FINAL PERFORMANCE REPORT
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Project Title: Investigating dispersal of the invasive red swamp crayfish (*Procambarus clarkii*) and its effects on the distribution and status of native crayfish populations.

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Project Goal: To determine the current abundance and distribution of native and invasive crayfish in South Carolina's northeastern coastal plain and assess dispersal pathways for the invasive red swamp crayfish *Procambarus clarkii.*

Progress: Completed

Objectives:

1. To characterize the current distribution and abundance of native and non-native crayfish in the swamps, streams and wetlands of Horry, Marion, Dillon and Georgetown counties using multiple field sampling approaches (e.g., dipnetting, trapping, backpack shocking);
2. To improve understanding of introduction and dispersal events leading to the recent expansion of *P. clarkii* using population genetic approaches.

Accomplishments

The establishment and spread of invasive species are multi-step processes that include both anthropogenic introduction and natural dispersal mechanisms. Multiple introduction pathways can lead to the establishment of non-native populations of invasive species, including unsecure commercial or recreational aquaculture practices, releases from the pet trade, and the use of non-native species as fishing bait, among others. Once introduced and established, natural dispersal can lead to the spread of these species throughout a given area. Distinguishing between multiple anthropogenic introductions within a given region and natural dispersal from established populations can be difficult but is vital for developing management strategies for mitigating the negative impacts of invasive species. Field surveys are an important tool for documenting the establishment and spread of invasive species, and when coupled with molecular analysis, these efforts can highlight the degree of connectivity among populations, providing insights into the relative importance of anthropogenic introductions and natural dispersal mechanisms that lead to the spread of invasive species.

The red swamp crayfish, *Procambarus clarkii*, is native to the United States Gulf coastal plain from Florida to Mexico but is now well-established in many areas worldwide outside of its native range, including South Carolina, USA. Once introduced and established in an ecosystem, *P. clarkii* have the ability to spread rapidly within watersheds, displace native taxa (Twardochleb *et al*. 2013, Huang *et al*. 2017) and alter structural and functional components of the invaded ecosystems (Shin-ichiro *et al.* 2009).

In South Carolina (SC), *P. clarkii* has been established since at least the 1970s. The existence of aquaculture ponds in the lower Pee Dee/Winyah watershed (near Georgetown, SC) in the 1970s and 1980s represent important potential sources for *P. clarkii* that are currently established in natural streams and wetlands in this area. For the upper Waccamaw and Little Pee Dee watersheds, however, it has been unclear if the *P. clarkii* populations located in these watersheds are the result of a series of human-mediated introductions, or if these *P. clarkii* resulted from a single introduction event, followed by

subsequent dispersal throughout the watershed. Understanding the relative importance of these dispersal mechanisms is vital for developing effective strategies for mitigating the effects of invasive species on native taxa.

Objective 1

Task 1: Distribution of native and invasive crayfishes

Prior to 2000, available data from Eversole and Foltz (2015) and state records on the location of crayfish farms, indicate that *P. clarkii* was recorded at <10 sites (Figure 1A). To better assess the distribution and abundance of crayfish in the Pee Dee basin as part of this project, SCDNR biologists surveyed a total of 156 locations from 2018-2021 across the Waccamaw, Little Pee Dee, and Winyah basins. Sites were sampled using dip nets, seine nets, baited traps, and burrow excavation. During this time, *P. clarkii* was recorded at 84 (54%) of the 156 sampled sites, with 4,714 individuals collected, representing both sexes and all life stages. Within the Waccamaw subbasin, *P. clarkii* was collected at 35 of 49 (71%) of sampled locations, yielding a total of 1,666 individuals. Within the Little Pee Dee subbasin, *P. clarkii* was recorded at 28 of 53 (53%) sampled locations, wherein 2,170 individuals were collected. Within the Lower Pee Dee basin, *P. clarkii* was recorded at 21 of 54 (39%) sampled locations, wherein 878 individuals were collected. To provide a holistic view of the spread of *P. clarkii* in this region, data from multiple sources, including this project, as well as data from federal grant numbers SC-T-F17AF01207, F18AP00261, and F18AS00099, are plotted in Figure 1B to show the recent distribution of *P. clarkii* in this region.

According to the 2015 SC State Wildlife Action Plan (SWAP), there are five crayfish species of conservation priority documented from the coastal plain habitats of the Little Pee Dee and Waccamaw subbasins, namely the Waccamaw crayfish, *Procambarus braswelli*, the Cedar Creek crayfish, *P. chacei*, the Carolina sandhills crayfish, *P. pearsei*, the coastal plain crayfish, *P. ancylus*, and the Santee crayfish, *P. blandingii*. All five of these conservation priority species were collected during this project. In addition to these conservation priority species, researchers also collected the white river crayfish, *P. acutus*, the eastern red swamp crayfish, *P. troglodytes*, the devil crayfish, *Lacunicambarus diogenes,* and the digger crayfish, *Creaserinus fodiens*. For the purposes of this report, *P. acutus* and *P. blandingii* are grouped together due to their overlapping morphological characteristics, especially of juvenile and non-reproductive (Form 2) males, in SC. For sampling associated with this project, each major drainage basin yielded at least 5 native species during sampling. The drainage basin with the greatest abundance of native crayfish (n = 1,082) was the Little Pee Dee subbasin, where native species were collected at 81% of sampling locations. The five most abundant native species collected were *P. acutus / blandingii* (n = 660), *P. troglodytes* (n = 635), *P. pearsei* (n = 195), *C. fodiens* (n = 141), and *P. ancylus* (n = 75). When limiting sampling efforts to those for which a given species was present (rather than including sites with zero abundance), the catch per unit effort (CPUE) was greatest for *P. clarkii* (Figure 2). This is consistent with existing knowledge that this species occurs at high abundances (Larsen *et al.* 2017). For native taxa, *P. pearsei* and *P. troglodytes* were both caught at relatively high abundances compared with other native taxa (Figure 2).

Seasonality of occurrence was tracked using CPUE metrics for three commonly occurring species (Figure 3). Results show that *P. clarkii* was least prevalent in spring months, but most prevalent in winter months. This pattern is likely driven by the high number of juvenile specimens of *P. clarkii* collected during winter months. In contrast, the sister species to *P. clarkii*, the native *P. troglodytes*, showed the highest abundance in summer months.

To further investigate the effects of *P. clarkii* on native crayfish, native crayfish species richness was compared between sites at which *P. clarkii* was either present or absent. Native crayfish richness was found to be significantly lower when *P. clarkii* was present (Likelihood ratio test, Deviance = 13.79, $P < 0.001$; Figure 4). Native crayfish richness was also compared across land uses. Land use classifications were designated by referencing the National Vegetation Classification subclass field of the US Geological

Survey Land Cover Gap Analysis Project data in ArcGIS Pro. The data layer was clipped to the watershed and converted to a vector file to assist in site comparison. Sites were designated "agricultural" if they were located within an "Herbaceous Agricultural Vegetation" polygon, "forested/shrubland" if they were located within a "Temperate & Boreal Shrubland & Grassland" or a "Temperate Forest" polygon, and as "urban/developed" if they were located within either "Developed & Urban" or "Recently Disturbed or Modified" polygons. Land use did not have a significant effect on native crayfish richness (Likelihood ratio test, Deviance = 1.83, $P = 0.40$; Figure 4).

Populations of *P. pearsei, P. braswelli,* and *P. ancylus* were compared to those of *P. clarkii* and analyzed for potential extirpation and continued co-occurrence (Figure 5). While other species of conservation concern (such as *C. fodiens, P. troglodytes,* and *P. acutus/blandingii*) were also collected during the study, historic data were not sufficient to allow for adequate comparisons in occurrence trends across time. Since numerous records were taken within close proximity to one another, sites were considered to overlap if they were within a one-mile radius of each other. Given the relatively recent description of *P. braswelli* (Cooper 1998) and its morphological similarities to *Procambarus chacei*, two indeterminate observations (either *P. chacei* or *P. braswelli*) were also leveraged for analyses in the *P. braswelli / P. chacei* accounts.

To provide a comprehensive and current view of the effects of *P. clarkii* in this region, data from multiple sources, including this project, as well as data from federal grant #'s SC-T-F17AF01207, F18AP00261, and F18AS00099, were used to compare distributions of *P. clarkii* and species of conservation priority, as shown in Table 1. Of the documented historical records of *P. braswelli / P. chacei*, there are 5 historic sites where *P. braswelli / P. chacei* was previously collected in which only *P. clarkii* have been collected in recent years. Similarly, there are 5 historic *P. ancylus* populations and 4 historic *P. pearsei* sites in which *P. clarkii* appears to have completely extirpated these native taxa. Documented co-occurrences range from 4 (*P. pearsei*) to 12 (*P. braswelli / P. chacei*) and are documented alongside known populations, suspected extirpations, and the relative proportions of each to all populations of each of the three species across the study area in Table 1. These numbers show that a quarter of all documented *P. ancylus* populations in the study region have been potentially extirpated by the spread of *P. clarkii*. Similarly, 14% of *P. pearsei* and 19% of *P. braswelli / P. chacei* populations within the study area have also been extirpated by the spread of *P. clarkii* across the upper Waccamaw and Little Pee Dee drainages. Given this information, and the fact that *P. ancylus* and *P. braswelli / P. chacei* both co-occur with *P. clarkii* at over half of all remaining documented locations within the study region, *P. clarkii* appears to be poised to significantly affect the remaining extent of at least two of South Carolina's conservation-priority native crayfish species.

