Revista Brasileira de Entomologia
http://dx.doi.org/10.1590/S0085-56262013005000039

SHORT COMMUNICATION

Exon-primed intron-crossing (EPIC) markers as a tool for ant phylogeography

Patrícia R. Ströher[1], Chenhong Li[2] & Marcio R. Pie[1]

[1] Laboratório de Dinâmica Evolutiva e Sistemas Complexos, Departamento de Zoologia, Universidade Federal do Paraná, 81531–990 Curitiba-PR, Brazil. patrícia.stroher@gmail.com; pie@ufpr.br
[2] College of Fisheries and Life Science, Shanghai Ocean University, 999 Hucheng Huan Road, Shanghai 201306, China.

ABSTRACT. Exon-primed intron-crossing (EPIC) markers as a tool for ant phylogeography. Due to their local abundance, diversity of adaptations and worldwide distribution, ants are a classic example of adaptive radiation. Despite this evolutionary and ecological importance, phylogeographical studies on ants have relied largely on mitochondrial markers. In this study we design and test exon-primed intron-crossing (EPIC) markers, which can be widely used to uncover ant intraspecific variation. Candidate markers were obtained through screening the available ant genomes for unlinked conserved exonic regions interspersed with introns. A subset of 15 markers was tested *in vitro* and showed successful amplification in several phylogenetically distant ant species. These markers represent an important step forward in ant phylogeography and population genetics, allowing for more extensive characterization of variation in ant nuclear DNA without the need to develop species-specific markers.

KEYWORDS. Formicidae; molecular markers; phylogeny; population genetics.

Ants are a dominant component of terrestrial animal communities worldwide (Hölldobler & Wilson 1990). For instance, ants and termites account for nearly one third of the entire animal biomass of the Brazilian Amazon forest (Fittkau & Klinge 1973). Moreover, ant density in some temperate localities can exceed 140 workers per m^2 (Kajak *et al*. 1971). The diversity of life histories in ants is also unparalleled, including solitary foragers, seed harvesters, plant obligate mutualists, fungus-growers, and group predators (Hölldobler & Wilson 1990). As a consequence, measuring levels of ant population differentiation in a given biome should therefore provide a representative picture of what the remaining animal communities in a given region might have experienced during their evolutionary past. Finally, the availability of efficient sampling methods that could uncover dozens of species from a given location in a time-scale of hours (*e. g*. Agosti & Alonso 2000) suggests that ants are among the best available model systems to investigate patterns of animal diversity and distribution of species in terrestrial ecosystems.

Until recently, most of the studies in ant phylogeography were based exclusively on mitochondrial markers (*e. g*. Goropashnaya *et al*. 2007; Solomon *et al*. 2008; Resende *et al*. 2010; Leppänen *et al*. 2011; but see Pringle *et al*. 2012). Such reliance on linked and uniparentally inherited loci might be problematic, given that several mechanisms such as introgression, selective sweeps, and the stochastic nature of the coalescent process might cause a mitochondrial tree not to be representative of the underlying level of population differentiation of a given species (Avise 2000; Wiens *et al*. 2010). More robust inferences on population structure should therefore include additional unlinked loci, leading to the prevalent use of microsatellites (Schlötterer 2004). However, given that microsatellite development is a laborious process that can only be applied to at most a few closely related species, their applicability to ant phylogeography and population genetics has been limited (Goropashnaya *et al*. 2007; Ross *et al*. 2007). A promising alternative method to uncover nuclear genetic variation is the use of exon-primed intron-crossing (EPIC) markers (Lessa 1992; Slade *et al*. 1993; Palumbi 1996), which has been successfully used in a variety of vertebrate taxa (*e. g*. Li *et al*. 2010; Silva-Oliveira *et al*. 2012). In this approach, primers bind to conserved exonic regions flanking an intron, which tends to accumulate mutations at a higher rate than transcribed regions (Graur & Li 2000). The availability of nuclear genomes for phylogenetically distinct ant species, *Harpegnathos saltator* Jerdon, 1851 *Camponotus floridanus* (Buckley, 1866), *Linepithema humile* (Mayr, 1868), *Pogonomyrmex barbatus* (Smith, 1858), *Atta cephalotes* (Linnaeus, 1758) and *Solenopsis invicta* Buren, 1972 (Bonasio *et al*. 2010; Smith *et al*. 2011a; Smith *et al*. 2011b; Suen *et al*. 2011; Würm *et al*. 2011, respectively), provides an ideal situation in which candidate EPIC markers can be selected that could potentially allow for successful amplification in a variety of ant lineages. In this study we use a recently developed pipeline (Li *et al*. 2010) to screen for candidate EPIC markers based on the genomes of six ant

