**Supplemental Information**

**Table S1.** List of collection sites of *Reticulitermes* specimens used as a “test panel” when assessing performance of the new PCR-RFLP assay. Spatial coordinates are in decimal degrees, and elevation is reported in meters above sea level. State abbreviations are: Mississippi, MS; Alabama, AL; Georgia, GA; Tennessee, TN; South Carolina, SC; North Carolina, NC; Virginia, VA; and West Virginia, WV. Region abbreviations are: National Forest, NF; National Park, NP; State Forest, SF; State Park, SP; and Wildlife Management Area, WMA.

| Site No. | Latitude  | Longitude  | Elevation | State | Region               |
|----------|-----------|------------|-----------|-------|----------------------|
| 1        | 38.82585  | −79.38506  | 548       | WV    | Monongahela NF       |
| 2        | 38.82374  | −79.38618  | 528       | WV    | Monongahela NF       |
| 3        | 38.80508  | −78.18149  | 755       | VA    | Shenandoah NP        |
| 4        | 38.72694  | −79.48494  | 936       | WV    | Monongahela NF       |
| 5        | 38.62592  | −78.34060  | 1032      | VA    | Shenandoah NP        |
| 6        | 38.29123  | −78.64308  | 761       | VA    | Shenandoah NP        |
| 7        | 38.12902  | −78.78368  | 814       | VA    | Shenandoah NP        |
| 8        | 38.04052  | −79.34980  | 784       | VA    | Washington NF        |
| 9        | 37.34757  | −80.54509  | 1121      | VA    | Jefferson NF         |
| 10       | 36.31100  | −82.07211  | 648       | TN    | Cherokee NF          |
| 11       | 36.13606  | −84.48829  | 496       | TN    | Frozen Head SP       |
| 12       | 36.12452  | −84.74478  | 378       | TN    | Clear Crk            |
| 13       | 35.77140  | −83.21343  | 575       | TN    | Great Smoky Mtn NP   |
| 14       | 35.70232  | −83.35717  | 653       | TN    | Great Smoky Mtn NP   |
| 15       | 35.65682  | −83.51849  | 780       | TN    | Great Smoky Mtn NP   |
| 16       | 35.61933  | −83.66993  | 593       | TN    | Great Smoky Mtn NP   |
| 17       | 35.59535  | −82.48742  | 722       | NC    | Blue Ridge Pkwy      |
| 18       | 35.52117  | −83.31077  | 666       | TN    | Great Smoky Mtn NP   |
| 19       | 35.39384  | −87.52677  | 304       | TN    | Natchez Trace Pkwy   |
| 20       | 35.34883  | −84.24760  | 327       | TN    | Cherokee NF          |
| 21       | 35.34534  | −84.19383  | 425       | TN    | Cherokee NF          |
| 22       | 35.32969  | −83.59187  | 593       | NC    | Nantahala NF         |
| 23       | 35.10896  | −84.62477  | 530       | TN    | Cherokee NF          |
| 24       | 35.01376  | −83.05563  | 887       | SC    | Sumter NF            |
| 25       | 34.94523  | −83.08929  | 744       | SC    | Sumter NF            |
| 26       | 34.93135  | −84.65486  | 485       | GA    | Chattahoochee NF     |
| 27       | 34.87866  | −84.71137  | 354       | GA    | Chattahoochee NF     |
| 28       | 34.86200  | −83.10755  | 536       | SC    | Oconee SP            |
| 29       | 34.84695  | −85.49971  | 315       | GA    | Cloudland Canyon SP  |
| 30       | 34.77972  | −84.63805  | 764       | GA    | Chattahoochee NF     |
| 31       | 34.77755  | −83.31242  | 469       | SC    | Sumter NF            |
| 32       | 34.77507  | −84.33880  | 730       | GA    | Chattahoochee NF     |
| 33       | 34.75931  | −84.69140  | 804       | GA    | Chattahoochee NF     |
| 34       | 34.74192  | −83.73265  | 766       | GA    | Chattahoochee NF     |
| 35       | 34.72782  | −83.22783  | 394       | SC    | Sumter NF            |
| 36       | 34.68311  | −84.25093  | 810       | GA    | Chattahoochee NF     |
| 37       | 34.64336  | −85.21630  | 386       | GA    | Johns Mtn WMA        |
Table S1. Cont.