Objective 1

Task 2: Comparison of sampling methods

In addition to the surveys we conducted in the Pee Dee basin to assess the distribution and abundance of *P. clarkii* in relation to native crayfish, we also sampled in this region to compare the efficacy of backpack electrofishers and dip nets across stream and wetland systems. In our earlier sampling for the project, we used multiple sampling approaches, including dip nets, seine nets, baited traps, and burrow excavation. Baited traps are the most common sampling methodology for quantitative crayfish collection but tend to be biased toward larger males and more aggressive species and/or individuals (Alonso 2001, Barnett and Adams 2018). There is less information available regarding the efficacy of the additional methods listed above (Price and Welch 2009).

A quantitative method available for sampling crayfish is time-constrained electrofishing (Rabeni *et al.* 1997, Price and Welch 2009). Electrofishing can be used to obtain qualitative and quantitative metrics about crayfish population structure and has been used in some coastal plain systems (Price and Welch 2009; Budnick *et al.* 2018; Barnett et al. 2020). Alonso (2001) showed that low voltage backpack shocking in small creeks in Spain is an effective method of assessing crayfish diversity and richness in

aquatic systems, but with limitations, such as risk to the sampling team, water depth, and water clarity. Additional efforts by Barnett *et al.* (2020) indicate that electrofishing surveys are a viable method for successfully and precisely assessing population density of assorted species of crayfish in larger stream systems.

The objective of this portion of the project was to assess the catch rates of electrofishing in comparison to dip netting, another commonly used capture method. We also focused our efforts on adding to our knowledge of the distribution and catchability of native crayfish species within our study area. There is limited knowledge of the complete geographic distribution of many of SC's native crayfish (Eversole and Foltz 2015), and every observation adds additional records that can be useful in conducting population assessments. Electrofishing has been shown to be an effective sampling method for documenting species richness (Price and Welch 2009) and thus has the potential to produce more robust results than perhaps methods such as baiting (Alonso 2001, Barnett and Adams 2018). Given the prevalence of the invasive *P. clarkii* in the coastal plains of SC and the continuing efforts of SCDNR researchers to catalog the richness, diversity, and abundance of native species in the same region, assessing the catchability of *P. clarkii* and competing native species by the collection methods described above can contribute additional information necessary to develop not only plans to manage invasive species, but also to assist in the conservation of their native counterparts.

Methods

We conducted a series of sampling events in November and December of 2021 in coastal plain streams in parts of the Great Pee Dee River basin (Figure 6). We conducted side-by-side dipnet and electrofishing comparisons at each location, with each collection method separated by a road crossing for all but five collections. Collections 1-4 were conducted along a single stream reach, while Collection 5 was conducted on separate sides of a single wetland (Figure 2). Sites were selected based on uniformity and habitat type. Sampling focused on stream and wetland systems, and only sites in which a single habitat type was represented on each side of the road crossing were surveyed. Gear type was chosen at random for the upstream/downstream region at the initial site for each sampling day and was alternated for each successive site throughout that day. Sites that did not maintain their integrity across the sampling region (i.e. stream on one side of the road and wetland on the other) were not included in the study. With the exception of the first day of sampling, in which collection times were 10 person-minutes (Collections 1-4) and 33 person-minutes (Collection 5), we standardized the amount of time for each collection method (dip net or backpack shocker) to 20 person-minutes. One researcher equipped with either a backpack electrofisher or a dip net systematically sampled the aquatic habitat upstream of the road, while the other researcher did the same downstream of the road with the alternative sampling method. Sampling direction was chosen at random at the onset of the sampling trip and alternated by collection method for the duration of each day's sampling effort. All crayfish for each sampling method were tallied, preserved in 95% ethanol, and later sorted to species level. Abundance is reported as catch per person-hour or catch per unit effort (CPUE).

Results

Crayfish abundance patterns

In total, staff collected a total of 235 crayfish consisting of five different species across 31 paired surveys (Table 1). Collections ranged from 0 to 33 crayfish per effort for dipnet efforts ($\bar{X}$=3.83) and from 0 to 10 crayfish per effort for electroshocker efforts ($\bar{X}$=1.64). Our analyses indicate significant effects of gear type (z= -6.79, P <0.001), habitat (z= -9.681, P<0.001), and a significant interaction between these factors (z=2.52, P =0.012) when tested using generalized linear mixed modelling, with collection day as a random effect (Std.Dev.=0.18; Figure 7). These results are consistent with previous work by Price and Welch (2009) in that the effectiveness of sampling methodologies can be habitat-specific. The Pee Dee basin consists of many aquatic habitat types, from clear-flowing, sandy-bottomed creeks to deep-water swamps and wetlands, but dip-netting appears to be a more consistent approach than electrofishing.

Species-specific data

Five species of crayfish were collected during the gear type comparison sampling: the red swamp crayfish, *P. clarkii (*n=124), the Waccamaw crayfish, *P. braswelli* (n=27), the white river crayfish, *P. acutus* (n=26)*,* the Carolina sandhills crayfish, *P. pearsei* (n=19)*,* and the coastal plain crayfish, *P. ancylus* (n=1). Among species, *P. clarkii* represents the greatest number of crayfish collected for both gear types, comprising 53% of all crayfish collected across the course of the gear comparison study. The mean CPUE was also greatest for *P. clarkii* (approximately 6 crayfish per per-person-hour), which is consistent with numerous studies that indicate their high occurrence. Collection distributions and mean CPUE for all five species are shown in Table 2. Furthermore, *P. clarkii* was collected in 22 of 31 dip net trials (71%), and in 17 of 31 backpack electroshocker trials (55 %). Three native species of conservation concern — *P. ancylus, P. braswelli,* and *P. pearsei* — were also collected in varying abundance as shown in Table 2.

Of the five species collected, *P. clarkii* had the greatest number of occurrences at survey sites across both gear types, followed by *P. acutus, P. braswelli, P. pearsei,* and *P. ancylus*, which was only collected at one site and only by dip netting (Figure 5).

When subdivided by species and gear type, collections followed a similar trend. A total of 84 *P. clarkii* were collected by dip net across all trials (50%), and a total of 40 *P. clarkii* were collected by the backpack electrofisher (68%). This is again the greatest number of specimens collected for any one species across either gear type; however, these data do not take into account the distribution of species across sample sites and reflect only overall abundances across the study rather than relative abundance across collection sites. The mean CPUE of species when separated by gear type is a more accurate reflection of species captured per relative effort and demonstrates a substantially greater mean catch per unit effort for *P. clarkii* for both dip net and backpack electroshocker collections (Figure 8). Similarly, though a relatively large number of *P. braswelli* were collected, occurrences were limited to a much lower number of sites.

Multiple studies have discussed catchability as a function of sex, size, and/or life stage (Alonso 2001, Price and Welch 2009, Barnett *et al.* 2020). Price and Welch (2009) found that dip netting tended to be biased toward smaller individuals while electrofishing yielded a more robust collection of species, individuals, and sizes. Similar trends can be observed in our sampling (Figure 9). Of the specimens we collected by dip net, 37% were juveniles and 42% were females of varying size; Form one males comprised only 7% of dip net collections, and Form 2 males comprised only 14% of collections. Backpack shocker collections yielded 60% female crayfish, 5% Form 1 males, 20% Form 2 males, and only 15% juveniles. Dip net collections in our study were inclined to favor female and juvenile specimens (GLMM with Poisson distribution; $P < 0.001$), while there was no significant relationship between gear type, habitat, and the proportion of adult male crayfish.

Anecdotally, we noticed marginally greater success in backpack shocker collections across specific wetland habitats. These sites had clear, shallow (<3') water and successful collections were typically matched with sunny conditions and minimal canopy obstruction. In the right conditions, crayfish response to shocking was readily observed and the stunned crayfish were easy to collect. Nevertheless, our data and statistical analyses support dip net collections in coastal plains systems over backpack electroshocker collections. Only dip netting yielded all five of the species collected; *P. ancylus* was not collected in any backpack shocker collections. This could, however, be a component of numerous factors unrelated to gear type, including but not limited to water quality, habitat surveyed, available cover, and collection site.

Overall, our gear comparison study indicated that dip nets are more effective than backpack shockers in sampling not only for *P. clarkii,* but also for native species of conservation concern. This held true across habitat type (creek vs. wetland) and across sex and life stage. While we would have also liked to have assessed the catch rate of Form 1 males (given their usefulness in species identification), we had too small a sample size of male specimens to provide any conclusive statistical data. Our study also further

illustrated the dominance of *P. clarkii* within the systems in which it has established itself. Of the five species collected, *P. clarkii* was collected more frequently and in greater quantities across both gear types and in both habitats. While there are habitats and occasions where backpack electrofishing is an effective means of sampling for crayfish species (Alonso 2001, Price and Welch 2009, Barnett *et al.* 2020), the results of this specific survey indicate that time and resources are best allocated to dip net collections over backpack shocker collections.