species. Primers were synthesized for a subset of 15 EPIC markers, which were then tested *in vitro*. These markers are ready to be widely used, providing an important tool to increase the quality and accuracy in studies on ant phylogeography, as well as the inference of phylogenetic relationships among closely related ant species.

We screened the complete nuclear genome of six ant species representing a phylogenetically diverse sample of the family: *H. saltator* (Ponerinae), *L. humile* (Dolichoderinae), *C. floridanus* (Formicinae), *S. invicta* (Myrmicinae), *P. barbatus* (Myrmicinae), and *A. cephalotes* (Myrmicinae) to identify conserved exons of single-copy genes. First, we retrieved coding sequences from *H. saltator* using its genome annotation information. We then compared the selected coding sequences of *H. saltator* with the genome of other ant species to identify single-copy and conserved exon among all six species. We screened for EPIC makers that are flanked by two single-copy and conserved exons. After this initial screening, potential markers were further selected based on the level of conservation of the flanking exons (to maximize the chance of cross-species successful amplification) and the size of the intron (450–650 pb, to allow for the amplification of the entire intron in a single reaction), from which 15 candidate markers were chosen for primer design and used for further analyses (Table I).

All primers were tested *in vitro* with the following ant species: *Gnamptogenys striatula* Mayr, 1884 (Ectatomminae), *Hylomyrma reitteri* (Mayr, 1887) (Myrmicinae), *Brachymyrmex* sp. (Formicinae), *Pheidole incisa* Mayr, 1870 (Myrmicinae) and *Linepithema* sp. (Dolichoderinae). We chose those species because they form a phylogenetically diverse sample and they belong to different species from those whose genomes were used in the initial screening. The phylogenetic relationships among all species involved in the present study are shown in Fig. 1. Total genomic DNA was extracted from entire ants using the PureLink™ Genomic DNA kit (Invitrogen™, USA), according to manufacturer's instructions. Thermocycling conditions were: 5 min at 95°C, 35 cycles of 92°C for 1 min, 58–60°C for 1 min and 70°C for 2 min, followed by final extension at 72°C for 6 min. Reactions were done in 25-μL reactions with 2 units of AmpliTaq DNA polymerase, 1x PCR buffer, 1.5mM of $MgCl_2$, 0.5 mM of dNTPs and 0.5 μM of each primer. PCR products were electrophoresed on 1.5% agarose gels with E-Gel® 1 Kb Plus DNA Ladder (to evaluate the size of the fragments), stained with ethidium bromide and visualized under UV light. Samples of the PCR products were purified using PEG 8000. All sequenced samples were obtained in both directions. The sequencing reaction protocol was performed in 10 μL: 0.5 μL ABI Prism® BigDye™ v3.1 (Applied Biosystems Inc., Foster City, CA), 1.0 μL 5x buffer and 1 μL each (3.2pmol) primer. The ultra-pure water and template to give 40–50 ng of DNA in each reaction was composed in the remainder of the mixture. The cycle sequencing reaction protocol contained an initial denaturation step of 96°C for 1 min, followed by 35 cycles of 10 s at 96°C denaturation,

