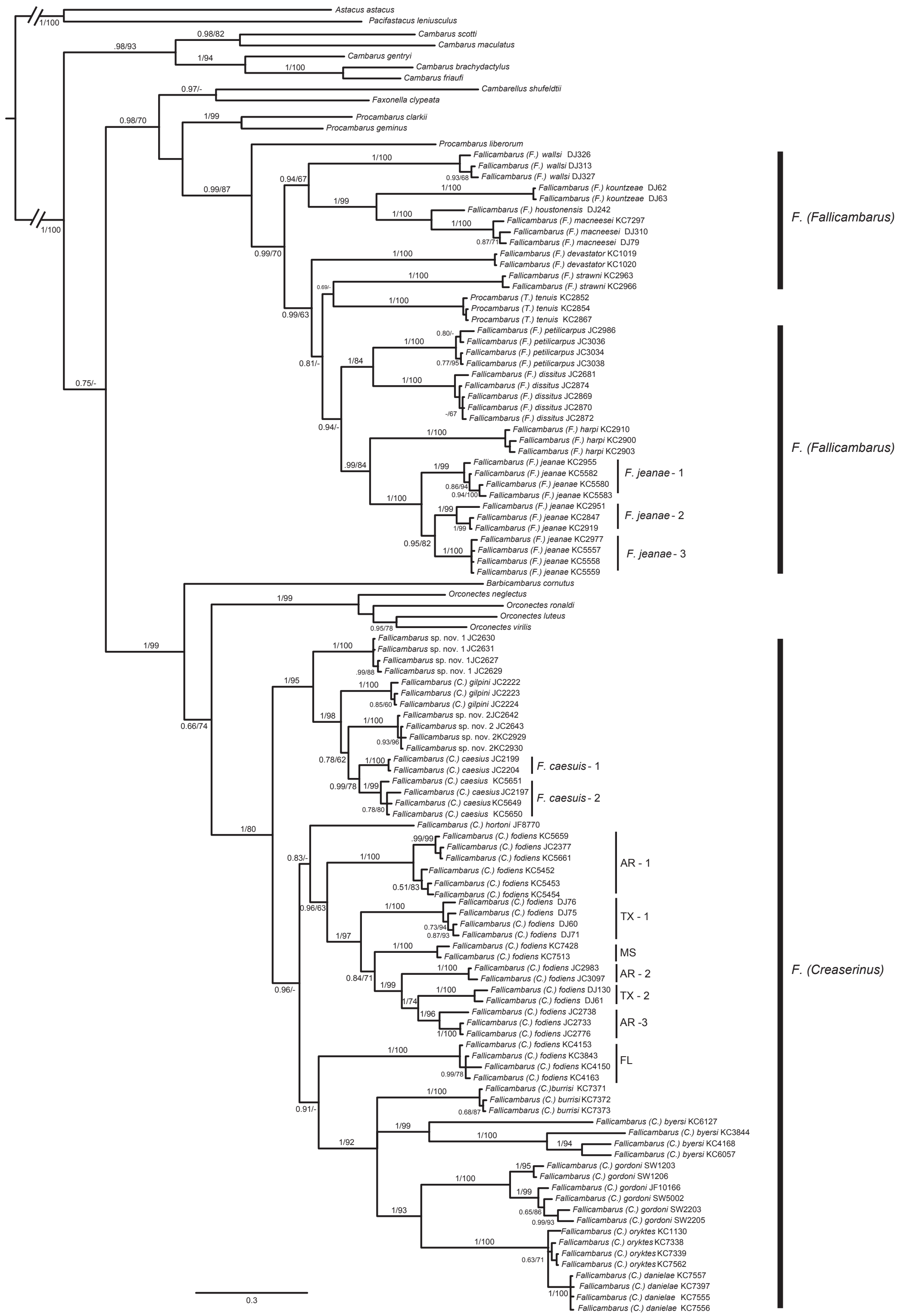

Fig. 3 Bayesian molecular phylogram of the genus *Fallicambarus* including outgroups species. For species with multiple samples the taxa names are followed by the tissue number. The inner most vertical bars show groups of taxa used in the *BEAST species tree analysis, and in the case of *Fallicambarus (C.) fodiens*, it indicates the state abbreviation from which the samples were collected. Bayesian posterior probability followed by ML bootstrap values is on braches leading to the node of support.

yet no trees in the Bayesian posterior distribution contain this group as monophyletic ($P = 0\%$). The recently described species *Fallicambarus (F.) wallsi* (Johnson 2011) falls out sister to *F. (F.) kountzeae*, *F. (F.) houstonensis* and *F. (F.) macneesei*. Also, the species *Fallicambarus (F.) jeanae* shows a considerable amount of diversity with three separate clades representing the species. In our constrained analysis for the monophyly of the subgenus *Fallicambarus*, *F. (F.) strawni* is the basal species of the subgenus as a result of forcing *Procambarus (T.) tenuis* to fall outside the subgenus.

