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INVESTIGAÇÃO DA BASE MOLECULAR E HISTÓRIA EVOLUTIVA DO MELANISMO EM FELÍDEOS SELVAGENS

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Orientador: Dr. Eduardo Eizirik

Porto Alegre

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ALEXSANDRA SCHNEIDER

Investigação da Base Molecular e História Evolutiva do Melanismo em Felídeos Selvagens

Tese apresentada como requisito para obtenção do grau de Doutor pelo Programa de Pós-Graduação em Biologia Celular e Molecular da Faculdade de Biociências da Pontifícia Universidade Católica do Rio Grande do Sul.

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Aprovada em: ____ de _____ de _____.

BANCA EXAMINADORA:

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Porto Alegre
2013

Aos felinos, melânicos ou não.

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"Some qualities nature carefully fixes and transmits, but some, and those the finer, she exhales with the breath of the individual as too costly to perpetuate. But I notice also that they may become fixed and permanent in any stock, by painting and repainting them on every individual, until at last nature adopts them and bakes them into her porcelain."

— Ralph Waldo Emerson

RESUMO

O melanismo é um polimorfismo de coloração bastante comum em felinos, definido como a ocorrência de uma acentuada produção de melanina escura que gera o escurecimento geral do tegumento do organismo. Sabe-se que esta variação da coloração em mamíferos é frequentemente regulada pela ação de dois genes e seus produtos: *MC1R* e *ASIP*. A eumelanina (pigmento escuro) é produzida quando o receptor de melanocortina-1 (MC1R) é ativado pelo hormônio estimulante de melanócito (α-MSH). Ao contrário, a ativação do MC1R é inibida pela ligação de um antagonista chamado 'proteína sinalizadora de agouti' (ASIP), cuja ação leva à troca da produção de eumelanina para feomelanina (pigmento claro). O melanismo foi documentado em 13 das 37 espécies atuais de felídeos e, em alguns casos, alcança altas frequências em nível populacional. Um estudo anterior indicou que o fenótipo surgiu múltiplas vezes em Felidae, com três espécies diferentes apresentando mutações independentes associadas ao mesmo (EIZIRIK et al., 2003). Assim, este estudo teve por objetivo identificar as mutações responsáveis por esta característica em outras cinco espécies de felídeos e analisar a dinâmica evolutiva do melanismo na família Felidae. No primeiro artigo, revelamos dois casos adicionais de mutações espécie-específicas envolvidas no melanismo de felídeos asiáticos, *Panthera pardus* e *Pardofelis temminckii* e discutimos o papel do gene *ASIP* na evolução deste fenótipo mutante. No segundo manuscrito, uma abordagem em nível genômico foi utilizada para analisar a evolução do melanismo em uma linhagem endêmica de felídeos neotropicais pertencentes ao gênero *Leopardus*. Nesta linhagem estão inclusas três espécies de pequenos felídeos que foram o principal foco desta análise: *L. colocolo*, *L. guigna* e *L. geoffroyi*. A presença do melanismo nestas espécies evolutivamente próximas, associada a frequências relativamente altas (20-30%) deste fenótipo em determinadas áreas de suas distribuições geográficas, sugere que a seleção natural pode estar envolvida na origem e evolução dessa característica. Neste contexto, identificamos três novas mutações nos genes *ASIP* e *MC1R*, cada uma delas fortemente associada ao melanismo em uma das espécies investigadas, e revelamos que a seleção natural parece ter influenciado a história evolutiva deste fenótipo nesta linhagem de felídeos.

Palavras-chave: Melanismo, pigmentação, *ASIP*, *MC1R*, felinos.

ABSTRACT

Melanism is a very common coat color polymorphism in felids, and has been defined as an increased production of dark melanin which generates a general darkening of the organism's tegument. Such coloration variation in mammals is often be regulated by the action of two genes and their products: *MC1R* and *ASIP*. Eumelanin (dark pigment) is produced when the Melanocortin-1 receptor (MC1R) is activated by the binding of the Alpha Melanocyte Stimulating Hormone (α-MSH). In contrast, MC1R activation is inhibited by the binding of the antagonist peptide ASIP (Agouti Signaling Protein), whose action leads to a switch to pheomelanin (light pigment) synthesis. Melanism has been documented in 13 out of 37 extant felid species, in some cases reaching high frequencies at the population level. A previous studies has indicated that this phenotype arose multiples times in the Felidae, with three different species exhibiting unique mutations associated with this trait (EIZIRIK et al., 2003). In this context, the present study aimed to identify the mutations implicated in this phenotype in five other felid species, and to investigate in more detail the evolutionary dynamics of melanism in the Felidae. In the first article, we revealed two additional cases of species-specific mutations involved in melanism in Asian wild cats, *Panthera pardus* and *Pardofelis temminckii*, and discuss the role of the *ASIP* gene in the evolution of this mutant phenotype. In the second manuscript, we analyzed the evolution of melanism in an endemic lineage of Neotropical felids belonging to the genus *Leopardus*. This lineage includes three species of small wild cats that were the focus of this study: *L. colocolo*, *L. guigna* e *L. geoffroyi*. The presence of melanism in these closely-related species, along with relatively high frequencies (ranging from 20% to 30%) of this phenotype observed in some areas of their geographic distribution, suggests that natural selection may be involved in the origin and evolution of this trait. In this context, we identified three novel mutations in the *ASIP* and *MC1R* genes, each of them strongly associated with melanism in one of the analyzed species, and revealed that natural selection may have played a role in the evolutionary history of melanism in this lineage of felids.

Keyword: Melanism, pigmentation, *ASIP*, *MC1R*, felids.

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CAPÍTULO I:
INTRODUÇÃO GERAL

1. Variação da Coloração em Vertebrados

A ampla diversidade de cores observada em animais é uma das principais características fenotípicas na natureza, e suas bases genéticas são de grande interesse para muitos cientistas a fim de compreender os mecanismos que geram e mantêm essa variação. Observações desta diversidade primeiramente em laboratório e mais tarde na natureza têm um papel essencial na compreensão de muitos processos biológicos em nível molecular, celular e do desenvolvimento.

O interesse por elucidar a base molecular e o significado adaptativo destas características fenotípicas há muito tempo intriga pesquisadores na área da biologia evolutiva. Estudos com esta abordagem permitem uma melhor compreensão de como a interação entre os mecanismos genéticos e a variação fenotípica é influenciada por processos evolutivos. Além disso, estes estudos fornecem importantes informações acerca de quantos genes são responsáveis pela variação de coloração em populações naturais, se estão os mesmos genes envolvidos em fenótipos convergentes, como a seleção natural afeta essa diversidade fenotípica e, ainda, se podemos detectar evidências de seleção em nível molecular.

A coloração animal tem relevância comportamental e ecológica, desempenhando papel adaptativo em alguns contextos, sendo propostas basicamente três hipóteses para a função da coloração em mamíferos: (i) camuflagem, através da coloração críptica (em que o animal se confunde com a coloração do ambiente dificultando a detecção visual de potenciais predadores ou presas) ou coloração disruptiva (como manchas ou listras para quebrar os contornos do animal, como parece ocorrer no leopardo ou na zebra); (ii) comunicação intraespecífica, que pode ajudar os animais a manter contato visual, como entre mãe e filhote ou alertar os coespecíficos que predadores estão próximos, e a comunicação interespecífica, incluindo a coloração aposemática; (iii) regulação de processos fisiológicos, como a termorregulação (CARO, 2005).

A coloração é uma das características fenotípicas mais evidentes em mamíferos, e representa um modelo promissor de estudo sobre os mecanismos que determinam o fenótipo (HUBBARD et al., 2010). Apesar do interesse no tema, relativamente poucos estudos abordaram a associação entre genótipo e fenótipo em populações naturais tentando investigar os processos evolutivos envolvidos na geração e manutenção da diversidade de padrões de coloração, bem como também elucidar o significado adaptativo da pigmentação em mamíferos (VAGE et al., 1997; CADIEU et al., 2009; CANDILLE et al., 2007; HOEKSTRA,

2006; ISHIDA et al., 2006; RIEDER et al., 2001; RITLAND; NEWTON; MARSHALL, 2001).

O estudo da pigmentação em mamíferos permite determinar quais genes podem afetar a produção dos diferentes tipos de melanina e com isso causar mudanças fenotípicas na coloração, se esta alteração é causada principalmente por mutações na região codificante ou regulatória de genes e, finalmente, se fenótipos semelhantes são devido às mesmas mudanças genéticas. Cabe ressaltar a importância da identificação não apenas dos genes envolvidos, mas também das mutações exatas implicadas nestas características e seus efeitos em funções protéicas e/ou regulatórias. Tal conhecimento viabilizaria uma compreensão mais detalhada dos mecanismos responsáveis pela variabilidade de fenótipos de coloração em diferentes táxons de vertebrados, incluindo episódios marcantes de convergência evolutiva (HOEKSTRA, 2006; HOFREITER; SCHÖNEBERG, 2010; HUBBARD et al., 2010).

A evolução convergente, ou seja, a evolução independente de uma determinada característica (usualmente servindo a uma mesma função ecológica) em dois ou mais táxons pode ser gerada por processos atuando em diferentes níveis: mutações, genes e função gênica (MANCEAU et al., 2010). No primeiro caso, uma mesma mutação em um determinado gene pode causar a convergência fenotípica entre diferentes espécies, como relatou um estudo com base em DNA antigo de um espécime de mamute da Sibéria (*Mammuthus primigenius*) de aproximadamente 43.000 anos (RÖMPLER et al., 2006). Neste estudo, os pesquisadores sequenciaram a região codificante do gene *MC1R* (*Receptor de Melanocortina-1*) e descobriram a mesma mutação associada à pigmentação clara em camundongos silvestres da Costa do Golfo (*Peromyscus polionotus* [HOEKSTRA et al. 2006]). Este resultado levanta a hipótese de que mamutes do Pleistoceno eram polimórficos para a cor do pelo, porém, sua relevância ecológica permanece um mistério. Outros estudos envolvendo o gene *MC1R* demonstram que os pássaros *Cambacica* (*Coereba flaveola*), uma espécie de codorna japonesa e as espécies domésticas de galinha e camundongo compartilham a mesma mutação responsável pelo fenótipo de coloração negra (LING et al., 2003; NADEAU; MINVIELLE; MUNDY, 2006; THERON et al., 2001).

Em contrapartida, diferentes mutações no mesmo gene também podem produzir fenótipos semelhantes. Neste caso, as mutações podem ou não afetar a função ou expressão gênica de maneiras diferentes. A convergência fenotípica da coloração clara observada no dorso de duas espécies de lagartos que habitam as dunas no Novo México é demonstrada em nível genético, sendo que as mutações espécie-específicas estão localizadas no mesmo gene (*MC1R*), porém o fenótipo é produzido por mecanismos funcionais diferentes

(ROSENBLUM, 2006). Em uma espécie, o efeito da mutação é semelhante ao observado em camundongos silvestres da Costa do Golfo (HOEKSTRA; NACHMAN, 2003), ou seja, uma deficiência do ligante em se ligar ao receptor e, consequentemente, a redução na sinalização do MC1R, diminuindo a produção do pigmento escuro. Na segunda espécie, a mutação também causa a perda parcial de função do MC1R, mas a função do receptor é principalmente comprometida pela incorreta integração do MC1R na membrana do melanócito impedindo a transmissão de sinal.

Por outro lado, existem diversos casos de mutações em diferentes genes associadas ao mesmo fenótipo. A evolução independente da coloração de pelagem clara de populações geograficamente isoladas de camundongos silvestres (*P. polionotus*) nas dunas da Costa do Atlântico na Flórida (EUA) é um dos exemplos mais conhecidos (HOEKSTRA et al. 2006). Tipicamente, esses roedores habitam solos argilosos com densa vegetação no sudeste dos EUA, no qual eles apresentam uma pelagem predominantemente escura. No entanto, os roedores que colonizaram as regiões arenosas da Costa do Golfo e da Costa do Atlântico da Flórida têm uma coloração significativamente mais clara, e constituem populações filogeograficamente distintas da mesma espécie. A mutação implicada na perda parcial de função do MC1R associada à coloração de pelagem clara em roedores da Costa do Golfo não foi identificada na população da Costa do Atlântico da Flórida. Análises adicionais de mudanças de expressão dos genes *ASIP* e *Corin* fortemente sugerem a associação do fenótipo claro a um desses genes nos roedores da Costa do Atlântico, indicando que existem mecanismos genéticos distintos para a evolução independente da camuflagem nestas populações de roedores.

Estes estudos demonstram que diferentes mecanismos genéticos são responsáveis pela evolução convergente da pigmentação em nível intra- ou interespecífico. No entanto, o termo convergência merece atenção nos casos em que se afirma que uma mesma mutação (isto é, convergência em nível de mutação) está envolvida na produção de fenótipos similares em subpopulações de uma mesma espécie ou entre espécies relativamente próximas. Isto porque a mesma mutação não necessariamente surgiu independentemente mais de uma vez. As possíveis causas alternativas incluem os casos em que a mutação consiste em uma variante ancestral e foi mantida através dos processos de especiação que geraram os táxons atuais (BARRETT; SCHLUTER, 2008; COLOSIMO et al., 2005), ou surgiu em uma das linhagens e foi transferida à outra através de processos de hibridação e introgressão (ANDERSON et al., 2009). Nestes casos, é esperado que os haplótipos carregando o alelo mutante formem um mesmo grupo monofilético, enquanto que o caso de convergência em nível de mutação prediz

a sua alocação em grupos haplotípicos distintos, cuja formação não seja explicável por recombinação. A análise detalhada destas distintas possibilidades é ainda muito pouco explorada na literatura abordando este tipo de fenômeno, e consiste de um tema muito interessante para investigações aprofundadas da evolução molecular de fenótipos polimórficos.

2. Melanismo

Dentre as diversas características influenciando a coloração da pelagem de mamíferos, o melanismo evoluiu em uma ampla variedade de formas de vida (MAJERUS, 1998), sendo classicamente documentado tanto em experimentos de laboratório com camundongo (*Mus musculus*; BARSH, 1995, 1996; SILVERS, 1979) quanto em muitas populações naturais de animais (SEARLE, 1968).

O melanismo é um polimorfismo de coloração definido como um escurecimento da pigmentação superficial (ou seja, do tegumento) do organismo, devido a uma acentuada produção de melanina escura (MAJERUS, 1998). Em espécies que apresentam padrões de manchas/pintas, como é o caso de muitos felinos (ver abaixo), o melanismo implica um escurecimento da coloração de fundo da pelagem, que usualmente é mais clara (SCHNEIDER et al., 2012).

Dos diversos locos gênicos envolvidos na pigmentação, dois deles são os principais responsáveis pela ampla variedade de coloração em mamíferos: *Receptor de Melanocortina-1* (*MC1R*) e *Proteína Sinalizadora de Agouti* (*ASIP*), os quais eram originalmente conhecidos como *agouti* e *extension* quando identificados por estudos com camundongos. Ambos apresentam um papel essencial na regulação da síntese de melanina durante o desenvolvimento do pelo, e são bem caracterizados em nível molecular em organismos modelo como o camundongo (BULTMAN; MICHAUD; WOYCHIK, 1992; PERRY et al., 1996; ROBBINS et al., 1993).

O gene *MC1R* codifica um receptor acoplado a proteína G contendo sete hélices transmembrana, que é expresso em melanócitos da pele, folículo de pelos e em células do sistema imune (MOUNTJOY et al., 1992; SMITH et al., 2001). Ao se ligar ao hormônio estimulante de melanócito (α-MSH), o MC1R ativa a síntese de AMP cíclico (cAMP) intracelular induzindo a síntese de eumelanina (pigmento escuro: preto, marrom). Ao contrário, a ativação do MC1R é inibida pelo ASIP, um peptídeo parácrino produzido no folículo de pelos e que se comporta como antagonista ao MC1R, impedindo sua ativação pelo

α-MSH e, assim, induzindo a troca da síntese de eumelanina para feomelanina (pigmento claro: amarelo, avermelhado; Figura 1).

A maioria dos mamíferos apresenta um padrão distinto na regulação desses dois genes entre a pigmentação do corpo dorsal e ventral, tipicamente caracterizada por um ventre claro e um dorso mais escuro. Os pelos dorsais apresentam uma ou mais bandas de feomelanina flanqueada por bandas de eumelanina, produzindo o fenótipo denominado 'agouti' (também referido como fenótipo selvagem na maior parte das espécies já analisadas, Figura 2). Esse padrão de pelo bandeado é causado por pulsos de expressão do *ASIP* durante o crescimento do pelo (BARSH, 1996; JACKSON, 1994).

Desta forma, o melanismo é influenciado pelos genes *MC1R* e *ASIP*, cujos produtos interagem na regulação da produção de melanina. Foi observado que os fenótipos melânicos em camundongos frequentemente se devem a mutações dominantes associadas com a proteína MC1R super ou constitutivamente ativa (JACKSON, 1994), ou a mutações de herança recessiva causando a perda parcial ou total da função da proteína ASIP (ROBBINS et al., 1993). Em outras palavras, ganho-de-função do *MC1R* ou perda-de-função do *ASIP* induzem o melanismo.

Além dos genes *MC1R* e *ASIP*, outros três sabidamente envolvidos em melanismo já foram documentados: *mahogany* (ATRN) em camundongo e loco K em lobos cinza da América do Norte (*Canis lupus*). A função do primeiro gene ainda não é bem entendida, embora se saiba que a proteína ATRN apresente um importante papel em estabilizar a interação entre ASIP e MC1R na membrana plasmática do melanócito (HE et al., 2001).

Diversos polimorfismos de coloração identificados em mamíferos domésticos e selvagens têm sido atribuídos à variação nos genes candidatos *ASIP* e *MC1R*. Dentre os exemplos, podemos citar as mutações no gene *ASIP* implicados no melanismo de raposa (VAGE et al., 1997), rato (KURAMOTO et al., 2001), cavalo (RIEDER et al., 2001), gato doméstico (EIZIRIK et al., 2003) e rato-veadeiro (KINGSLEY et al., 2009). Em contrapartida, os polimorfismos no *MC1R* estão associados ao melanismo na vaca (KLUNGLAND et al., 1995), galinha (TAKEUCHI et al., 1996), raposa (VAGE et al., 1997), porco (KIJAS et al., 1998), ovelha (VÅGE et al., 1999), pássaro *Cambacica* (THERON et al., 2001), onça-pintada e jaguarundi (EIZIRIK et al., 2003), *rock pocket mice* (NACHMAN; HOEKSTRA; D'AGOSTINO, 2003), mico-leão-dourado (MUNDY; KELLY, 2003) e esquilo cinza (MCROBIE; THOMAS; KELLY, 2009).

Apesar dos diversos exemplos de mutações associadas ao melanismo identificadas nestes dois genes, muito pouco ainda é conhecido sobre os processos evolutivos envolvidos

na origem e manutenção deste fenótipo (AYOUB et al., 2009; EIZIRIK et al., 2003; MUNDY; KELLY, 2003), bem como a sua relevância adaptativa em diferentes contextos ecológicos.

3. Melanismo em Felinos

Entre os mamíferos, a família Felidae é um grupo bastante interessante para o estudo da evolução da variação fenotípica em populações naturais. Variações marcantes entre espécies e polimorfismo intra-específico são observados em gatos domésticos e felídeos selvagens, e serviram de base para hipóteses clássicas de adaptação e associações ecológicas (ORTOLANI AND CARO, 1996)**.** A ocorrência de pigmentação polimórfica é comum em gatos domésticos e selvagens, incluindo variação na cor de fundo (do branco ao amarelo, cinza, vermelho ou marrom escuro) e também na presença, forma, coloração e distribuição de manchas (pintas, listras, ocelos ou rosetas). Em várias espécies de felídeos a aparente segregação de cores polimórficas foi utilizada para descrever diferentes subespécies ou populações locais historicamente distintas. Em vários casos, é plausível supor que estas variações sejam produzidas por adaptação local a ambientes distintos, sendo assim importantes componentes na história de vida dessas espécies.