Task 3: Sequencing of native Procambarus

During the collection of native crayfish species in both North Carolina and South Carolina, opportunistic sequencing of native *Procambarus* species was combined with sequencing efforts for other projects to develop preliminary clustering relationships within and among *Procambarus* species (Figure 10). DNA was extracted from individuals of the genus *Procambarus* collected primarily from South Carolina and North Carolina by the SCDNR or North Carolina Museum of Natural Sciences (NCMNS) staff and/or affiliates. In addition, we extracted DNA from several historic *Procambarus* specimens – also from South Carolina and North Carolina – housed in the NCMNS Non-Molluscan Invertebrates Collection.

A region of the mitochondrial gene cytochrome c oxidase I (COI; "barcoding region") was sequenced, as well as the mitochondrial gene 16S rDNA and the nuclear gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) for a subset of individuals. Data were obtained for several species of conservation concern, including *P. pearsei*, *P. ancylus*, and *P. braswelli*, and several species that have presented considerable taxonomic confusion, including *P. acutus*, P. *blandingii*, and *P. troglodytes*.

Results clearly showed the utility of genetic data helping delimit species boundaries. We observed no genetic ambiguity among species that have been historically morphologically problematic to identify. In contrast, the *Procambarus* species sampled not only appear to be genetically distinct—with one exception (see below)—we have identified previously unknown diversity in several species; this "hidden" diversity warrants a more thorough assessment using an integrative approach to ensure adequate conservation measures are in place to confront encroaching threats, such as invasive species, habitat alteration, etc.

Procambarus acutus, both alone and in tandem with *P. blandingii*, presents a long-standing taxonomic conundrum in South Carolina, and North Carolina, and well beyond. The species exhibits wide variation in color pattern and several taxonomically informative morphological characters, which appear to be decoupled from geography. Yet, genetic data suggest that *P. acutus* in Virginia, North Carolina, and South Carolina comprises two distinct clades that appear to separate along a north-south axis. Furthermore, *P. blandingii* seems to be clearly separate from both *P. acutus* clades. Additional sampling, examining genetics, morphology, color pattern, behavior, etc., is needed to confidently establish the taxonomic status, distribution, and conservation needs of these taxa.

Similar to *P. acutus*, *P. troglodytes* has presented substantial taxonomic confusion in the region. Indeed, unpublished efforts by Horton H. Hobbs Jr. in the 1940s indicated that the species might be a species complex, composed of at least two potential subspecies. As above for *P. acutus*, additional integrative efforts are needed to confidently assess patterns of diversity and structure within *P. troglodytes* within a taxonomic framework; however, preliminary data show intraspecific genetic structure that may suggest the species harbors evolutionarily significant units, even if not at the level of species.

Genetic data indicate that *P. ancylus*, a species of conservation concern in South Carolina and North Carolina, may be two distinctly different species, one of which occurs in the Cooper River basin and the other in the Pee Dee River basin (Figure 11). While John E. Cooper had previously identified distinct spinose versus non-spinose morphs of *P. ancylus*, questioning the presence of more than one species, he believed that the morphological differences were driven by habitat (burrows versus stream-dwelling) rather than taxonomy. While he appears to have been correct in his assessment of spinosity, he appears to have missed a combination of characters that together correlate with observed genetic divergence.

Additional targeted sampling may be helpful in delimiting these two taxa, *P. ancylus sensu stricto* and *P.* sp. nov., and assess conservation status for each.

An unexpected result of this project was the discovery that *P. braswelli* appears to be genetically indistinguishable from *P. chacei* (Figure 12). Preliminary genetic data suggested this to be the case, and subsequent morphological examination provides confirmation. The junior synonym status of *P. braswelli* relative to *P. chacei* was suggested by Bill Poly (SC SWAP 2015, Supplemental Volume: species of special concern, Mimic Crayfish species account). This taxonomic act would affect the overall conservation status of both species, substantially increasing the distribution of *P. chacei*; however, we urge that a conservative approach be taken in state listings, given the detrimental effects that the spread of *P. clarkii* has had on the northern drainages in which *P. chacei* / *P. braswelli* occurs.

The genetic data, used here in combination with field surveys and morphology-based identifications, have been invaluable in highlighting specific species and/or areas that should be targeted for future efforts necessary for effective conservation of South Carolina's *Procambarus* species. In addition, we have shown that we can obtain genetic data from museum specimens. Such data provide a historical perspective on distribution and diversity that, when compared with data from recent collections, can give us critical insight into how populations have changed over time, e.g., range expansion or contraction. While not all museum specimens lend themselves to being used in this fashion, based on, for example, preservative used, storage conditions, etc., and in some cases, representative specimens are few, we suggest that these resources be used whenever possible.

Objective 2: Improve understanding of introduction and dispersal events using genetic approaches

Task 1. Development and optimization of microsatellite markers for *P. clarkii*

Initial optimization of microsatellite markers comprised testing three DNA isolation methods and two tissue types (tail muscle and gill) for efficiency and effectiveness. The first method tested followed Yue & Orban's (2005) paper which describes a "simple and affordable method" for DNA extraction and isolation in high-throughput format. This method involves adding tissue to a digestion solution containing 5% Chelex 100 resin and 0.07μg/μl proteinase K in a PCR plate followed by a one hour incubation at 55°C with a subsequent heat-inactivation step at 95°C for 10 minutes. Following the two-step incubation process, the plate was spun at 2,000 rpm for 2 minutes in order to pellet the Chelex 100 resin. The clear supernatant containing total DNA was removed and stored in a new PCR plate. The second method tested was the Promega Wizard SV Genomic DNA Isolation system following the manufacturer's protocols (standard spin-column isolation). The third method was a modified spin-column isolation (same as above) which included a 5% Chelex 100 resin treatment in 5% SDS at 95°C for 5 minutes before an overnight proteinase K digestion. DNA from the three methods were evaluated for quality/quantity and PCR inhibition using gel electrophoresis and qPCR, respectively. While none of the methods showed signs of PCR inhibition using a standard qPCR assay, the unmodified spin-column isolations yielded higher qualities and quantities of DNA when visualized on a 1.5% multi-purpose agarose gel than the other two methods for both tissue types (Figure 13). Furthermore, when using the unmodified spin-column isolation, gill tissue yielded the highest quantity of total DNA (Figure 13). Therefore, we recommend using the unmodified spin-column following the manufacturer's protocols for all subsequent DNA isolations.

Following DNA isolation optimization, DNA from one individual (Pcl-00071) was isolated and sent to Applied Biological Materials Inc. for 300 base pair paired-end whole genome sequencing using an Illumina MiSeq V3 Kit (600 cycles, 25M reads, **split with one other sample** not all reads recovered were expected to be from *P. clarkii*). A total of 4,350,549 *P. clarkii* reads were delivered from the sequencing run. FastQC (Babraham Bioinformatics) was used to visualize overall read quality. TRIMMOMATIC (Bolger *et al.* 2014) was used to trim adaptor sequences and filter reads by quality scores. FLASH was to merge paired reads resulting in 3,625,700 reads ranging from 10-592 base pairs. Finally, poor-quality base pairs were trimmed from both ends of the reads resulting in a FASTQ file

containing 3,625,700 high-quality reads ranging from 2-562 base pairs. The FASTQ file was converted to FASTA format to reduce file size and the resulting FASTA file was searched for microsatellites using the program MSATCOMMANDER (Faircloth 2008). MSATCOMMANDER returned 23,082 microsatellites with PCR primers for each.

The resulting microsatellite loci were sorted by primer melting temperature and repeat motif length. A total of 92 standard oligo primers were ordered and tested for amplification and polymorphisms using end-point PCR followed by visualization in 3% high-resolution Metaphor Agarose. Of the initial 92 primer pairs tested, 34 were ordered with fluorescent labels for visualization using capillary electrophoresis on Beckman CEQ/GeXP automated DNA sequencers. These 34 primer pairs were selected based on the potential to multiplex PCRs given the observed allelic ranges from the high-resolution agarose gels. Thirteen of the labeled primers were reliably scoreable, amplified in multiplex reactions, and were polymorphic.

Our final optimized reaction conditions for each multiplexed PCR group (MPG) were as follows using standard thermal cycling conditions of 95°C for 5 mins, followed by 35 cycles of denaturing at 95°C for 30s, annealing at 60°C for 30s and extension at 65°C for 30s, followed by a final extension step at 65°C for 60 mins: MPG1 – 1 X HotMaster buffer (5Prime), 0.2 mM dNTPs each, 3 mM MgCl, 0.04 mg/ml BSA, 0.3 µM forward and reverse primers, 0.4 U HotMaster *Taq* polymerase (5Prime), and 1 µl of DNA template. MPG2 – 1 X HotMaster buffer (5Prime), 0.2 mM dNTPs each, 1.5 mM MgCl, 0.04 mg/ml BSA, 0.3 µM forward and reverse primers, 0.3 U HotMaster *Taq* polymerase (5Prime), and 1 µl of DNA template. MPG3 -- 1 X HotMaster buffer (5Prime), 0.2 mM dNTPs each, 3 mM MgCl, 0.08 mg/ml BSA, 0.3 µM forward and reverse primers, 0.4 U HotMaster *Taq* polymerase (5Prime), and 1 µl of DNA template.