| Site No. | Latitude       | Longitude     | Elevation | State | Region               |
|---------|----------------|---------------|-----------|-------|----------------------|
| 38      | 34.60502       | −88.19299     | 177       | MS    | Tishomingo SP        |
| 39      | 34.57297       | −85.06536     | 450       | GA    | Johns Mtn WMA        |
| 40      | 34.56515       | −85.24268     | 408       | GA    | Johns Mtn WMA        |
| 41      | 34.56416       | −85.24043     | 427       | GA    | Johns Mtn WMA        |
| 42      | 34.54107       | −85.25067     | 341       | GA    | Johns Mtn WMA        |
| 43      | 34.45540       | −85.58357     | 395       | AL    | Little River Canyon  |
| 44      | 34.41979       | −87.33273     | 321       | AL    | Bankhead NF          |
| 45      | 34.17659       | −87.27680     | 248       | AL    | Bankhead NF          |
| 46      | 34.14676       | −85.84679     | 188       | AL    | Shinbone Ridge       |
| 47      | 34.12260       | −85.26428     | 232       | GA    | Lyons Ridge          |
| 48      | 33.96340       | −85.45730     | 300       | AL    | Talladega NF         |
| 49      | 33.91858       | −85.49764     | 257       | AL    | Talladega NF         |
| 50      | 33.57157       | −85.69391     | 328       | AL    | Talladega NF         |
| 51      | 33.56059       | −85.70074     | 425       | AL    | Talladega NF         |
| 52      | 33.47105       | −85.80658     | 621       | AL    | Talladega NF         |
| 53      | 33.46215       | −85.81731     | 485       | AL    | Talladega NF         |
| 54      | 33.40451       | −85.87318     | 460       | AL    | Talladega NF         |
| 55      | 33.20150       | −86.07201     | 291       | AL    | Talladega NF         |

Table S2. List of GenBank accessions of mtCOII used for initial design of a PCR-RFLP assay for identification of eastern United States Reticulitermes termite species.

| Species     | State | GenBank accession(s)                      | Original reference |
|-------------|-------|-------------------------------------------|--------------------|
| *R. flavipes* | DE    | JQ280721–JQ280727                          | [1]                |
|             | FL    | AF525321                                    | [2]                |
|             | FL    | AY808077, AY808082–AY808086                  | [3]                |
|             | FL    | JQ280662–JQ280694, JQ280745–JQ280746        | [1]                |
|             | GA    | AF107479–AF107482, AF107484                  | [4]                |
|             | GA    | AY027477                                    | [5]                |
|             | GA    | EF206316–EF206317                           | [6]                |
|             | GA    | JQ280701                                    | [7]                |
|             | IN    | AY168203, AY168210–AY168211                  | [1]                |
|             | IN    | JQ280705                                    | [8]                |
|             | MA    | DQ493725                                    | [1]                |
|             | MS    | JQ280622–JQ280626, JQ280741–JQ280744        | [8]                |
|             | NC    | DQ493728                                    | [1]                |
|             | NC    | JQ280716–JQ280720                           | [1]                |
|             | OH    | JQ280702                                    | [1]                |
|             | SC    | JQ280711–JQ280715                           | [1]                |
|             | TN    | JQ280703                                    | [1]                |
|             | VA    | JQ280728–JQ280736                           | [1]                |
|             | GA    | JF796216                                    | [9]                |
| Species            | State | GenBank accession(s) | Original reference |
|--------------------|-------|----------------------|--------------------|
| *R. flavipes*      | GA    | JF796217             | [9]                |
|                    | ?     | JF796218             | [9]                |
|                    | FL    | JF796220             | [9]                |
|                    | GA    | JN207492             | [9]                |
| *R. hageni*        | GA    | AF107486             | [4]                |
|                    | GA    | AY027478             | [5]                |
|                    | GA    | AF525328             | [2]                |
|                    | GA    | EU689026             | [10]               |
|                    | NC    | DQ493729             | [8]                |
|                    | GA    | JF796224             | [9]                |
|                    | GA    | JF796225             | [9]                |
| *R. malletei*      | GA    | GU550074             | [10]               |
|                    | GA    | JF796226             | [9]                |
|                    | GA    | JF796227             | [9]                |
|                    | NC    | JF796228             | [9]                |
| *R. nelsonae*      | GA    | EU689013             | [9]                |
|                    | NC    | JF796229             | [9]                |
|                    | GA    | JF796230             | [9]                |
|                    | FL    | JF796231             | [9]                |
|                    | GA    | JF796232             | [9]                |
|                    | FL    | JF796233             | [9]                |
|                    | GA    | JF796235             | [9]                |
|                    | GA    | JF796236             | [9]                |
| *R. virginicus*    | FL    | AF525356             | [2]                |
|                    | FL    | AY808096             | [3]                |
|                    | FL    | AY808098             | [3]                |
|                    | FL    | EF206319             | [6]                |
|                    | GA    | AF107487             | [4]                |
|                    | GA    | AY027479             | [5]                |
|                    | GA    | EF206318             | [6]                |
|                    | GA    | EU689027             | [10]               |
|                    | NC    | DQ493743             | [8]                |
|                    | VA    | AF525357             | [2]                |
|                    | GA    | JF796221             | [9]                |
|                    | GA    | JF796222             | [9]                |
|                    | GA    | JF796223             | [9]                |
|                    | FL    | JF796234             | [9]                |
Table S3. Consensus mtCOII sequences for each five *Reticulitermes* termite species, showing polymorphic nucleotide positions within a 658-bp alignment. IUPAC ambiguity codes reflect intraspecific variability, based on the GenBank accessions from which consensus sequences were generated (Table S2). Polymorphisms that could potentially be used for species identification are color-coded according to whether only two alternative nucleotide states exist (red = one state is unique to a particular species, or green = one state is characteristic of a subset of species) vs. three alternative nucleotide states (blue). In each case, underlined nucleotides indicate potentially diagnostic character(s). The ✓ and ✗ symbols indicate whether DNA sequence polymorphisms do or do not generate restriction site polymorphisms, respectively. Dark grey shading indicates locations of PCR primers that we designed (*i.e.*, RetCo2-F and RetCo2-R) to amplify the most information-rich region (pale grey).