O melanismo é um dos muitos polimorfismos de coloração de felídeos, sendo comprovadamente documentado em 13 das 37 espécies da família (SCHNEIDER et al., 2012), representando sete das oito linhagens evolutivas reportadas por JOHNSON et al., 2006 (Figura 2). Embora seja bastante comum em Felidae e alcance frequências relativamente altas em algumas populações naturais (KAWANISHI et al., 2010), apoiando a ideia de que este fenótipo possa ser adaptativo em alguns contextos ecológicos, muito pouco se conhece sobre seu valor ecológico/comportamental ou sua história evolutiva em qualquer das espécies de felídeos.

A base molecular do fenótipo melânico em múltiplas espécies da mesma família de organismos foi inicialmente investigada por EIZIRIK et al., 2003. Os autores caracterizaram os genes *ASIP* e *MC1R* em espécies da família Felidae e encontraram três deleções independentes associadas com a coloração melânica em três diferentes espécies de gatos (*Felis catus, Panthera onca* e *Puma yagouarondi*). Uma deleção de dois pares de base no gene *ASIP* foi identificada em gatos domésticos pretos (*F. catus*), confirmando o modo de herança recessivo do alelo, enquanto outras duas deleções de herança dominante de 15 e 24 pares de base no gene *MC1R* induzem melanismo em onça-pintada (*P. onca*) e jaguarundi (*P.*

yagouarondi), respectivamente. Os mesmos autores reportaram a ausência dessas mutações em indivíduos melânicos de cinco outras espécies (*Leopardus tigrinus, L. geoffroyi, L. colocolo, Panthera pardus* e *Pardofelis temmincki)* sugerindo que o melanismo surgiu independentemente pelo menos quatro vezes na família.

Um aspecto interessante acerca das espécies do gênero *Leopardus* mencionadas acima, cuja base molecular do fenótipo melânico não foi documentada no estudo inicial, é a ocorrência de hibridação entre as mesmas (TRIGO et al., 2008). Estes autores revelaram fortes evidências de que existe uma zona híbrida entre *L. geoffroyi* e *L. tigrinus* na região central do Estado do Rio Grande do Sul, no sul do Brasil, apresentando complexos padrões de introgressão genética bidirecional. Segundo este mesmo estudo, a ocorrência de hibridação foi também documentada entre *L. tigrinus* e um terceiro felídeo neotropical proximamente relacionado, *L. colocolo*. Desta forma, é possível que o melanismo possa ter sido introduzido nestas espécies através de eventos de hibridação. Caso similar foi documentado através da descoberta de uma deleção de três pares de bases no gene CDB103 ou loco K (β-defensina), que confere a cor negra em cães domésticos e está presente também em lobos negros da América do Norte e Itália (ANDERSON et al., 2009). Neste estudo, foi proposto que a variante de coloração negra foi introduzida na população de lobos através da hibridação e introgressão com cães domésticos. Neste contexto, três diferentes hipóteses podem explicar a ocorrência de melanismo nas quatro espécies de felídeos do gênero *Leopardus* mencionadas acima: a primeira é a de que o melanismo ocorre como um polimorfismo trans-específico, que surgiu no ancestral deste grupo, sendo mantido por seleção natural; a segunda postula que uma mutação gerando este fenótipo ocorreu independente em cada uma destas espécies; e a última sugere que eventos de hibridação podem ser a causa da ocorrência deste fenômeno, visto que há evidências de hibridação entre estes felídeos.

Até o momento, poucos estudos abordaram a base molecular de fenótipos de coloração em múltiplas espécies da mesma família de organismos, tentando investigar aspectos da sua história evolutiva e significado adaptativo. Portanto, destaca-se o potencial dos felídeos como um excelente modelo de investigação para tentar desvendar os complexos processos envolvidos na evolução da pigmentação da pelagem.

4. Espécies-foco deste estudo

As espécies-alvo deste estudo pertencem a três linhagens evolutivas distintas, cada uma sendo reconhecida atualmente como um gênero: *Panthera*, *Pardofelis* e *Leopardus*.

4.1. *Panthera pardus*

O leopardo (*P. pardus*) é uma das quatro espécies de grandes felinos do gênero *Panthera* cuja distribuição geográfica estende-se da África, principalmente na região subsaariana (ao sul do Deserto do Saara) a regiões na Índia e em maior extensão na Ásia meridional. Morfologicamente, o leopardo é bastante semelhante à onça-pintada, porém a última tem uma aparência mais robusta, com a cabeça maior e membros mais vigorosos. Ambas as espécies apresentam o padrão de coloração da pelagem similar, ou seja, uma coloração amarelada com a presença de manchas escuras formando rosetas (ocelos), mas, em geral, as rosetas dos leopardos são menores e mais numerosas, não contendo pontos pretos no seu interior. O melanismo em leopardos é sabidamente uma característica mendeliana de herança recessiva em relação à coloração amarelada com rosetas (ROBINSON, 1970). No entanto, observa-se que o melanismo afeta primariamente a coloração de fundo, sendo que as rosetas permanecem visíveis, sendo ainda mais negras que a coloração de fundo.

A espécie explora uma ampla variedade de habitats, ocupando desde áreas de florestas tropicais, bosques e selvas até áreas mais abertas como savanas e ambientes rochosos, evitando apenas áreas de desertos (JOHNSINGH; PANWAR; RODGERS, 1991). Observações sugerem que o melanismo em populações naturais de leopardos alcança frequências relativamente altas em florestas tropicais do sudeste da Ásia, principalmente na Península da Malásia, podendo ser comum também em Java. Ao contrário, indivíduos melânicos raramente são vistos na África (KAWANISHI et al., 2010).

4.2. *Pardofelis temminckii*

A segunda espécie-foco deste estudo pertence ao gênero *Pardofelis,* previamente conhecido como *Catopuma*. O gato-dourado-asiático (*P. temminckii*) apresenta uma pelagem altamente polimórfica, desde um padrão liso e uniforme sem marcas até a presença de manchas escuras semelhantes ao padrão da jaguatirica (*Leopardus pardalis*). A coloração de fundo pode variar entre os tons castanho-avermelhado, marrom-amarelado e acinzentado, ou

ainda apresentar a forma melânica. A espécie está distribuída na Índia, China, Tibete e Nepal (SUNQUIST; SUNQUIST, 2002), assim como em Butão, Bangladesh, Mianmar, Tailândia (NOWELL; JACKSON, 1996), Laos, Camboja, Vietnã, Península da Malásia, Sumatra e Indonésia (SUNQUIST; SUNQUIST, 2002).

O gato-dourado-asiático pode ser encontrado em ambientes de florestas tropicais e subtropicais úmidas, e ocasionalmente em áreas mais abertas como savanas. Na região do Himalaia, a espécie foi registrada em altitudes de até 3.960 metros (BASHIR et al., 2011). A frequência do fenótipo melânico nesta espécie é difícil de estimar, visto a escassez de conhecimento acerca de aspectos básicos da biologia e comportamento da espécie, mas a ocorrência tem sido reportada como ocasional ao longo da sua distribuição (GHIMIREY; PAL, 2009). O estudo com armadilhas fotográficas conduzido por BASHIR et al., (2011) observou que indivíduos melânicos eram mais comuns do que a forma lisa ou com manchas em uma região da Índia. Na China e Tibete, os indivíduos com manchas parecem predominar sobre a forma lisa e uniforme (NOWELL; JACKSON, 1996).

4.3. *Leopardus* spp.

No gênero *Leopardus*, que divergiu dos gêneros mais próximos há cerca de oito milhões de anos (Ma) estão inclusas três espécies de pequenos felídeos que são o principal alvo do segundo capítulo deste trabalho: *L. colocolo*, *L. guigna* e *L. geoffroyi*. As três espécies são consideradas felídeos de pequeno porte, sendo que em todas é registrada a ocorrência de melanismo. O padrão de pelagem é um dos principais caracteres diagnósticos de cada espécie, sendo *L. colocolo* a mais diferenciada, com uma pelagem mais longa e áspera, principalmente na região do dorso, e a presença de listras largas e escuras nas patas anteriores e posteriores. A coloração varia desde um cinza-amarelado ao cinza-escuro ou marrom-avermelhado, podendo ou não apresentar manchas no corpo. A pelagem de *L. geoffroyi* e *L. guigna* caracteriza-se pela presença de pintas sólidas e pretas no corpo, com *L. geoffroyi* apresentando uma coloração entre o cinza-claro e o amarelo-ocráceo e *L. guigna* variando de marrom-acinzentado ao marrom-avermelhado. A presença de estreitos anéis pretos na cauda de *L. guigna* o diferencia de *L. geoffroyi,* e alguns indivíduos têm proeminentes bandas escuras no pescoço e marcas na face e cabeça. A incidência de melanismo em ambas é tida como bastante comum (NOWELL; JACKSON, 1996; OSGOOD, 1943). No caso de *L. guigna,* a frequência do fenótipo aumenta com a latitude e é particularmente comum na Isla Grande Chiloé e nas Ilhas Guaitecas no Chile (FREER, 2004). Estas informações acerca da frequência

de melanismo nas populações naturais destes felídeos constituem-se basicamente em registros de ocorrência, sendo extremamente escassa a existência de estudos mais aprofundados.

Os três felídeos sofreram uma diversificação relativamente rápida e recente (nos últimos 2.4 Ma), e estão distribuídos exclusivamente na América do Sul (ver Figura 2 no capítulo III). O gato-do-mato-grande (*L. geoffroyi*) ocorre desde a Bolívia e o chaco paraguaio até o sul do Chile, com presença em toda a Argentina, o Uruguai e o sul do Rio Grande do Sul, Brasil (EISENBERG; REDFORD, 1999; OLIVEIRA, 1994). O gato-palheiro (*L. colocolo*) é simpátrico com *L. geoffroyi* em boa parte de sua distribuição, ocorrendo desde o Chile, cobrindo praticamente toda Argentina, Paraguai e Uruguai até regiões da Bolívia, Equador e região central do Brasil (NOWELL; JACKSON, 1996; OLIVEIRA, 1994). Apenas o guiña (*L. guigna*) não ocorre em território brasileiro, sendo uma espécie de distribuição restrita ao Chile e à Argentina, ocupando uma área adjacente à de *L. geoffroyi*. No entanto, há registro de simpatria dessas espécies na floresta da Patagônia Andina, na parte mais ao leste da distribuição de *L. guigna* (LUCHERINI; VIDAL; BELDOMENICO, 2001).

Quanto à associação de habitats, *L. geoffroyi* e *L. colocolo* parecem ocupar predominantemente áreas mais abertas de cerrado e campos, com cobertura arbustiva incluindo matas pouco densas e banhados (NOWELL; JACKSON, 1996; OLIVEIRA; CASSARO, 1999), enquanto *L. guigna* está fortemente associado com os ambientes de florestas úmidas temperadas (NOWELL; JACKSON, 1996). Em algumas áreas, *L. geoffroyi* também pode ser encontrado em áreas florestais mais densas (OLIVEIRA; CASSARO, 1999). No entanto, para as três espécies existem registros de ocorrência em diversos ambientes, até mesmo em florestas secundárias, plantações de *Pinus* e eucaliptos, áreas próximas a plantações e altamente afetadas por desmatamentos (NOWELL; JACKSON, 1996; OLIVEIRA; CASSARO, 1999; SANDERSON; SUNQUIST; IRIARTE, 2002).

5. As novas técnicas de *next generation sequencing* e suas aplicações em estudos genômicos

A tecnologia de sequenciamento de DNA teve papel essencial no avanço da biologia molecular (GILBERT, 1981). Em 1977, o método de sequenciamento de Sanger causou uma revolução na área da biologia com o surgimento de uma metodologia eficaz para a determinação da sequência de DNA de organismos. Importantes avanços no conhecimento biológico, incluindo o sequenciamento do genoma humano, foram possíveis devido à introdução dessa técnica.

Com a meta de decifrar o genoma humano no final da década de 1990 e início do século XXI, houve um aumento sem precedentes na escala do sequenciamento de DNA. Novas estratégias começaram a ser pensadas para o desenvolvimento de uma tecnologia mais eficiente e barata capaz de produzir dados genômicos em larga escala. Atualmente, uma nova revolução tem transformado a área da genômica com o advento das novas plataformas de sequenciamento de nova geração, denominadas *next-generation sequencing* (NGS). Estas promovem o sequenciamento em grande escala de milhões de sequências de DNA em uma única corrida, permitindo que genomas e transcriptomas sejam sequenciados de forma rápida e representativa por custos relativamente baixos.

O potencial desses métodos de sequenciamento muda não apenas o panorama de novos projetos de sequenciamento de genomas como também introduz novas oportunidades de estudos genômicos complexos até então inimagináveis. Diversos são os contextos em que os instrumentos de *next-generation sequencing* vêm sendo utilizados nos últimos anos. As aplicações mais importantes incluem: (1) o sequenciamento *de novo*, que consiste na geração inicial de sequências genômicas de um organismo; (2) o resequenciamento, considerado o uso mais comum das plataformas de NGS (DAVIES, 2001) para a identificação de SNPs, indels, variações no número de cópias de DNA (*copy-number-variation* - CNVs) e variações estruturais em genomas, ou o resequenciamento para a descoberta de mutações em regiões genômicas alvo; (3) análises transcriptômicas e anotação de transcritos através do sequenciamento de RNA mensageiro ou micro-RNAs, assim como análises de expressão gênica e *splicing* alternativo; (4) análises epigenéticas, como a caracterização de padrões de metilação de DNA, o mapeamento de interações entre DNA-proteína e as modificações de histonas e nucleossomos; e (5) metagenômica, que envolve a análise genômica de microrganismos através da extração de DNA de uma comunidade viral e/ou microbiana ambiental, permitindo a identificação de espécies e/ou a descoberta de genes nelas contidos.

A ampla aplicabilidade das plataformas de NGS tem sido demonstrada em diversas áreas da ciência como, por exemplo, no sequenciamento de genomas completos de animais, plantas e fungos (por ex. DIGUISTINI et al., 2009; HUANG et al., 2009; LI et al., 2010; VELASCO et al., 2007) no avanço na compreensão de cânceres (MARDIS; WILSON, 2009; MOROZOVA; MARRA, 2008; TAYLOR et al., 2007), na descoberta de vacinas (DHIMAN; SMITH; POLAND, 2009), em testes de diagnóstico molecular (CHIU et al., 2008; FAN et al., 2008), melhoramento genético de plantas como milho, trigo, *Pinus* e eucalipto (VARSHNEY et al., 2009), diversidade genética microbiana humana, do solo e da biosfera marinha (p. ex.

(EDWARDS et al., 2006; FIERER et al., 2007), variação genética em vários organismos (p.ex. IMELFORT et al., 2009), dentre outros.

O advento dessas tecnologias de alto desempenho tem viabilizado a descoberta de dezenas de milhares de polimorfismos de nucleotídeo único (SNPs) em organismos não-modelo (ELLEGREN; SHELDON, 2008; ELLEGREN, 2008), incluindo espécies ameaçadas de extinção ou espécies de interesse ecológico, agronômico ou médico, assim como estudos mais complexos em organismos poliplóides, com grande quantidade de sequências repetitivas, genomas comparativos e genes diferencialmente expressos.

O crescente interesse na utilização de SNPs para o estudo de populações naturais tem se expandido nas últimas décadas devido ao potencial desses marcadores moleculares em abordar inúmeras questões sobre ecologia e evolução (LUIKART et al., 2003; NIELSEN, 2005). O uso de um grande número de SNPs permite a caracterização da diversidade genômica e a investigação genética aprofundada das relações evolutivas entre espécies, como a divergência recente com ou sem fluxo gênico secundário entre populações, ou a ocorrência de hibridação e introgressão entre espécies (ANDERSON et al., 2009). A análise de múltiplos locos, incluindo representação de herança biparental, é necessária para que possa ser realizada uma inferência abrangente sobre os padrões e processos evolutivos atuando sobre o genoma (EIZIRIK, E, JOHNSON, WE AND O'BRIEN; SJ, 2006), o que ressalta o potencial destas novas metodologias neste campo.

Uma abordagem genômica que se beneficiou com a chegada das tecnologias de nova geração é a identificação de genes e mutações que determinam fenótipos e a caracterização dos mecanismos ecológicos e evolutivos subjacentes a esses efeitos. Elucidar a base genética de características funcionalmente importantes tem se tornado mais viável através do uso de estudos de associação em nível genômico (*genome-wide association studies* – GWAS). Este tipo de estudo tem sido empregado em muitos organismos (especialmente humanos) visando a uma melhor compreensão e tratamento de doenças, assim como em plantas para dissecar características complexas relacionadas à adaptação a diferentes ambientes naturais (p.ex. (HIRSCHHORN; DALY, 2005).

Diversos métodos têm sido usados nestes estudos de associação, incluindo o método de genes candidatos (TABOR; RISCH; MYERS, 2002), análises de expressão gênica através de sequenciamento de ESTs (*expressed sequence tags*), hibridização baseada em *microarray*, mapas de ligação de características quantitativas (QTL; SLATE, 2005) e a busca por regiões genômicas sujeitas a pressões seletivas (STORZ, 2005). Dentre os métodos mencionados, o último tem revelado ser promissor e envolve a identificação de regiões cromossômicas sob

pressões seletivas através de padrões de polimorfismos de DNA, medindo seus níveis de diferenciação entre múltiplos locos não-ligados. Além disso, esta abordagem apresenta várias vantagens quando comparada aos métodos baseados em cruzamentos. Primeiro, ele pode ser aplicado a qualquer organismo não-modelo, enquanto os estudos de mapeamento de QTL são restritos as espécies que podem ser cruzadas em laboratório. Segundo, é possível identificar locos que apresentam sinais de um processo de seleção natural antiga e fraca e, por último, o método pode ser usado para identificar locos sob seleção natural sem a prévia informação sobre variação fenotípica.

Embora a caracterização da diversidade genômica tenha progredido significativamente nos últimos anos, o número de estudos testando os efeitos de fenótipos na natureza ainda é incipiente. Uma das limitações destes estudos de associação entre genótipo-fenótipo em espécies selvagens não-modelo é a ausência de conhecimento sobre os níveis de desequilíbrio de ligação (DL) (BACKSTRÖM et al., 2006). Em teoria, os níveis esperados de DL entre os alelos são dados pela idade da mutação e a taxa de recombinação entre os locos (STUMPF; MCVEAN, 2003). Quando o DL se estende ao longo de amplas regiões genômicas, ocorre uma maior chance de se descobrir uma associação entre um gene envolvido em algum determinado fenótipo e um marcador molecular. Um exemplo clássico do uso destes testes de associação com mapeamento de DL é o de genes do complexo maior de histocompatibilidade (MHC) associados com resposta imune em uma ampla diversidade de organismos (BERNATCHEZ; LANDRY, 2003; GARRIGAN; HEDRICK, 2003).

A partir da expressiva aplicação das novas tecnologias de sequenciamento em estudos genômicos como mencionado acima, torna-se evidente o potencial destas em auxiliar na identificação dos mecanismos genéticos e processos evolutivos envolvidos na determinação do fenótipo melânico em organismos não modelo como os felídeos. Neste sentido, o presente estudo se propôs a iniciar o uso deste tipo de abordagem neste grupo, como será descrito no Capítulo III.

6. Seleção natural investigada a partir de marcadores moleculares

A convergência fenotípica entre populações e espécies sob pressões ambientais semelhantes fortemente sugere que essas características evoluíram por seleção natural (HARVEY; PAGEL, 1991). Tal raciocínio apresenta um tema de investigação bastante interessante do ponto de vista da evolução do melanismo, especialmente em grupos nos quais este fenótipo surgiu múltiplas vezes de forma independente, como é o caso da família Felidae.

Assim sendo, o estudo da evolução desta característica neste grupo pode representar um modelo bastante rico no âmbito da investigação mais ampla do papel da seleção natural na ocorrência de convergência evolutiva envolvendo fenótipos polimórficos.