All samples that successfully genotyped at 10 or more loci were included in initial marker testing and final analyses. The data set was then evaluated for Hardy Weinberg equilibrium (HWE) and linkage disequilibrium among loci and sampling locations using ARLEQUIN 3.5.1.2 (Excoffier and Lischer 2010) and GENEPOP 4.7.2 (Rousset 2008). No loci were identified to be consistently linked across locations. Two loci, Pcl-36 and Pcl-30, were consistently out of HWE and were excluded from final analyses. The remaining 11 loci in all sampling locations were in HWE with the expectation of one locus in two different sampling locations; therefore all 11 were retained for final analyses.

Task 2. Population genetic analyses to improve understanding of introductions and dispersal of P. clarkii.

Samples of *P. clarkii* were collected from sampling locations throughout each of the 3 study areas (Figure 14). Standard population genetic statistical analyses were applied to the resulting sample data set. Diversity statistics were generated in ARLEQUIN and GENEPOP. Population genetic structure throughout the collection range was assessed via evaluations of pairwise F_{ST} statistics and Garza-Williamson (G-W) indices calculated in ARLEQUIN and with the clustering algorithms implemented in STRUCTURE 2.3.4 (Pritchard *et al.* 2000). The clustering model assignment employed in the program STRUCTURE using a hierarchical approach with the assistance of the web-based software StructureSelector (Li and Liu 2018) was used to identify the most appropriate number of distinct populations (K) of each run. Simulations were run with both the locprior (collection location) and no locprior parameter settings for all analyses, with 5 replicates for each K, the length of the burn-in period set at 100,000, and number of Markov chain Monte-Carlo reps after burn-in set at 100,000. All analyses were conducted from K=1 to K= 23, the number of collection locations. Samples that showed homogenous ancestry patterns were removed from the data set and STRUCTURE was run iteratively until K=1 was the most appropriate assignment for each cluster (based on combined evaluation of the Evanno method and log likelihood plots).

A total of 594 samples met the genotyping inclusion criteria, resulting in an average of 10.9 loci genotyped across all sampling locations. Initial pairwise Fst comparisons of all collection locations showed a highly structured genetic landscape with no indication of geneflow between the 3 river basins.

Within basin, the Winyah remained highly structured having significant Fst comparisons between every sampling location (Fst 0.051-0.225). Within the Little Pee Dee, Fst comparisons suggest substantial geneflow exists between Sites 1-2 (Fst= 0.01855), while comparisons of these sites with all others in the system were all significant (Fst =0.05543-0.10901). Little Pee Dee sites 3-8 also appeared to be genetically similar with ample gene flow indicated by no significant differences in Fst comparisons (Fst = -0.00303-0.02291). Within the Waccamaw, Sites 1-2 were genetically similar (Fst=0.00298) and distinct from all other Waccamaw sampling locations (Fst=0.02173-0.09097). Likewise, Sites 7-8 were genetically similar (Fst=0.01185) and distinct from all other Waccamaw sampling locations (Fst=0.02589-0.09097). Comparisons of Waccamaw Sites 4-6 were not significant (Fst=0.00223-0.01175) while Waccamaw Site 3 appeared to have slightly elevated Fst values suggesting limited geneflow between Sites 4-5 (Fst~0.0141). Garza-Williamson indices were low (0.12-0.21) with no apparent trends among sampling locations.

Our STRUCTURE analysis, including all 23 sampling locations, resulted in the most likely scenario of 13 genetic clusters (k=13; Figure 15). The genetic groups recovered (Figure 16) were concordant with our Fst comparisons, showing a highly structured genetic landscape. Each Winyah site was distinct from all other sites and there were no shared ancestries between the 3 river basins. The largest homogeneous cluster was recovered in Little Pee Dee sites 3-8, and when run alone in STRUCTURE, results in 2 genetic groups, Sites 3-4 and 5-8. Little Pee Dee Sites 3-4 appear to have minimal shared ancestry with Little Pee Dee Sites 1-2 suggesting some mixing has occurred. In the Waccamaw, the furthest upstream Sites, 1-2, form a unique genetic cluster and slight gradient of mixed ancestry from Sites 2-3. This gradient continues through Waccamaw Sites 3-6, with Site 5 being the most differentiated. Waccamaw Sites 7-8 mostly share a common unique ancestry with Site 8 mixing with Sites 4-6.

Overall, our results show a highly structured genetic landscape between the three river basins. In the Winyah, an area of historic *P. clarkii* aquaculture activities and recorded higher densities of *P. clarkii* prior to the year 2000, we found the highest level of genetic structure in the data set, with every sampling location being genetically distinct. It is unclear whether these 7 genetic lineages are the result of independent introductions related to farming and reflect the diversity of potential source populations or whether these populations, after likely being established prior to the 1970s, have diverged due to a lack of gene flow/dispersal. While *P. clarkii* is capable of rapid long-distance dispersal, negative density-dependent dispersal limitations of large/densely established populations may prevent genetic mixing.

Our results for the Little Pee Dee system show a rather stark contrast to the Winyah with only two genetically distinct clusters over a large geographic area and a gradient of mixing between them. Prior to the year 2000, there were no records of *P. clarkii* in the drainage; however, our surveys from 2018 – 2021 document this species throughout the Little Pee Dee, and show they are dominate at most sites where they are found. While it is unclear where *P. clarkii* was first introduced to the system, the genetically homogenous group spanning Sites 3-8 is indicative of a founder effect and subsequent rapid range expansion through the area. Given the history of successful *P. clarkii* invasions worldwide, positive density-dependent dispersal into the previously uncolonized Little Dee Pee system may have been facilitated by habitat similarity to its native range. Little Pee Dee Sites 1-2 are genetically distinct from the downstream sampling locations. Such differentiation could be a result of isolation and drift over time; or given the timeframe of expansion (<20 years), perhaps may represent an independent introduction.

The Waccamaw system was also colonized with *P. clarkii* within the past 20 years and our results show a slightly more structured landscape than that of the Little Pee Dee. The 2 furthest upstream Sites (1-2) are genetically distinct which, again, could be from isolation and genetic drift. Anecdotally, however, through interactions with the public, we are aware of at least one moderately large introduction of commercially sourced *P. clarkii* around Waccamaw Site 1, which occurred ~20 years ago. While Sites 1-2 are unique, a gradient of genetic mixing in our STRUCTURE plot (Figure 16) is apparent from Sites 2-6. An increase in the average number of alleles is observed in the Waccamaw which likely explains the increase in assigned shared ancestries found with STRUCTURE. These results suggest the colonization of the

Waccamaw was either from a more diverse source population or more independent introductions followed by rapid dispersal have occurred than in the Little Pee Dee.

Discussion

Contemporary sampling and comparison with historical data indicate a rapid spread of *P. clarkii* throughout much of the Pee Dee basin in SC. Microsatellite data show strong population genetic structure among sites in the Winyah region, but sites within the Little Pee Dee and within the Waccamaw show less population genetic structure. The relative lack of population genetic structure in the Little Pee Dee and Waccamaw, as compared with the Winyah region, suggests that natural dispersal is likely an important factor leading to the recent spread of *P. clarkii* in the recently invaded watersheds. Given that, we can likely expect to see these populations continue to spread and disperse throughout these regions and beyond, and thus may need to consider management approaches and strategies accordingly.

High genetic structure in the Winyah region is likely due to the historic pattern of introductions in this region. In both the Little Pee Dee and Waccamaw watersheds, we see similar patterns of gene flow throughout each of the two watersheds. The Little Pee Dee's two genetically distinct clusters, as well as the slightly more structured genetic landscape of the Waccamaw, indicate at least some degree of gene flow is occurring over the course of the last quarter century. This is particularly notable when one considers that, prior to the year 2000, there were no apparent populations of *P. clarkii* in either basin. This does not mean that they were definitively not present, but the fact that there were no documented records during this time indicate they were very low in abundance if present at all. There are now at least 28 (Little Pee Dee) and 35 (Waccamaw) recorded populations of *P. clarkii*. These metrics, as well as the genetically substantiated hypothesis that *P. clarkii* populations in these regions are rapidly dispersing, indicate that rapid spread is possible by this species in South Carolina's coastal plain, potentially threatening native species in other watersheds in the region as well.

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Table 1. Comparison of known populations of three crayfish species of conservation concern in relation to current populations of *P. clarkii*.

Species	Documented Populations	Potential Extirpations	% Populations Extirpated	Remaining Populations	Co-Occurrence	% Co-Occurrence
P. ancylus	20	5	25%	15	10	67%
P. braswelli	26	5	19%	21	12	57%
P. pearsei	28	4	14%	24	4	17%

Table 2. Distribution of crayfish across collections sorted by total number collected and total number of species per gear type. Asterisks indicate that collections were not time-standardized.