| Position (bp) | 6 | 12 | 18 | 19 | 22 | 23 | 24 | 27 | 28 | 32 | 36 | 39 | 42 | 58 | 60 | 72 | 75 | 78 | 82 | 90 | 93 | 105 | 107 | 111 | 114 | 117 | 123 | 129 |
|--------------|---|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|
| R. flavipes  | R | A | H | M | A | S | R | W | C | R | A | Y | Y | R | Y | Y | T | H | Y | R | T | Y | Y | C | ✓ | ✓ |
| R. hageni    | A | A | C | A | A | G | A | A | C | A | A | A | T | C | G | Y | C | Y | T | C | A | Y | C | C | C | A | R | A | T |
| R. malletei  | A | R | C | A | A | G | A | A | C | A | R | G | T | C | G | T | C | T | T | Y | A | C | C | C | C | R | R | A | C |
| R. nelsonae  | A | A | Y | A | G | A | A | C | A | W | A | Y | C | R | C | C | Y | T | T | A | C | C | C | C | A | R | A | Y |
| R. virginicus| A | A | C | A | R | G | A | W | S | A | A | A | C | C | R | Y | C | T | T | Y | A | T | Y | C | Y | A | G | A | C |
| Cut site     | ✓ | ✓ |
|              | 135 | 137 | 138 | 141 | 144 | 147 | 150 | 152 | 153 | 162 | 163 | 165 | 166 | 171 | 177 | 178 | 186 | 189 | 195 | 201 | 202 | 207 | 213 | 217 | 219 | 228 | 230 | 231 | 234 |
| R. flavipes  | R | Y | T | R | T | A | Y | Y | Y | R | C | C | R | A | H | A | Y | R | T | R | A | Y | Y | R | ✓ | T | C | A | A |
| R. hageni    | A | T | Y | R | T | A | C | C | T | T | A | T | T | A | R | C | R | Y | A | T | G | A | C | C | A | T | C | A | A |
| R. malletei  | A | T | T | A | T | A | C | C | C | C | C | A | T | A | A | M | A | C | A | T | G | A | C | C | A | T | C | G | A |
| R. nelsonae  | A | T | T | A | Y | R | Y | C | T | Y | A | Y | Y | A | R | Y | R | C | A | Y | G | A | C | C | A | Y | C | R | R |
| R. virginicus| A | T | T | A | T | R | C | C | T | T | A | T | T | A | A | C | A | Y | A | T | G | R | C | C | A | T | S | R | A |
| Cut site     | ✓ |
|              | 240 | 249 | 250 | 252 | 255 | 256 | 258 | 264 | 268 | 270 | 273 | 279 | 280 | 288 | 289 | 291 | 294 | 297 | 300 | 303 | 306 | 309 | 312 | 318 | 323 | 324 | 327 | 330 | 333 |
| R. flavipes  | Y | Y | C | R | Y | Y | A | Y | A | Y | Y | A | A | Y | C | R | R | D | D | R | Y | R | A | A | D | T | A | Y | M |
| R. hageni    | C | C | C | A | Y | C | A | C | A | C | C | R | A | T | C | A | A | A | A | A | A | A | C | A | ✓ | R | A | T | A | Y | A |
| R. malletei  | C | T | C | A | T | C | A | C | A | C | A | A | C | A | A | A | A | A | A | A | C | A | ✓ | G | A | T | A | C | A |
| R. nelsonae  | C | T | Y | A | T | C | A | C | W | Y | Y | A | R | C | Y | A | A | A | A | A | C | R | A | A | A | T | R | T | A |
| R. virginicus| Y | T | C | A | T | C | W | C | A | C | Y | A | A | C | C | A | A | A | R | A | C | A | ✓ | R | A | Y | A | C | A |
| Cut site     | ✓ |
| Position (bp) | 339 | 348 | 354 | 360 | 363 | 369 | 372 | 375 | 381 | 382 | 388 | 389 | 394 | 396 | 399 | 406 | 408 | 417 | 420 | 421 | 426 | 430 | 432 | 441 | 444 | 450 | 454 | 456 | 458 |
|--------------|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| R. flavipes  | Y A Y Y Y C R R W W S C R Y Y Y A Y T C T H A Y H Y C R Y | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| R. hageni    | C A T T C T A R A A A A C A C C Y A C C C Y C A C A C Y A T | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| R. malletei  | C A T T C T A A A A A G C A C C T A C C C T C A T A C T A T | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| R. nelsonae  | C R T T C T R A A A A R C C C C T R Y C C T C A Y A Y Y A T | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| R. virginicus| C A T T C T A R A A A A Y A C C C A C C Y S T C S T A C T A T | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| Cut site     | ✓   | ✓   | ✓   | ✓   | ✓   | ✓   | ✓   | ✓   | ✓   | ✓   | ✓   | ✓   | ✓   | ✓   | ✓   | ✓   | ✓   | ✓   | ✓   | ✓   | ✓   | ✓   | ✓   | ✓   | ✓   | ✓   | ✓   | ✓   | ✓   | ✓   | ✓   |