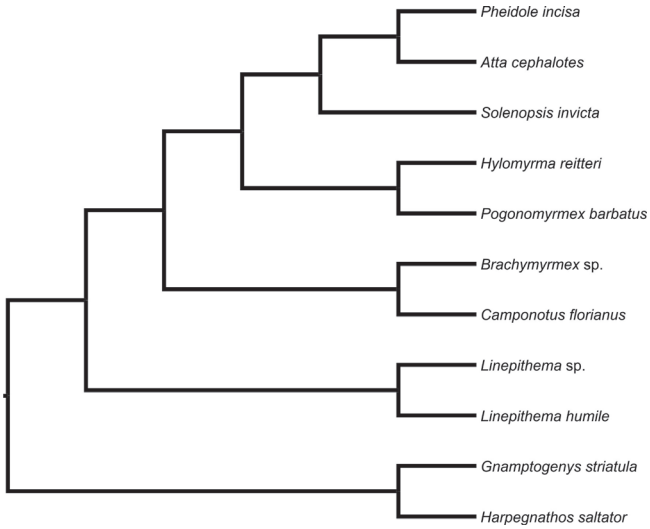

Fig. 1. Phylogenetic relationships among all species involved in the present study, based on Brady *et al*. (2006) and unpublished results on *Hylomyrma reitteri*.

Table I. EPIC primers developed in the present study and the corresponding position in the genome of *Harpegnathos saltator*.

Locus	The position of exon 1 in *Harpegnathos saltator* genome	The position of exon 2 in *Harpegnathos saltator* genome	Average size of the intron across six species used for designing primers	Identity of the exon sequence across six species used for designing primers
ant.1076	scaffold1076:271056:270920	scaffold1076:269369:269215	616.0	94.0
ant.1087	scaffold1087:158069:157789	scaffold1087:157182:157026	938.0	96.0
ant.1225	scaffold1225:12532:12329	scaffold1225:11932:11705	432.0	94.5
ant.1281	scaffold1281:238506:238274	scaffold1281:237845:237709	406.0	94.5
ant.1401	scaffold1401:28851:28726	scaffold1401:27273:27163	678.0	94.5
ant.1503	scaffold1503:164517:164731	scaffold1503:166007:166262	932.0	94.0
ant.1	scaffold1:871727:871884	scaffold1:872436:872847	349.0	93.5
ant.202	scaffold202:749629:749750	scaffold202:750173:750299	461.0	94.5
ant.263	scaffold263:19788:19657	scaffold263:19271:19145	390.0	95.5
ant.346	scaffold346:68688:68870	scaffold346:69142:69264	291.0	96.5
ant.384	scaffold384:473216:473086	scaffold384:472110:471826	833.0	96.5
ant.389	scaffold389:104575:104358	scaffold389:103782:103644	94.5	508.0
ant.505	scaffold505:742686:742795	scaffold505:743318:743431	95.5	408.0
ant.839	scaffold839:296212:296350	scaffold839:297615:297720	97.0	1042.0
ant.965	scaffold965:696156:696310	scaffold965:696571:696741	94.0	259.0

15 s at annealing 50°C and 4 min at 60°C. The final DNA precipitation was performed with isopropanol and run on an ABI 3500 sequencer to assess the level of intraspecific sequence variability. Chromatograms were edited in the StadenPackage (Staden *et al*. 2000) and unambiguously aligned by hand using BioEdit program version 7.1.3 (Hall 1999), all sequences being available in GenBank (accession numbers KF738831 to KF738842).

The initial screening of potential fragments smaller than 3 kbp indicated 16,089 candidate loci (mean = 232.8 bp, SD = 381.4). Although not explored in the present study, shorter fragments (<200 bp) could be particularly useful in the context of next-generation sequencing (Puritz *et al*. 2012). We were able to successfully amplify all loci selected for the *in vitro* tests (Table II). Amplified product size varied between loci and species, from 400 to 1,000 bp. The size discrepancy for the same amplification product across species ranged from 50 to 500 bp. (mean = 196.6, Table II).