Site	CPUE electrofisher	CPUE Dip Net	Richness electrofisher	Richness Dip Net
1*	3	31	2	3
2*	1	12	1	3
3*	0	9	0	2
4*	2	5	1	2
5*	2	1	2	1
6	0	0	0	0
7	0	2	0	1
8	2	10	1	2
9	10	6	1	2
10	1	3	1	1
11	0	3	0	2
12	0	3	0	2
13	1	1	1	1
14	2	0	0	0
15	2	14	2	2
16	3	1	1	1
17	1	2	1	1
18	0	17	0	1
19	2	2	1	1
20	0	2	2	0
21	2	0	1	0
22	0	0	0	0
23	5	0	2	0
24	1	5	2	2
25	3	33	1	3
26	1	4	1	2
27	0	0	0	0
28	0	2	0	1
29	1	0	1	0
30	9	11	1	1

Table 3. Comparison of occurrences, collection numbers, and CPUE for the five species of crayfish collected during sampling.

Species	Sites (Dip Net)	Sites (Electrofisher)	Abun. (Dip Net)	Abun. (Electrofisher)
Procambarus acutus	10	5	25	12
Procambarus ancylus	1	0	1	0
Procambarus braswelli	7	3	42	4
Procambarus clarkii	22	17	90	40
Procambarus pearsei	1	1	16	3

Table 4. Distribution of crayfish life forms across species for electrofishing / dip net collections, respectively.

Species	Form 1 Male	Form 2 Male	Female	Juvenile
P. clarkii	2 / 6	8 / 14	24 / 43	7 / 27
P. acutus	1 / 2	3 / 3	8 / 15	0 / 6
P. braswelli	0 / 4	0 / 6	3 / 15	0 / 17
P. pearsei	0 / 0	1 / 0	1 / 1	1 / 15
P. ancylus	0 / 0	0 / 1	0 / 0	1 / 0
Total	3 / 12	12 / 24	36 / 74	9 / 65

Table 5. Microsatellite loci currently incorporated into multiplexed PCR: repeat motif, size ranges, number of alleles, number of samples included and respective multiplex groups (MPG).

Locus Name	Repeat Motif	Size Range	Number of Alleles	Number of Samples	Multiplex Group
Pcl-12	AGC	93-174	27	593	MPG1
Pcl-34	ACC	207-249	14	590	
Pcl-70	ACAG	229-337	37	579	
Pcl-57	AGGC	251-343	40	588	
Pcl-43	AAT	306-339	15	571	
Pcl-39	AGAT	142-210	22	593	MPG2
Pcl-53	ACTC	150-286	36	590	
Pcl-64	AGGC	206-298	21	593	
Pcl-30†	AAT	244-301	14	570	
Pcl-36†	AG	117-167	39	588	MPG3
Pcl-79	ACTC	122-184	17	592	
Pcl-46	AAT	151-193	21	593	
Pcl-52	AGAT	188-252	28	584	

†Excluded from final analyses

Table 6**.** Diversity statistics by sampling location. Sample size, average number of alleles (N_a), expected and observed heterozygosity (H_o & H_e), Garza-Williamson index (G-W), and inbreeding coefficient (F_{is}).

Site Name	Sample Size	N_a	H_o	H_e	G-W	F_{is}
Little Pee Dee 1	30	9.0	0.72926	0.77272	0.17351	0.0654
Little Pee Dee 2	23	9.2	0.70931	0.77688	0.17707	0.0937
Little Pee Dee 3	26	10.0	0.75325	0.79894	0.17936	0.1064
Little Pee Dee 4	19	9.0	0.74313	0.78118	0.17416	0.1122
Little Pee Dee 5	29	8.0	0.71357	0.77749	0.15447	0.1494
Little Pee Dee 6	30	8.1	0.71306	0.76644	0.15507	0.1145
Little Pee Dee 7	21	7.3	0.67834	0.76839	0.14654	0.1817
Little Pee Dee 8	15	7.2	0.70862	0.77493	0.14324	0.1455
Winyah 1	27	10.5	0.76068	0.81749	0.19282	0.1467
Winyah 2	30	9.5	0.7488	0.75905	0.17645	0.0501
Winyah 3	25	7.5	0.73636	0.66585	0.12507	-0.0024
Winyah 4	30	8.5	0.76343	0.77362	0.16705	0.0475
Winyah 5	25	10.3	0.72727	0.74924	0.17713	0.0372
Winyah 6	29	6.5	0.63771	0.67781	0.15768	0.0738
Winyah 7	19	7.3	0.71186	0.75419	0.15391	0.1056
Waccamaw 1	27	10.3	0.79979	0.82237	0.18195	0.0511
Waccamaw 2	30	10.7	0.76897	0.82987	0.18639	0.1298
Waccamaw 3	30	14.1	0.80627	0.82524	0.19891	0.0832
Waccamaw 4	30	14.5	0.77168	0.857	0.21078	0.1172
Waccamaw 5	30	14.0	0.83325	0.86535	0.20916	0.0718
Waccamaw 6	30	13.9	0.75731	0.84555	0.21793	0.1532
Waccamaw 7	20	7.5	0.711	0.71544	0.16277	0.1079
Waccamaw 8	19	10.5	0.74003	0.77666	0.2031	0.1173

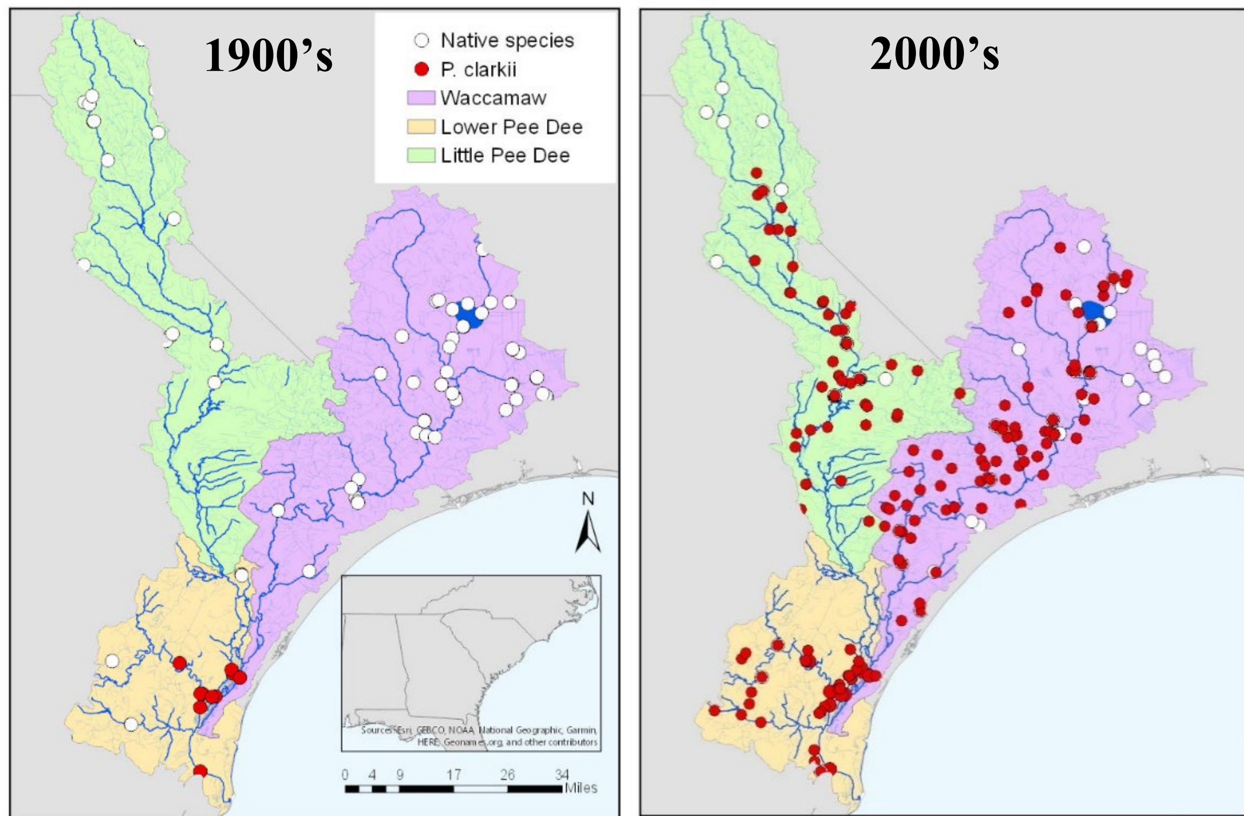

Figure 1. A) Historic (1900's) records and B) Contemporary (2000's) records of native crayfish and *P. clarkii* in portions of the Great Pee Dee watershed in North Carolina and South Carolina compiled from data collected as part of this, and other projects (see text for details).

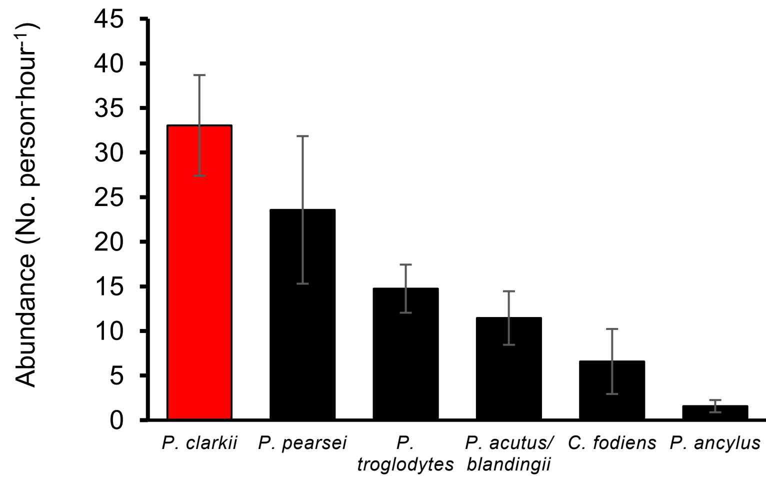

Figure 2. Mean (±SE) catch per unit effort (CPUE) of the six most collected crayfish species from January 1, 2020 to December 3, 2020.