Independent runs for the species tree for each subgenus converged and ESS values were above 200 for our set burn-in of 3.555×10^6 for the subgenus *Fallicambarus* and at 2.7×10^7 for the subgenus *Creaserinus*. Species tree estimation for the subgenus *Fallicambarus* (Fig. 4a) resulted in an identical topology as the ML and Bayesian analyses. The species tree for subgenus *Creaserinus* (Fig. 4b) resulted in fairly similar topology estimations as the ML and Bayesian analyses with the only difference being the location of the Florida *F. (C.) fodiens*. In the Bayesian and ML topologies the Florida *F. (C.) fodiens* was basal to *Fallicambarus (C.) burrisi*, *F. (C.) byersi*, *F. (C.) gordoni*, *F. (C.) oryktes* and *F. (C.) danielae*, and in the *BEAST species tree Florida *F. (C.) fodiens* moves deeper in the tree and is also basal to *Fallicambarus (C.) hortoni* and the rest of the *F. (C.) fodiens*. A monophyletic *F. (C.) fodiens* was not strongly supported in the species tree Bayesian analysis with a *P*p = 3.7%.

Discussion

The genus *Fallicambarus* is statistically supported as paraphyletic using a multi-gene phylogeny with Bayesian and ML topology test. *Fallicambarus* was estimated to be paraphyletic in each individual gene tree as well as the concatenated analysis. This rejects the hypothesis that the genus *Fallicambarus* is a monophyletic group. Therefore, we conclude the genus *Fallicambarus* as invalid as the two subgenera form independent monophyletic clades with clear evolutionary separation and represent two distinct evolutionary lineages.

The subgenus *Creaserinus* is strongly supported as monophyletic (*P*p = 1, BS = 80) and is evolutionarily distinct from the subgenus *Fallicambarus*. Our data appear to support elevating the subgenus *Creaserinus* to genus level. However, we will address this in a subsequent systematics paper with greater outgroup representation to confirm the monophyly of the group and determine the appropriate sister taxon. A broad scale phylogenetic analy-