A hipótese de evolução molecular neutra propõe que a maioria da variabilidade genética contida em populações naturais é devida a mutações neutras (KIMURA, 1968). Sob evolução neutra, novas mutações requerem um tempo suficientemente longo para alcançar altas frequências na população, e o desequilíbrio de ligação (DL) ao redor da nova mutação irá decair substancialmente durante este período devido à taxa de recombinação. A mudança nas frequências alélicas de uma população sob evolução neutra é devida ao efeito aleatório da deriva genética (KIMURA, 1991). Entretanto, em situações reais a seleção natural também pode contribuir para a mudança na frequência de alelos sendo, no entanto, devida à adaptação diferencial dos organismos aos seus respectivos ambientes.

A seleção natural atua em três modos: (i) direcional ou positiva, diminuindo a variabilidade genética ao favorecer um alelo vantajoso; (ii) purificadora ou negativa, eliminando alelos deletérios; e (iii) balanceadora, a qual mantém a variação genética no loco através de três possíveis mecanismos (vantagem do heterozigoto, seleção dependente de frequência, ou seleção espacialmente heterogênea). Cada um destes tipos de seleção opera mudando as frequências alélicas e deixando assinaturas específicas na variação genômica adjacente ao loco afetado. As assinaturas dependem do tipo, idade e da força destes eventos seletivos.

Quando a seleção positiva leva uma mutação vantajosa à fixação ou ao aumento significativo de sua frequência na população, ocorrerá um decréscimo na variabilidade de alelos neutros em locos ligados à mesma, e o valor de desequilíbrio de ligação entre estes sítios aumentará substancialmente (MAYNARD SMITH; HAIGH, 1974; STEPHAN; WIEHE; LENZ, 1992). A eliminação (ou redução) de variação neutra em regiões adjacentes e ligadas a um alelo com vantagem seletiva é conhecida como varredura seletiva (*selective sweep*, MAYNARD SMITH; HAIGH, 1974), sendo observada usualmente em regiões sobre seleção positiva recente.

A busca por regiões alvo de seleção natural positiva e recente é de interesse não só para elucidar questões relacionadas à evolução de populações e espécies (ANDERSON et al., 2009; CHAN et al., 2010; SABETI et al., 2002; SCHLÖTTERER, 2002), mas também por aumentar as evidências de seleção atuando sobre genes que apresentam alguma vantagem adaptativa ao ambiente (ANDERSON et al., 2009; KAYSER; BRAUER; STONEKING, 2003; VASEMÄGI; PRIMMER, 2005) ou que estejam potencialmente associados a doenças e

a resistência de drogas ou pesticidas (CATANIA et al., 2004; CLARK et al., 2003; KOHN; PELZ; WAYNE, 2000; NAIR et al., 2002), ou ainda em casos de domesticação (PALAISA et al., 2004; RUBIN et al., 2010; VIGOUROUX et al., 2002). Detectar varreduras seletivas a partir de dados genômicos é complicado e desafiador, pois os efeitos de seleção podem ser confundidos com os efeitos de fatores demográficos que causam impactos semelhantes nas frequências alélicas de uma população. A fim de discerni-los, é necessário analisar os padrões de variabilidade genética em múltiplos locos não-ligados, uma vez que a premissa de métodos multi-locos é a de que os processos demográficos terão efeitos uniformes por todo o genoma, enquanto que os efeitos de seleção são locos-específicos e podem ser inferidos a partir de padrões de variação em locos ligados (LEWONTIN; KRAKAUER, 1973).

Diversos métodos são utilizados para detectar sinais de seleção natural em nível molecular. Os testes estatísticos tradicionais incluem D de Tajima (TAJIMA, 1989), F de Fu & Li (FU; LI, 1993), H de Fay & Wu (FAY; WU, 2000), McDonald & Kreitman (MCDONALD; KREITMAN, 1991) e Hudson-Kreitman-Aguadè (HKA; HUDSON; KREITMAN; AGUADE, 1987). A premissa destes testes de neutralidade assume uma população em equilíbrio e sob um regime de evolução neutra. Assim, qualquer desvio da neutralidade teórica sugere que a população sofreu influências de processos demográficos e/ou seletivos. Entretanto, estes testes parecem ser menos sensíveis em detectar efeitos de seleção mais recente quando comparados a métodos que analisam a estrutura haplotípica através de medidas de DL de um alelo em função da distância, como a abordagem definida como *extended haplotype homozygosity* (EHH, SABETI et al., 2002). A EHH consiste na probabilidade em que dois cromossomos carregando um alelo de interesse são escolhidos randomicamente e apresentam um excesso de homozigosidade ao longo da estrutura haplotípica (SABETI et al., 2002). Desta forma, haplótipos com longos EHH e com alta frequência na população indicam a presença de uma mutação recente e positivamente selecionada. Em um estudo sobre a história evolutiva do melanismo em lobos-cinza da América do Norte (ANDERSON et al., 2009), a construção de haplótipos foi baseada em 36 SNPs distribuídos ao longo de uma região cromossômica de aproximadamente 150 kb. As análises revelaram um EHH de cerca de 60 kb ao redor do alelo mutante em indivíduos melânicos, com os haplótipos alcançando altas frequências em ambientes de florestas e revelando, portanto, evidências moleculares de seleção positiva e recente.

A redução de variabilidade genética em locos ligados localizados em regiões de baixa recombinação pode também ser explicada pela seleção do tipo purificadora. Este tipo de seleção consiste na eliminação de alelos deletérios que surgem por mutação

(CHARLESWORTH; MORGAN; CHARLESWORTH, 1993) e, da mesma forma que a seleção positiva, causa a redução na variação genética de alelos neutros ligados ao loco focal. Enquanto a seleção positiva leva uma mutação vantajosa à fixação através do seu aumento significativo de frequencia, a seleção purificadora constantemente remove alelos deletérios impedindo que estes se fixem na população. Assim, alelos neutros ligados aos mesmos também são eliminados, causando um excesso de alelos ancestrais na população. No entanto, alelos deletérios podem fixar-se devido à deriva genética, ou podem ser negativamente selecionados, mas permanecer em baixas frequências no *pool* gênico. É o caso de muitos alelos recessivos causadores de doenças. Outra característica relevante deste tipo de situação é que os genes mais sujeitos à seleção purificadora tendem a apresentar reduzidas taxas de mutação não-sinônima e, portanto, são mais conservados entre diferentes espécies.

Ao contrário, a ocorrência de uma força seletiva mantendo a diversidade genética na população é conhecida como seleção balanceadora, que atua mantendo dois ou mais alelos em frequências similares no *pool* gênico. Quando estes diferentes alelos persistem por longos períodos na população, a seleção balanceadora mantém a diversidade genética do loco sofrendo os efeitos da seleção e aumenta a diversidade em locos ligados a este (que acumulam mutações e sofrem menor ação da deriva genética). Portanto, um excesso de polimorfismo fornece evidências de seleção balanceadora (CHARLESWORTH, 2006). Porém, detectar estes sinais de seleção balanceadora de longo prazo é difícil, uma vez que a seleção é específica em regiões genômicas e pode ser confundida devido às taxas de recombinação (CHARLESWORTH; NORDBORG; CHARLESWORTH, 1997).

Existem três formas em que a seleção balanceadora pode atuar, como mencionado acima. No primeiro, o genótipo heterozigoto possui valor adaptativo mais alto do que os dois genótipos homozigotos; no entanto, esta forma foi documentada até o momento em poucos casos, como por exemplo, o da anemia falciforme em seres humanos (PASVOL; WEATHERALL; WILSON, 1978). Já a seleção dependente de frequência ocorre quando o valor adaptativo de um alelo é afetado por sua frequência na população, ou seja, o fenótipo mais comum é desfavorecido. Um exemplo clássico deste tipo de seleção é o dos peixes ciclídeos comedores de escamas demonstrado pelo comportamento de fuga da presa ao perceber a aproximação do predador (HORI, 1993). Como a frequência de dois fenótipos oscila em uma dada população, indivíduos de fenótipo raro têm mais sucesso do que os de fenótipo mais comum. Desta forma, o alelo, ao aumentar sua frequência, diminui seu valor adaptativo. Por fim, pode haver seleção espacialmente heterogênea, em que alelos distintos apresentam vantagem adaptativa em ambientes adjacentes e conectados. O fluxo gênico entre

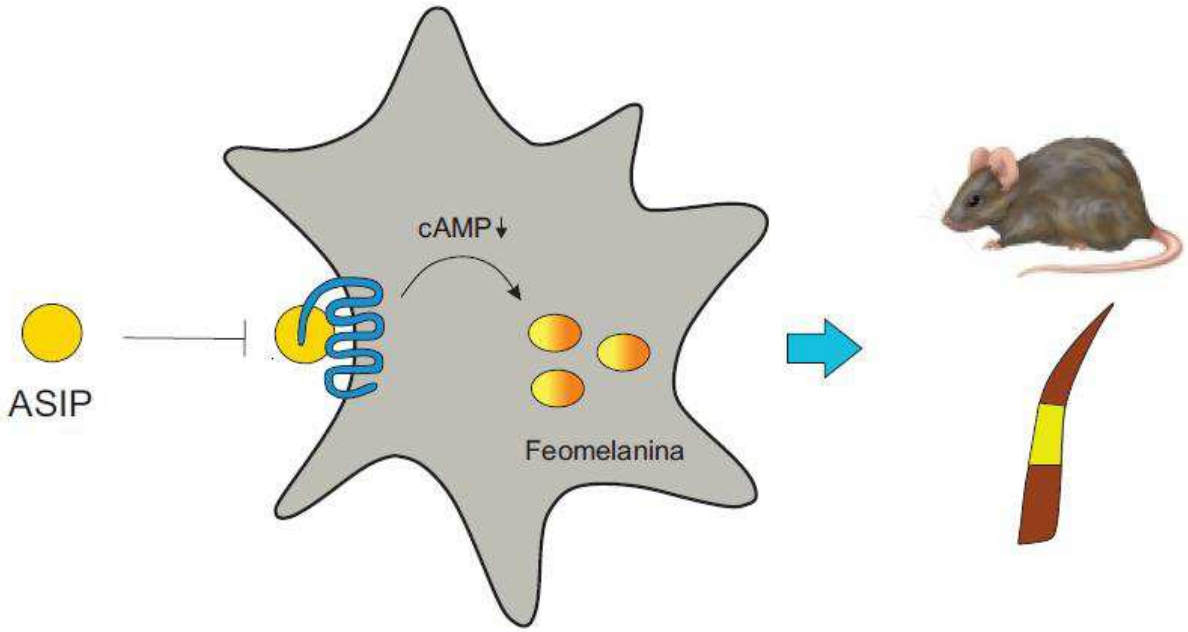
os dois ambientes mantém a população geneticamente coesa e ambos os alelos segregando em frequências similares, devido à pressão seletiva distinta que favorece cada um deles em uma parte da distribuição.

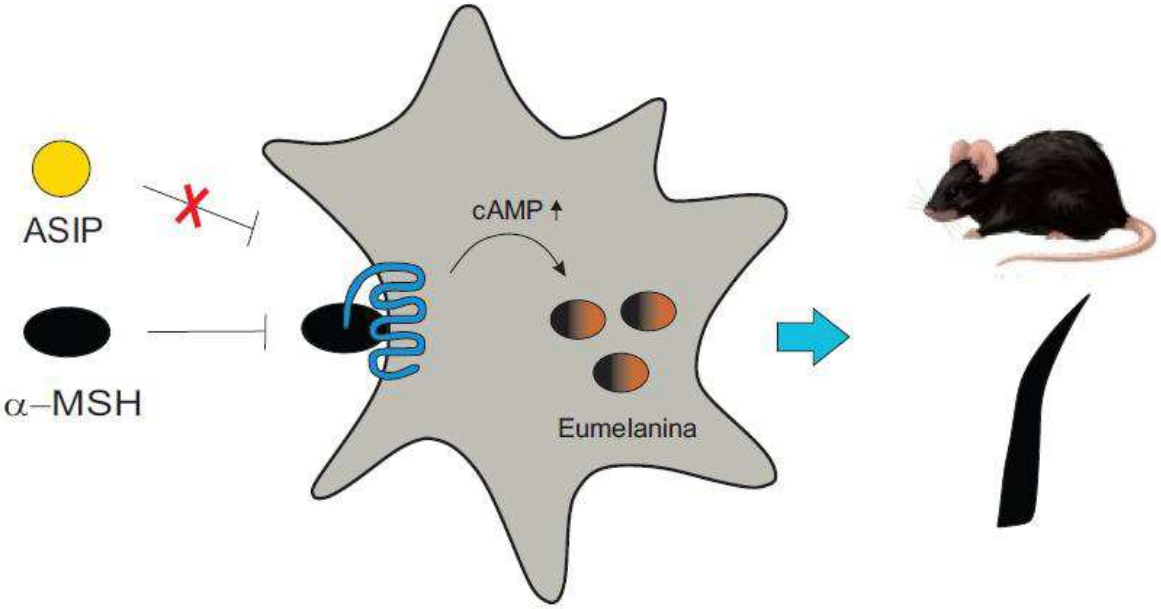
As inferências moleculares podem ser úteis no estudo sobre seleção natural e adaptação. Isto porque as sequências de DNA são registros informativos sobre sua própria história evolutiva. O sequenciamento de genomas completos combinado com uma crescente caracterização da variabilidade em nível genômico intra- e interespecífico de múltiplos grupos de organismos, permite que estimativas cada vez melhores sejam realizadas sobre os processos históricos atuando sobre as populações, o que facilita a investigação de assinaturas de seleção sobre um determinado loco. À medida que mais genes envolvidos em fenótipos sejam descritos e sua diversidade genética analisada, maior será nossa compreensão sobre o impacto da seleção natural na história evolutiva de características observadas em populações naturais. Neste contexto, o presente estudo se propõe a investigar a evolução do melanismo em felídeos, incluindo o uso de abordagens genômicas para analisar a ocorrência de seleção natural afetando a dinâmica histórica deste fenótipo em populações naturais.

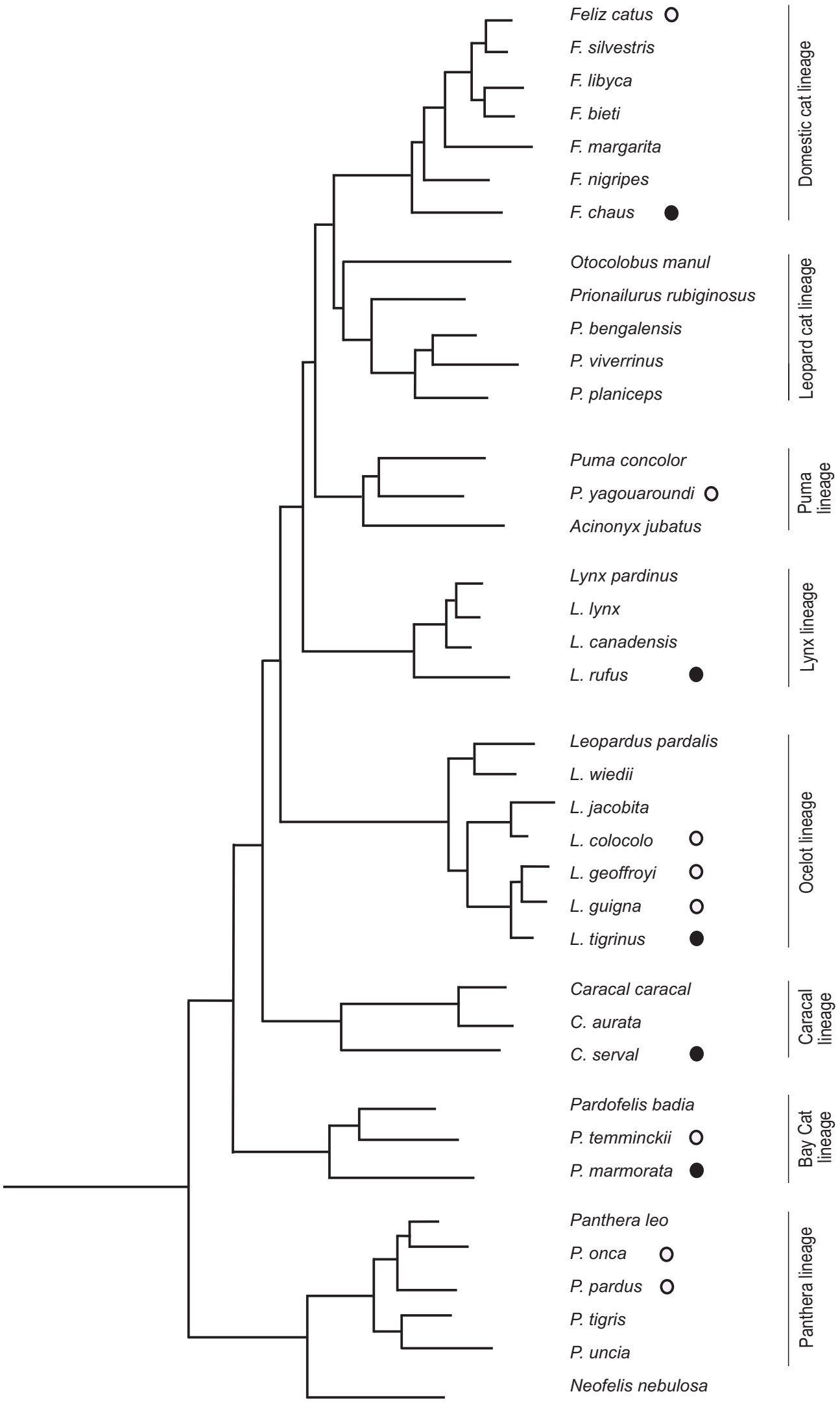
LEGENDA DAS FIGURAS

Figura 1. O gene *MC1R* codifica um receptor acoplado a proteína G contendo sete hélices transmembrana, que é expresso em melanócitos da pele, folículo de pelos e em células do sistema imune. Ao se ligar ao hormônio estimulante de melanócito (α-MSH), o MC1R ativa a síntese de AMP cíclico (cAMP) intracelular induzindo a síntese de eumelanina (pigmento escuro: preto, marrom). Ao contrário, a ativação do MC1R é inibida pelo ASIP, um peptídeo parácrino produzido no folículo de pelos e que se comporta como antagonista ao MC1R, impedindo sua ativação pelo α-MSH e, assim, induzindo a troca da síntese de eumelanina para feomelanina (pigmento claro: amarelo, avermelhado).

Figura 2. Filogenia da família Felidae mostrando a ocorrência de melanismo. Os círculos ao lado do nome de cada espécie indicam as 13 espécies de felinos com evidências confirmadas de melanismo. Círculos abertos indicam as oito mutações associadas com o fenótipo já identificadas neste e em estudos prévios, enquanto que os círculos pretos são as espécies em que ainda não se conhece a base molecular do fenótipo melânico.

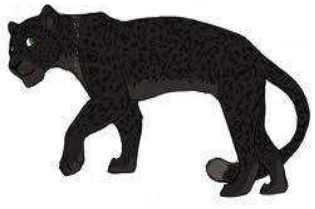

cAMP↓
ASIP
Feomelanina


cAMP↑
ASIP
α–MSH
Eumelanina


Feliz catus
F. silvestris
F. libyca
F. bieti
F. margarita
F. nigripes
F. chaus
Domestic cat lineage
Otocolobus manul
Prionailurus rubiginosus
P. bengalensis
P. viverrinus
P. planiceps
Leopard cat lineage
Puma concolor
P. yagouaroundi
Acinonyx jubatus
Puma lineage
Lynx pardinus
L. lynx
L. canadensis
L. rufus
Lynx lineage
Leopardus pardalis
L. wiedii
L. jacobita
L. colocolo
L. geoffroyi
L. guigna
L. tigrinus
Ocelot lineage
Caracal caracal
C. aurata
C. serval
Caracal lineage
Pardofelis badia
P. temminckii
P. marmorata
Bay Cat lineage
Panthera leo
P. onca
P. pardus
P. tigris
P. uncia
Neofelis nebulosa
Panthera lineage

CAPÍTULO II:

1º ARTIGO CIENTÍFICO



How the Leopard Hides its Spots: *ASIP* Mutations and Melanism in Wild Cats

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How the Leopard Hides Its Spots: *ASIP* Mutations and Melanism in Wild Cats

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Abstract

The occurrence of melanism (darkening of the background coloration) is documented in 13 felid species, in some cases reaching high frequencies at the population level. Recent analyses have indicated that it arose multiple times in the Felidae, with three different species exhibiting unique mutations associated with this trait. The causative mutations in the remaining species have so far not been identified, precluding a broader assessment of the evolutionary dynamics of melanism in the Felidae. Among these, the leopard (*Panthera pardus*) is a particularly important target for research, given the iconic status of the 'black panther' and the extremely high frequency of melanism observed in some Asian populations. Another felid species from the same region, the Asian golden cat (*Pardofelis temminckii*), also exhibits frequent records of melanism in some areas. We have sequenced the coding region of the *Agouti Signaling Protein* (*ASIP*) gene in multiple leopard and Asian golden cat individuals, and identified distinct mutations strongly associated with melanism in each of them. The single nucleotide polymorphism (SNP) detected among the *P. pardus* individuals was caused by a nonsense mutation predicted to completely ablate ASIP function. A different SNP was identified in *P. temminckii*, causing a predicted amino acid change that should also induce loss of function. Our results reveal two additional cases of species-specific mutations implicated in melanism in the Felidae, and indicate that *ASIP* mutations may play an important role in naturally-occurring coloration polymorphism.