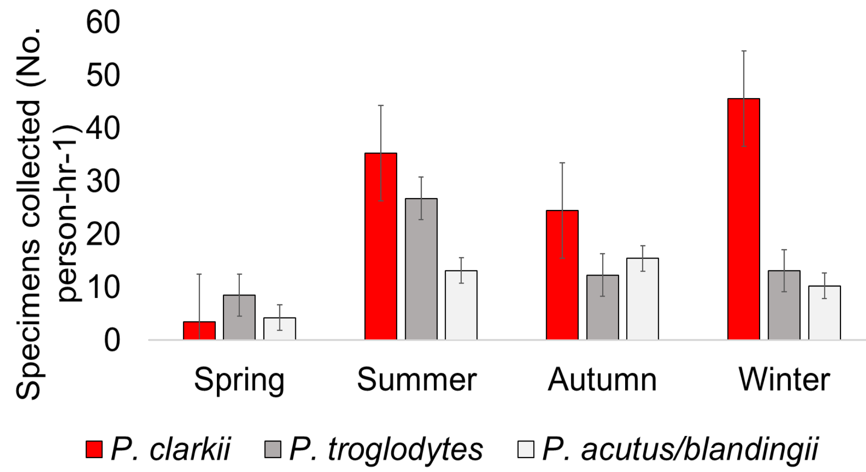

Figure 3. Mean (±SE) catch per unit effort (CPUE) of three commonly collected species compared with season.

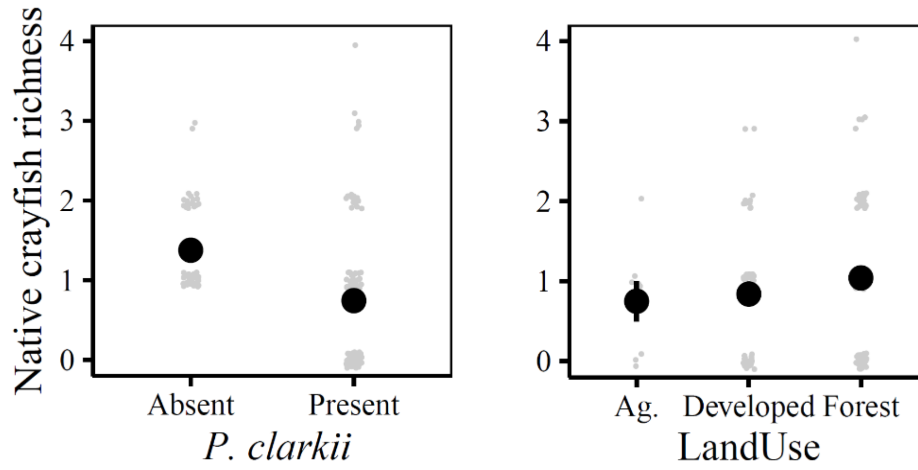

Figure 4. Patterns of native crayfish species richness when *P. clarkii* is present or absent (left) and under different land use regimes (right).

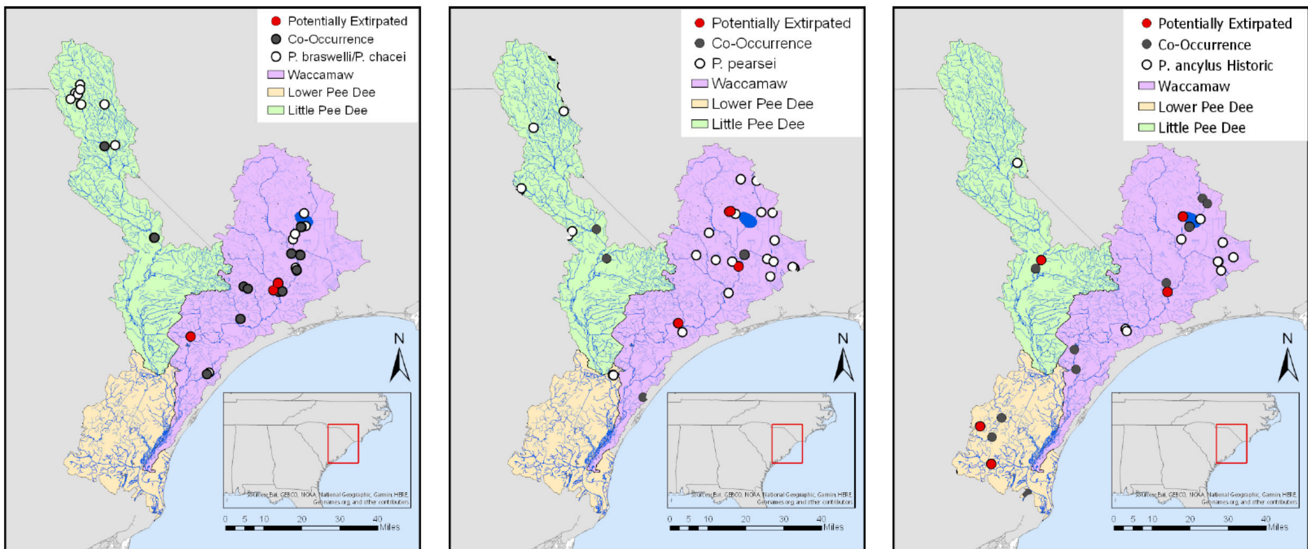

Figure 5. Distribution patterns of crayfishes of conservation priority status in SC and how they related to patterns of occurrence of *P. clarkii*. This includes sites where the two species co-occur (grey points) and sites where the native species previously occurred but has not recently been collected.

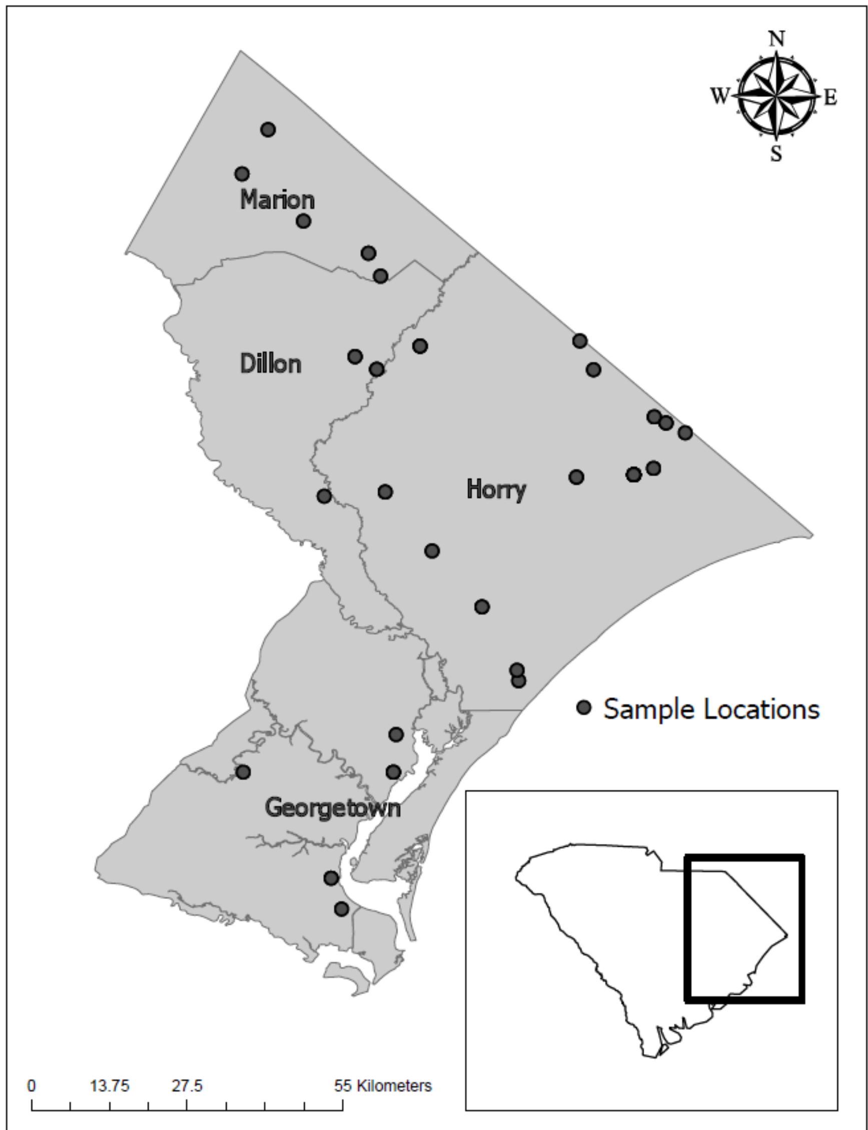

Figure 6. Sampling locations in Dillon, Georgetown, Horry, and Marion Counties in November and December 2021.

