

Distribution of Karyotypes of the *Cryptocercus punctulatus* Species Complex (Blattodea: Cryptocercidae) in Great Smoky Mountains National Park

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Abstract

During the period between 1999 and 2006, wood-feeding cockroaches in the *Cryptocercus punctulatus* Scudder species complex were collected throughout Great Smoky Mountains National Park, USA. The chromosome numbers of insects from 59 sites were determined, and phylogenetic analyses were performed based on mitochondrial COII and nuclear ITS2 DNA. The distribution of the three male karyotypes found in the park ($2n=37$, 39, and 45) is mapped and discussed in relation to recent disturbances and glacial history. Clades of the three karyotype groups meet near the ridgeline separating North Carolina from Tennessee in the center of the park, suggesting that these may have originated from separate lower elevation refugia after the last glacial maximum. The timing of divergence and a significant correlation between elevation difference and genetic distance in two of the clades supports this hypothesis. The ecological role of the cockroaches in the park is discussed.

Key words: All Taxa Biodiversity Inventory, refugia, ecological service, coarse woody debris

Great Smoky Mountains National Park (GSMNP) is home to one of the oldest and most ecologically diverse mountain chains in the world, and is an internationally recognized hotspot of temperate forest biodiversity (Nichols and Langdon 2007). In an effort to catalogue this diversity, a comprehensive inventory of all life forms in the GSMNP, the All Taxa Biodiversity Inventory (ATBI), was initiated and charged with the goal of discovering the identity and distribution of as many species as possible that occur in the park (Sharkey 2001, White and Langdon 2006). As part of this effort, the wingless wood-feeding cockroach *Cryptocercus punctulatus* Scudder was recovered from seven locations in the park, representing seven watersheds (Discover Life in America website, accessed 14 November 2016: https://www.dlia.org/atbidata/MapTaxon.php?taxon=Species&tname=Cryptocercus_punctulatus). *Cryptocercus punctulatus* in the eastern United States, however, is a cryptic-species complex currently divided into four known karyotype groups, three of which have been reported from 12 sites previously sampled in the park (Nalepa et al. 2002, Everaerts et al. 2008, Maekawa and Nalepa 2011). The karyotypes of males consist of 18–22 pairs of autosomes and a single X chromosome. The $2n=37$ and $2n=39$ male karyotypes were found in the North Carolina section of the park; the $2n=45$ karyotype was identified along the crest separating North Carolina and Tennessee in the

eastern half of the park, and also at the eastern border near Waterville Lake. The Tennessee half of the park was largely unsampled.

These karyotype groups have been described as separate species (Burnside et al. 1999). The validity of the proposed species-level status was questioned, however, because chromosome numbers were known for only part of the sample, the evolutionary relationships among members of the species complex were unclear, and although morphological variation was apparently present, it had not been demonstrated that this variation consistently distinguished the proposed species (Nalepa et al. 2002, Everaerts et al. 2008, Maekawa and Nalepa 2011). Lineages that share a male chromosome number may represent different species, subspecies, or races (Everaerts et al. 2008, Che et al. 2016). Consequently, *Cryptocercus* found along the Appalachian Chain in the eastern United States is best thought of as a species complex that requires additional study before its taxonomy can be delineated.

Despite the genetic differences among karyotype groups of *C. punctulatus*, there are no known differences in their ecology within their examined range; nor is there evidence of clear divergence in life history, biology or behavior. All members of the genus are fairly large, subsocial insects that are wingless and log-dependent; i.e., all

stages nest in coarse woody debris (CWD) that serves as both food and shelter. The distribution of the cockroach is therefore intimately tied to the distribution of their log hosts. Any event that has an impact on mature forests, including deforestation and glaciation, will have an impact on the distribution of *C. punctulatus* (Nalepa 2001; Nalepa et al. 2001, 2002). Because 95% of GSMNP is forested, with downed logs at all stages of decay on the forest floor (Martin 1992, Sharkey 2001), the cockroach is expected to be more or less continuously distributed within the park.

The goal of this study is to begin documenting the spatial organization of the three known karyotypes of *C. punctulatus* within GSMNP, and to determine whether the $2n=43$ karyotype, not previously detected in the park, can be found there. This information would contribute to the existing ATBI database, and aid in the search for concordant patterns of distribution in other taxa with low vagility and similar ecological requirements. If diverse organisms had retreated to and shared the same refugial areas during glacial cycles of the Pleistocene, some degree of geographic patterning would be expected (Howden 1985, Vermeij 1986, Cranston and Naumann 1991, Soltis et al. 2006). Evidence from a variety of sources indicates that there were one or more refugia in the Southern Appalachians during the glacial cycles of the Pleistocene (Tilley 1997, Church et al. 2003, Soltis et al. 2006, Sokolov et al. 2007, Walker et al. 2009, Rissler and Smith 2010, Garrick 2011).

Materials and Methods

Insect Sampling

Cryptocercus cockroaches were collected between 1999 and 2006, in some cases in conjunction with other studies of the genus. Four collection sites were previously reported in Nalepa et al. (2002) and eight additional sites were detailed in Everaerts et al. (2008) (see Table 1). Some location-coordinates may have shifted slightly from those originally in the literature; more precise location data have become available as technology has advanced. Here we combine data on those 12 sites with data collected on cockroaches from an additional 47 sampling locations within the park (total = 59) to give a more comprehensive summary of the geographic distribution of the different karyotypes within the park. The samples include three sites from the Foothills Parkway (sites #81–83) and two sites just outside park boundaries (sites #39, 85).

At each site, cockroaches were sampled from rotting logs until at least three adult males were collected for chromosome preparations. In some locations these males were all from a single log. At other times more logs had to be sampled; however, the sampled logs were always located within an area of $\leq 50\text{ m}^2$. In a few cases (sites #126, 127, 128, 141), the karyotype and molecular analyses were done using the same male specimen; in most, however, females and nymphs from the same collections were used for molecular analyses. Collections were limited to locations reached by round-trip hiking in one day, so there is some bias associated with accessibility of the sampling sites. The more interior locations were under-sampled, particularly in the western half of the park.

Cockroaches were classified by chromosome number without implications of taxonomic status, and were collected under permits GRSM-99-063, GRSM-2001-SCI-0024, and GRSM-2005-SCI-0084.

Chromosome Counts

For each sampled location, chromosome preparations were made from the testes of three adult males using the technique of Luykx (1983). Meiotic chromosomes were counted and are reported as the male diploid count.