In order to test if the developed EPIC markers would provide sufficient intraspecific variability, we sequenced a subset of four samples from the amplified products of *G. striatula* obtained at locations between 600 km and 2,900 km apart (Brazilian states of Santa Catarina, São Paulo, Sergipe and Pernambuco). Different loci provided varying levels of divergence, from 0.22–0.56% (ant.1F/R, ant.202F/R) to 1.1–1.4% (ant.263F/R, ant.965F/R, and ant.1281F/R), with a maximum of 8.9% (ant.346F/R). None of the amplified sequences showed heterozygous indel positions. However, that is indeed a possibility with EPIC markers. Given the broad variety of product sizes among species, preliminary tests on a variety of loci might be necessary to select those with sufficiently small lengths such that these issues are minimized. Given that we specifically chose phylogenetically distant species, it is not surprising that sequences from different species cannot be aligned. However, the levels of intraspecific variability detected in the present study suggest that at least some loci might be valuable to assess the phylogenetic relationships of closely-related species.

The EPIC markers developed in the present study represent an important addition to the ant phylogeography toolbox, allowing for unprecedented new sources of information from nuclear markers. However, given that ants are a remarkably diverse and ancient lineage, it is possible that some markers might not be equally useful in all cases. Future studies should therefore include an initial assessment of fragment size and sequence variability for the species in question before proceeding to more extensive sampling. However, the fact that we obtained successful amplification of all loci for a phylogenetically diverse sample of ant lineages strongly suggests that these markers should be applicable to ants in general.

Table II. EPIC primers developed in the present study and the corresponding estimated fragment sizes based on gel electrophoresis.

Primer name	Length	Sequence (5'to 3')	*Brachymyrmex* sp.	*Pheidole incisa*	*Hylomyrma reitteri*	*Gnamptogenys striatula*
ant.1076F	24	AAAATCTNATGTGGAACACGGTCA	600	600	550	550
ant.1076R	23	CCGTATCTGATYTCCATGTAGCA				
ant.1087F	21	ACCAGCAGAGGCTGGACGTGA	700	650	800	700
ant.1087R	27	GCCAAGTTGATTGTGTACGAACTTTCT				
ant.1225F	26	TAATACRACTGAAGAGAGACCAGGAG	500	600	650	1000
ant.1225R	27	GACTAGATCCTAAGCTAGAGAGRCTGG				
ant.1281F	23	GACGCAGGTTGYAACGAAATCAC	500	500	400	550
ant.1281R	24	GCCRCTAATATCCAGCTTCACGAG				
ant.1401F	22	GYAGGAAGGACGCTCTTAATCT	700	550	600	700
ant.1401R	26	AAGCTTATCTCTAGGAAACTCCCATC				
ant.1503F	21	GRTTYGCCTTCCAGGAGATCA	800	700	800	800
ant.1503R	23	AAGTAGTCCAGGCAGAACCACAC				
ant.1F	27	CCTTCGTGCCTAYGAGAATAGYGTTAC	600	450	400	650
ant.1R	21	AACGACGTCSGACGGTTCCAT				
ant.202F	26	CCYATCAACTCTGTTAATATCGAACG	650	500	550	600
ant.202R	22	GACACAATGTTGGAAGCCCTTG				
ant.263F	27	GACTAGCTCAGAATCACACTCTTCCAC	450	550	550	600
ant.263R	24	GTTGTTTTGGWGGCAATATTGGAG				
ant.346F	23	GTGGTCCACCATCCGTKGGATCT	400	400	400	500
ant.346R	26	GGATTGTTTTGTGTAATCTGCGTTCG				
ant.384F	27	TAGTAGTCGAAGGAGTCATACCAAAGG	850	650	850	800
ant.384R	20	TGYGTGTTCGATGCCGTTGA				
ant.389F	23	ACGGACCCCACATTGAGAAGAAC	600	650	650	850
ant.389R	21	CYTTACCCACCTCCTCCACCA				
ant.505F	24	CCTCAGATGAAGTTYCGAGTTTCC	650	600	600	600
ant.505R	26	TAAYCCGRACACCCTCACTTTATACG				
ant.839F	25	CAATGGCGATTTACAACGAATTTCT	450	450	850	400
ant.839R	22	CAGGCANAGCAGCAATGTGACG				
ant.965F	24	AGTTCAAGGTTCACCGGTGCCTAA	400	500	400	650
ant.965R	25	GAGAAGGYGAAYTTAAAGACTGATG				