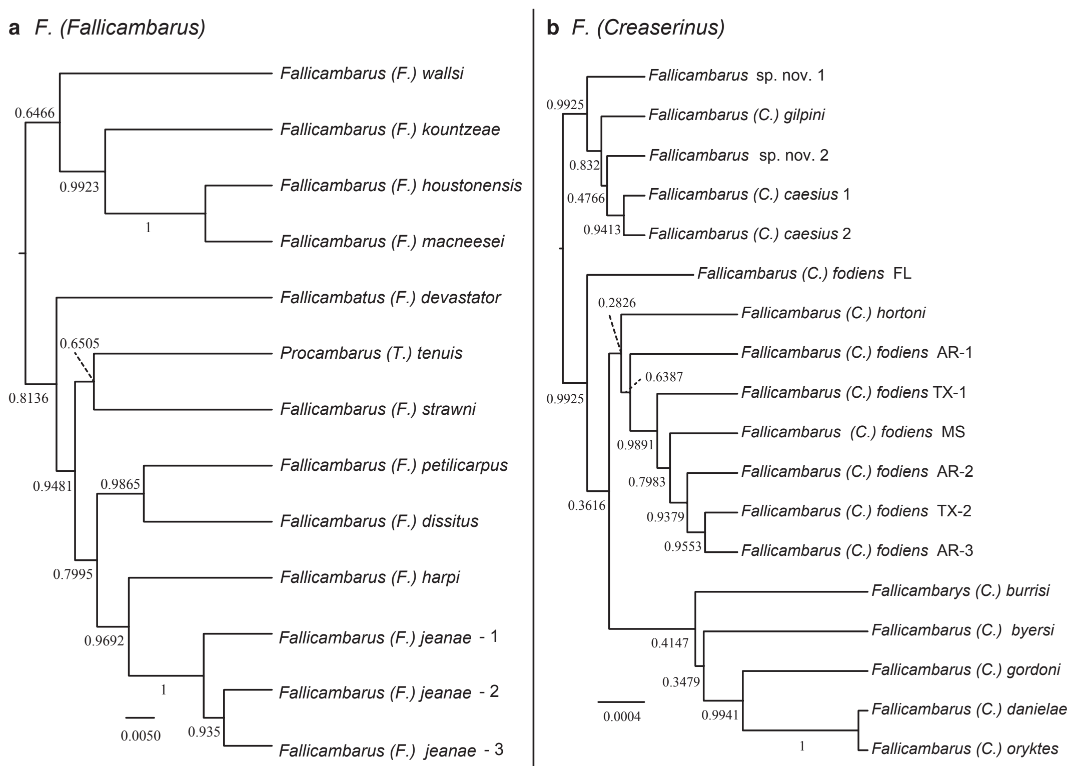

Fig. 4 Species tree estimation of the relationship in the subgenus *Fallicambarus* (4a) and the subgenus *Creaserinus* (4b) estimated in *BEAST. Bayesian posterior probability values are on branches leading to the node of support.

sis of Cambaridae will be needed before we can be confident that *Creaserinus* should be elevated to genus status. *Fallicambarus (C.) danielae* and *F. (C.) oryktes* are very difficult to distinguish from each other, and further work should be done to determine whether they truly are separate species. Our *F. (C.) gordoni* samples are restricted to Camp Gordon, and because the species is split into two distinct clades, further sampling and analysis of the entire range of this species needs to be completed.

The *F. (C.) fodiens* complex is difficult to completely resolve with our data. The concatenated analysis (Fig. 3) and the species tree analysis (Fig. 4b) returned different topologies for *F. (C.) fodiens*. Even within these analyses, the branches are significantly deeper than the other species within the genus *Fallicambarus*. It is significant that this lineage is so old especially considering that our samples only cover a fraction of the range of the species. Hobbs & Robison (1989) reclassified this species to describe what had been three separate species previously arguing that there were not enough morphological differences to justify separate species. However, our genetic data suggest multiple species exist within this complex. Further sampling needs to be carried out, including sampling the type locality in Canada and the disjunct populations in the northeastern United States, to resolve the evolutionary history of this species complex.

The subgenus *Fallicambarus* appears to be paraphyletic with *Procambarus (Tenuicambarus) tenuis* falling inside the subgenus. This result is not surprising as Hobbs (1973) notes that the number of similarities between *Fallicambarus* and *Procambarus (T.) tenuis* is far too numerous to be owing to convergence in independent lineages. Hobbs (1973) lists 12 morphological similarities that unite *Procambarus (T.) tenuis* and *Fallicambarus* species. In addition, our molecular results support moving *Procambarus (T.) tenuis* (Hobbs 1972) to the genus *Fallicambarus*.

The fact that both of these groups, *Fallicambarus* and *Creaserinus*, are distinguished morphologically by the terminal elements of the pleopod bent caudally at an angle >90° is significant. Our results show outgroups that do not share this character in between these two subgenera. This suggests convergent evolution of this feature. If the feature did evolve independently two separate times, then it would be a conflicted character to use to identify taxa. Our data are the best resource available to distinguish individuals within the genus. Using our data, females and form II males could be accurately identified through molecular analyses.

Our study clearly demonstrates the non-monophyly of the genus *Fallicambarus*. With the addition of *Procambarus (Tenuicambarus) tenuis* to the subgenus *Fallicambarus* (i.e., *Fallicambarus (Fallicambarus) tenuis* (new combination)), the two subgenera form robust and independent (meaning other genera fall between these two clades suggesting evolutionarily independent origins) monophyletic clades. Future work on this group is needed to elevate the subgenus *Creaserinus* to genus status (if justified with additional data and analyses) and to explore the various species complexes identified through our study.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Appendix S1. Maximum likelihood tree estimated from the three mitochondrial genes (COI, 16S, 12S). Bootstrap values above 60 are on branches leading to the node of support.

Appendix S2. Maximum likelihood tree estimated from the 28S gene. Bootstrap values above 60 are on branches leading to the node of support.

Appendix S3. Maximum likelihood tree estimated from the H3 gene. Bootstrap values above 60 are on branches leading to the node of support.