Molecular Analysis

DNA Extraction, Amplification, Purification, and Sequencing. Total DNA was extracted from the leg tissue of individuals preserved in 80–100% ethanol by using DNeasy Tissue Kit (Qiagen, Tokyo, Japan). Each individual was considered to be representative of the population in each location. The fragments of mitochondrial COII (448 bp) and nuclear ITS2 (~400 bp) were amplified using PCR. Primer sequences for the amplifications of COII and ITS2 are shown in Park et al. (2004) and Everaerts et al. (2008), respectively. The temperature profile for amplifications of COII and ITS2 was 94°C for 3 min, followed by 35 cycles of 94°C for 1 min, 50°C for 1 min and 70°C for 2 min. Amplified PCR products were purified by using the Mag Extractor Kit (Toyobo, Osaka, Japan) or ExoSAP-IT (Affymetrix, Santa Clara, CA, USA), and they were used as templates for sequencing performed by the DNA sequencer (ABI 373 or 3130 Genetic Analyzer; Applied Biosystems, Carlsbad, CA, USA).

Sequence Alignments. For alignments, MUSCLE in MEGA version 6.06 (Tamura et al. 2013) was used. To consider the possibility of mitochondrial introgression, we analyzed mitochondrial (COII) and nuclear (ITS2) data separately. For the mitochondrial dataset, *Cryptocercus clevelandi* Byers was included as an outgroup (GenBank accession no. AB078557). For the nuclear dataset, however, as shown in Everaerts et al. (2008), there were many insertions and deletions in DNA sequences between *C. clevelandi* and cockroaches from the southern Appalachians. Thus, the ITS2 tree is an unrooted tree.

Phylogenetic Analysis. We obtained estimations of tree topologies under the Bayesian inference (BI), maximum likelihood (ML) and maximum parsimony (MP) methods. For BI, the most appropriate model of sequence evolution was determined using MEGA version 6.06 model selection option (Tamura et al. 2013). The T92 + G model was selected from both COII and ITS2 data. Parameters for the selected model of substitution were estimated from the data. In total, 100,000 trees were obtained (ngen = 10,000,000, samplefreq = 100) using MrBayes version 3.2.6 (Ronquist and Huelsenbeck 2003), and the first 25% of these (25,000) were discarded as the burn-in. A 50%-majority-rule consensus tree of the remaining trees was produced. Two independent runs under the same model of sequence evolution were performed. For ML, 1,000 bootstrap replicates were performed based on the same model of sequence evolution as BI in MEGA 6.06 (Tamura et al. 2013). Initial trees for the heuristic search were obtained by applying the NJ method to a matrix of pairwise distances estimated using the Maximum Composite Likelihood approach. For MP analysis, all characters were included and weighted equally, and 1,000 bootstrap replicates were performed using MEGA version 6.06 (Tamura et al. 2013). Subtree-Pruning-Regrafting algorithm with search level 1, in which the initial trees were obtained by the random addition of sequences (10 replicates), was used.

Estimation of Divergence. As in previous studies of Korean *Cryptocercus* spp. (Park et al. 2004), we did a preliminary estimation of divergence times between each phylogenetic group (I–VI in Figs. 1 and 2) based on the COII sequences using transversion (TV) distances (0.13–0.30%/million years (Myr)). Mean TV distances were calculated as the Jukes–Cantor formula modified by Beckenbach et al. (1993).

Principal Component Analysis (PCA)

To understand the correlation of the chromosome numbers and phylogenetic groups with the sampling location (latitude, longitude and elevation shown in Table 1), we did a PCA using the statistical

Table 1. Collection localities, chromosome number of males, and Genbank accession numbers of *Cryptocercus* collected in Great Smoky Mountains National Park

Collection no.	Site no.	Location	Male 2n	County	Latitude	Longitude	Elev. (m)	Published?	COII Accession no.	ITS2 Accession no.
1	49	Wolf Ridge	37	Swain, NC	36.475	83.880	480	Nalepa et al. 2002	LC218170	LC218221
2	50	Thomas Divide	37	Swain, NC	35.466	83.419	666	Nalepa et al. 2002	LC218171	LC218222
3	81	Chilhowee	37	Blount, TN	35.559	84.011	317		LC218172	LC218223
4	82	Look Rock	37	Blount, TN	35.633	83.942	797		LC218173	LC218224
5	83	Mt. Nebo	37	Blount, TN	35.717	83.821	360		LC218174	LC218225
6	84	Cades Cove	37	Blount, TN	35.607	83.779	627		LC218175	LC218226
7	85	Townsend	37	Blount, TN	35.668	83.716	398		LC218176	LC218227
8	86	Elkmont	37	Sevier, TN	35.663	83.600	629		LC218177	LC218228
9	87	Chimneys	37	Sevier, TN	35.636	83.493	837		LC218178	LC218229
10	88	Greenbrier	37	Sevier, TN	35.733	83.415	438		LC218179	LC218230
11	91	Clingmans Dome	37	Swain, NC	35.558	83.493	1,895		LC218180	LC218231
12	109	Ramsay Cascades	37	Sevier, TN	35.710	83.319	856		LC218181	LC218232
13	111	Maddron Bald Trail	37	Cocke, TN	35.769	83.267	549		LC218182	LC218233
14	113	Collins Creek Trail	37	Swain, NC	35.569	83.339	742		LC218183	LC218234
15	122	Noland Divide Trail	37	Swain, NC	35.566	83.475	1,742	Everaerts et al. 2008	AB425865	AB425887
16	123	Keg Drive Branch	37	Swain, NC	35.577	83.449	1,480	Everaerts et al. 2008	AB425866	AB425888
17	124	Fork Ridge Trailhead	37	Swain, NC	35.590	83.470	1,794		LC218184	LC218235
18	129	Deep Low Gap	37	Swain, NC	35.512	83.348	1,103	Everaerts et al. 2008	AB425864	AB425886
19	130	Mingus Mill	37	Swain, NC	35.520	83.309	597		LC218185	LC218236
20	147	Old Sugarlands	37	Sevier, TN	35.674	83.493	686		LC218186	LC218237
21	153	Roaring Fork	37	Sevier, TN	35.681	83.462	768		LC218187	LC218238
22	51	Kephart Prong Trail	39	Swain, NC	35.590	83.371	897	Nalepa et al. 2002	LC218188	LC218239
23	93	Smokemont	39	Swain, NC	35.556	83.310	711		LC218189	LC218240
24	94	Palmer Creek	39	Haywood, NC	35.628	83.175	1,377		LC218190	LC218241
25	95	Straight Fork	39	Swain, NC	35.581	83.243	815		LC218191	LC218242
26	98	Cataloochee Divide	39	Haywood, NC	35.632	83.045	1,251		LC218192	LC218243
27	106	Sunkota Ridge	39	Swain, NC	35.546	83.369	1,442		LC218193	LC218244
28	107	Kanati Fork Trail	39	Swain, NC	35.572	83.385	1,505		LC218194	LC218245
29	108	Thomas Divide Trailhead	39	Swain, NC	35.585	83.399	1,417		LC218195	LC218246
30	112	Deep Creek Trailhead	39	Swain, NC	35.600	83.424	1,416		LC218196	LC218247
31	140	Kephart Shelter	39	Swain, NC	35.611	83.369	1,078		LC218197	LC218248
32	141	Cabin Flats	39	Swain, NC	35.609	83.333	891	Everaerts et al. 2008	AB425871	AB425893
33	39	Waterville Lake	45	Haywood, NC	35.699	83.041	867	Nalepa et al. 2002	LC218198	LC218249
34	89	Cosby	45	Cocke, TN	35.783	83.217	517		LC218199	LC218250
35	90	Big Creek	45	Haywood, NC	35.766	83.109	507		LC218200	LC218251
36	92	Newfound Gap	45	Sevier, TN	35.611	83.424	1,534		LC218201	LC218252
37	114	Sweat Heifer	45	Sevier, TN	35.621	83.404	1,777	Everaerts et al. 2008	AB425882	AB425904
38	115	Boulevard Trail	45	Sevier, TN	35.630	83.392	1,853		LC218202	LC218253
39	116	Anakeesta Knob	45	Sevier, TN	35.637	83.411	1,792		LC218203	LC218254
40	117	West Point View	45	Sevier, TN	35.652	83.444	1,932		LC218204	LC218255
41	118	Arch Rock	45	Sevier, TN	35.636	83.439	1,439		LC218205	LC218256
42	119	Grassy Patch	45	Sevier, TN	35.629	83.449	1,250		LC218206	LC218257
43	125	Road Prong	45	Sevier, TN	35.610	83.448	1,618		LC218207	LC218258
44	126	Mt. Collins	45	Sevier, TN	35.595	83.476	1,698	Everaerts et al. 2008	AB425881	AB425903
45	127	Indian Grave Flats	45	Sevier, TN	35.620	83.470	1,235		LC218208	LC218259
46	128	Chimney Tops	45	Sevier, TN	35.634	83.470	1,024		LC218209	LC218260
47	134	Camelback	45	Cocke, TN	35.726	83.207	1,428	Everaerts et al. 2008	AB425884	AB425906
48	135	Upper Low Gap	45	Cocke, TN	35.737	83.182	1,293		LC218210	LC218261
49	136	Cosby Creek	45	Cocke, TN	35.753	83.206	671		LC218211	LC218262
50	137	Mt. Sterling	45	Haywood, NC	35.700	83.098	1,134		LC218212	LC218263
51	138	Double Gap	45	Haywood, NC	35.724	83.087	854		LC218213	LC218264
52	142	Laurel Top	45	Sevier, TN	35.664	83.328	1,680	Everaerts et al. 2008	AB424883	AB425905
53	143	Dry Sluice Gap	45	Sevier, TN	35.638	83.369	1,655		LC218214	LC218265
54	148	Balsam Point	45	Sevier, TN	35.653	83.479	1,469		LC218215	LC218266
55	149	West Point	45	Sevier, TN	35.657	83.450	1,814		LC218216	LC218267
56	150	LeConte North	45	Sevier, TN	35.665	83.439	1,653		LC218217	LC218268
57	151	Trillium Gap	45	Sevier, TN	35.674	83.433	1,439		LC218218	LC218269
58	152	Grotto Falls	45	Sevier, TN	35.674	83.449	1,106		LC218219	LC218270
59	161	Double Spring Gap	45	Swain, NC	35.564	83.534	1,332		LC218220	LC218271