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Introduction

Melanism is a remarkable polymorphic phenotype observed in multiple animal groups, whose occurrence may be influenced by differential adaptation to varying environments or to distinct inter-specific interactions [1–3]. In the cat family (Felidae), melanism is quite common, having been reported in 13 of 37 extant species (Table 1). Although such darkened pelage reaches considerably high frequencies in some cat species [4], supporting the notion that this phenotype may be adaptive in some contexts, still little is known about its evolutionary history and ecological/behavioral significance in any felid. Initial molecular analyses have revealed that melanism arose multiple times in the Felidae, with three different mutations being implicated in this phenotype in distinct species [5].

As is the case in other vertebrates [1,6], felid melanism was found to be influenced by two different genes whose products interact in the regulation of melanin production. Eumelanin (dark pigment) is produced when the Melanocortin-1 receptor (MC1R) is activated by the binding of Alpha Melanocyte Stimulating Hormone (α-MSH). In contrast, MC1R activation is inhibited by the binding of the antagonist peptide ASIP (Agouti Signaling Protein), whose action leads to a switch to pheomelanin (light pigment) synthesis [2,7,8]. Therefore, gain of function in MC1R or loss of function in ASIP induce melanism. In felids, both genes were found to be implicated, with *MC1R* variants underlying melanistic phenotypes in two different wild cat species (*Panthera onca* and *Puma yagouaroundi*), and a mutation in *ASIP* inducing black color in domestic cats [5].

Since that initial study, no additional mutation involved in melanism has been identified in any of the remaining felid species exhibiting this trait, hampering a broader assessment of its evolutionary history and adaptive significance. Such lack of knowledge is remarkable, as it extends to well-known and iconic animals such as the 'black panther', the melanistic form of the leopard (*Panthera pardus*) that is very common in some regions of southeastern Asia and often seen in zoos and museums. Other wild cats exhibiting melanism are less known, and the molecular analysis of melanism-inducing mutations would provide relevant

Table 1. Available information on the occurrence of melanism in felid species.

Species	Strongest evidence and original references	Proposed mode of Inheritance	No. of offspring analyzed in the original literature source
Felis catus	**Visual** [30,31]	**Recessive** [5,30,31]	**1 black offspring from a pair of wild type parents** [30,31]
Felis chaus	**Photograph** [32]	**Dominant** [32]	**1 wild-type offspring from a pair of melanistic parents** [32]
Felis silvestris, F. lybica	Anecdotal [32,33]	-	-
Prionailurus bengalensis	Anecdotal [34,35]	-	-
Panthera pardus	**Visual** [36,37]	**Recessive** [36,37]	**Total of 439 offspring** [36,37]
Panthera onca	**Visual** [32]	**Dominant** [5,32]	**Total of 81 offspring** [32]
Panthera leo	Anecdotal [32]	-	-
Panthera tigris	Anecdotal [34,38]	-	-
Panthera uncia	Anecdotal [39]	-	-
Neofelis nebulosa	Anecdotal [40,41]	-	-
Lynx rufus	**Photograph** [34]	-	-
Leopardus geoffroyi	**Visual** [42]	-	-
Leopardus guigna	**Photograph** [32,43,44,45]	-	-
Leopardus tigrinus	**Visual** [32,46]	-	-
Leopardus colocolo	**Photograph** [32]	**Recessive** [32]	**2 black offspring from a pair of wild-type parents** [32]
Acinonyx jubatus	Anecdotal [40,47]*	-	-
Puma concolor	Anecdotal [48]	-	-
Puma yagouaroundi	**Visual** [5]	**Co-dominant** [5]	-
Leptailurus serval	**Video** [33,34,39]	**-**	**-**
Caracal caracal	Anecdotal [34]	-	-
Caracal aurata	Anecdotal [49]	-	-
Pardofelis temminckii	**Photograph** [34,35]	**Recessive****	**-**
Pardofelis marmorata	**Photograph** [50]	**-**	**-**

Bold types indicate species for which reliable evidence of melanism exists (including direct visual observation by E.E., photograph, or video). Numbers refer to bibliographic sources (see References).
*Reference to melanism is not explicit.
**Based on results from this study.
doi:10.1371/journal.pone.0050386.t001

insights into even basic aspects of the biology of this polymorphic phenotype in the wild.

In this study we report two novel mutations associated with melanism in wild felids, demonstrating that this mutant phenotype arose at least five times independently in the cat family. We show that two different variants of the *ASIP* gene are implicated in melanistic phenotypes in the leopard and in the Asian golden cat (*Pardofelis temminckii*). We discuss these findings in the context of the evolution of melanism, as well as the relative roles of *ASIP* and *MC1R* in the origin of such pigmentation variants.

Materials and Methods

Ethics statement

Biological samples used in this study were available in the tissue collection held at the Laboratory of Genomic Diversity, National Cancer Institute, National Institutes of Health (USA), having been collected previously in the context of collaborations with the South East Asian Zoological Park and Aquarium Association (SEAZA), the Chinese Association of Zoological Gardens (CAZG) and multiple captive breeding institutions from several countries (listed on Table 2). The purpose of those collaborations was to collect biological materials from a representative sample of Southeast Asian wild felids to allow studies on their taxonomy, genetics, evolution, and epidemiology, whose results would be incorporated into the design and implementation of conservation strategies on behalf of these species. Samples were collected by trained and certified veterinarians in the course of general health check-ups, following protocols approved by the scientific and/or ethics committees of each captive breeding institution. After collection, samples were imported into the USA under CITES permit number 12US694126/9, issued to the Laboratory of Genomic Diversity, National Institutes of Health, USA.

Methods

The study was performed on the basis of biological material (blood or skin samples) of *P. pardus* and *P. temminckii* collected from captive animals of Asian origin (Table 2). In order to minimize any impact of population structure on the association studies, we strived to only include samples that were originated from the same geographic region or nearby locations for each of the species.

DNA extraction from all samples was performed using standard phenol/chloroform protocols [9–11]. To identify potential molec-

Table 2. Samples of *Panthera pardus* and *Pardofelis temminckii* included in the present study, including their respective genotypes for *ASIP*.

Sample ID[a]	Origin	Institution/Contact	Coat Color	*ASIP*	
				Genotype	**positions**
				333	384
Ppa-221	Jenderak, Malaysia	Melaka Zoo, Malaysia	Melanistic	A/A	C/C
Ppa-222	Negeri Sambilay, Malaysia	Melaka Zoo, Malaysia	Melanistic	A/A	C/C
Ppa-223	Perak, Malaysia	Melaka Zoo, Malaysia	Melanistic	A/A	C/C
Ppa-224	Jenderak, Malaysia	Melaka Zoo, Malaysia	Melanistic	A/A	C/C
Ppa-225	Dungun, Malaysia	Melaka Zoo, Malaysia	Melanistic	A/A	C/C
Ppa-227	Taiping, Malaysia	Taiping Zoo/Kevin Lazarus	Melanistic	A/A	C/C
Ppa-228	Taiping, Malaysia	Taiping Zoo/Kevin Lazarus	Melanistic	A/A	C/C
Ppa-230	Pehang Pekan, Malaysia	Negara Zoo	Melanistic	A/A	C/C
Ppa-231	Johor, Malaysia	Negara Zoo	Melanistic	A/A	C/C
Ppa-284	Guamurang, Malaysia	Khao Kheow Open Zoo	Melanistic	A/A	C/C
Ppa-288	Chiangmai Zoo, Thailand	Warren Johnson	Melanistic	A/A	C/C
Ppa-277	Probably Thailand	Khao Kheow Open Zoo	Wild-type	C/A	C/C
Ppa-283	Probably Thailand	Khao Kheow Open Zoo	Wild-type	C/C	C/C
Ppa-285	Chonburi, Thailand	Khao Kheow Open Zoo	Wild-type	C/C	C/C
Ppa-286	Chonburi, Thailand	Khao Kheow Open Zoo	Wild-type	C/C	C/C
Pte-038	Bangkok, Thailand	Dusit Zoo	Melanistic	C/C	G/G
Pte-051[b]	Yunnan, Ruili Region, China	Kunming Zoo	Melanistic	C/C	G/G
Pte-052[b]	Gansu Province, Tianshui Region, China	Lanzhou Zoo	Wild-type	C/C	C/C
Pte-053[b]	Gansu Province, Tianshui Region, China	Lanzhou Zoo	Wild-type	C/C	C/C

Melanistic individuals are highlighted in bold.
[a]Code names indicate species identification of each sample: Ppa = *Panthera pardus*; Pte = *Pardofelis temminckii*.
[b]Individuals shown in Figure 2: Pte-051 in panel E, Pte-052 in panel D and Pte-053 in panel C.
doi:10.1371/journal.pone.0050386.t002

ular variants associated with melanistic coat color in these species we characterized the candidate gene *ASIP*. The coding region of the gene was amplified by PCR (*Polymerase Chain Reaction*; [12]) from each sample, using primers designed with the software Primer3 (http://frodo.wi.mit.edu; see Table S2 for primer sequences) [13] on the basis of the domestic cat genomic sequence (U. *California - Santa Cruz*, http://genome.ucsc.edu/; *GARFIELD*, http://lgd.abcc.ncifcrf.gov/cgi-bin/gbrowse/cat/). PCR reactions for *ASIP* exon 2 and exon 3 were performed in a 10 μL final volume containing 2.0 mM $MgCl_2$, 0.2 mM dNTPs, 0.5 U of AmpliTaq Gold DNA polymerase (Applied Biosystems), 0.2 μM each of the forward and reverse primers, and 10 ng of DNA. Thermal cycling used a touchdown profile with the annealing temperature decreasing from 60°C to 51°C in 10 cycles, followed by 30 or 40 cycles with annealing at 50°C for exons 2 and 3, respectively. Amplification of *ASIP* exon 4 was carried out with Takara LA Taq with GC Buffer (Takara Bio Inc.), following the guidelines provided by the manufacturer and the same thermal cycling conditions as exon 3.

PCR products were purified with Exonuclease I and Shrimp Alkaline Phosphatase, and sequenced for both strands using BigDye chain terminator chemistry (Applied Biosystems). Sequencing products were purified using Sephadex G-50 plates and analyzed with an ABI 3700 automated DNA sequencer. All resulting sequences were analyzed with Sequencher 4.2 (GeneCodes Corporation, Ann Arbor, MI), and every polymorphism was carefully inspected for confirmation. Nucleotide and amino acid sequences of *ASIP* were aligned with multiple mammalian homologs using ClustalW (http://www.ebi.ac.uk/Tools/msa/clustalw2/), with alignments being subsequently inspected and verified by hand. The DNA sequences reported here have been deposited in GenBank (accession numbers JX845175-JX845178).

Results and Discussion

Identification of *ASIP* mutations

Sequencing of the coding region of *ASIP* revealed that it was highly conserved within each species, with all individuals exhibiting an identical sequence except for a single nucleotide site (Figures 1 and S1). The single nucleotide polymorphism (SNP) detected among the *P. pardus* individuals was caused by a non-synonymous mutation located in exon 4 (C333A) predicted to introduce a stop codon at amino acid position 111. All 11 analyzed melanistic leopards (Figure 2) were homozygous for this mutation, while the wild-type individuals (i.e. bearing a yellowish background coloration with black rosettes; see Figure 2) were either homozygous for the ancestral 'A' allele or heterozygous. This finding reveals a significant association between melanism and a homozygous AA genotype ($\chi^2 = 14.95$, d.f. = 1, $p<0.005$), which is consistent with a recessive mode of inheritance of this trait in leopards, as suggested by previous breeding studies performed in captivity (Table 1).

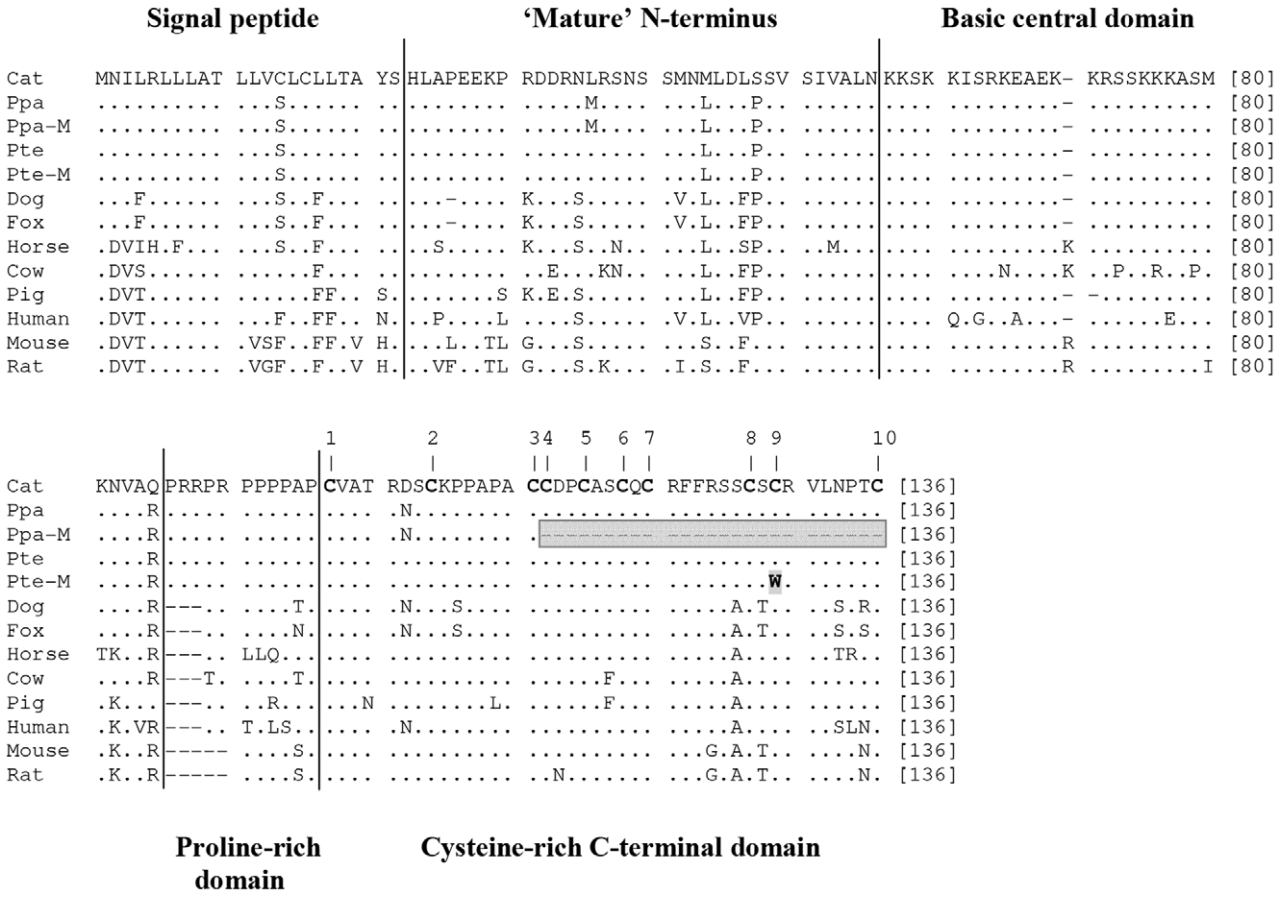

Figure 1. Amino acid alignment of ASIP, including the novel *Panthera pardus* and *Pardofelis temminckii* sequences. Wild-type and melanistic sequences of each wild cat species are shown. Dots indicate identity to the top sequence; amino acid positions are shown at the end of each line. Vertical lines demarcate the boundaries among the five functional domains proposed for ASIP ([17]), named above or below the sequences. Dashes represent insertion/deletion (indel) variants. Numbers 1–10 refer to the 10 conserved cysteine residues present in the C-terminal domain. The premature stop codon in melanistic *P. pardus* is shaded (dashes indicate deleted sites). The non-synonymous mutation in melanistic *P. temminckii* is indicated in bold and shaded as well.
doi:10.1371/journal.pone.0050386.g001

A different SNP was identified in exon 4 of *P. temminckii*. The ancestral allele was identified by comparison to sequences from other species, and consists of a 'C' at position 384 (see Figure S1). The mutant allele derives from a non-synonymous substitution (C384G) predicted to cause a cysteine-tryptophan substitution at codon 128 (see Figure 1). This mutant allele was perfectly associated with black coat color in the Asian golden cat ($\chi^2 = 4.00$, d.f. = 1, $p<0.05$). The melanistic individuals (n = 2; see Figure 2E) were homozygous for the mutant allele, whereas two non-melanistic animals (one of which was plain agouti-colored and the other bearing dark rosettes; see Figure 2C, 2D) were homozygous for the ancestral allele. Given that *ASIP*-associated melanism is always inherited as a recessive trait [14,15] we can infer that this is the mode of inheritance in Asian golden cats, as observed in leopards and also in domestic cats (see Table 2). As *P. temminckii* has been the focus of very few genetic studies, so far the inheritance mode of this prominent coloration polymorphism had remained unknown for this species.

Comparative analysis of *ASIP* variation

We aligned our *ASIP* coding sequences to those generated previously for other mammals (see Table S1). The alignment consisted of 408 bp (136 codons) that exhibited heterogeneous patterns of variation. Some sites were highly conserved across mammals, whereas other segments were quite variable at the nucleotide and amino acid levels (see Figure 1 and Figure S1). A highly variable region, including multiple substitutions as well as insertion/deletion (indel) sites, was located between nucleotide coding positions 240 and 290, at the boundary between the basic (lysine-rich) and proline-rich central domains. At the amino acid level, this region was also considerably variable, but even higher diversity was observed in portions of the signal peptide and the mature N-terminus. Such variation may be due to relaxation of functional constraints in these regions, or to diverging selective pressures across lineages. Testing these hypotheses would help understand the historical pressures shaping *ASIP* diversity in mammals, and could be accomplished with structural and molecular evolutionary analyses targeting these particular regions of the gene.

In contrast to these highly variable segments, some regions were quite conserved across mammals, including sites that have remained identical in all the species sampled so far (Figure 1 and Figure S1). Some conserved amino acid sites are particularly noteworthy, as they have been the subject of direct experimentation assessing their functional relevance [16,17]. All the amino acid residues in which replacements have been experimentally shown to cause loss or decrease of *ASIP* function are completely

Figure 2. Coat color phenotypes of the leopard (*Panthera pardus*) (top) and Asian golden cat (*Pardofelis temminckii*) (bottom). (A) Typical non-melanistic leopard individual. (B) Melanistic leopard or 'black panther'. (C, D, E) Polymorphic coat color of *P. temminckii*: (C) plain agouti with few markings; (D) tan background with dark rosettes; (E) melanistic phenotype. The individuals shown in C, D and E were actually typed in this study (see Table 2). Photo credits to Kae Kawanishi (A), Bruce Kekule (B), Warren Johnson and Sujin Luo (C, D, E).
doi:10.1371/journal.pone.0050386.g002

conserved across mammals. In particular, these experiments revealed that non-synonymous mutations involving each of the 10 cysteine residues of the C-terminal Cys-rich domain negatively affected ASIP activity. Eight out of 10 substitutions (at cysteine sites 1–4 and 6–9 (see Figure 1)) abolished ASIP activity, while two others (at sites 5 and 10) resulted in partial loss of protein function. Therefore, these cysteine residues were found to be critical for protein activity and receptor binding [16–19].

