PCR primers used for *Gambusia* sequencing:

**CytB—400 bp:**
1. L14724 (forward) $5’$- CGA AGC TTG ATA TGA AAA ACC ATC GTT G- 3’
2. H15149 (reverse) $5’$- AAA CTG CAG CCC CTC AGA ATG ATA TTT GTC CTC A- 3’

Great efficacy across a wide range of species (*affinis*, *caymanensis*, *gaigei*, *hubbsi*, *lemaitrei*, *melapleura*, *nicaraguensis*, *nobilis*)

**ND2—1100 bp:**
1. GamMet (forward) $5’$- AAG CTT TCG GGC CCA TAC CC- 3’
2. GamTrp (reverse) $5’$- GCT TAG GGC TTT GAA GGC CC- 3’
3. GamH4917 $5’$- ATG GTT AAT GCT ATT GCG- 3’
4. GamL546 $5’$- AAT TGC CCA CCT CGG GTG AA- 3’
5. GamH1047 $5’$- TAA RGG YRG GGG TAA YAG GG- 3’

3 is an alternate reverse primer for *G. hubbsi* used in the Evolution 2007 paper.

Overall good efficacy; a second band around 400bp is amplified in a few species (*nobilis*, some *affinis* [RBL 121], *nicaraguensis*) and around 1000 bp for *manni* specimens and some *G. hubbsi* specimens. Annealing temp for *G. nicaraguensis* of 66C is best to amplify upper (target) band.

**Rag2—1000bp:**
1. 1297R (reverse) $5’$- TCG GTG GAG TAG TAA GGC TCC CA- 3’
2. 277F (forward) $5’$- GAC CCC GAG YGY TAC CTC ATC C- 3’

Overall good efficacy, but requires gel extraction for many samples; almost all have a faint second band amplified around 400bp. Primers not effective for *B. belizanus* due to high non-specific binding.

**S7—800bp:**
1. S7RP1F.RBL (forward) $5’$- CTC TTC CTT GGC CGT CGC TG- 3’
2. S7RP2R.RBL (reverse) $5’$- TTA CCT GGG AGA TTC CAG ACT CAA A- 3’
3. S7RPEX2R (reverse) $5’$- AAC TCG TCT GGC TTT TCG CC- 3’
4. S7RPEX1F (forward) $5’$- TGG CCT CTT CCT TGG CCG TC- 3’
5. S7RPEX3R (reverse) $5’$- GCC TTC AGG TCA GAG TTC AT- 3’

*from Chow and Hazema 1998
6. S7RPEX1F.RKR (forward) $5’$- CTC TTC CCA GGC CGT CGT TG- 3’

*from Reachel Remington
7. S7RPEX3R.10 (reverse) $5’$- TCA GAG TTC ATC TCC AGC TC- 3’

*from Peter Unmack
Carlos’s nested approach with 4-7 gives best results and completely eliminates amplification of second band around 600bp. Primers 1-2 almost always amplify a second gene around 600bp in size, sometimes not amplifying the 800bp target at all; 3-4 give fair results.

**PCR primers used for *Gobiomorus* sequencing:**

**ND2:**

- **GOBYL4919** 5’- CCC ATA CCC CGA AAA TGA TG- 3’
- **GOBYH6064** 5’- CTC CTA CTT AGA GCT TTG AAG GC- 3’
- **GOBYL4575** 5’- TGA GGN GGY YTM AAY CAA ACH CAA -3’
- **GOBYH5513** 5’- GAG TAG GCT AGG ATT TTW CGA AGY TG -3’
- **GOBYL4NEW** 5’- TGC AAG CTC TCA CTG ACT CC -3’
- **GOBYH986NEW** 5’- AAA GGG TGA AGA GGG CAG TT -3’

GOBYL4 and GOBYH986 seem to be the most effective; a second 1500bp band will appear along with the desired 1100bp band at some annealing temperatures.