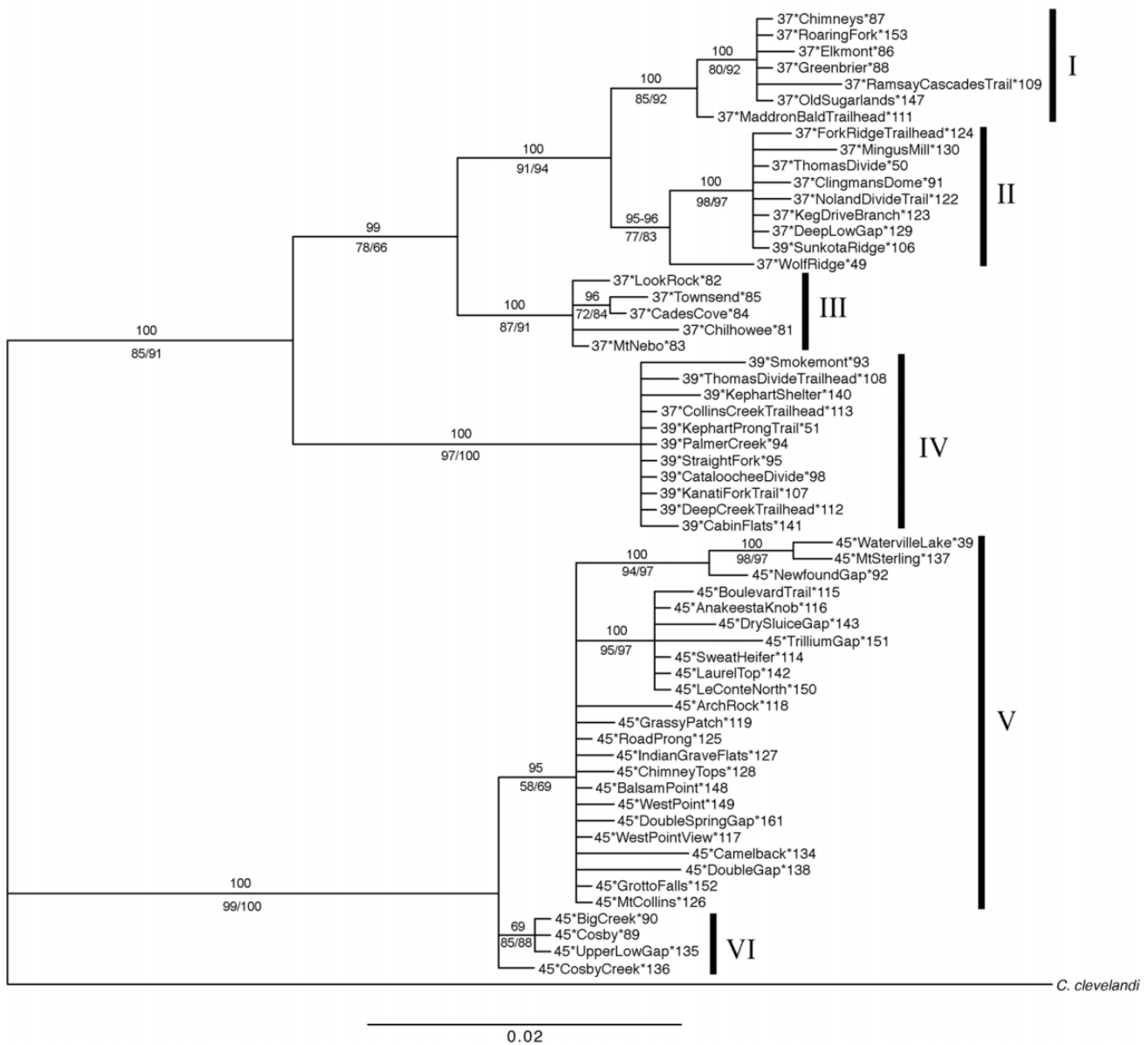


Fig. 1. Phylogenetic relationships of the *Cryptocercus punctulatus* species complex distributed in Great Smoky Mountain National Park based on the mitochondrial COII gene sequences (448 bp). The topology and branch lengths shown were obtained by Bayesian inference method. Posterior probabilities (PP) are shown above branches to indicate the level of support for each node. Only one number is given if PP was identical at that node in the two different runs. Numbers below each branch indicate the percentage of bootstrap support (1,000 replicates) in the maximum likelihood (ML) and maximum parsimony (MP) methods, respectively. An asterisk indicates a node that was not supported by ML and MP methods. Clades labels I–VI correspond to those in Figures 2 and 3b.

software Mac Multivariate Analysis ver. 2.0 (Esumi, Tokyo, Japan). On the basis of the PCA analysis, we compared elevation difference and genetic distance (using COII sequence divergence based on the T92 + G model) between two locations within each phylogenetic group. Pearson's correction coefficient was calculated using Microsoft Excel for Mac 2011 ver. 14.7.2. *P* values < 0.05 were considered statistically significant.

Results

Karyotype and Molecular Analysis

As previously reported (Nalepa et al. 2002, Everaerts et al. 2008), the $2n = 37$, 39 and 45 karyotypes were found within the boundaries of GSMNP; more intense sampling did not result in the discovery of additional karyotypes of *C. punctulatus* within the park.