Such direct experimental evidence facilitates the interpretation of novel mutations affecting some of these conserved residues. The amino acid change associated with melanism in *P. temminckii* affects the 9th conserved cysteine residue (see Figure 1), which was shown in mice to be required for ASIP function, and whose loss led to melanism [16]. Even stronger impacts are expected from mutations that induce stop codons in this region, as they can remove more than a single conserved cysteine residue. In mice, a mutation affecting the 5th cysteine introduced a stop codon that led to a null phenotype [17], while mutations inducing premature stop codons (also removing conserved cysteines) in other species were associated with melanistic phenotypes as well [5,20]. In this context, the mutation identified in black leopards is inferred to have a substantial functional impact, eliminating most of the C-terminal domain, from the 4th conserved cysteine onward. Overall, these observations reinforce our inference that both mutations detected in wild cats are likely to cause melanism due to loss of ASIP function.

Melanism Evolution in the Felidae

Although it is often difficult to demonstrate a clear association between coat color polymorphism and SNP variation [21,22], there have been several examples of success in identifying mutations implicated in melanism. In almost every case they were variants of the *ASIP* or *MC1R* genes, which were associated with darkened phenotypes in domestic and wild populations [6,23–25]. In this context, a particular group that has been found to harbor species-specific mutations in these genes that are strongly associated with melanism is the family Felidae.

Our present results reveal two novel mutations implicated in melanism in felids. Taken together with the previous findings reporting three additional mutations [5], we conclude that this mutant phenotype arose at least five times independently in the cat family. Interestingly, three of these mutations are located in *ASIP*, indicating that this gene is equally or more often involved in felid melanism than *MC1R*.

This observation contrasts with the view that *MC1R* is more frequently implicated in melanism than *ASIP* [1,8]. Kingsley et al. [20] have hypothesized that the perceived higher frequency of *MC1R*-induced melanism in natural populations, relative to *ASIP*-induced darkening, may be due to either lower pleiotropic effect of mutations in the former, or to differential effects of natural selection on variants of each gene. Given current knowledge on their biology, it is unclear whether *ASIP* mutations would have substantially more pleiotropic effects than those in *MC1R*. In effect, the *ASIP* coding region is quite variable across taxa (see Figure 1), suggesting that functional constraints on this gene are not very stringent. Additional functional studies are thus required to assess in more detail the pleiotropic effects of both loci. In addition, it remains possible that, due to lineage-specific genetic features, *ASIP* mutations are less affected by pleiotropic effects in felids, allowing this gene to be less constrained and thus more often involved in melanistic phenotypes. This hypothesis can be tested by investigating differential patterns of expression and activity of *ASIP* in felids relative to other groups.

Another interesting aspect pertains to the relevance of regulatory vs. coding mutations in the context of *ASIP*-induced melanism. Although it has been proposed that *ASIP*-related melanism is more often caused by regulatory mutations [8,21], our results show a high incidence of coding mutations leading to pelage darkening in felids. Again, this may be a consequence of felid-specific changes in the pleiotropic effect of *ASIP* mutations, which is likely stronger when the coding region is affected [20]. Remarkably, the three different *ASIP* mutations found so far to induce melanism in felids seem to cause complete loss of gene function, and might therefore induce strong pleiotropic effects. Nevertheless, there is so far no evidence of pleiotropic effects associated with melanism in domestic or wild felids, suggesting that loss of ASIP function only affects pigmentation, or can be compensated in other systems by the activity of other proteins.

The second hypothesis raised by Kingsley et al. [20] to explain the apparent difference in *ASIP vs. MC1R* involvement in melanism pertains to differential effects of natural selection on these loci. Since melanism is dominant when induced by *MC1R*, it is more easily detected by natural selection, and would more quickly rise in frequency when favorable. On the other hand, *ASIP*-induced melanism is recessive, and would thus take more time to rise in frequency when favorable, but also linger in the population for a longer period when negatively selected. Kingsley et al. [20] thus hypothesized that *MC1R*-induced melanism would be prevalent when this trait is adaptive, but *ASIP*-induced darkening might be expected when the trait is deleterious. This would more often occur when melanism is present at low frequencies, as was the case in the *Peromyscus* populations analyzed by Kingsley et al. [20]. In contrast, *ASIP*-induced melanism can reach very high frequency in some felid populations, suggesting that this trait may be adaptive or at least neutral.

Such a pattern is particularly noticeable in the case of leopards from the Malay Peninsula, where melanism approaches fixation [4]. Using samples from this very region (see Table 2), we show here that *ASIP* is implicated in this mutant phenotype. Although we have shown that this near fixation may have been caused by genetic drift over a long period of time [4], this would be very unlikely if the trait was deleterious. Moreover, such high frequency would be much more quickly achieved if the trait was favorable, and therefore driven to near fixation by natural selection. The identification of the molecular basis of this phenotype now opens up new avenues to investigate its evolutionary history and adaptive significance in the wild.

Another interesting point regarding leopard melanism is the observation that black rosettes are still visible in spite of the much darkened background coloration (see Figure 2B). This indicates that rosettes are still darker than the essentially black background, and are not obliterated by the melanism-inducing mutation. Such observation supports the hypothesis that pattern formation on mammalian coats is induced by two separate processes, encompassing considerably more complexity than the well-established ASIP-MC1R interplay [26–28]. Although it could be hypothesized that localized differences in ASIP and/or MC1R expression/function could induce the presence of spots/stripes on mammalian coats, observations such as the presence of these 'ghost rosettes' argue otherwise. Moreover, the results from this study indicate that melanism in leopards is caused by complete loss of ASIP function, which would imply no action of this antagonist peptide and thus maximum MC1R signaling for dark melanin across the whole body. The fact that rosettes are even darker than this background strongly argue for the action of a distinct pigmentation pathway [28], which has so far not been characterized in any mammal bearing ASIP-null mutations [7,25,29]. Interestingly, in black domestic cats (also inferred to be induced by loss of ASIP function [5]), 'ghost' tabby markings are mostly visible in the juvenile, and become indistinguishable from the darkened background in the adult. Dissecting the molecular and developmental pathways affecting coat patterning *vs.* background melanogenesis in these and other felid species promises to shed unprecedented light onto the genetic basis and evolutionary history of pigmentation diversity in mammals.

Supporting Information

Figure S1 Nucleotide variation in the *ASIP* coding region among mammals, including sequences of *Panthera pardus* and *Pardofelis temminckii*, shown for a wild-type and a melanistic individual (indicated by the letter 'M'). Asterisks indicate the nucleotide position for the mutant alleles associated with melanism. Dots indicate identity to the top sequence; vertical lines demarcate boundaries between exons. Shaded segments containing dashes indicate insertion/deletion (indel) regions.
(DOC)

Table S1 GenBank accession numbers for mammalian sequences included in the *ASIP* alignments analyzed in this study.
(DOC)

Table S2 Primers developed in this study for PCR amplification and sequencing of *ASIP* in felids.
(DOC)

Acknowledgments

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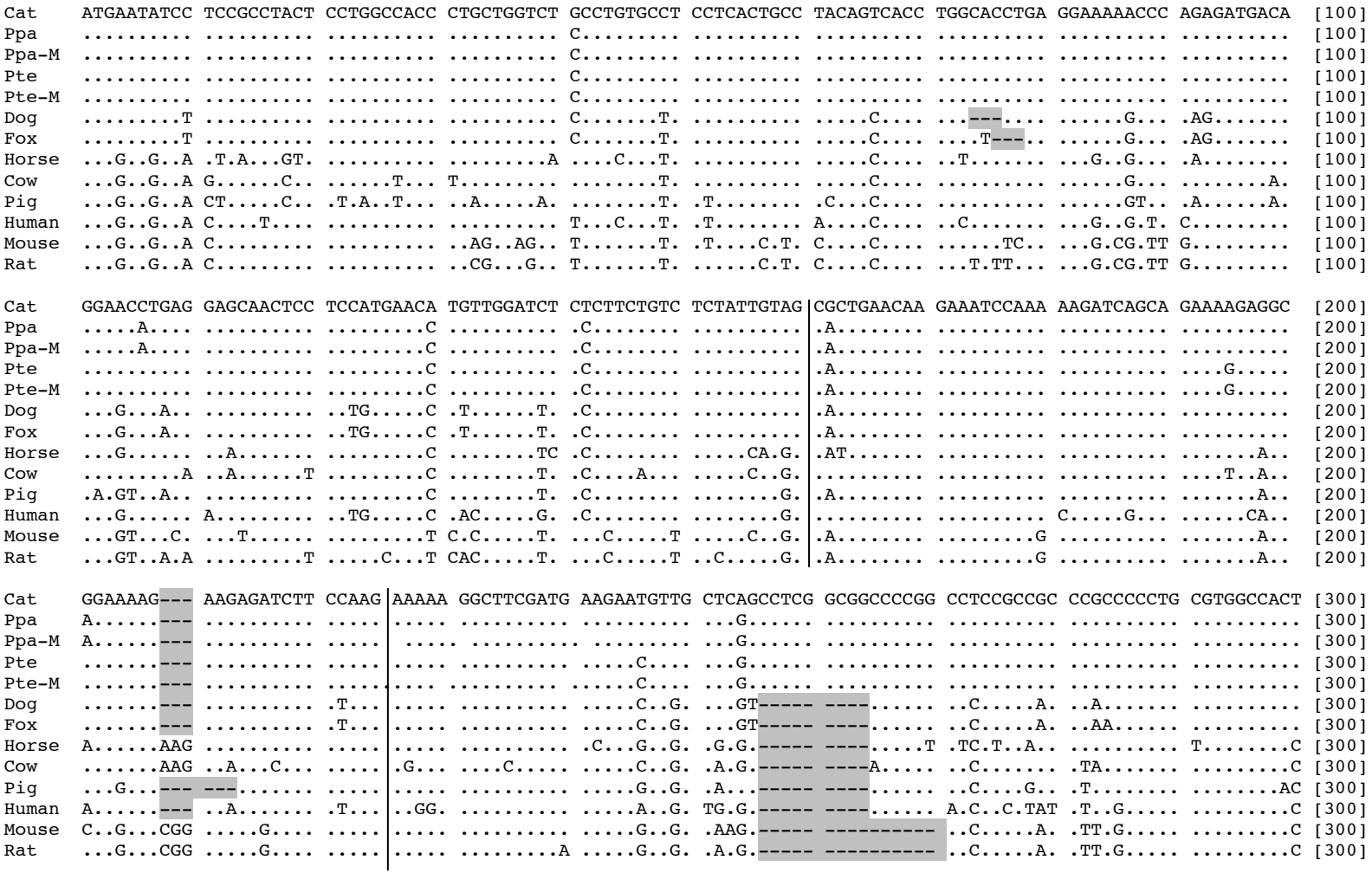
Author Contributions

Conceived and designed the experiments: EE AS. Performed the experiments: AS VAD. Analyzed the data: AS VAD EE. Contributed reagents/materials/analysis tools: WEJ MMR GSB SJO. Wrote the paper: AS EE.

References

1. Hubbard JK, Uy JAC, Hauber ME, Hoekstra HE, Safran RJ (2010) Vertebrate pigmentation: from underlying genes to adaptive function. Trends in Genetics. 26: 231–239.
2. Majerus MEN, Mundy NI (2003) Mammalian melanism: natural selection in black and white. Trends in Genetics. 19: 585–588.
3. Caro T (2005) The Adaptive Significance of Coloration in Mammals. BioScience. 55: 125–136
4. Kawanishi K, Sunquist ME, Eizirik E, Lynam AJ, Ngoprasert D, et al. (2010) Near fixation of melanism in leopards of the Malay Peninsula. Jour of Zoology 282: 201–206.
5. Eizirik E, Yuhki N, Johnson WE, Menotti-Raymond M, Hannah SS, et al. (2003) Molecular Genetics and Evolution of Melanism in the Cat Family. Curr Biology 13: 448–453.
6. Theron E, Hawkins K, Bermingham E, Ricklefs RE, Mundy NI (2001) The molecular basis of an avian plumage polymorphism in the wild: a melanocortin-1-receptor point mutation is perfectly associated with the melanic plumage morph of the bananaquit, *Coereba flaveola*. Curr Biol 11: 550–557.
7. Barsh GS (1996) The genetics of pigmentation: from fancy genes to complex traits. Trends in Genetics. 12: 299–305.
8. Hoekstra HE (2006) Genetics, development and evolution of adaptive pigmentation in vertebrates. Heredity. 97: 222–234.
9. Sambrook J, Fritsch EF, Maniatis T (1989) Molecular Cloning: a laboratory manual, Second Edition. New York: Cold Spring Laboratory Press.
10. Palumbi S, Martin A, Romano S (1991) The simple fool's guide to PCR, version 2.0. Dept. of Zoology, University of Hawaii, Honolulu.
11. Hillis DM, Mable BK, Larson A, Davis SK, Zimmer EA (1996) Nucleic acid IV: Sequencing and Cloning. In DM Hillis, C Moritz, BK Mable, editors. Molecular Systematics. Sunderland, MA: Sinauer Associates, pp. 321–381.
12. Palumbi SR (1996) Nucleic acid II: The polymerase chain reaction. In DM Hillis, C Moritz, BK Mable, editors. In Molecular Systematics. Sunderland, MA: Sinauer Associates, pp. 205–247.
13. Rozen S, Skaletsky HJ (2000) Primer3 on the WWW for general users and for biologist programmers. In S Krawetz, S Misener editors, Bioinformatics Methods and Protocols: Methods in Molecular Biology. Totowa, NJ: Humana Press, pp. 365–386.
14. Barsh GS (2006) Regulation of pigment type-switching by Agouti, Melanocortin signaling, Attractin, and Mahoganoid. In JJ . Nordlund, RE . Boissy, VJ . Hearing, RA . King, WS . Oetting, JP . Ortonne, editors. The Pigmentary System. Blackwell: Oxford. pp. 395–410.
15. Searle AG (1968) Comparative Genetics of Coat Colour in Mammals. London: Logos Press. 308 p.
16. Perry WL, Nakamura T, Swing DA, Secrest L, Eagleson B, et al. (1996) Coupled site-directed mutagenesis/transgenesis identifies important functional domains of the mouse agouti protein. Genetics 144: 255–264.
17. Miltenberger RJ, Wakamatsu K, Ito S, Woychik RP, Russell LB, et al. (2002) Molecular and phenotypic analysis of 25 recessive, homozygous-viable alleles at the mouse agouti locus. Genetics 160: 659–674.
18. Dinulescu DM, Cone RD (2000) Agouti and agouti related protein: analogies and contrasts. Journal of Biol Chemistry. 275: 6695–6698.
19. McNulty JC, Jackson PJ, Thompson DA, Chai B, Gantz I, et al. (2005) Structures of the agouti signaling protein. Journal of Molecular Biology 346: 1059–1070.
20. Kingsley EP, Manceau M, Wiley CD, Hoekstra H (2009) Melanism in *Peromyscus* is caused by independent mutations in agouti. PLoS One 4(7): e6435. doi:10.1371/journal.pone.0006435
21. Mundy N, Kelly J (2006) Investigation on the role of agouti signaling protein gene (*ASIP*) in coat color evolution in primates. Mamm Genome. 17: 1205–1213.
22. MacDougall-Shackleton EA, Blanchard L, Gibbs HL (2003) Unmelanized plumage patterns in old world leaf warblers do not correspond to sequence variation at the Melanocortin-1 receptor locus (*MC1R*). Mol Biol Evol. 20: 1675–1681.
23. Klungland H, Våge DI, Gomez-Raya L, Adalsteinsson S, Lien S (1995) The role of the melanocyte-stimulating hormone (MSH) receptor in bovine coat color determination. Mamm Genome. 6: 636–639.
24. Våge DI, Lu D, Klungland H, Lien S, Adalsteinsson S, et al. (1997) A non-epistatic interaction of agouti and extension in the fox, *Vulpes vulpes*. Nat Genetics. 15: 311–315.
25. Rieder S, Taourit D, Langlois B, Guerin G (2001) Mutations in the agouti (ASIP), the extension (MC1R), and the brown (TYRP1) loci and their association to coat color phenotypes in horses (*Equus caballus*). Mamm. Genome. . 12: 450–455.
26. Eizirik E, David VA, Buckley-Beason V, Roelke ME, Schaffer AA, et al. (2010) Defining and Mapping Mammalian Coat Pattern Genes: Multiple Genomic Regions Implicated in Domestic Cat Stripes and Spots. Genetics. 184: 267–275.
27. Kaelin C, Barsh G (2010) Tabby pattern genetics – a whole new breed of cat. Pigm Cell Melan Res. 23: 514–516.
28. Kaelin CB, Xu X, Hong LZ, David VA, McGowan KA, et al. (2012) Specifying and Sustaining Pigmentation Patterns in Domestic and Wild Cats. Science. 337: 1536–1541.
29. Kerns JA, Newton J, Berryere TG, Rubin EM, Cheng JF, et al. (2004) Characterization of the dog *Agouti* gene and a *nonagouti* mutation in German Shepherd Dogs. Mamm Genome. 15: 798–808.
30. Whiting PW (1918) Inheritance of coat colour in cats. J Exp. Zool. 25: 539–570.
31. Robinson R (1959) Genetics of the domestic cat. Bibliographia Genetica XVIII: 273–362.
32. Dittrich L (1979) Die vererbung des melanismus beim jaguar (*Panthera onca*). Zool. Garten 49: 417–428.
33. Angwin D (1975) Melanistic serval cats, melanistic wild cat and bongo. Bull E Afr. Nat Hist. Soc. 3.
34. Ulmer FA Jr (1941) Melanism in the Felidae, with special reference to the genus *Lynx*. J Mamm. 22: 285–288.
35. Robinson R (1976) Homologous genetic variation in the *Felidae*. Genetica 46: 1–31.
36. Robinson R (1969) The breeding of spotted and black leopards. J Bombay Nat. Hist. Soc. 66: 423–429.
37. Robinson R (1970) Inheritance of the black form of the leopard *Panthera pardus*. Genetica 41: 190–197.
38. Burton RG (1928) Black tigers. Field 152: 656–657.
39. Lönnberg E (1898) Über eine melanistische Varietät vom Serval nebst Bemerkungen über andere melanistische Säugetiere. Zool. Jb. 10: 569–595.
40. Sunquist ME, Sunquist F (2002) Wild Cats of the World. Chicago, Univ. of Chicago Press.
41. Gibson-Hill CA (1950) Notes on the clouded leopard [*Neofelis nebulosa* (Griffith)]. J Bombay Nat. Hist. Soc. 19: 375–391.
42. Coleman LE (1974) Melanistic phases of Felidae in captivity. Preliminary survey results. Carnivore Genet. Newsl. 2: 209–211.
43. Junge C (1975) Chillan Viejo, Chile. Int. Zoo News 21: 38.
44. Sunquist M, Sanderson J (1998) Ecology and behavior of the kodkod in a highly-fragmented, human-dominated landscape. Cat News 28:17–18.
45. Dunstone N, Durbin L, Wyllie I, Rose S, Acosta G (1998) Ecology of the kodkod in Laguna San Rafael National Park, Chile. Cat News 29: 18–20.
46. Weigel I (1961) Das Fellmuster der wildlebenden Katzenarten und der Hauskatze in vergleichender und stammesgeschichtlicher Hinsicht. Säugetierkdl. Mitt. 9 (Sonderheft): 1–120.
47. Wrogemann N (1975) Cheetah under the sun. McGraw-Hill, New York.
48. Young SP, Goldman EA (1946) The Puma, Mysterious American Cat. Stockpole, Harrisburg.
49. Lamotte M (1942) La fauna mammalogique du Mont Nimba (Haute Guinée). Mammalia 6: 114–119.
50. Wibisono HT, McCarthy J (2010) Melanistic marbled cat from Bukit Barisan Selatan National Park, Sumatra, Indonesia. Cat News 52: 9–10.

