*Ann. Zool. Fennici* 45: 478–482 ISSN 0003-455X (print), ISSN 1797-2450 (online)
Helsinki 30 December 2008 

# PCR multiplexing for maximising genetic analyses with limited DNA samples: an example in the collared flycatcher, *Ficedula albicollis*

Nikoletta Karaiskou[1,2] & Craig Primmer[2,*]

[1)] *Department of Genetics, Development and Molecular Biology, School of Biology, Aristotle University of Thessaloniki, 54 124 Thessaloniki, Macedonia, Greece*
[2)] *Department of Biology, FI-20014 University of Turku, Finland (*corresponding author's e-mail: craig.primmer@utu.fi)*

*Received 12 Nov. 2007, revised version received 2 Jan. 2008, accepted 7 Jan. 2008*

Karaiskou, N. & Primmer, C. 2008: PCR multiplexing for maximising genetic analyses with limited DNA samples: an example in the collared flycatcher, *Ficedula albicollis*. — *Ann. Zool. Fennici* 45: 478–482.

In population and evolutionary genetics it is commonly recognised that more reliable results are often obtained when the number of loci analysed is increased, however lack of DNA is one of several factors which can limit the possibility to increase the number of loci assessed in a given study. A promising way to overcome the problem is the simultaneous amplification of several loci within the same reaction i.e. multiplex PCR. The purpose of the present work was to develop a series of microsatellite multiplexes using a recently released commercial multiplex buffer in order to demonstrate the potential usefulness of PCR multiplexing in a non-model organism. We developed ten multiplex sets of primers for the amplification of 77 microsatellite markers in collared flycatchers, each reaction requiring 30 ng of genomic DNA. The multiplexed microsatellite markers provide an easy and cost effective method for collection of genotype data thereby reducing the quantity of reagents and importantly reducing the quantity of DNA required for obtaining successful amplification to less than 4 ng per locus.

## Introduction

In population and evolutionary genetics it is commonly recognised that more reliable results are often obtained when the number of loci analysed is increased (Takazaki & Nei 1996, Koskinen *et al*. 2004, Vähä & Primmer 2006). Also in conservation genetics, maximising the number of loci analysed will generally increase the reliability of any management conclusions drawn from a study. Factors limiting the analysis of larger numbers of loci in non-model organisms include a lack of available loci, lack of funds, insufficient time and/or lack of sufficient DNA. When sufficient loci are available, the remaining problems can be solved by multiplex PCR, whereby two or more loci are simultaneously amplified in the same reaction (Chamberlain *et al*. 1988, Edwards & Gibbs 1994). By using multiplex reactions, the total number of reactions per experiment, the quantity of reagents and also the required DNA quantity can be reduced dramatically. This can be particularly important in studies where hundreds of markers are to be analysed

e.g. in linkage mapping or when the amount of DNA available is severely limited as may be the case in many conservation genetic studies. Despite these potentially attractive features, application of large scale multiplex PCR analyses in non-model organisms have rarely been reported. This is most likely due to complications involved resulting in non-specific amplification when > 3–4 primer pairs are included in the same reaction (pers. obs.). However, the recent release of commercial multiplex buffers appear to have overcome these problems, thus enabling a broader application of this potentially resource saving technique. Given the limited resources often available for conservation genetic studies, in particular limited starting amounts of genomic DNA, a re-assessment of the potential benefits of multiplex PCR is particularly relevant.

In this study we focus on a passerine bird, the collared flycatcher (*Ficedula albicollis*) which has been frequently studied in evolutionary and ecological research (e.g. Ellegren *et al*. 1995). The purpose of the present work was to develop a series of microsatellite multiplexes using a recently released commercial multiplex buffer in order to demonstrate the potential usefulness of PCR multiplexing in a non-model organism.