In both phylogenetic trees (Figs. 1 and 2) the sampled cockroaches split into two major clades: one that included *C. punctulatus* with chromosome counts of $2n = 37$ and $2n = 39$, and one that included those with chromosome counts of $2n = 45$. All cockroaches with a chromosome count of $2n = 45$ were clearly monophyletic in both the COII and ITS2 trees. In the clades that include the $2n = 37$ and $2n = 39$ karyotypes, the chromosome counts of individuals from two locations were at odds with the molecular signatures used to construct the trees. Collection #106 (Sunkota Ridge) had a chromosome count of $2n = 39$ but fell into the $2n = 37$ clade in both the COII and ITS2 analyses. Just one of the three slides made from testes of adult males from this location was suitable for chromosome analysis; counts of chromosomes of meiotic cells on that slide were made independently by the first and last authors and both came to the conclusion of $2n = 39$. The second discrepancy was collection #113

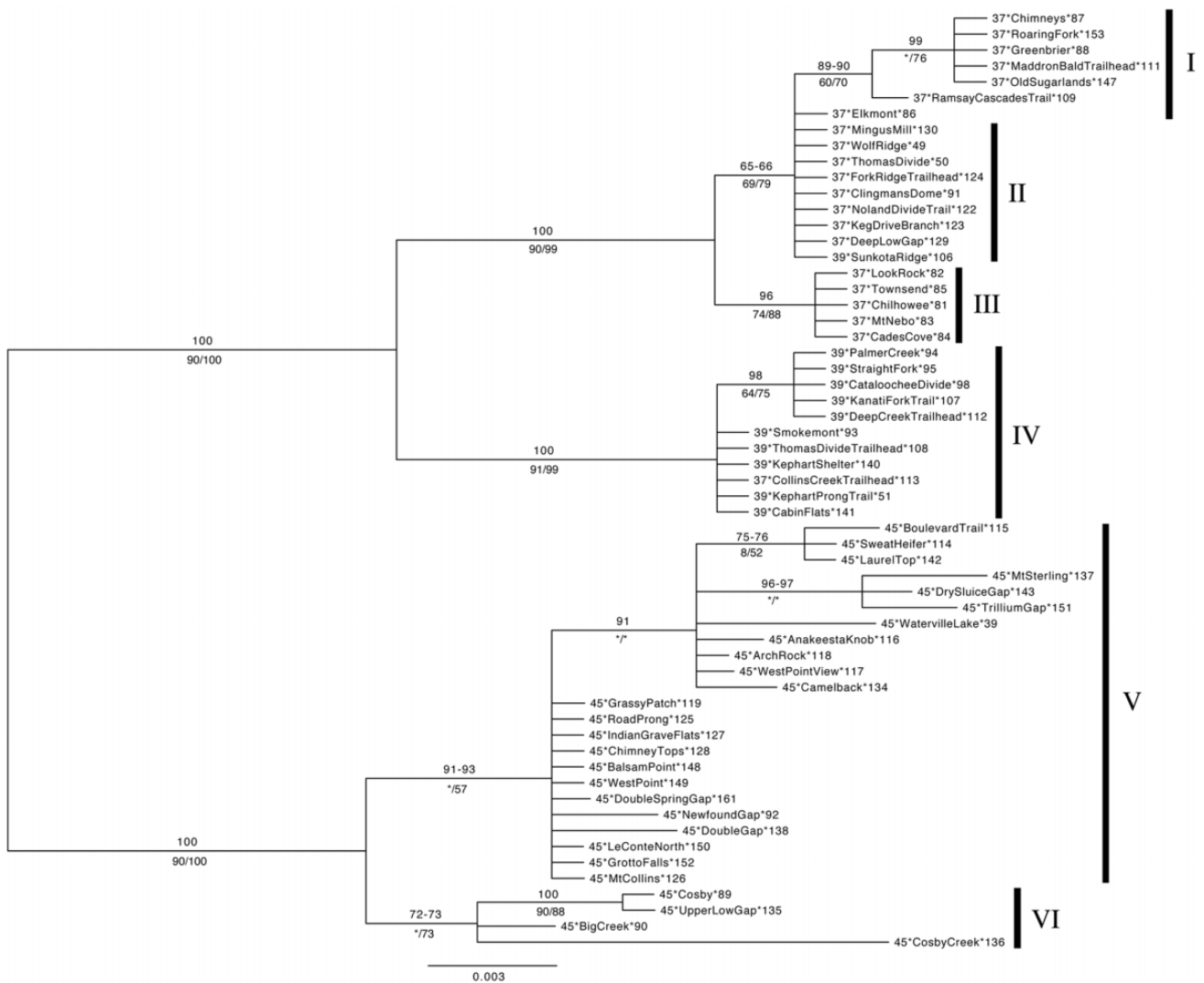


Fig. 2. Phylogenetic relationships of the *Cryptocercus punctulatus* species complex distributed in Great Smoky Mountain National Park based on the nuclear ITS2 sequences (424 bp including gaps). The topology and branch lengths shown were obtained by Bayesian inference method. Note that this tree is unrooted. Posterior probabilities (PP) are shown above branches to indicate the level of support for each node. Only one number is given if PP was identical at that node in the two different runs. Numbers below each branch indicate the percentage of bootstrap support (1,000 replicates) in the maximum likelihood (ML) and maximum parsimony (MP) methods, respectively. An asterisk indicates a node that was not supported by ML and MP methods. Clades labels I–VI correspond to those in Figures 1 and 3b.