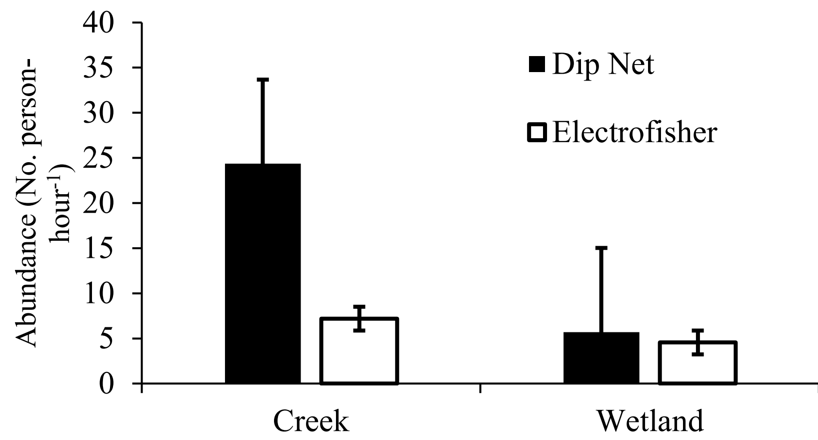

Figure 7. Mean (±SE) catch rates of crayfish collected by dip net and electrofisher collections across habitat.

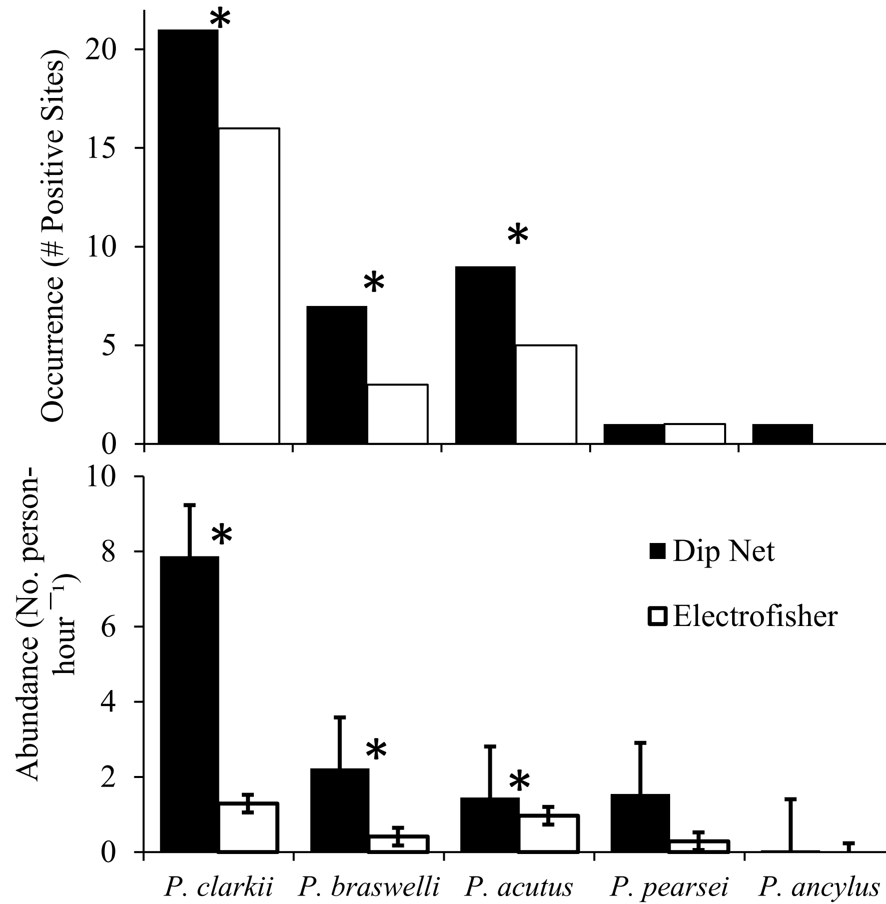

Figure 8. Occurrence (top panel) and mean abundance (± SE; bottom panel) of crayfish species collected by gear type. Due to the low number of individuals collected, neither occurrences nor CPUE were statically compared for *P. pearsei* or *P. ancylus*.

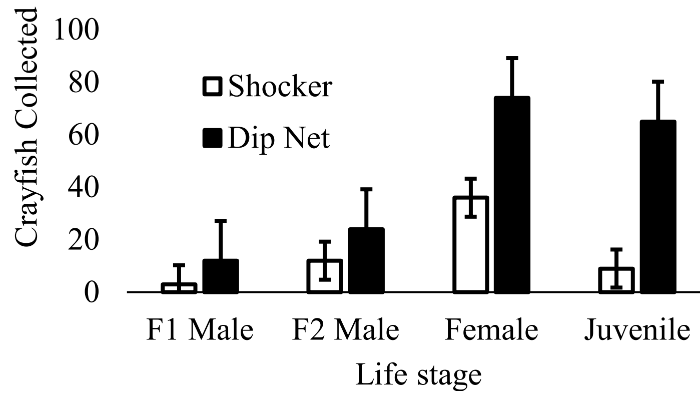

Figure 9. Comparison (±SE) of crayfish collections for each form across gear type.