ACKNOWLEDGMENTS

We thank Rogério R. Silva for providing specimens used in the *in vitro* tests. We also thank Paula F. B. Bassi for assistance during laboratory work. This study was partly funded by a grant from CNPq/MCT (571334/2008-3) to MRP.

REFERENCES

Agosti, D. & Alonso, L.E. 2000. The ALL Protocol: A Standard Protocol for the Collection of Ground-Dwelling Ants, p. 204–206. In: Agosti, D., Majer, J.D., Alonso, L.E. & Schultz, T.R. (Eds.). **Ants: Standard Methods for Measuring and Monitoring Biodiversity.** Biological Diversity Handbook Series. Washington, Smithsonian Institution Press, 280 p.

Avise, J.C. 2000. **Phylogeography: The History and Formation of Species.** Cambridge, Harvard University Press, 464 p.

Brady, S.G., Schultz, T.R., Fisher, B.L. & Ward, P.S. 2006. Evaluating alternative hypotheses for the early evolution and diversification of ants. **Proceedings of the National Academy of Sciences of the United States of America 103**: 18172–18177.

Bonasio, R., Zhang, G., Ye, C., Mutti, N.S., Fang, X., Qin, N., Donahue, G., Yang, P., Li, Q., Li, C., Zhang, P., Huang, Z., Berger, S.L., Reinberg, D., Wang, J. & Liebig, J. 2010. Genomic comparison of the ants *Camponotus floridanus* and *Harpegnathos saltator*. **Science 329**: 1068–1071.

Fittkau, E.J. & Klinge, H. 1973. On biomass and trophic structure of the central Amazonian rain forest ecosystem. **Biotropica 5**: 2–14.

Goropashnaya, A.V., Fedorov, V.B., Seifert, B. & Pamilo, P. 2007. Phylogeography and population structure in the ant *Formica exsecta* (Hymenoptera, Formicidae) across Eurasia as reflected by mitochondrial DNA variation and microsatellites. **Annales Zoologici Fennici 44**: 462–474.

Graur, D. & Li, W.-H. 2000. **Fundamentals of Molecular Evolution**. Second Edition. Massachusetts, Sunderland, 481 p.

Hall, T.A. 1999. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. **Nucleic Acids Symposium Series 41**: 95–98.

Hölldobler, B. & Wilson, E.O. 1990. **The Ants**. Cambridge, Belknap Press, 732 p.

Kajak, A., Breymeyer, A. & Petal, J. 1971. Productivity investigation of two types of meadows in the Vistula valley. XI Predatory arthropods**. Polish Journal of Ecology 19**: 223–233.

Leppänen, J., Vepsäläine, K. & Savolainen, R. 2011. Phylogeography of the ant *Myrmica rubra* and its inquiline social parasite. **Ecology and Evolution 1**: 46–62.

Lessa, E.P. 1992. Rapid surveying of DNA sequence variation in natural populations. **Molecular Biology and Evolution 9**: 323–330.

Li, C., Riethoven, J.-J.M. & Ma, L. 2010. Exon-primed intron-crossing (EPIC) markers for non-model teleost fishes. **BMC Evolutionary Biology 10**: 90.