## Material and methods

Seventy microsatellite markers which had been characterised from a pied flycatcher (*F. hypoleuca*) enriched microsatellite library (Leder *et al*. 2008), 75 EST-linked microsatellites identified from zebra finch EST sequences (Karaiskou *et al*. 2008) as well as six microsatellite markers previously described (Ellegren 1992, Primmer *et al*. 1996, Rubenstein 2005), were initially screened for polymorphism in eight unrelated collared flycatcher individuals. We chose to multiplex the most highly polymorphic markers in order to produce multiplex sets of primers with the best resolution for linkage mapping. Additionally, the selection of the markers was based on the allele range, the primer compatibility and the genotyping performance when amplified by multiplex PCR. Loci labelled with the same fluorescent tag (FAM, NED, PET or VIC) were separated by at least 50 bp in order to avoid potential size range overlap. A number of different primer pair combinations were tested by excluding and adding different markers in order to identify the optimal primer combinations. Once a suitable multiplex PCR protocol was identified, individual microsatellite primer pair concentrations were modified as necessary to obtain relatively equal fluorescent intensity signals for each microsatellite marker in the multiplex (Table 1). There were cases in which specific loci amplified very well in a single PCR reaction but did not amplify in any of the multiplex protocols and thus they were excluded from the analyses. Multiplex PCRs were performed in 10 $\mu$l reactions which included 5 $\mu$l of Qiagen Buffer from the Qiagen multiplex PCR kit, 1 $\mu$l of the primer mix (a mix with the optimal concentration of the primer pairs included in each multiplex protocol; *see* Table 1) and 30 ng of genomic DNA. Thermocycling was performed using an initial step of 95 °C for 15 min, followed by 35 cycles of 94 °C for 30 sec, 55–59 °C (specific for each multiplex protocol; *see* Table 1) for 1.5 min, 72 °C for 1 min with a final extension in 60 °C for 15 min. The PCR products were diluted 1/60 after which 2 $\mu$l of each dilution was mixed with 10 $\mu$l of formamide and 1 $\mu$l of internal size standard GS 600LIZ (Applied Biosystems). The resulting mixture was denatured for 5 min at 100 °C and electrophoresed for 20 min on an ABI3130xl Genetic Analyser. Genotypes were scored using Genemapper 4.0 software (Applied Biosystems).

## Results and discussion

The genotyping performance of the PCR multiplex compared with separate PCR amplification of single loci was tested by screening eight unrelated individuals. In all cases, multiplex PCRs produced the same genotypes as the single PCR for each locus. In total, 77 microsatellite loci were amplified in ten different multiplex sets (Table 1). The number of loci in each protocol ranged from six to nine. All the loci in the ten different multiple reactions revealed clearly separated fragment peaks with strong signal intensities. EST-linked microsatellites often revealed only low levels of polymorphism and/or failed to amplify when included in the reaction with anonymous mic-

**Table 1.** Multiplex sets with fluorescent dye and primer concentrations for each locus in the primer mix, and annealing temperature and allele size range for each multiplex set. In addition, polymorphism data ($A$ = number of alleles, $H_o$ = observed heterozygosity and Pr(Ex) = second parent exclusion probability for the multiplex) based on 8 unrelated individuals are listed.