(Collins Creek Trailhead), which had a chromosome count of $2n = 37$, but grouped with the $2n = 39$ clade in both trees. In this location all three chromosome preparations were suitable for chromosome counts, and were consistently $2n = 37$.

Distributions

The $2n = 45$ karyotype is found almost exclusively in the Tennessee half of the park and along the ridgeline separating Tennessee from North Carolina (Fig. 3). The four sites with this chromosome number found in North Carolina are all located on the eastern boundary of GSMNP (sites # 39, 90, 137, 138). The karyotype $2n = 39$ forms a fairly cohesive group in the southeastern quadrant except for the intrusion of the karyotype anomaly at site #113. The distribution of the $2n = 37$ group appears to wrap the boundary of the park except in the easternmost quarter; it also may be common in the interior of the largely un-sampled western third of the park. The three distinct clades of the $2n = 37$ karyotype in the COII and ITS2 phylogenetic trees are also geographically distinct (clades I, II and III: Fig. 3b), as

are the two clades of the $2n = 45$ karyotype group (clades V and VI: Fig. 3b). All three karyotypes potentially come into contact in the middle of the park, in the high-elevation area between Newfound Gap and Clingmans Dome.

The present results support the suggestion that the different karyotypes are not separated along altitudinal gradients (Nalepa 2003). The elevation of the $2n = 37$ karyotype in GSMNP ranged from 317 to 1,895 m; $2n = 39$ ranged from 711 to 1,505 m, and $2n = 45$ ranged from 507 to 1,932 m. Each karyotype, then, is represented in the range of elevations found in the park, 300 m to over 1,800 m (Nichols and Langdon 2007).

Estimation of Divergence. Using rates of 0.13–0.30%/Myr and mean pairwise TV distances, clades I–IV (male $2n = 37$ or 39) and V + VI (male $2n = 45$) diverged 8.83–20.38 Myr ago (mean TVs = 2.65%, $n = 864$; Table 2). Among the I–IV groups, divergence events between I and III (male $2n = 37$) and IV (male $2n = 39$), and I + II and III (within male $2n = 37$) occurred almost at the same time (2.29–5.29 Myr ago, mean TVs = 0.69%, $n = 231$ and

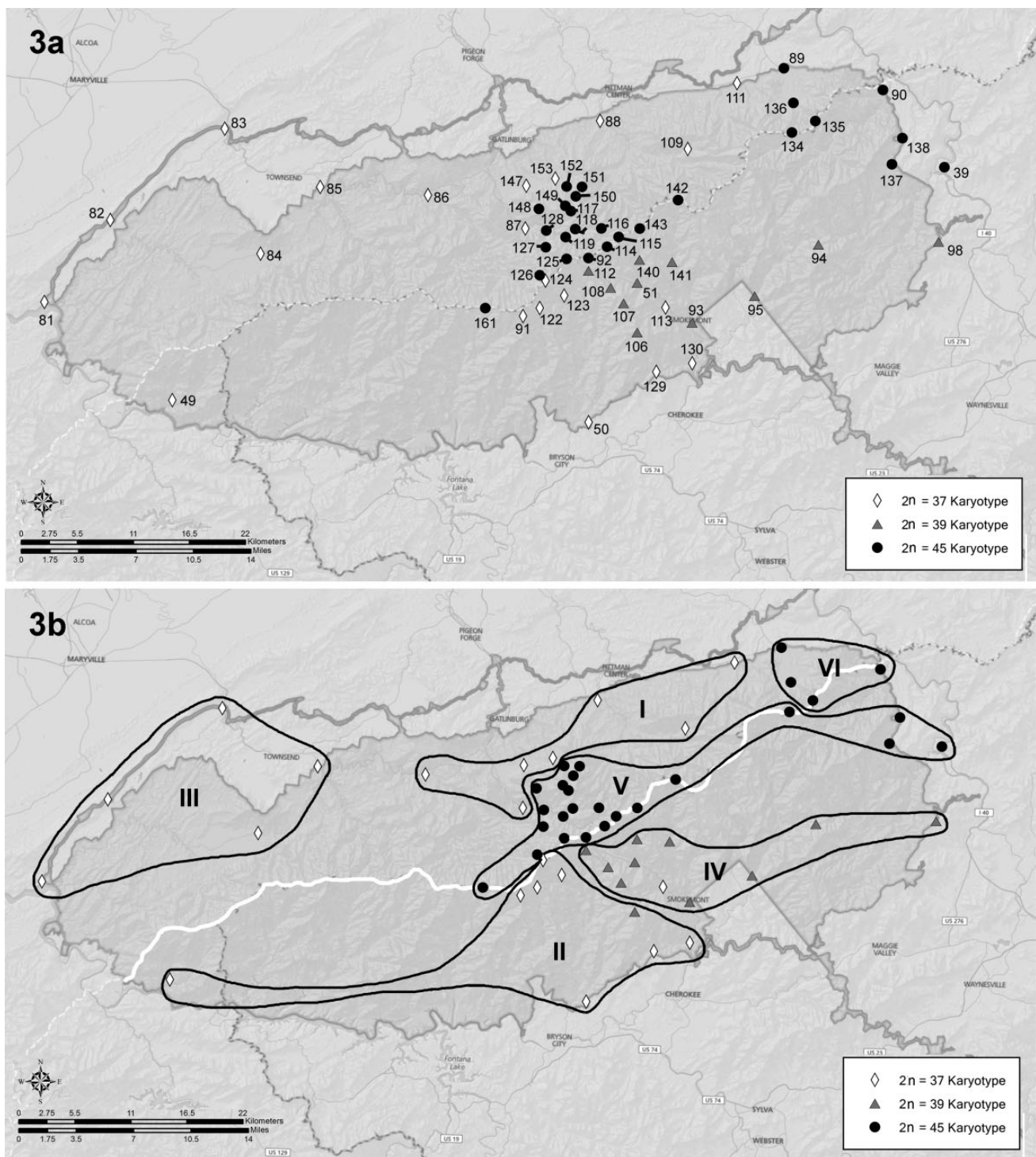


Fig. 3. Map of karyotype groups of the *Cryptocercus punctulatus* species complex in Great Smoky Mountains National Park, North Carolina and Tennessee, USA. (a) Location of sample collections; site numbers correspond to those listed in Table 1. (b) Geographic distribution of clades I–VI as indicated in Figures 1 and 2. The white line indicates the ridgeline between North Carolina and Tennessee.