Figure S1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Ppa .... C..... .... [100]
Ppa-M .... C..... .... [100]
Pte .... C..... .... [100]
Pte-M .... C..... .... [100]
Dog .....T .... C.....T. ....C.... ...---.... ......G... .AG..... [100]
Fox .....T .... C.....T. ....C.... ....T---.. ......G... .AG..... [100]
Horse ...G..G..A .T.A...GT. ....A ....C...T. ....C.... ..T..... ...G..G... .A..... [100]
Cow ...G..G..A G......C.. ......T... T..... .....T. ....C.... ....G... .....A. [100]
Pig ...G..G..A CT.....C.. .T.A..T... ..A.....A. .....T. .T..... .C...C.... ....GT.. .A.....A. [100]
Human ...G..G..A C....T.... .... T...C...T. .T..... A....C.... ...G..G.T. C..... [100]
Mouse ...G..G..A C..... ....AG..AG.. T.....T. .T....C.T. C....C.... ......TC.. ...G.CG.TT G..... [100]
Rat ...G..G..A C..... ....CG...G.. T.....T. ......C.T. C....C.... ...T.TT... ...G.CG.TT G..... [100]
Cat GGAACCTGAG GAGCAACTCC TCCATGAACA TGTTGGATCT CTCTTCTGTC TCTATTGTAG|CGCTGAACAA GAAATCCAAA AAGATCAGCA GAAAAGAGGC [200]
Ppa .....A.... ....C ..... .C..... .....|.A..... .... [200]
Ppa-M .....A.... ....C ..... .C..... .....|.A..... .... [200]
Pte ....C ..... .C..... .....|.A..... ....G..... [200]
Pte-M ....C ..... .C..... .....|.A..... ....G..... [200]
Dog ...G...A.. ....TG.....C .T.....T. .C..... .....|.A..... .... [200]
Fox ...G...A.. ....TG.....C .T.....T. .C..... .....|.A..... .... [200]
Horse ...G..... ..A..... .....C ....TC .C..... .....CA.G. .AT..... ....A.. [200]
Cow .....A ..A.....T .....C ....T. .C....A... .....C..G. ....T..A.. [200]
Pig .A.GT..A.. ....C ....T. .C..... .....G. .A..... ....A.. [200]
Human ...G..... A..... ..TG.....C .AC.....G. .C..... .....G. .... C.....G... ......CA.. [200]
Mouse ...GT...C. ...T..... .....T C.C.....T. ...C.....T .....C..G. .A..... ....G ....A.. [200]
Rat ...GT..A.A .....T .....C...T CAC.....T. ...C.....T ..C.....G. .A..... ....G ....A.. [200]
Cat GGAAAAG--- AAGAGATCTT CCAAG|AAAAA GGCTTCGATG AAGAATGTTG CTCAGCCTCG GCGGCCCCGG CCTCCGCCGC CCGCCCCCTG CGTGGCCACT [300]
Ppa A.....--- .... |..... ....G..... .... [300]
Ppa-M A.....--- .... | ....G..... .... [300]
Pte .....--- .... |..... ....C.... ...G..... .... [300]
Pte-M .....--- .... |.... ....C.... ...G..... .... [300]
Dog .....--- ..... .T... |..... ....C..G. ...GT----- ----..... ..C.....A. ..A..... ..... [300]
Fox .....--- ..... .T... |..... ....C..G. ...GT----- ----..... ..C.....A. ..AA..... ..... [300]
Horse A.....AAG .... |..... ..... .C...G..G. .G.G.----- ----.....T .TC.T..A.. ..... T.....C [300]
Cow .....AAG ..A...C... ..... |.G... ....C..... .....C..G. .A.G.----- ----A..... ..C..... .TA..... .....C [300]
Pig ...G...--- ---..... ..... |..... ....G..G. .A...----- ----..... ..C....G.. .T..... .....AC [300]
Human A.....--- ..A..... .T... |..GG. ....A..G. TG.G.----- ----..... A.C..C.TAT .T..G..... .....C [300]
Mouse C..G...CGG .....G.... ..... |..... ....G..G. .AAG.----- ---------- ..C.....A. .TT.G..... .....C [300]
Rat ...G...CGG .....G.... ..... |..... .....A .....G..G. .A.G.----- ---------- ..C.....A. .TT.G..... .....C [300]

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                                                *                                                                 *
Cat    CGTGACAGCT GCAAGCCGCC GGCGCCCGCC TGCTGCGACC CGTGCGCCTC CTGCCAGTGC CGCTTCTTCC GCAGCTCCTG CTCCTGCCGA GTGCTCAACC  [400]
Ppa    ...A...... .......... .......... .......... .......... .......... .......... .......... .......... ..........  [400]
Ppa-M  ...A...... .......... .......... .....A.... .......... .......... .......... .......... .......... ..........  [400]
Pte    ..A....... .......... .......... .......... .......... .......... .......... .......... .......... ..........  [400]
Pte-M  ..A....... .......... .......... .......... .......... .......... .......... .......... ......G... ..........  [400]
Dog    ..CA...... .....T.C.. .......... .....T.... .C........ .......... .......... .....G.... .A.......C ..T....GT.  [400]
Fox    ..CA...... .....T.C.. .......... .....T.... .C........ .......... .......... .....G.... .A.......C ..T....GT.  [400]
Horse  ..C....... .......... .......... .......... .......... .........T .......... .....G.... .........T .......CT.  [400]
Cow    ..C....... .......T.. A......... .......... .........T .......... .......... .....G.... .........C ..........  [400]
Pig    ..C....... .......T.. .....T.... .......... .........T .......... .......... ....TG.... .........C ..........  [400]
Human  ..CA...... .......... ...A...... .......... .......... .......... .......... .....G.... .........C .......G..  [400]
Mouse  ..C....... .......A.. C..A...... .......... .......... .......... ..T......G .....G.... .A....T... ..A.......  [400]
Rat    ..C....... .......... T......... ......A... .......... .......... ..T......G .....G.... .A.T.....C ..A.......  [400]