| Multiplex protocol | Marker[a] | Dye labeling | Primer concetration (pmol µl$^{-1}$) | Annealing temperature (°C) | Allele size range (bp) | $A$ | $H_o$ | Pr(Ex) |
|---|---|---|---|---|---|---|---|---|
| Set 1 | Tgu-EST10[1] | PET | 0.15 | 59 | 155–167 | 6 | 0.375 | 0.978 |
| | Tgu-EST75[1] | PET | 0.8 | | 309–315 | 3 | 0.286 | |
| | Tgu-EST9[1] | VIC | 0.15 | | 400–408 | 4 | 0.475 | |
| | GGC25[1] | FAM | 0.15 | | 222–231 | 3 | 0.429 | |
| | Tgu-EST31[1] | FAM | 0.5 | | 342–347 | 3 | 0.500 | |
| | Tgu-EST85[1] | NED | 0.15 | | 248–261 | 4 | 0.600 | |
| | Tgu-EST86[1] | NED | 0.6 | | 177–188 | 6 | 0.671 | |
| | Tgu-EST16[1] | NED | 0.4 | | 309–313 | 4 | 0.250 | |
| Set 2 | Fhy404[2] | FAM | 2 | 56 | 300–327 | 7 | 0.286 | 0.999 |
| | Fhy407[2] | FAM | 2 | | 221–243 | 7 | 1.000 | |
| | Fhy328[2] | NED | 0.1 | | 134–186 | 5 | 1.000 | |
| | Fhy329[2] | NED | 1 | | 257–307 | 8 | 0.500 | |
| | Fhy339[2] | NED | 0.5 | | 351–423 | 12 | 1.000 | |
| | Fhy336[2] | PET | 0.3 | | 154–183 | 7 | 0.500 | |
| | Fhy356[2] | PET | 0.2 | | 374–428 | 10 | 0.875 | |
| | Fhy341[2] | VIC | 0.05 | | 174–202 | 7 | 0.750 | |
| | Fhy344[2] | VIC | 6 | | 392–468 | 8 | 0.714 | |
| Set 3 | Fhy402[2] | FAM | 7 | 55 | 149–170 | 6 | 0.429 | 0.984 |
| | Fhy326[2] | NED | 0.4 | | 334–341 | 3 | 0.500 | |
| | Tgu-EST46[1] | NED | 0.3 | | 221–227 | 3 | 0.250 | |
| | Fhy350[2] | NED | 0.1 | | 165–180 | 4 | 0.375 | |
| | Fhy361[2] | NED | 0.3 | | 401–403 | 2 | 0.375 | |
| | Fhy310[2] | PET | 0.2 | | 328–360 | 8 | 0.750 | |
| | Fhy342[2] | VIC | 6 | | 272–292 | 6 | 0.500 | |
| Set 4 | Fhy215[2] | FAM | 8 | 55 | 359–388 | 7 | 0.375 | 0.997 |
| | Fhy216[2] | FAM | 0.1 | | 145–161 | 7 | 0.875 | |
| | Fhy307[3] | FAM | 8 | | 255–279 | 4 | 0.333 | |
| | Fhy467[2] | VIC | 8 | | 255–328 | 9 | 0.750 | |
| | Fhy464[2] | PET | 0.3 | | 149–186 | 7 | 0.833 | |
| | Fhy415[2] | PET | 6 | | 362–382 | 4 | 0.400 | |
| | Fhy321[2] | NED | 4 | | 244–261 | 5 | 0.429 | |
| Set 5 | Fhy301[2] | VIC | 1.5 | 56 | 130–166 | 4 | 0.250 | 0.999 |
| | Fhy304[2] | VIC | 1.5 | | 228–274 | 6 | 0.714 | |
| | Fhy413[2] | VIC | 0.4 | | 441–557 | 4 | 0.333 | |
| | Fhy306[2] | FAM | 8 | | 447–475 | 8 | 0.750 | |
| | Fhy401[2] | FAM | 2.5 | | 278–312 | 8 | 1.000 | |
| | Fhy403[2] | FAM | 6.0 | | 168–202 | 9 | 0.875 | |
| | Fhy408[2] | NED | 1.5 | | 135–191 | 9 | 1.000 | |
| | Fhy409[2] | NED | 0.3 | | 194–239 | 6 | 0.375 | |
| | Fhy405[2] | PET | 6 | | 122–152 | 6 | 0.667 | |
| Set 6 | FhU2[4] | VIC | 0.3 | 25 cycles at | 137–163 | 7 | 0.333 | 0.998 |
| | SS12[5] | VIC | 2 | 55 | 224–245 | 7 | 0.750 | |
| | FhU4[6] | FAM | 5.5 | | 163–202 | 9 | 0.750 | |
| | FhU5[6] | NED | 3.0 | 15 cycles at | 155–268 | 11 | 0.875 | |
| | FhU3[6] | PET | 7 | 53 | 169–179 | 5 | 0.625 | |
| | Fhy468[3] | PET | 0.15 | | 206–221 | 5 | 0.267 | |