80, respectively). On the other hand, I and II (within male $2n = 37$) diverged relatively recently (1.29–2.97 Myr ago, mean TVs = 0.39%, $n = 63$), and divergence events between V and VI (within male $2n = 45$) occurred 0.46–1.06 Myr ago (mean TVs = 0.14%, $n = 92$).

PCA

PCA was performed using the chromosome numbers ($2n = 37, 39, 45$), the phylogenetic groups obtained (I–VI shown in Figs. 1 and 2)

and the sampling locations (latitude, longitude and elevation shown in Table 1). The first principal component accounted for 51.25% of the total variance, and positively reflected chromosome numbers and phylogenetic groups (eigenvector: 0.54 and 0.55, respectively). Within sampling locations, elevation had a higher eigenvector (0.31) than latitude (-0.02) or longitude (-0.07). We then compared elevation difference and genetic distance (using COII sequence divergence based on the T92 + G model) between two locations within each

Table 2. Estimated divergence times between each clade inferred from COII gene sequences (Figs. 1, 3b)

Comparison	Mean TVs (%)	0.3%/Myr	0.13%/Myr
I vs. II ($n = 63$)	0.386	1.287	2.969
I + II vs. III ($n = 80$)	0.687	2.290	5.284
I + II + III vs. IV ($n = 231$)	0.687	2.290	5.285
V vs. VI ($n = 92$)	0.138	0.460	1.061
I + II + III + IV vs. V + IV ($n = 864$)	2.649	8.831	20.380

TV = transversion distances.

phylogenetic clade (I–VI). The results indicate that genetic distance was significantly correlated with elevation within two clades: clade II ($2n = 37$) and clade V ($2n = 45$; Fig. 4).

Discussion

Incongruence Between Karyotype and Molecular Analyses

The karyotype and molecular analyses were largely congruent, with two exceptions. Cockroaches from site #106 (Sunkota Ridge) had a $2n = 39$ chromosome count but a molecular profile of the $2n = 37$ group, and site #113 (Collins Creek Trailhead) was the opposite: $2n = 37$, but it grouped with the $2n = 39$ clade in the COII and ITS2 trees. Both sites are in the central region of the North Carolina side of the park, where populations of the two karyotype groups are in close contact and may interdigitate or overlap. There is therefore the possibility of sampling error, because in both cases the chromosome counts and the molecular analyses were done on different individuals. Karyotype groupings are suspected to meet or overlap on very fine spatial scales; for example, individuals of the $2n = 39$ and $2n = 43$ karyotypes (inferred, however, from mtDNA sequencing data) have been reported from the same log in the southeastern corner of Tennessee (Garrick 2016), although that location is nowhere near the currently known distribution of the $2n = 43$ group. The closest to that Tennessee site that a $2n = 43$ karyotype (based on actual chromosome counts) is known to occur is 230 km northeast in Yancey Co., NC, and 280 km east-northeast in Burke Co., NC (Nalepa et al. 2002). Further work is required to determine if the Tennessee finding represents an isolated, disjunct population of that karyotype. The $2n = 43$ karyotype was not found in GSMNP during this study.

Alternative explanations to possible sampling error are that the karyotype discrepancies in this study—one $2n = 39$ individual in a $2n = 37$ clade, and a $2n = 37$ individual in a $2n = 39$ clade—might be explained either as the result of occasional Robertsonian chromosomal rearrangements (of the same kind that produced the karyotypic population-differences in the first place) or, more likely, as first- or second-generation hybrids between different chromosomal populations. As noted, both of these “tree-discrepant karyotypes” were found in locations that could be boundary- or overlap-regions between the different karyotypes. Ongoing studies on *Cryptocercus* have shown that crosses between different karyotypes sometimes produce viable offspring (Nalepa, Maekawa and Luykx, in prep.). There is evidence for at least partial reproductive compatibility between naturally occurring karyotypic variants in other insects, as well as in fish and mammals (Capanna et al. 1976, Luykx and Syren 1981, Herzog and Harrington 1991, Castiglia and Capanna 1999, Choochote et al. 2002, Tanuja et al. 2003, Pazza et al. 2006, Horn et al. 2012).

Taxonomic subdivision of cockroaches has been indicated by cuticular hydrocarbons (Brown et al. 1997, Everaerts et al. 2008), but in the case of *Cryptocercus* the significance of different cuticular

hydrocarbon profiles is not yet clear. Although there are disparities between hydrocarbon profile and chromosome number in other parts of the range, in the current study they are concordant in the sites in which hydrocarbon profiles are known (Everaerts et al. 2008). In four sites of $2n = 45$ that have been analyzed for cuticular hydrocarbons (#114, 126, 134, 142), all fall into the same hydrocarbon group (HcG IV), as do those of the $2n = 37$ karyotype (#122, 123, 129; HcG II). The one site of the $2n = 39$ karyotype group that was analyzed (#141) fell into the HcG V group. Analysis of cuticular hydrocarbons of insects from the two sites with karyotype-molecular disparities (#106 and 113) would be informative.

That genetically different clades may have the same karyotype suggests that DNA-sequence changes do not take as long to become established as do karyotype changes (e.g., clades V and VI both have $2n = 45$). This may be because the first-generation progeny of an individual with the initial karyotype change would be karyotypic heterozygotes, and may suffer a loss of fertility as a result of irregularities in meiosis. Only when two such heterozygotes interbred, producing new karyotypic homozygotes among their progeny, would normal meiotic pairing and normal fertility be restored in a new sub-population. Changes in DNA sequences, on the other hand, may be neutral in their consequences (ITS2 sequences are not translated into protein), or possibly even have some selective advantage in the heterozygous condition (e.g., COII sequences could affect energy metabolism and therefore have relevance for adaptations to temperature during glacial versus interglacial periods).

Geographic Distribution and Biogeography of Karyotypes. The results indicate that the different karyotypes are currently geographically adjacent, with fairly abrupt transitions in their natural contact zones and no apparent geographic barriers between them. Interactions between cockroaches of different karyotypes are therefore inevitable. Lineage boundaries may be maintained by general reproductive incompatibility between karyotype groups; however, different hydrocarbon groups of the same karyotype (see Everaerts et al. 2008), and widely separated populations or clades within the same karyotype also may be infertile. The analysis of Everaerts et al. (2008), for example, indicates the $2n = 43$ karyotype group is not a monophyletic group, based on mtDNA, nuclear DNA, and cuticular hydrocarbons.

An open question is whether these geographic distributions are currently stable or are moving as one group invades areas previously occupied by another. It is relevant in this regard that in their broader range, the distribution of $2n = 39$ group is split by a population of $2n = 37$. It is the northern population of the $2n = 39$ karyotype group that is in GSMNP; another population is known from the extreme southwest corner of North Carolina extending into northern Georgia (Nalepa et al. 2002; Fig. 2). These two populations are separated by a swath of the $2n = 37$ karyotype group that is contiguous with sites #49 and #50 reported here.