Cat    CCACCTGCTGA [411]
Ppa    ........... [411]
Ppa-M  ........... [411]
Pte    ........... [411]
Pte-M  ........... [411]
Dog    ...GA...... [411]
Fox    ...G....... [411]
Horse  G.......... [411]
Cow    ........... [411]
Pig    ........... [411]
Human  T..A....... [411]
Mouse  ...A....... [411]
Rat    ...A....... [411]
```

Table S1. GenBank accession number of mammalian sequences included in the *ASIP* alignments analyzed in this study.

Mammal species	GenBank Acession Number
Domestic cat (*Felis catus*)	NP_001009190.1
Dog (*Canis familiaris*)	NP_001007264.1
Red fox (*Vulpes vulpes*)	Y09877.2
Horse (*Equus caballus*)	AF288358.1
Cow (*Bos taurus*)	X99692.1
Pig (*Sus scrofa*)	AJ427478.2
Human (*Homo sapiens*)	NM_001672
Mouse (*Mus musculus*)	NM_015770.3
Rat (*Rattus rattus*)	NM_052979.1

Table S2. Primers developed in this study for PCR amplification and sequencing of *ASIP* in felids.

Primer	**Sequence (5' – 3')**
ASIP exon2-F	TCTGTTCCACTCAGGCCTTC
ASIP exon2-R	GGGTCAAGCTGGGCTACTTA
ASIP-exon3-F	CTCTTCTCCCACACCCTGAG
ASIP-exon3-R	CACCCCCACAATGAAAACTC
ASIP-exon4-F	GAGCAGACCCCGCTTTTC
ASIP-exon4-R	GCCTTGGAGGTGGGTGAG

CAPÍTULO III:

2º ARTIGO CIENTÍFICO

Recurrent Evolution of Melanism in an Endemic Lineage of Wild Cats

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Recurrent Evolution of Melanism in an Endemic Lineage of Wild Cats

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Running Head: Recurrent evolution of melanism in wild cats

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Abstract

Identifying the molecular basis of convergent phenotypes can provide insights into the mechanisms generating diversity, as well as reveal the evolutionary history underlying the origin and maintenance of such features. Among the traits that comprise coloration diversity in animals, melanism (darkening of the background coloration) is a powerful system to assess the molecular mechanisms and evolutionary processes that underlie the origin of phenotypic convergence. To address such issues, it is interesting to investigate evolutionary lineages that contain multiple species bearing polymorphic melanism, as is the case of the Neotropical felid genus *Leopardus*. Melanism is rather common in some species belonging to this group, reaching high frequencies in some populations of the closely related *L. colocolo, L. guigna* and *L. geoffroyi*. Aiming to understand the evolutionary history of melanism in this lineage, the present study investigated the molecular basis of this phenotype by characterizing the candidate genes *ASIP* (*Agouti Signaling Protein*) and *MC1R* (*Melanocortin-1 receptor*) in multiple individuals of each of these species. We also performed an extensive analysis of the genomic regions surrounding these loci using next-generation sequencing. Our findings show distinct mutations strongly associated with melanism in each of the assessed species, and demonstrate that melanism has evolved independently at least eight times in the Felidae. We then assessed the evolutionary history of these molecular variants by analyzing a large data set of single nucleotide polymorphisms (SNPs) sampled in large genomic regions surrounding these genes, aiming to test the presence of signatures of natural selection. This analysis supported the inference of recent and positive selection favoring the mutation implicated in melanism in at least one of the species, and provided suggestive evidence of selection for the other two. Therefore, our results support for the first time in felids the hypothesis that melanism evolution may be driven by natural selection.

Introduction

The evolution of phenotypic diversity is a complex subject that has attracted the attention of scientists working on a wide variety of organisms [1–4]. Recent analyses have revealed many instances of rapid, divergent phenotypic evolution among closely related species, as well as episodes of phenotypic convergence among lineages whose relationship is more distant [5–10]. In this context, the identification of the molecular basis of phenotypic traits allows the assessment of contrasting hypotheses regarding their origin and spread in populations and species. Such analyses have revealed cases in which similar phenotypes evolved via very distinct genetic mechanisms (e.g. [5,11], while in other instances the very same gene or even mutation was repeatedly implicated in convergent traits [3,6,12,13].

Although the molecular assessment of such processes has progressed significantly in recent years, the understanding of the underlying basis of phenotypic evolution is still in its infancy. So far one of the systems that have been most often investigated in this regard is the evolution of coloration diversity, for which several of the implicated genes have already been identified (e.g. [7,14–18]. Among the traits influencing coat color variation, melanism has been the focus of several studies addressing the molecular basis of this phenotype, including analyses of domestic animals, laboratory models and wild populations (e.g. [19–23]).

Melanism is a polymorphic phenotype in which the background coloration is darkened, and which has been found to be often influenced by two different genes whose products interact in the regulation of melanin production. When the Melanocortin-1 receptor (MC1R) is activated by the binding of Alpha-Melanocyte Stimulating Hormone (α-MSH), it signals the melanocyte to produce eumelanin (dark pigment). In contrast, the Agouti Signaling Protein (ASIP) is a peptide which acts as an antagonist to the MC1R, preventing its activation by the α-MSH and thus inducing a switch from eumelanin to pheomelanin (light pigment) synthesis.

Among felids, melanism arose independently at least five times, and has been recorded as a rather common phenomenon in several species [24]. In this context, a particularly interesting group is an endemic lineage of Neotropical wild cats which includes multiple species exhibiting melanism as a naturally occurring phenotypic variant. This lineage comprises seven species of small cats belonging to the genus

109 *Leopardus*¸ which seem to have recently diversified after the colonization of South
110 America by a common ancestor *ca*. 3 million years ago (MYA) [25]. Three of these
111 species (*Leopardus colocolo*, *L. guigna*, *L. geoffroyi*) have been reported to include
112 melanistic individuals in at least a portion of their geographic distributions (Figures 1
113 and 2), in some cases occurring at appreciable frequencies. For example, field data
114 collected so far indicate that melanism may be present in up to ~30 % of the individuals
115 in some *L. colocolo* and *L. guigna* populations, and ~20% in the case of *L. geoffroyi* (L.
116 Silveira, pers. comm., [26], E.E. and collaborators, unpublished data). Although it is
117 presently unknown if and how melanism affects fitness in these wild cats, the presence
118 of this polymorphic phenotype in multiple, closely related species is quite intriguing,
119 and raises the possibility that it could be involved in adaptive processes such as
120 camouflage or thermoregulation [27,28].
121 In an attempt to understand the evolution of melanism in these species, we have
122 raised three hypotheses that could account for this shared feature: (1) melanism arose
123 only once in this group, in the common ancestor of the three species, having been
124 maintained during their divergence as a trans-specific polymorphism; (2) given previous
125 evidence of inter-species hybridization and introgression in this genus [29], melanism
126 could have arisen in one of the species, and then spread into the others due to
127 admixture; and (3) melanism evolved independently in each of these species, having
128 subsequently risen in frequency to the presently observed levels. These three hypotheses
129 allow distinct predictions in terms of the molecular variants underlying this trait: in the
130 first case, a single shared mutation would be present in all species; in the second
131 scenario, one or more shared variants would be present, with a genealogical pattern
132 reflecting introgression; and in the last case, species-specific mutations would be
133 expected. Interestingly, all three scenarios appear to be highly improbable under
134 neutrality, given the presence of the trait at rather high frequencies in three distinct
135 species. Therefore, in addition to testing the hypotheses raised above, it is relevant to
136 investigate whether natural selection may have played a role in the evolution of this
137 phenotype in this lineage.
138 To address these issues, we investigated the molecular basis of melanism in *L.*
139 *colocolo, L. guigna* and *L. geoffroyi* by characterizing the candidate genes *ASIP* and
140 *MC1R* in these species, including association studies of identified coding variants, as
141 well as in-depth analyses of their surrounding genomic regions. Our results demonstrate

that melanism is associated with a distinct mutation in each species, indicating that it is a case of remarkable phenotypic convergence in a recently diversified lineage. Moreover, the analysis of genomic data allowed a detailed assessment of the evolutionary history of these variants, in at least one case supporting the inference of positive selection favoring the mutation implicated in melanism. The other two species also showed patterns suggestive of selective signatures, albeit their cases could not be established as clearly. Overall, the observed patterns reveal a complex evolutionary history of these two genomic segments in this lineage, and provide insights that may be relevant for similar assessments of phenotypic evolution in other taxa.

Materials and Methods

Sample collection and DNA extraction

The study employed biological samples (blood or tissue) collected from multiple individuals from each of the focal species. Blood samples were collected from wild-caught individuals in the context of field ecology studies, or from captive specimens of known geographic origin, held in accredited zoological institutions. Tissue samples were obtained from road-killed animals encountered during routine wildlife surveys. In all cases, sampling was performed following appropriate national regulations for the handling of animals and biological materials.

In order to minimize biases derived from population structure in the association studies (see below), we only included, for each species, samples that were originated from the same geographic region. Overall, we analyzed a total of 57 samples: 18 *L. colocolo*, 17 of which were from Emas National Park, located in Goiás state, central Brazil; 16 *L. guigna*, all from Chiloé Island, Chile; and 23 *Leopardus geoffroyi*, all from the southern portion of Rio Grande do Sul state, southernmost Brazil (Table S1; Figure 2). For each of these species, both the melanistic and non-melanistic phenotypes were sampled (*L. colocolo*: 10 melanistic and 8 non-melanistic; *L. guigna*: 5 melanistic and 11 non-melanistic; *L. geoffroyi*: 7 melanistic and 16 non-melanistic; see Table S1).

Genomic DNA was extracted from blood and tissue samples using standard phenol/chloroform protocols [30]. After DNA extraction, quality control and quantification using agarose gels and a NanoDrop device (Thermo Scientific), two

different sets of experiments were performed: (1) a PCR (polymerase chain reaction)-based characterization of the candidate genes *ASIP* and *MC1R*, searching for molecular variants potentially associated with melanism in each species; and (2) an in-depth analysis of the genomic region surrounding each of these two genes, based on a hybrid capture assay followed by next-generation DNA sequencing. Each of these two approaches will be described in more detail below.

Characterization of candidate genes

To investigate whether the *ASIP* and *MC1R* genes were implicated in melanism in these wild cats, an initial characterization of these genes was conducted to identify molecular variants potentially associated with this phenotype in each species. We first performed PCR and sequencing of a subset of samples from each species, including four individuals of each phenotype (i.e. melanistic and non-melanistic; Data set1, see Table S1). After identifying potential genotype-phenotype associations in this initial subset of samples, we expanded this PCR-based approach to test the relevant gene in all available samples for each of the three species (Data set 2, Table S1).

To characterize the *ASIP* gene, we used the PCR primers originally reported by Schneider et al. 2012, which span the three coding exons of this locus. To analyze the *MC1R* gene we developed novel primers using the Primer3 tool [31]; http://primer3.wi.mit.edu/) on the basis of the domestic cat and domestic dog genomic sequences (*University of California - Santa Cruz*, http://genome.ucsc.edu/; *GARFIELD*, http://lgd.abcc.ncifcrf.gov/cgi-bin/gbrowse/cat/). As *MC1R* comprises a single coding exon of *ca*. 1 kb, it was divided into two segments of similar length to facilitate amplification and sequencing (see Table S2 for *MC1R* primer sequences).

Polymerase chain reactions (PCRs) for *ASIP* were performed as previously described [24]. PCRs targeting both amplicons of *MC1R* were carried out using Takara LA Taq with GC Buffer (Takara Bio Inc.), following the guidelines provided by the manufacturer. The thermal cycling conditions were the same for both amplicons, except for the total number of cycles (40 for fragment 1 and 30 for fragment 2).

PCR products were purified using the enzymes Exonuclease I and Shrimp Alkaline Phosphatase. Purified PCR products were sequenced using BigDye chain terminator chemistry (Applied Biosystems) and subsequently analyzed with an ABI

3700 automated DNA sequencer. Sequence electropherograms were visually verified and manually corrected with Sequencher 4.2 (GeneCodes Corporation, Ann Arbor, MI), and every polymorphism was carefully inspected for confirmation. Homologous nucleotide and amino acid sequences of each gene from additional mammalian species were obtained from GenBank (Table S3) and aligned using ClustalW (http://www.ebi.ac.uk/Tools/msa/clustalw2/). Final alignments were checked by hand. The DNA sequences reported here have been deposited in GenBank (Accession numbers xxx-xxx).

Targeted DNA resequencing using next-generation approaches

Illumina library construction

Concentrations of all input genomic DNAs were determined with a Picogreen assay (Life Technologies). Input genomic DNA quality was assessed by running 1-3 μL on a 1% agarose gel that contained 1X Sybr Green I dye (Life Technologies). Typical input DNA quantity for Illumina library construction ranged from 700 ng to 2.5 μg. Depending on the level of DNA degradation before shearing, the quantity of input DNA was increased. Genomic DNAs were sheared to a size range of 100-500 bp with a Bioruptor XL sonicator (Diagenode) with a refrigerated recirculator containing 50% ethylene glycol solution. Shearing conditions were 4 cycles of 30s on and 30s off for 10 min each. Genomic DNAs were end-repaired, adenylated, and ligated according to standard Illumina protocols with inline barcoded adapters. Library concentrations were determined with an Agilent Bioanalyzer high sensitivity DNA assay using 1:50 diluted libraries.

Targeted capture enrichment

For targeted sequencing, the 'clone-adapted targeted capture hybridization for sequencing' (CATCH-Seq) method was used (Day et al., manuscript in preparation). Fosmid clones derived from the domestic cat genome were selected to prepare probes for sequencing enrichment targeting the *ASIP* and *MC1R* loci, as well as surrounding regions (Table S4). Fosmid DNAs were selected from an available library previously

mapped onto the domestic cat genome (*Garfield browser;* [32]; clone identities were verified by PCR. Fosmids were pooled by mass based on their individual percentage of the composite target size (684.8kb) multiplied by 1.5 μg of input pooled fosmid DNA for shearing under the same conditions as genomic DNAs. Sheared fosmids were enzymatically processed similarly to the preparation of Illumina libraries, and ligated with T7 promoter-containing adapters for biotinylated RNA probe synthesis using a Megascript T7 kit (Ambion) and biotin-11-UTP (Life Technologies) for 1.5h. Completed in vitro transcriptions were DNAse treated, followed by addition of 1 μL 0.5M EDTA, and heat inactivation for 10 min at 75ºC. Reactions were pooled and a 3-μL aliquot was mixed with gel loading buffer (heated to 95ºC for 2 min) and loaded on a 1.5% agarose gel with 10 μg/mL ethidium bromide to assess the quality of the transcription. Unincorporated nucleotides were removed with NucAway gel filtration spin columns (Ambion). Probe concentration was determined with a Qubit RNA assay (Life Technologies) and verified with an Agilent Bioanalyzer.

Inline barcoded Illumina libraries were pooled equally according to their concentrations in 4-plex sets to a total of 500 ng of library for capture processing. Hybrid selection, washes, and captures were detailed in a previous report using a 26 μL volume hybridization reaction [33]. A probe solution was prepared to deliver 50 ng of probe per hybridization in 6 μL containing 20 U SUPERase-In (Ambion). For capture, 20 μL of input Dynabeads MyOne Streptavidin C1 were used per hybridization (Life Technologies). Final captured libraries were enriched by PCR using 0.5 μL of each standard Illumina primer (25 μM each), 5 μL 5M Betaine (Sigma), 2.5 μL 10 mM dNTP mix (New England Biolabs), 5 μL 10x PCR buffer, 2 μL 50 mM $MgCl_2$, 1 μL Platinum Taq, library, and water up to a 50 μL volume. Cycling conditions were 98ºC for 1 min, followed by 20 cycles of 95ºC for 30s and 62ºC for 3 min. Concentrations of 4-plex captures were determined with an Agilent Bioanalyzer High Sensitivity DNA assay and also by real time PCR with a library quantification kit (KAPABiosystems). We multiplexed 12 representative libraries per HiSeq lane by mixing three pooled 4-plex captures. Individual 4-plex libraries were pooled based on concentration, and final 12-plex set concentrations were determined by an additional real time PCR reaction. Paired-end 50 bp sequencing on an Illumina HiSeq platform was performed according to standard procedure. Sequencing reads from individual cats were demuxed based on their inline barcode sequences.

274 Bioinformatic analyses of sequence data
275
276 Our analytic strategy aimed to identify, for each of the three species, a set of
277 high quality genotype markers to be used in comparative population-level analyses of
278 the *ASIP* and *MC1R* regions. For this purpose we devised a custom variant discovery
279 pipeline from Illumina sequence reads, supported by some of the tools available in
280 *Genome Analyzer Toolkit* (*GATK*; [34]. A preliminary sequence reference for the two
281 studied regions was extracted from a 6x draft assembly of the domestic cat genome
282 (unpublished, available at http://genome.wustl.edu/genomes/detail/felis-catus/) and used
283 for read alignment purposes. The sequence coordinates used throughout this study refer
284 to this genomic reference. In this draft assembly, the *ASIP* gene is located on
285 chromosome A3, while *MC1R* is located on chromosome E2 (corroborating the
286 mapping results reported by Eizirik et al. [2003]). This preliminary sequence reference
287 was masked for transposable elements and low complexity regions with the
288 *RepeatMasker* tool [35]. Raw sequence reads were then mapped against the preliminary
289 domestic cat reference using the *Burrows-Wheeler Aligner* (*BWA*) with default
290 parameters [36]. These alignments were then used to specifically reassemble the target
291 regions in each of the three species, by applying *samtools* to compute consensus
292 sequences [37], in order to minimize subsequent genotyping errors related to inter-
293 species differences in allele structure and distribution. Sequence reads were then
294 remapped to these species-specific reassembled consensus sequences to support a
295 variant discovery approach, which was guided by the best practices for variant detection
296 provided with *GATK* (http://www.broadinstitute.org/gatk/guide/topic?name=best-
297 practices#best-practices15). Briefly, we started by applying alignment quality control
298 procedures available in *GATK* to detect sequence intervals with low quality mappings
299 (i.e. possibly related to the presence of sequence variants, such as small insertions or
300 deletions, in subsets of the analyzed samples). In all such cases a thorough local
301 realignment of the reads was performed to minimize the number of mismatching bases.
302 The *GATK Unified Genotyper* (UG) tool was applied on realigned reads to infer the
303 genotype structure simultaneously across all samples for each species. Raw genotype
304 calls were subjected to a filtering procedure by imposing thresholds on a set of quality
305 criteria including minor allele frequency MAF>10%, Phred-scaled mapping quality
306 MQ>40, UG quality by depth QD>2 and UG HaplotypeScore>13. The filtered calls

were further restricted to a small subset of high quality calls, so as to satisfy an average variant density threshold of ~1 per 1kb of target sequence. Finally, the *BEAGLE* genetic analysis software package [38] was used to check genotype consistency across all samples of each species, and to infer the haplotype phase of selected variants.

The curated genotype and haplotype data were used for downstream population genetic analyses. Among them, the nucleotide diversity values and different neutrality tests such as Tajimas's D, Fu and Li's D and Fu and Li's F were computed with DnaSP 5.10 [39]. Long-range linkage disequilibrium tests, including measures of extended haplotype homozygosity (EHH; [40] and haplotype bifurcation plots, were computed with the *Sweep* software tool (version 1.1, available at http://www.broadinstitute.org/mpg/sweep/index.html) and the *rehh* package (version 1.0, [41] available in the *R environment for statistical computing* (http://www.R-project.org). Finally, haplotype network analyses were generated using Network 4.5.0.0 (www.fluxus-engineering.com).

Results and Discussion

Identification of genotype-phenotype association

PCR-based sequencing of the *ASIP* and *MC1R* coding regions revealed high conservation within and among the focal species. In *ASIP*, the alignment of the entire coding region comprised 408 bp, six of which were variable at the inter- and/or intraspecific levels (Table S5). In the initial screen of 24 individuals (i.e. including four melanistic and four non-melanistic samples from each species – Data set1, see Table S1), we did not detect any variable site within each species on exons 2 and 3. Inter-specific divergence was also low in these two exons, with only two fixed differences (one synonymous and one non-synonymous) between *L. colocolo* and the other two species (sites 6289 and 6299; see Table S5). We observed more diversity on exon 4, with four variable sites in the inter-species alignment. Three of them were non-synonymous substitutions, one of which was present as a heterozygous site in a single *L. geoffroyi* individual, and the other two segregated within *L. colocolo* and within *L. guigna*, respectively (see Table S5). There was also a synonymous variant fixed between *L. colocolo* and the other two species.

The missense mutation segregating in *L. colocolo* (C10585T) caused an arginine to cysteine replacement at *ASIP* codon 120 (R120C), and was strongly associated with the melanistic phenotype ($\chi^2 = 17.96$, d.f.= 1, $p<0.005$, based on Data set 2 – see Table S6). All ten melanistic individuals were homozygous for the mutant 'T' allele, while all eight non-melanistic individuals carried one or two copies of the ancestral 'C' allele.

A different missense mutation (G10604A) was detected on *ASIP* exon 4 of some *L. guigna* individuals. All wild-type individuals (n = 11) were homozygous for the ancestral 'G' allele or heterozygous at position 10604, while all melanistic individuals (n = 5) were homozygous for the mutant 'A' allele. This mutation caused a cysteine to tyrosine substitution at amino acid position 126 (C126Y), and was perfectly associated with the melanistic phenotype in this species ($\chi^2 = 16.03$, d.f.= 1, $p<0.005$, based on Data set 2 – see Table S6).

Both of the *ASIP* mutations found here to be associated with melanism involve residues that are known to be critical for the function of the resulting protein [42]. The substitution identified in *L. guigna* removes the eighth of the 10 cysteine residues of the C-terminal Cys-rich domain that are completely conserved across mammals, and whose presence is critical for ASIP activity [43,44]. In contrast, the substitution identified in *L. colocolo* introduces an extra cysteine residue in this region, likely affecting the stability and/or topology of the disulfide bonds that characterize this domain. Moreover, the arginine residue itself (which is removed by this substitution) is part of the triplet Arg-Phe-Phe (RFF) that forms a central loop which plays a crucial role in protein activity [42]. This loop is closed by a disulfide bond between the Cys119 and Cys126 residues, with the latter happening to be the site affected by the *L. guigna* mutation (see above). Given the important role of the disulfide bonds, as well as the central loop, we can conclude that the amino acid changes observed in both *L. colocolo* and *L. guigna* do have a strong functional impact, likely reducing or ablating ASIP function. As demonstrated experimentally in mice (e.g. [43] and corroborated by association studies in several other species (e.g. [5,45], such loss of function is invariably implicated in the causation of recessively-inherited melanism.

A similar approach targeting *MC1R* also revealed interesting patterns. The alignment of the full coding exon of this locus in *Leopardus* spp. comprised 954 bp (317 codons), and showed low levels of variation within and among species. Interestingly, the coding sequence of *L. guigna* was identical to one of the haplotypes

373 observed in non-melanistic *L. geoffroyi* individuals, a finding which is consistent with
374 the recent history of divergence between these species (see below). Overall, there were
375 twelve variable sites, two of which (sites 32669 and 32937; see Table S5) were fixed
376 between *L. colocolo* and the other two species. A third site (position 32658) was fixed
377 for different alleles in *L. colocolo* and *L. guigna*, but polymorphic in *L. geoffroyi*. Six
378 other variable sites were identified as within-species polymorphisms that showed no
379 obvious association with melanism. Four of them (two synonymous [sites 32141 and
380 32159] and two non-synonymous [sites 32098 and 32175]) were identified in *L.*
381 *colocolo*, and two (one synonymous and one non-synonymous [sites 32453 and 32945,
382 respectively) in *L. geoffroyi*.

383 Finally, there were also four non-synonymous substitutions between melanistic
384 and non-melanistic *L. geoffroyi* (Table S5). Two of them (C32608T and A32609G)
385 occurred in the second and third positions of codon 177, changing a threonine to
386 isoleucine residue, while a third non-synonymous substitution (A32658G) induced a
387 serine to glycine substitution at codon 194. We found little evidence that these amino
388 acid substitutions may be involved in coat color changes in *L. geoffroyi* because they are
389 non-conservative amino acids sites across mammals. In contrast, the non-synonymous
390 mutation T32451C was more likely implicated causally in this phenotype, as it induced
391 a cysteine to arginine substitution at amino acid position 125 (C125R). All six
392 melanistic *L. geoffroyi* analyzed here were heterozygous for this mutation, while the 10
393 wild-type individuals were homozygous for the ancestral 'T' allele. There was thus a
394 significant association between melanism and the CT genotype ($\chi^2 = 16.00$, d.f.= 1,
395 $p<0.005$, based on Data set 2 – see Table S7), consistent with a dominant mode of
396 inheritance of this trait in *L. geoffroyi*, which is expected in cases where *MC1R* is
397 implicated [5,21,23]. This is a relevant inference, since the mode of inheritance of
398 melanism had not been previously reported for this species. In addition, an interesting
399 argument reinforcing the hypothesis that this mutation is implicated in the melanistic
400 phenotype of *L. geoffroyi* is that it consists of exactly the same mutation previously
401 reported to cause dark pigmentation in the fox (*Vulpes vulpes*) by constitutively
402 activating *MC1R* [46].
403
404
405

406 Allele frequency estimation
407
408 Given the observed association between melanism and specific alleles identified
409 at the *ASIP* or *MC1R* loci in the three surveyed species, we set out to estimate the
410 frequency of such variants in the sampled populations. In all three cases, the frequency
411 of the allele associated with melanism was appreciably high (Table 1). This was
412 especially the case of *L. colocolo*, in which the frequency of the R120C allele among
413 the wild-caught individuals was remarkable (0.71). All such specimens were sampled
414 from the same natural population, in Emas National Park, central Brazil (see
415 supplementary Table 1), with no known bias in capture probability towards either of the
416 coloration phenotypes. Therefore, we infer that such estimate of allele frequency indeed
417 reflects the occurrence of this molecular variant in the field. We tested for Hardy-
418 Weinberg equilibrium (HWE) at this locus, and found support for this assumption (see
419 Table 1), with both the observed and expected frequency of melanism in our sample
420 being *ca*. 50%. Although lower than this estimate, the field-based assessment of the
421 melanism frequency in the sampled area was also quite high, reaching *ca*. 30% (see
422 Table 1). Interestingly, this is the only pampas cat population in which the occurrence
423 of melanism has been confidently demonstrated (L. Silveira, pers. comm.), so it is
424 intriguing to observe that this coloration variant reaches such high frequency at this site.
425 Likewise, field-observed frequencies of melanism are quite high for *L. guigna*,
426 ranging from 15.8% to 28.6% in the area where our samples were collected (Isla Grande
427 de Chiloé [[26,47]]). On the basis of our molecular data, the frequency of the melanism-
428 associated C126Y allele was 0.5, and the expected frequency of melanism assuming