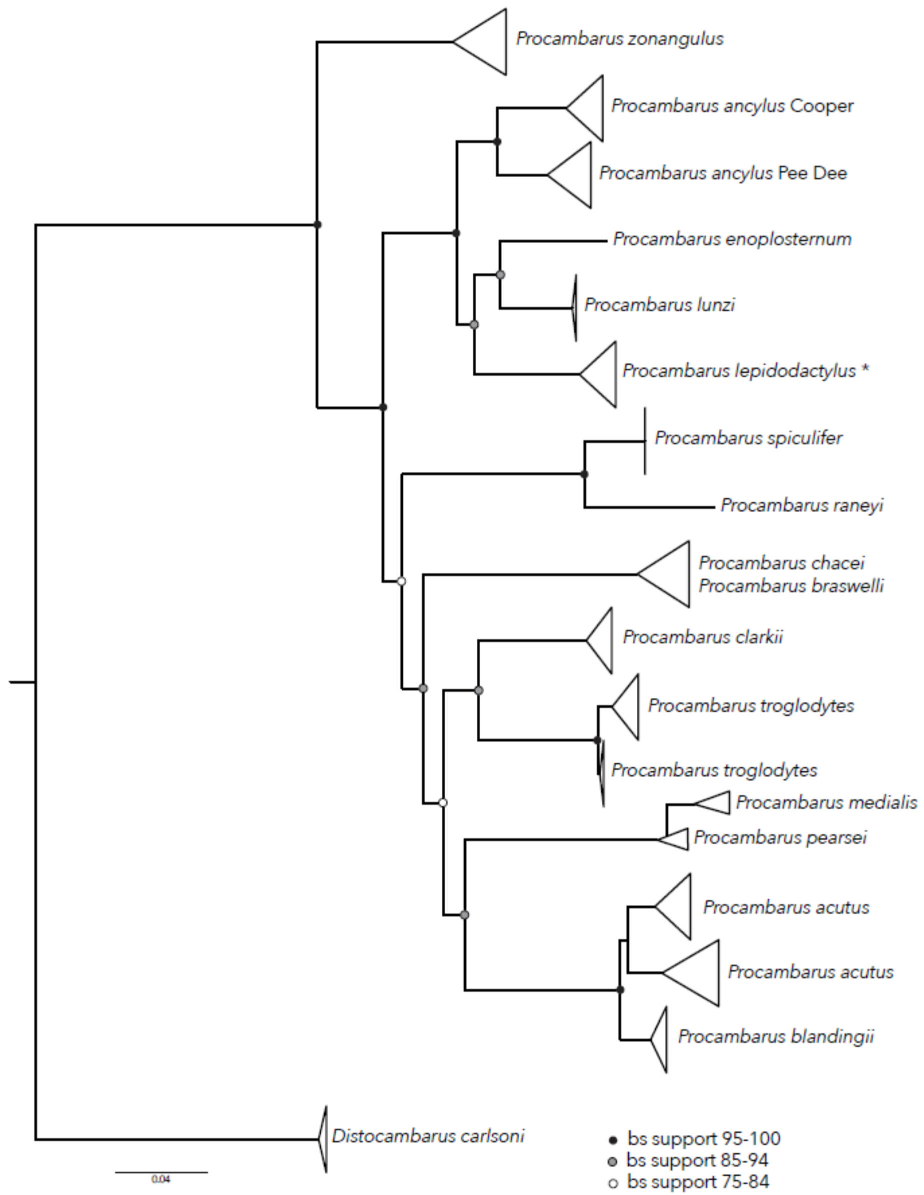

Figure 10. … Sequence data for procams…

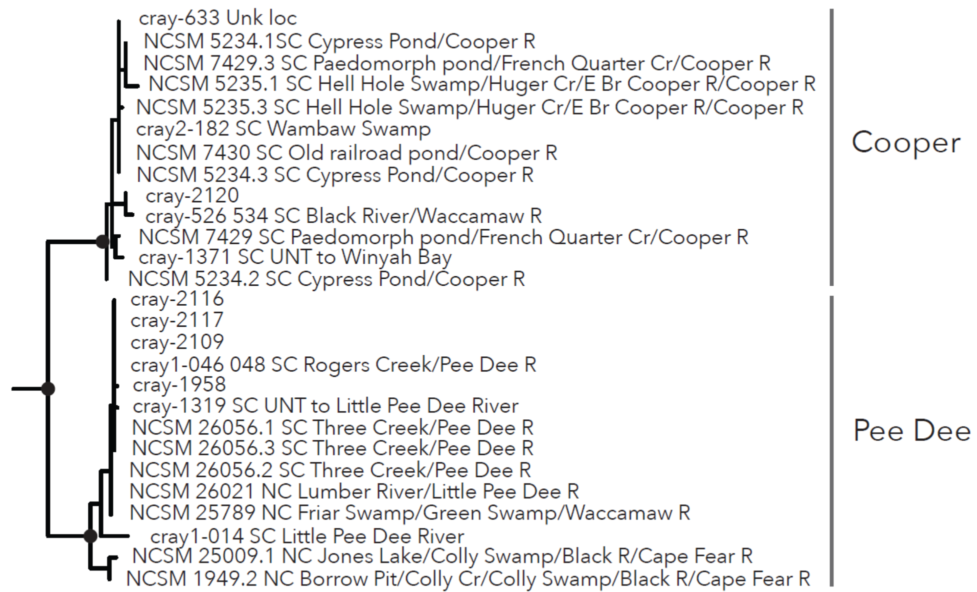

Figure 11. … Sequence data for ancylus…

Figure 12. … Sequence data for chacei/braswelli…

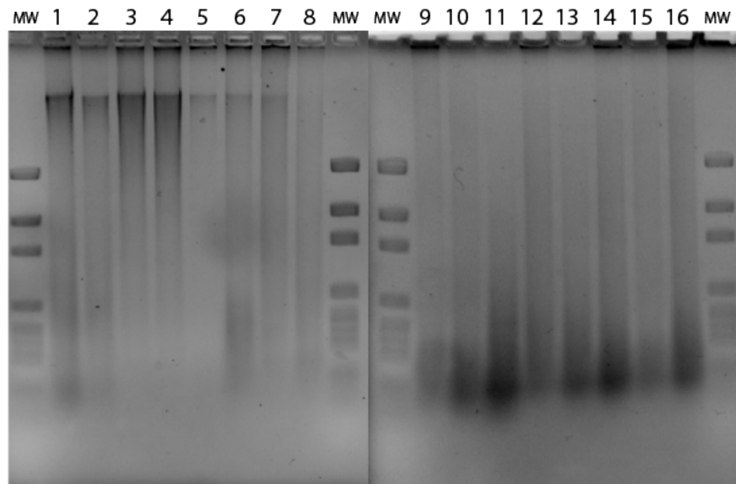

Figure 13. Gel image visualizing relative DNA qualities and quantities. Lanes 1-2 are tail muscle with unmodified spin-coulmn; 3-4 are gill with unmodified spin-column; 5-6 are tail muscle with modified spin-column; 7-8 are gill with modified spin-column; and 9-16 are gill with Yue & Orban (2005) method. MW is the pgem molecular weight ladder with the top band representing 2,645 base pairs. Lanes 3-4 show the strongest signal of high molecular weight genomic DNA.

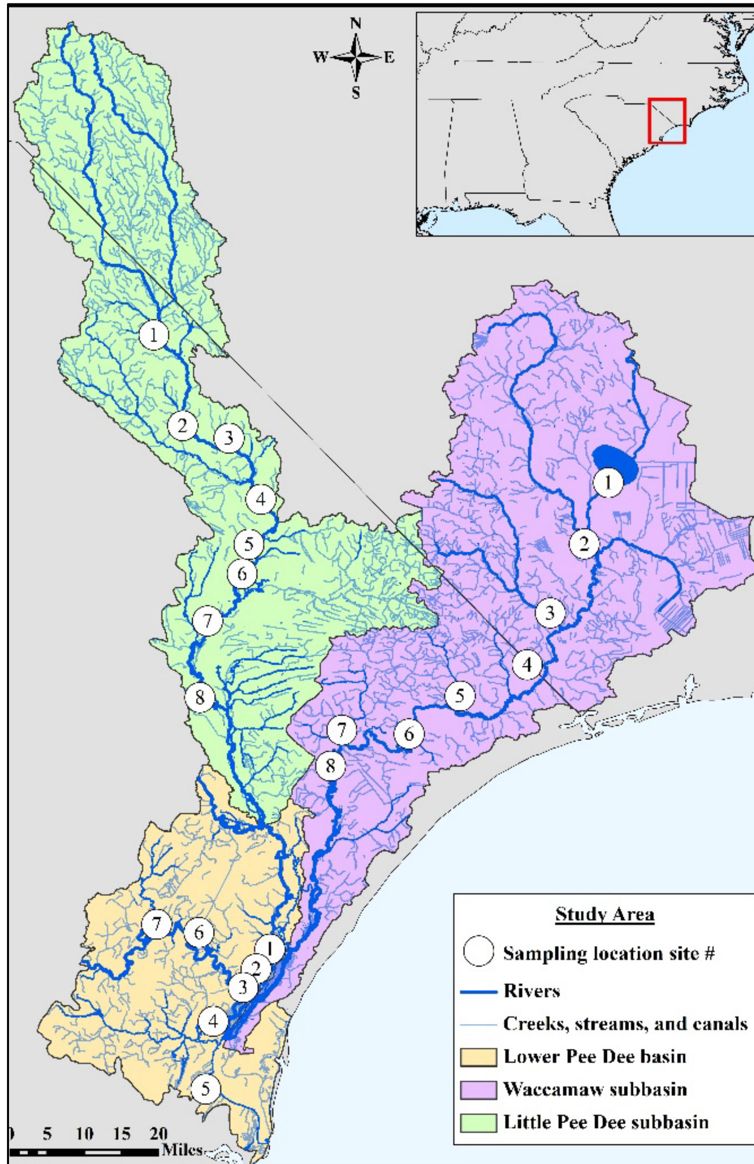

Figure 14. Sampling locations designed to address research objective 2 relating to the introduction and dispersal patterns of *P. clarkii* within the Pee Dee basin.

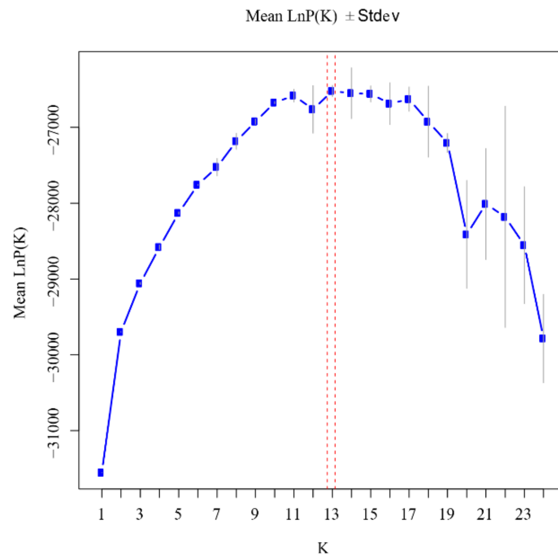

Figure 16. Mean log likelihood plot generated by StructureSelector. Dotted red lines bound the most likely scenario k=13.

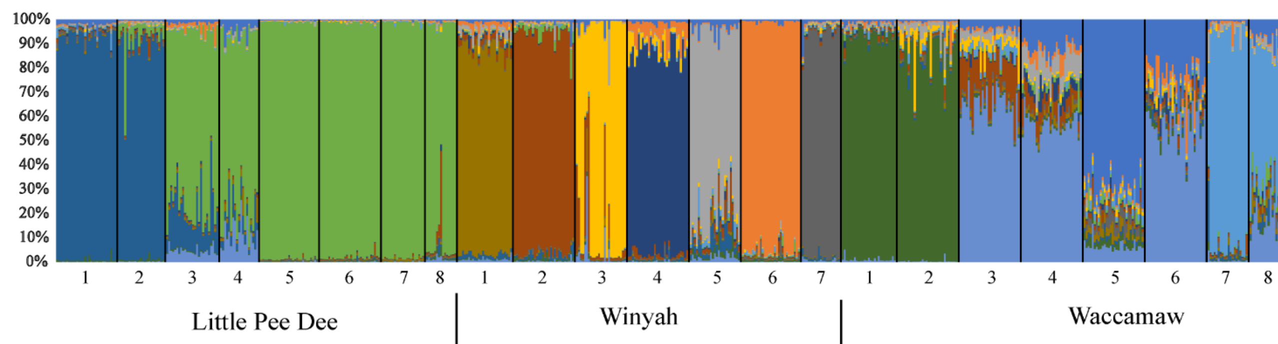

Figure 15. STRUCTURE plot of most likely scenario of K=13. Bar plot: each bar represents an individual and colors represent different ancestries. Percent ancestry is along the y-axis.