*continued*

**Table 1.** Continued.

| Multiplex protocol | Marker[a] | Dye labeling | Primer concetration (pmol µl$^{-1}$) | Annealing temperature (°C) | Allele size range (bp) | *A* | $H_o$ | Pr(Ex) |
|---|---|---|---|---|---|---|---|---|
| Set 7 | Fhy217[2] | NED | 0.3 | 55 | 122–144 | 7 | 0.714 | 0.999 |
| | Fhy370[2] | NED | 0.1 | | 256–272 | 5 | 0.571 | |
| | Fhy428[2] | FAM | 0.2 | | 269–305 | 7 | 0.875 | |
| | Fhy453[2] | FAM | 0.3 | | 387–435 | 10 | 0.875 | |
| | Fhy429[2] | VIC | 0.3 | | 296–377 | 9 | 0.667 | |
| | Fhy427[2] | VIC | 0.3 | | 244–286 | 8 | 0.857 | |
| | Fhy450[2] | PET | 0.2 | | 411–515 | 9 | 0.857 | |
| | Fhy454[2] | PET | 2 | | 232–260 | 8 | 0.714 | |
| Set 8 | Fhy431[2] | NED | 0.3 | 56 | 218–237 | 8 | 1.000 | 0.998 |
| | Fhy432[2] | NED | 0.3 | | 294–327 | 5 | 0.500 | |
| | Fhy444[2] | VIC | 0.3 | | 451–467 | 5 | 0.750 | |
| | Fhy452[2] | VIC | 0.3 | | 290–374 | 10 | 0.857 | |
| | Fhy466[2] | VIC | 0.3 | | 148–162 | 5 | 0.750 | |
| | Fhy448[2] | PET | 0.3 | | 186–235 | 7 | 0.714 | |
| | Fhy458[2] | PET | 0.3 | | 345–440 | 10 | 0.571 | |
| | Fhy455[2] | FAM | 5 | | 262–415 | 11 | 1.000 | |
| | Fhy465[2] | FAM | 3 | | 170–191 | 7 | 0.750 | |
| Set 9 | Fhy230[2] | VIC | 0.6 | 56 | 337–376 | 10 | 0.750 | 0.998 |
| | Fhy224[2] | NED | 0.6 | | 360–440 | 8 | 0.500 | |
| | Fhy225[2] | PET | 0.6 | | 374–402 | 5 | 0.500 | |
| | Fhy227[2] | PET | 0.3 | | 227–250 | 5 | 0.625 | |
| | Fhy220[2] | FAM | 0.3 | | 279–322 | 7 | 0.571 | |
| | Fhy223[2] | FAM | 0.3 | | 128–148 | 5 | 0.714 | |
| | Fhy237[2] | FAM | 3 | | 250–258 | 4 | 0.875 | |
| Set10 | Fhy221[2] | VIC | 0.5 | 56 | 154–190 | 7 | 0.625 | 0.998 |
| | Fhy234[2] | VIC | 0.5 | | 279–298 | 6 | 0.625 | |
| | Fhy226[2] | NED | 0.5 | | 230–320 | 9 | 0.750 | |
| | Fhy236[2] | PET | 0.5 | | 154–190 | 9 | 1.000 | |
| | Fhy228[2] | PET | 0.5 | | 448–470 | 6 | 0.571 | |
| | Fhy231[2] | FAM | 0.5 | | 370–400 | 6 | 0.500 | |
| | Fhy235[2] | FAM | 0.5 | | 200–210 | 4 | 0.625 | |

[a] References for primer sequences: [1]Karaiskou *et al.* (2008), [2]Leder *et al.* (2008), [3]Primer sequences (5´–3´) as follows: Fhy307-F: TGGAATGAATGAATGAATAA, Fhy307-R: GTTTCACATGCTGTCTTACACT, Fhy468-F: TTTAATTAGAGCCTATGTGT, Fhy468-R: GTTTCTGCCTTTATTCTTATTAC, Accession numbers EU368595-6, [4]Ellegren 1992, [5]Rubenstein 2005, [6]Primmer *et al.* 1996.

rosatellites (data not shown). This may be due to some mismatches in the primer sequences of the zebra finch (*Taeniopygia guttata*) derived EST markers resulting in the flycatcher derived anonymous markers annealing more specifically when in competition. Therefore, we designed one multiplex reaction with eight EST-microsatellites (Tgu-EST10, Tgu-EST75, Tgu-EST9, GG-C25, Tgu-EST31, Tgu-EST85, Tgu-EST86, Tgu-EST16) and an additional EST-microsatellite (Tgu-EST46) was included in multiplex set 3.