We can only speculate on the relative importance of the multiple biotic, abiotic and historical factors that created the present day geographic pattern, as it is the result of the complex interaction of intrinsic, lineage-specific traits and the exogenous factors that affect their host logs on both an ecological and evolutionary scale. It has long been thought that the distribution of the genus *Cryptocercus* is linked to its limited capacity for dispersal (Mamaev 1973), a feature associated with regional endemism, conservation of patterns of genetic variation, easily fragmented distributions, and the tendency to form parapatric boundaries (Bull 1991, Cruzan and Templeton 2000, Yeates et al. 2002, Grove 2002). The genus is wingless, so movement of adults and large nymphs is limited by how far they can walk (Nalepa and Grayson

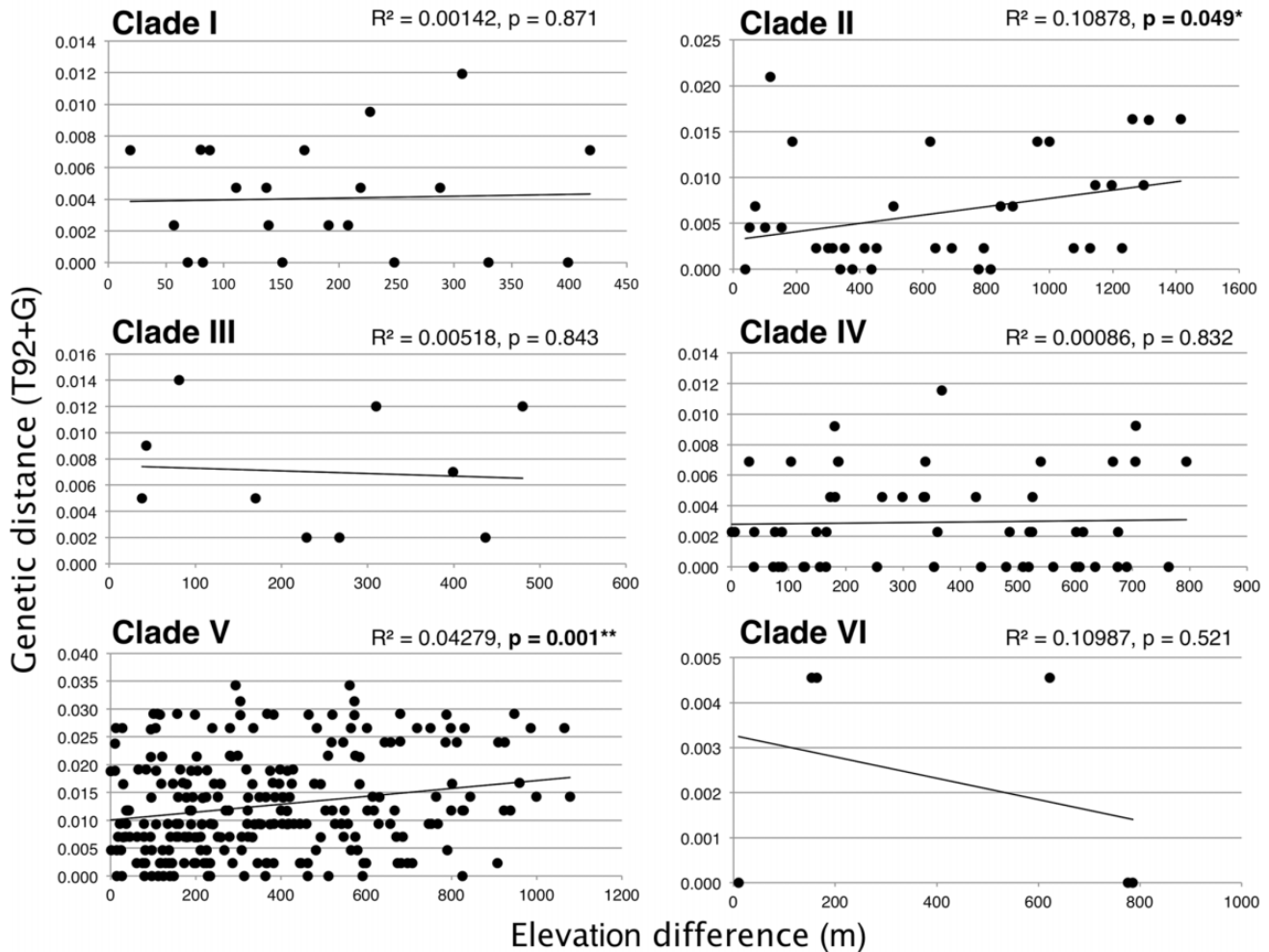


Fig. 4. Genetic distance as a function of elevational differences within each clade indicated in **Figures 1, 2 and 3b**. Genetic distance was calculated from COII sequence divergence based on the T92 + G model used in the phylogenetic analysis. The square of Pearson's correlation coefficient (R^2) and P values ($^* < 0.05$ and $^{**} < 0.01$) are shown at the upper right of each graph.

2011). The distance they range is unknown, but most adults within a population outbreed (Yaguchi et al. 2016). There is no evidence that cockroaches of any one karyotype have a better dispersal capacity than others. Another relevant feature of the insect is its low population growth linked to semelparity and an unusually long generation time for an insect. The time from hatch to hatch in the $2n=43$ population at Mountain Lake Biological Station, Virginia, is 5–6 years (Nalepa et al. 1997: Table 2); consequently, there have been only 13 or 14 generations since GSMNP became a national park in 1934.

The survival, reproduction and development of *Cryptocercus* depends on the presence of moist, rotted logs, with the log host-range determined by the tree species composition of the inhabited forest (Cleveland et al. 1934, Nalepa and Bandi 1999, Nalepa 2003). The cockroach is found in CWD on the floor of all major forest types in GSMNP, and current evidence suggests that cockroaches with different karyotypes are ecologically interchangeable. This lack of host-plant specificity has strong implications for the distribution of the insect in both ecological and evolutionary time. Specifically, within its range the location of the insect is determined by the presence of forest and the dynamics of CWD deposition, accumulation and depreciation; the vegetational composition of the forests is of little relevance.