Palumbi, S.R. 1996. Nucleic acids II: the polymerase chain reaction, p. 205–247. In: Hillis, D.M., Moritz, C. & Mable, B.K. (eds.). **Molecular Systematics**. Second Edition. Sunderland, Sinauer, 655 p.

Pringle, E.G., Ramírez, S.R., Bonebrake, T.C., Gordon, D.M. & Dirzo, R. 2012. Diversification and phylogeographic structure in widespread *Azteca* plant-ants from the northern Neotropics. **Molecular Ecology 21**: 3576–3592.

Puritz, J.B., Addison, J.A. & Toonen, R.J. 2012. Next-generation phylogeography: A targeted approach for multilocus sequencing of non-model organisms. **PLoS ONE** 7: e34241. doi:10.1371/journal.pone.0034241.

Resende, H.C., Yotoko, K.S.C., Delabie, J.H.C., Costa, M.A., Campiolo, S., Tavares, M.G., Campos, L.A.O.& Fernandes-Salomão, T.M. 2010. Pliocene and Pleistocene events shaping the genetic diversity within the central corridor of the Brazilian Atlantic Forest. **Biological Journal of the Linnean Society 101**: 949–960.

Ross, K.G., Krieger, M.J.B., Keller, L. & Shoemaker, D.D. 2007. Genetic variation and structure in native populations of the fire ant *Solenopsis invicta*: evolutionary and demographic implications. **Biological Journal of the Linnean Society 92**: 541–560.

Schlötterer, C. 2004. The evolution of molecular markers — just a matter of fashion? **Nature Reviews Genetics 5**: 63–69.

Silva-Oliveira, G.C., Silva, A.B.C., Oliveira, Y., Nunes, Z.P., Torres, R.A., Sampaio, I. & Vallinoto, M. 2012. New nuclear primers for molecular studies of Epinephelidae fishes. **Conservation Genetics Resourses 5**: 165–168.

Slade, R.W., Moritz, C., Heideman, A. & Hale, P.T. 1993. Rapid assessment of single-copy nuclear DNA variation in diverse species. **Molecular Ecology 2**: 359–373.

Smith, C.D., Zimin, A., Holt, C. *et al*. 2011a. Draft genome of the globally widespread and invasive Argentine ant (*Linepithema humile*). **Proceedings of the National Academy of Sciences of the United States of America 108**: 5673–5678.

Smith, C.R., Smith, C.D., Robertson, H.M. *et al*. 2011b. Draft genome of the red harvester ant *Pogonomyrmex barbatus*. **Proceedings of the National Academy of Sciences of the United States of America. 108**: 5667–5672.

Solomon, S.E., Bacci Jr., M. Martins Jr., J., Vinha, G.G. & Mueller, U.G. 2008. Paleodistributions and comparative molecular phylogeography of leafcutter ants (*Atta* spp.) provide new insight into the origins of Amazonian diversity. **PLoS ONE 3**: e2738.

Staden, R., Beal, K.F. & Bonfield, J.K. 2000. The Staden package, 1998. **Methods in Molecular Biology 132**: 115–130.

Suen, G., Teiling, C., Li, L., Holt, C., Abouheif, E. *et al*. 2011. The genome sequence of the leaf-cutter ant *Atta cephalotes* reveals insights into its obligate symbiotic lifestyle. **PLoS Genetics 7**: e1002007.

Wiens, J.J., Kuczynski, C.A. & Stephens, P.R. 2010. Discordant mitochondrial and nuclear gene phylogenies in emydid turtles: implications for speciation and conservation. **Biological Journal of the Linnean Society 99**: 445–461.

Würm, Y., Wang, J., Riba-Grognuz, O., Corona, M., Nygaard, S., Hunt, B.G., Ingram, K.K. *et al*. 2011. The genome of the fire ant *Solenopsis invicta*. **Proceedings of the National Academy of Sciences of the United States of America 108**: 5679–5684.

Received 15 April 2013; accepted 6 September 2013
Associate Editor: Eduardo A. B. Almeida