Tale S3. Cont.
Table S4. Original interpretive framework for identifying eastern United States *Reticulitermes* termite species using PCR-RFLP applied to the mtCOII gene, based on “training data” from NCBI’s GenBank database. Expected fragment sizes produced by three restriction enzyme digests (performed one-at-a-time) for each of five recognized taxa are represented by check marks. Following application to a “test panel” of Southern Appalachian samples, the interpretive framework was refined to accommodate new polymorphisms (see Table 1 in the main text).

| Restriction Enzyme | Fragment Sizes (bp) | *R. flavipes* | *R. hageni* | *R. malleoti* | *R. nelsonae* | *R. virginicus* |
|--------------------|---------------------|---------------|-------------|---------------|---------------|---------------|
| *Rsa I*            | 175, 201            | ✓             | ✓           | ✓             | ✓             | ✓             |
|                    | 48, 127, 201        | ✓             | ✓           | ✓             | ✓             | x             |
|                    | 86, 115, 175        | x             | ✓           | x             | x             | x             |
| *Taq I*            | 376                 | x             | ✓           | ✓             | ✓             | ✓             |
|                    | 153, 223            | x             | x           | x             | ✓             | x             |
|                    | 183, 193            | x             | x           | x             | x             | x             |
|                    | 67, 126, 183        | ✓             | x           | x             | x             | x             |
|                    | 30, 67, 126, 153    | ✓             | x           | x             | x             | x             |
| *Msp I*            | 376                 | ✓             | x           | x             | x             | ✓             |
|                    | 37, 339 *           | ✓             | x           | ✓             | x             | ✓             |
|                    | 77, 299             | x             | ✓           | x             | ✓             | x             |
|                    | 37, 40, 299         | x             | x           | ✓             | x             | x             |

* For *R. flavipes*, fragment sizes may instead be 38-bp and 338-bp, but since the 1-bp differences compared to those reported in the table are indistinguishable, only the shorter fragments are listed.
Figure S1. Phylogenetic relationships among *Reticulitermes* mtCOII sequences from 55 new and five reference specimens (n = 37 non-redundant haplotypes, 607-bp each), estimated using Maximum Likelihood and the HKY+I model of nucleotide substitution. Shown here is the tree with the highest log likelihood. Numbers above nodes are bootstrap support values. Open circles on nodes mark the most inclusive well-support clades that contain only one reference taxon (the same groups were recovered using Maximum Parsimony; see Figure 2 of the main text).
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