In undisturbed, mature forests of GSMNP the cockroaches likely find what they need within a modest ambit, as there is a relative stability of tree mortality and deposition of CWD to the forest floor in old growth temperate forests (Davis et al. 2015). Furthermore, accumulation of CWD in GSMNP is high for the region, particularly in cove forests; this is attributed to large bole size of canopy trees and their tendency to suffer higher mortality (Busing 2005 and refs. therein). Mature forests, however, tend to be patchworks in varying stages of succession due to both natural and anthropogenic disturbances (Harmon et al. 1986). Insects, disease, fire, wind throw, and ice storms can devastate entire stands and result in pulses of CWD that serve as habitat for *Cryptocercus*. In recent history, exotic pest species have been particularly significant in altering forests and CWD in the park (Davis et al. 2015, Tuttle and White 2016). The American chestnut [*Castanea dentata* (Marsh.) Borkh.], for example, covered a full 31% of the Smokies in the early 1900s, grew to mammoth sizes (Brown 2000), and served as important habitat for *Cryptocercus* (Cleveland et al. 1934, Hebard 1945). When that species was decimated by chestnut blight [*Cryphonectria parasitica* (Murrill) Barr.], there was a shift in trees that dominated the canopy (Elliot and Swank 2008), and *Cryptocercus* had no problem in moving into logs of alternative species on the forest floor (Nalepa 2003). A more recent example is the death of Fraser firs [*Abies fraseri*

(Pursh) Poir] from an infestation of balsam woolly adelgid [*Adelges piceae* (Ratzeburg)]; this has resulted in estimates of 70–90% mortality in GSMNP, with a magnitude of CWD among the highest reported in the literature for the eastern US (Houk 1993, Smith and Nicholas 1998, Rose and Nicholas 2008). Similar cases include the death of hemlock from hemlock woolly adelgid (*Adelges tsugae* Annand; Krapfl et al. 2011), and the near simultaneous death of pine stands across GSMNP between 1999 and 2003 from southern pine beetle (*Dendroctonus frontalis* Zimmerman; Webster and Jenkins 2005). Biological disturbances such as these provide bonanzas of host material for the cockroach, suggesting that local populations would take advantage of the surfeit of woody material and remain in a given area as long as other tree species eventually filled gaps in the canopy, allowing the abundant CWD to remain suitably moist. *Cryptocercus* can cross gaps in standing vegetation as long as some large logs remain on the substrate in treeless areas (see Fig. 10.5 in Bell et al. 2007).

Anthropogenic disturbances, on the other hand, typically involve the removal and destruction of CWD. The Great Smoky Mountains were clear-cut, burned, mined, settled, farmed, and grazed for more than a century before the area was established as a national park; much of the original forest cover was lost because of human activity (Pyle 1985, Houk 1993, Pierce 2000, Brown 2000, Linzey 2008). Although the park still contains a large tract of primary forest, ~80% is second growth recovering from logging and settlement, particularly in its western half (Jenkins 2007, Tuttle and White 2016). Some sites remain without continuous forest cover >90 years after logging, and large diameter CWD is least common in forests with a history of concentrated settlement (Webster and Jenkins 2005, Tuttle and White 2016). In this study, the central regions of the park where the three karyotype groups of *C. punctulatus* meet are in or near areas that were not logged intensively and are therefore high in virgin-forest attributes (Pyle 1985).

On an evolutionary scale, the divergence of different karyotype groups of the *C. punctulatus* species complex probably occurred prior to the Pleistocene glaciations, making it difficult to infer the sequence of events leading to their present distribution. The most recent estimate of the genetic structuring of the Appalachian populations of *Cryptocercus* is at ~18 mya, with the $2n = 37$ and 39 karyotypes diverging ~10 mya (Che et al. 2016). There have been an estimated 18–20 glaciations in the last two million years (Constantz 2004), and with each successive glacial cycle the species complex has apparently undergone range contraction and isolation, followed by expansion and secondary contact, potentially overriding or erasing previous distributional patterns. The current pattern suggests that during the last glacial maximum, when high elevations in the park were above treeline (King and Stupka 1950, Whittaker 1956, Watts 1980, Wright 1987, Delcourt and Delcourt 2000), clades within two karyotype groups examined in this study (I and II: $2n = 37$, and V and VI: $2n = 45$) may have harbored in separate refugia in ravines or slope habitats adjacent to river valleys on opposite sides of the ridgeline in central GSMNP. As the climate subsequently warmed, the cockroaches followed the timberline back up to the main divide separating North Carolina from Tennessee, which stands >1,524 m above sea level for 58 km (King and Stupka 1950). Advancing fronts of closely related taxa are expected to meet midway between former refugia (Endler 1982), and the timing of divergence of clades I versus II and V versus VI is consistent with divergence during the Pleistocene (Table 2). The significant correlation of genetic distance with elevation difference within clades II and V further supports the suggestion that lower elevation sites in GSMNP served as refugia or microrefugia during the ice ages (Rull 2009).

There would have been a time lag before *Cryptocercus* could repopulate high elevation sites when the climate began to warm, as there is a disequilibrium between climate change and forest expansion with the latter notably delayed because vegetation zone margins migrate only when seeds are blown into the treeless areas (Pielou 2008). Additional time would lapse before the migrating forests reached a successional stage where CWD suitable for *Cryptocercus* would be present; some ecologists estimate that it takes 200–1,000 years for a forest to achieve climax in the Appalachians (Constantz 2004). Recolonization of the cockroaches into a tree-decimated area is therefore a prolonged downstream effect of climate and topography in allowing reforestation and successional climax in a region previously denuded of vegetation.

In the opposite scenario, the extinction or exodus of the cockroaches from areas where trees have declined because of a severely cooling climate also would be associated with a time lag. In this case, however, the lag is associated with the pace of accumulation and then degeneration of CWD: a boom in resources as a large number of trees died, then a bust as the logs cycled through the decomposition process. Log residence has been estimated at 46–124 years, depending on a variety of factors (Davis et al. 2015 and refs. therein), one factor undoubtedly being *Cryptocercus* itself. Large-bodied saproxylic insects such as *Cryptocercus* accelerate log decomposition by channeling and fragmenting dead wood, and via their biotic interactions with microbes (Hanula 1993, Ulyshen 2016). It has been suggested that *Cryptocercus* may pulverize logs on a time scale exceeding that of termites (Bell et al. 2007: Fig 10.1).

The *C. punctulatus* species complex dominates the saproxylic guild in GSMNP and occupies the same niche as does the subterranean termite *Reticulitermes* spp. at lower elevations (Nalepa et al. 2002). Like termites (Collins 1989), these cockroaches have the potential to influence forest productivity by their effect on soil fertility. *Cryptocercus* produces abundant, large, fecal pellets, and their gut microbiota fix nitrogen at rates comparable to those of termites on a body-weight basis (Breznak et al. 1974, Breznak 1975, Tai et al. 2016), thus providing a mechanism for nitrogen return to the ecosystem (Nardi et al. 2002). In sum, *Cryptocercus* plays a critical role in decomposition of dead wood, contributes to soil formation and nutrient cycling (Speight 1989), and should be considered a long-term stabilizing force in the forest ecosystems of GSMNP.

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