Use of PCR multiplexing reduced the number of independent PCR reactions required to amplify all the studied microsatellite loci by 87%, from 77 to 10 which results in large savings on reagents and time, as well as reducing pressure on PCR machine reservations. An additional advantage of the technique described herein is the great reduction in the quantity of DNA used per locus- in this case 300 ng of DNA was required for genotyping all 77 loci i.e. 3.9 ng per locus. This is considerably less than in earlier

reported multiplex analyses (Heyen *et al*. 1997, Deng *et al*. 2000, Cargill *et al*. 2002, Bonhomme *et al*. 2005) and 13 times less than if single-locus PCRs had been used.

## Acknowledgements

We thank Hans Ellegren and Niklas Bäckström for supplying collared flycatcher DNA. The study was supported by the Academy of Finland (Centre of Excellence in Evolutionary Genetics and Physiology).

## References

Bonhomme, M., Blancher, A. & Crouau-roy, B. 2005: Multiplex microsatellite for rapid identification and characterization of individuals and populations of cercopithecidae. — *American Journal of Primatology* 67: 385–391.

Chamberlain, J. S., Gibbs, J. E., Ranier, E., Nguyen, P. N. & Caskey, C. T. 1988: Deletion screening of the Duchenne muscular dystrophy locus via multiplex DNA amplification. — *Nucleic acids Research* 16: 11141–11156.

Cargill, E. J., Clark, L. A., Steiner, J. M. M. & Murphy, K. E. 2002: Multiplexing of canine microsatellite markers for whole genome screens. — *Genomics* 80: 250–253.

Deng, H. W., Zhou, Y., Recker, R. R., Johnson, M. L. & Li, J. 2000: Fragment size difference between multiplex and singleplex PCR products and their practical implications. — *Biotechniques* 29: 298–308.

Ellegren, H. 1992: Polymerase chain reaction (PCR) of microsatellites — a new approach to studies of genetic relationships in birds. — *Auk* 109: 886–895.

Ellegren, H., Lifjeld, J. T., Slagsvold, T. & Primmer, C. R. 1995: Handicapped males and extrapair paternity in pied flycatchers: an experiment using microsatellite markers. — *Molecular Ecology* 4: 739–744.

Edwards, M. C. & Gibbs, A. R. 1994: Multiplex PCR: Advantages, development and applications. — *Genome Research* 3: 65–75.

Heyen, D. W., Beever, J. E., Da, Y., Evert, R. E., Green, C., Bates, S. R. E., Ziegle, J. S. & Lewin, H. A. 1997: Exclusion probabilities of 22 bovine microsatellite markers in fluorescent multiplexes for semi-automated parentage testing. — *Animal Genetics* 28: 21–27.

Karaiskou, N., Buggiotti, L., Leder, E. & Primmer, C. R. 2008: High degree of transferability of 86 newly developed zebra finch EST-linked microsatellite markers in 8 bird species. — *Journal of Heredity*. [In press].

Koskinen, M. T., Hirvonen, H., Landry, P. A. & Primmer, C. R. 2004: The benefits of increasing the number of microsatellites utilized in genetic population studies: an empirical perspective from analyses of grayling (*Thymallus thymallus*) evolutionary relationships. — *Hereditas* 141: 61–67.

Leder, E. H., Karaiskou, N. & Primmer, C. R. 2008: 70 new microsatellites for the pied flycatcher *Ficedula hypoleuca* and amplification in other passerine birds. — *Molecular Ecology Resources* 8: 874–880.

Primmer, C. R., Møller, A. P. & Ellegren, H. 1996: New microsatellites from the pied flycatcher *Ficedula hypoleuca* and the swallow *Hirundo rustica* genomes. — *Hereditas* 124: 281–283.

Rubenstein, D. R. 2005: Isolation and characterization of polymorphic microsatellite loci in the plural cooperatively breeding superb starling, *Lamprotornis superbus*. — *Molecular Ecology Notes* 5: 739–744.

Takezaki, N. & Nei, M. 1996: Genetic distances and reconstruction of phylogenetic trees from microsatellite DNA. — *Genetics* 144: 389–399.

Vähä, J.-P. K. & Primmer, C. R. 2006: Efficiency of model-based Bayesian methods for detecting hybrid individuals under different hybridization scenarios and with different numbers of loci. — *Molecular Ecology* 15: 63–72.