429 HWE was 0.25. This value was very close to the observed frequency of melanism in our
430 sample (0.31), and again supported the assumption of HWE in this data set (see Table
431 1). In this case, the field-based and molecular-derived estimates of melanism frequency
432 were even more concordant than in *L. colocolo*, and again there was support for high
433 values in the sampled population.
434 Finally, melanism frequencies were also high for the *L. geoffroyi* population
435 analyzed in this study. In this case, the sampled population was spatially broader,
436 comprising animals collected in Rio Grande do Sul state, southern Brazil. Field-based
437 estimates of melanism in this region reach *ca*. 20% (E.E. and collaborators, unpublished
438 data), and are similar to the values we derived from our molecular data set (observed:

0.3; expected under HWE: 0.28 [no departure from HWE detected]). Although lower than in the case of the other two species, the estimated frequency of the melanism-associated allele was still quite high (0.15), leading to considerable prevalence of this phenotype given the inferred dominant inheritance of this variant.

Patterns of sequence variation across the targeted genomic regions

A total of 52 DNA libraries of *Leopardus* spp. were successfully constructed and sequenced. The five attempts that failed (see Table S1) were due to low DNA quality. We analyzed large-scale genotype data obtained from the targeted resequencing of *ASIP* and *MC1R* genomic regions, which represents hundreds of SNPs distributed widely across these regions (Table S8). The target resequencing entirely confirmed the genotypes at the segregating loci identified initially by PCR-based sequencing (Data set 3). Furthermore, the haplotype structure identified in these regions by next-generation targeted resequencing and using only the high-quality genotype markers (Figure S2) showed interesting and very distinct patterns in the three analyzed species.

We first characterized the genetic diversity across the *ASIP* and *MC1R* genomic regions aiming to assess the occurrence of any atypical pattern in the regions surrounding the melanism-associated mutations. In general, the levels of nucleotide diversity were similar in both *ASIP* and *MC1R* genes for each species, except in *L. colocolo,* which showed higher diversity in the latter. Among the three species, *L. guigna* showed the lowest nucleotide diversity (Table S9). When all individuals of either coat color phenotype were considered together (Figure 3A, 3C and 3E; column ALL in Table S9), the nucleotide diversity indices were higher or intermediate, owing to the mixture of melanistic and non-melanistic alleles. Considering the analyses performed in *ASIP* segments for *L. colocolo* and *L. guigna,* comparison among non-melanistic and melanistic individuals revealed a significant reduction of genetic variation at the genomic segment carrying the mutations implicated in melanism, i.e., the AsipD segment in both species (Figure 3B and 3D, Figure S1). The observed levels were fifteen-fold and eight-fold less diversity in melanistic *vs*. non-melanistic *L. colocolo* and *L. guigna*, respectively (see NM and M columns of Table S9 for comparisons).

To investigate whether the observed low variability could be due to an effect of selective pressure acting on AsipD in *L. colocolo* and *L. guigna*, we performed several neutrality tests comparing this segment to all others. Tajima's D values were positive (in several cases significantly so) across most of *ASIP* and *MC1R* segments for both melanistic and non-melanistic individuals. Given that several of these segments can be safely assumed to be unlinked to each other (see Fig. S1 for the estimated distance among the sampled segments), the observation of this pervasive positive signal across both genomic regions can be inferred to be due to demographic causes, possibly representing an effect of past population structure (not due to current subdivision, given the local geographic scale of our sampling for these species). A striking exception to this pattern was observed for melanistic individuals at the AsipD segment, whose estimate of D was negative (-1.44 and -0.93 for *L. colocolo* and *L. guigna,* respectively) (Figure 4A e 4D). A negative value Tajima's D can be indicative of positive selection or population-size expansions, both of which produce an excess of low-frequency polymorphisms [48]. Given the localized occurrence of the negative values, a selective explanation is more likely. Fu and Li's D and F tests were consistent with Tajima's D, showing positive values across the genomic regions, but negative for AsipD (Fu and Li's D = -1.25 and Fu and Li's F = -1.50 for *L. colocolo* [Figure 4B-C]*;* and -0.95 and -0.96, respectively, for *L. guigna* [Figure 4E-F]). Such negative values are indicative of recent positive selection, an inference which is strengthened by their specific occurrence in melanistic individuals for the AsipD segment, where the implicated mutation lies.

An intriguing pattern was observed in the AsipE and AsipF segments of non-melanistic *L. guigna* individuals, with negative values (albeit non-significant) for all neutrality tests. This pattern is consistent with the observed lower nucleotide diversity in these individuals, compared to melanistic ones at the same segments (Figures 3C and 3D; Table S9). Although this observation could be due to some stochastic demographic event, its localized (and phenotype-associated) occurrence suggests otherwise. Selection-related explanations include two possibilities: (i) a long-range signal of historical selection favoring non-melanistic alleles at *ASIP* (which is consistent with a hypothesis of balancing selection in this species [see below]); or (ii) other genes located in these segments (see Figure S1), whose function is not completely characterized, play an adaptive role in non-melanistic individuals. Additional data will be required to adequately test these hypotheses.

Another instance of reduction in genetic diversity was observed in the AsipH segment in *L. colocolo* and *L. geoffroyi*, in this case not exhibiting any association with coat color (see Figure 3). No reliable data from *L. guigna* could be collected from AsipH (due to an insufficient number of called SNPs [not shown]), precluding an assessment of this species in this case. Although the proximate cause for the observed low variation is the small number of SNPs called in this segment (see Table S8), there is no obvious technical reason that explains this result, suggesting that some underlying evolutionary process has affected this segment. One possibility is the occurrence of background selection in that region, possibly related to negative selection targeting the conserved *Nuclear receptor coactivator 6* (*NCOA6*) gene, located in that segment. Such hypothesis remains to be tested in future genomic surveys of these and other species.

Interesting results were also obtained with equivalent analyses of *MC1R*, especially regarding the region containing the melanism-associated mutation in *L. geoffroyi*. Levels of genetic variability within *L. geoffroyi* were not significantly different between *ASIP* and *MC1R* (see column 'ALL' in Table S9), and the nucleotide diversity was similar when comparing the melanistic and non-melanistic haplotypes across the *MC1R* segments (Figure 3F). Still, an interesting trend was observed in the neutrality tests, pertaining to the genomic segment that contained the *MC1R* gene (Mc1rC) and another (Mc1rB) lying 2 kb upstream of it (see Figure S1). The initial analyses, with individuals categorized by their phenotype, revealed the same general trend towards positive values described above, likely reflecting a pervasive demographic signal. Interestingly, Tajimas's D and Fu and Li's D and F tests yielded negative values for the melanistic category in the Mc1rB segment, but not in Mc1rC (see Figures 4G, 4I, 4K). Given that our sample size was small (possibly reducing the power of these tests), and that all melanistic individuals in this case were heterozygotes (i.e. half of the haplotypes included in the 'melanistic' category were in fact 'non-melanistic' haplotypes, at least in the case of Mc1rC, where phasing could be conducted), we ran a second set of analyses re-categorizing the Mc1rC data. In this case, we allocated Mc1rC haplotypes (instead of individuals) to the melanistic *vs*. non-melanistic categories, thus improving the sample size for the former category, and also removing the noise induced by the heterozygotes. This approach revealed negative (albeit not statistically significant) values for the Mc1rC segment in all neutrality tests (-0.67 in Tajima's D and Fu and Li's D, -0.74 in Fu and Li's F test; see Figures 4H, 4J,

4L). These results suggest that there is indeed a signal for positive selection in the *L. geoffroyi MC1R* data, which is partially masked by a confounding demographic signal (as was also the case of *ASIP* in the other species). Intriguingly, the signal appears more clearly in the Mc1rB segment than in Mc1rC (which contains the focal gene), perhaps due to a stochastic effect derived from the variants available in the population at the time when a selective sweep occurred. Given the very close proximity of the two segments, they may be considered effectively linked, so that the hypothesis that Mc1rB is reflecting a historical signal that originated within Mc1rC seems plausible.

Haplotype Network Analysis

Haplotype networks were generated at two scales, one of them focusing on the coding region of the *ASIP* and *MC1R* genes, and the other on the broader genomic segment carrying these loci (AsipD and Mc1rC, respectively). We first describe and discuss the results obtained for *ASIP,* and in this case mostly focus on the AsipD analysis, since it allowed some interesting insights onto the evolution of melanism in *L. colocolo* and *L. guigna*).

The haplotype network generated from the AsipD segment (Figure S3-A) allowed the identification of three well-defined groups, one for each of the analyzed species (*L. colocolo, L. guigna* and *L. geoffroyi*). Fifty-two individuals (18 *L. colocolo,* 13 *L. guigna* and 21 *L. geoffroyi*) representing 104 chromosomes were allocated to 40 unique haplotypes defined by 171 variable sites. The network analysis revealed *L. colocolo* as the most divergent group, and the seven *L. guigna* haplotypes formed a distinct cluster which was nested within *L. geoffroyi* (see Figures S3-A and S3-B), supporting the hypothesis that the former species arose as an isolated population of the latter. These observed genealogical relationships among haplotypes were in agreement with the current understanding of the phylogeny of this Neotropical felid lineage [25].

Considering the *L. colocolo* clade, a total of 36 chromosomes (20 melanistic and 16 non-melanistic) distributed in ten haplotypes were defined by 16 variable sites. The relationships among some haplotypes were ambiguous, with reticulations suggesting the occurrence of recombination events at some sites. This included haplotypes bearing the melanism-associated mutation (10585). A visual evaluation of the network supported the expectation that the melanistic haplotypes (in black color, Figure S3-A) were

derived from a more basal, non-melanistic haplotype (in green color). This pattern is consistent with a recent origin of melanism in *L. colocolo*, relative the overall coalescence of the full set of haplotypes. Nevertheless, it shows that sufficient time has elapsed since the mutation occurred for subsequent mutation/recombination events to take place.

A different pattern was observed in the *L. guigna* clade (Figure S3-A). The seven haplotypes were sampled in 26 chromosomes (6 melanistic and 20 non-melanistic) defined by 11 polymorphic sites. The network structure suggested the occurrence of two distinct subgroups (non-melanistic *versus* melanistic) that have been historically separated for sufficient time to accumulate several distinctive mutations and to achieve reciprocal monophyly. This pattern may indicate that the melanism-associated mutation is older in *L. guigna* than in *L. colocolo*, and also suggests that balancing selection may be acting upon the former (i.e. maintaining those two divergent haplotypic lineages as a stable polymorphism).

In contrast to the AsipD results, the haplotype network generated with the Mc1rC genomic segment (not shown) could not be interpreted clearly, likely due to an excessive number of recombination events which confused the algorithm (see Figure S2 for a full depiction of the SNP data set and the implied recombination events in each segment). Thus, in this case we only show the haplotype network analysis based on the *MC1R* coding region (Figure S3-C). Fifty-five individuals (18 *L. colocolo,* 13 *L. guigna,* 23 *L. geoffroyi* and 1 *Felis catus* used as outgroup), representing 110 chromosomes, were allocated in 13 haplotypes defined by 25 variable sites. The network analysis revealed the same phylogenetic relationships among the cats as observed in the *ASIP* networks (Figures S3-A and S3-B). The most common haplotype was shared between *L. geoffroyi* and *L. guigna*, and was nested within the evolutionary diversity of the former species. A striking observation was that the haplotype associated with melanism in *L. geoffroyi* was quite distinct from all others (exhibiting a divergence that surpassed that observed in inter-specific comparisons). This haplotype was three mutational steps away from its nearest sampled relative, while the distance between all pairs of neighboring non-melanistic haplotypes was a single mutational step (see Figure S3-C). Even more remarkable, these three mutational steps all consist of non-synonymous mutations, which suggests that this haplotypic lineage may have undergone a selectively induced acceleration in its substitution rate. Although this pattern is different from that

603 observed in the melanism-associated mutations of *L. colocolo* and *L. guigna* (likely
604 involving an older origin of melanism in *L. geoffroyi*), again there is evidence
605 suggestive of natural selection having played a role in the evolution of this trait.
606
607 Using genomic haplotype properties to assess signals of natural selection
608
609 The measures of extended haplotype homozygosity (EHH) for the *ASIP* locus in
610 *L. colocolo* showed a large and very striking difference between melanistic and non-
611 melanistic chromosomes, demonstrating the existence of a long extended haplotype of
612 ~180 kb associated with the melanistic allele (Figure 5A). The haplotype bifurcation
613 plots (Figure 5B), built assuming as origin the SNP associated with melanism (position
614 10585), located in the AsipD segment, corroborated the EHH results. Indeed, these plots
615 depict a decreased haplotype diversity around and especially upstream of this locus in
616 the melanistic chromosomes, while the non-melanistic ones do not display any
617 particular pattern. These observations suggest that the mutation implicated in melanism
618 arose recently, and rapidly reached a high frequency (see above for estimates of
619 frequency in the field), inducing a selective sweep in the adjacent genomic regions
620 whose signature is still apparent.
621 In strong contrast with *L. colocolo*, the EHH measures computed for *L. guigna*
622 around the implicated mutation (position 10604) demonstrated a much shorter length
623 (~ 11 kb) of the haplotype shared by the melanistic chromosomes, distributed mostly
624 upstream of that locus and contained almost entirely within the AsipD segment (Figure
625 5C). This observation could suggest a more ancient occurrence of the melanistic
626 mutation in this species, which is also consistent with the haplotype network analysis
627 described above (Figure S3). In addition, the difference between melanistic and non-
628 melanistic chromosomes appeared less marked, confounded by several haplotype
629 segments with relatively high EHH observed in the non-melanistic chromosomes
630 around the implicated site. This latter pattern was also supported by haplotype
631 bifurcation plots (Figure 5D), and may be explained by the demographic history of *L.*
632 *guigna* individuals, which have possibly undergone a genetic bottleneck during the
633 founding of the Chiloé Island population. Such historical bottleneck is also consistent
634 with the observation of lower levels of genetic diversity in *L. guigna* relative to the

635 other species (see above), and warrants additional scrutiny employing additional
636 genomic regions.
637 Finally, the EHH measures obtained for *L. geoffroyi* around the melanism-
638 associated mutation (located in the Mc1rC segment; position 32451), demonstrate the
639 absence of a shared haplotype around this locus, with a similar degree of diversity in
640 both melanistic and non-melanistic chromosomes (Figure 5E). This observation was
641 also confirmed by the haplotype bifurcation plots, which were built using as origin the
642 focal site (Figure 5F). Taken together, these results suggest a more ancient origin of the
643 melanistic mutation in *L. geoffroyi* relative to the other species, thus allowing the
644 recombination-driven erosion of the long-range haplotypic patterns that were present at
645 the time of its occurrence.
646
647 Melanism Evolution in *Leopardus* spp.
648
649 Our results reveal three novel mutations implicated in melanism in *Leopardus*
650 spp., supporting the hypothesis of species-specific mutations inducing this phenotype in
651 these wild felids. Along with previous findings reporting five other mutations [5,24], we
652 conclude that this phenotype arose independently at least eight times in the Felidae.
653 The analysis of genomic data revealed distinct evolutionary histories of
654 melanism in these closely related species. First, we showed a strong association
655 between a molecular variant and black coat color in *L. colocolo*, in which the causal
656 allele (R120C) is quite common, reaching a frequency of 71% in the sampled
657 population. This remarkable estimate is supported by the high phenotype frequency (*ca*.
658 30%) observed in Emas National Park. In addition to the high frequency, we observed
659 negative values of Tajima's D and other neutrality tests in melanistic haplotypes of
660 exactly the region where *ASIP* lies, indicating an excess of low frequency
661 polymorphism compared to neutral expectations. In addition, melanistic haplotypes had
662 an unusually high EHH (*ca*. 180 kb) that associated with their estimated high population
663 frequency and the pattern observed in the bifurcation plots, is indicative of a recent
664 episode of positive selection favoring the mutation that induces this phenotype. Taken
665 together, these results strongly indicate that the melanistic mutation constitutes a
666 selectively advantageous allele, supporting the interpretation that melanism has an
667 adaptive significance in *L. colocolo*. This is the first time that natural selection is

implicated in the evolution of melanism in felids, and one of the first times for wild animals in general.

Although weaker and different, there was also a signal of natural selection in the *L. guigna* data. We did not find strong evidence of positive selection in the *ASIP* genomic region by analyzing the haplotype structure of these samples. In contrast to *L. colocolo,* the melanistic haplotypes of *L. guigna* shared a shorter-range haplotype of *ca.* 11 kb, indicating that, if a selective sweep has occurred in the past, it was either weaker (i.e. not inducing a pervasive signal) or older (so that local recombination rates have already eroded the signal). Overall, the frequency of the melanism-associated C126Y allele was estimated at 50% in our sampled area, which is consistent with the high field-based phenotype frequency (ranging from *ca.* 15 to 30 % in Chiloé Island). These high estimates are quite striking, since new variants require a long time to reach high frequency in a population under neutral expectations. Moreover, the results from the genomic patterns of diversity, neutrality tests and haplotype networks are all suggestive of the occurrence of balancing selection in this species. In contrast to what is seen in *L. colocolo*, here there is more diversity in the combined (ALL) data set for AsipD than in either the melanistic or non-melanistic categories (see Figure 3C), an observation which is perfectly explained by the pattern depicted in the haplotype network (Figure S3-A). This species exhibits two distinct lineages comprising melanistic vs. non-melanistic haplotypes, which have been maintained in the population for a considerable period of time (give the mutations they have accumulated after their divergence). Under such a scenario, the most likely drivers of such process would be frequency-dependent selection (possibly related to camouflage) or spatially heterogeneous selection (with melanistic and non-melanistic animals being favored in different but connected areas). Although these possibilities need to be tested with additional data (both molecular and field-based), they raise interesting and plausible hypotheses to be addressed in future studies.

Finally, our analysis of the *L. geoffroyi* data showed distinct patterns, some of which were also indicative of natural selection acting on the *MC1R* gene in connection with melanism. An initial indication that natural selection may also influence melanism in this species is the high field-based and molecular-derived estimates its frequency (20% and 15%, respectively), which could have arisen by chance but would more likely do so if selectively favored. Some of our analyses did not support this hypothesis, given

the similar levels of diversity in melanistic and non-melanistic haplotypes, and the absence of a long genomic stretch sharing extended haplotype homozygosity (EHH). On the other hand, the neutrality tests did yield results suggestive of a selective effect in this region, although the results were not as clear-cut as those observed in *ASIP* (especially for *L. colocolo*). One possible reason for this weaker pattern is that the melanistic mutation is older in *L. geoffroyi* than in the other two species, and/or that its recombination rates are higher in this region. Although there was indeed considerable evidence for plentiful recombination in this portion of the *L. geoffroyi MC1R* genomic region (see Figure S2), there was no conclusive indication that it was higher than in other sampled segments, or than in the other species. This observation may support the hypothesis of an older age for this mutation, which remains to be further investigated. The strongest piece of evidence suggesting non-neutral evolution of melanism in *L. geoffroyi* emerged from the analysis of the *MC1R* coding region. The occurrence of three non-synonymous mutations (and no synonymous ones) in the lineage associated with melanism is quite striking, and deviates completely from the pattern observed in the remaining haplotypes. Such result suggests the occurrence of an adaptive acceleration of the substitution rate in this lineage, implying that natural selection has favored an increased intensity of evolutionary change in the alleles associated with melanism. Although this process of acceleration could in itself mimic the effect of an older age of the mutation, these possibilities (accelerated non-synonymous rate and older age) are non-exclusive, and may both have shaped the observed patterns of molecular diversity.

Overall, our results strongly indicate that natural selection has played an important role in the evolution of melanism in at least one of the analyzed species (*L. colocolo*), and likely in all three of them. However, the genomic signals observed in each of them are clearly different, possibly due to distinct demographic histories affecting them, but also likely induced by different selective processes, as discussed above. Dissecting the demographic and selective effects underlying the evolution of melanism in these species promises to be a challenge for future studies, but also an exciting system to investigate the origin and maintenance of polymorphic phenotypes.

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References

1. Hoekstra HE, Nachman MW (2003) Different genes underlie adaptive melanism in different populations of rock pocket mice. Molecular Ecology 12: 1185–1194.

2. Shapiro MD, Marks ME, Peichel CL, Blackman BK, Nereng KS, et al. (2004) Genetic and developmental basis of evolutionary pelvic reduction in threespine sticklebacks. Nature 428: 717–723.

3. Mundy NI, Badcock NS, Hart T, Scribner K, Janssen K, et al. (2004) Conserved genetic basis of a quantitative plumage trait involved in mate choice. Science 303: 1870–1873.

4. Cohan FM (1984) Genetic divergence under uniform selection. 1. Similarity among populations of Drosophila melanogaster in their responses to artificial selection for modifiers of ciD. Evolution 38: 55–71.

5. Eizirik E, Yuhki N, Johnson WE, Menotti-Raymond M, Hannah SS, et al. (2003) Molecular genetics and evolution of melanism in the cat family. Current Biology 13: 448–453.

6. Römpler H, Rohland N, Lalueza-Fox C, Willerslev E, Kuznetsova T, et al. (2006) Nuclear gene indicates coat-color polymorphism in mammoths. Science 313: 62.

761 7. Protas ME, Hersey C, Kochanek D, Zhou Y, Wilkens H, et al. (2006) Genetic
762 analysis of cavefish reveals molecular convergence in the evolution of albinism.
763 Nature Genetics 38: 107–111.

764 8. Steiner CC, Römpler H, Boettger LM, Schöneberg T, Hoekstra HE (2009) The
765 genetic basis of phenotypic convergence in beach mice: similar pigment patterns
766 but different genes. Molecular Biology and Evolution 26: 35–45.

767 9. Rosenblum EB, Römpler H, Schöneberg T, Hoekstra HE (2010) Molecular and
768 functional basis of phenotypic convergence in white lizards at White Sands.
769 Proceedings of the National Academy of Sciences of the United States of
770 America 107: 2113–2117.

771 10. Zhang J, Kumar S (1997) Detection of convergent and parallel evolution at the
772 amino acid sequence level. Molecular Biology and Evolution 14: 527–536.

773 11. Hoekstra HE (2006) Genetics, development and evolution of adaptive
774 pigmentation in vertebrates. Heredity 97: 222–234.

775 12. Ling MK, Lagerstrom MC, Fredriksson R, Okimoto R, Mundy NI, et al. (2003)
776 Association of feather colour with constitutively active melanocortin 1 receptors
777 in chicken. European Journal of Biochemistry 270: 1441–1449.

778 13. Nadeau NJ, Minvielle F, Mundy NI (2006) Association of a Glu92Lys
779 substitution in MC1R with extended brown in Japanese quail (Coturnix
780 japonica). Animal Genetics 37: 287–289.

781 14. Rosenblum EB, Hoekstra HE, Nachman MW (2004) Adaptive reptile color
782 variation and the evolution of the Mc1r gene. Evolution 58: 1794–1808.

783 15. Steiner CC, Weber JN, Hoekstra HE (2007) Adaptive variation in beach mice
784 produced by two interacting pigmentation genes. PLoS Biology 5: e219.

785 16. Lamason RL, Mohideen M-APK, Mest JR, Wong AC, Norton HL, et al. (2005)
786 SLC24A5, a putative cation exchanger, affects pigmentation in zebrafish and
787 humans. Science 310: 1782–1786.

17. Gratten J, Beraldi D, Lowder B V, McRae a F, Visscher PM, et al. (2007) Compelling evidence that a single nucleotide substitution in TYRP1 is responsible for coat-colour polymorphism in a free-living population of Soay sheep. Proceedings Biological Sciences / The Royal Society 274: 619–626.

18. Roberts RB, Ser JR, Kocher TD (2009) Sexual conflict resolved by invasion of a novel sex determiner in Lake Malawi cichlid fishes. Science 326: 998–1001.

19. Robbins LS, Nadeau JH, Johnson KR, Kelly M a, Roselli-Rehfuss L, et al. (1993) Pigmentation phenotypes of variant extension locus alleles result from point mutations that alter MSH receptor function. Cell 72: 827–834.

20. Rieder S, Taourit S, Mariat D, Langlois B, Guérin G (2001) Mutations in the agouti (ASIP), the extension (MC1R), and the brown (TYRP1) loci and their association to coat color phenotypes in horses (Equus caballus). Mammalian Genome 12: 450–455.

21. Theron E, Hawkins K, Bermingham E, Ricklefs RE, Mundy NI (2001) The molecular basis of an avian plumage polymorphism in the wild: a melanocortin-1-receptor point mutation is perfectly associated with the melanic plumage morph of the bananaquit, Coereba flaveola. Current Biology 11: 550–557.

22. Hoekstra HE, Drumm KE, Nachman MW (2004) Ecological genetics of adaptive color polymorphism in pocket mice: geographic variation in selected and neutral genes. Evolution 58: 1329–1341.

23. Klungland H, Våge DI, Gomez-Raya L, Adalsteinsson S, Lien S (1995) The role of melanocyte-stimulating hormone (MSH) receptor in bovine coat color determination. Mammalian Genome 6: 636–639.

24. Schneider A, David V a, Johnson WE, O'Brien SJ, Barsh GS, et al. (2012) How the Leopard Hides Its Spots: ASIP Mutations and Melanism in Wild Cats. PloS One 7: 1-7.

814 25. Johnson WE, Eizirik E, Pecon-Slattery J, Murphy WJ, Antunes A, et al. (2006)
815 The late Miocene Radiation of Modern Felidae: A Genetic Assessment. Science
816 311: 73–77.

817 26. Napolitano CG (2012) Filogeografía, inferencia demográfica y genética de la
818 conservación del felino Leopardus guigna en el sur de sudamérica. Programa de
819 Doctorado en Ciencias Silvoagropecuarias y Veterniarias. Santiago, Chile:
820 Universidad de Chile.

821 27. Ortolani and Caro T (1996) The adaptative significance of color patterns in
822 carnivores: Phylogenetic test of classic hypotheses. In: JL G, editor. Carnivore
823 Behavior, Ecology and Evolution. New York: Cornell Univ. Press. pp. 132–188.

824 28. Caro T (2005) The Adaptive Significance of Coloration in Mammals. BioScience
825 55: 125–136.

826 29. Trigo TC, Freitas TRO, Kunzler G, Cardoso L, Silva JCR, et al. (2008) Inter-
827 species hybridization among Neotropical cats of the genus Leopardus, and
828 evidence for an introgressive hybrid zone between L. geoffroyi and L. tigrinus in
829 southern Brazil. Molecular Ecology 17: 4317–4333.

830 30. Sambrook J, Fritsch EF, Maniatis T (1989) Molecular Cloning: a laboratory
831 manual. Second Edi. New York: Cold Spring Laboratory Press.

832 31. Rozen S, Skaletsky HJ (2000) Primer3 on the WWW for general users and for
833 biologist programmers. In: S. K, Misener S., editors. Bioinformatics Methods and
834 Protocols: Methods in Molecular Biology. Totowa, NJ: Humana Press. pp. 365–
835 386.

836 32. Pontius JU, O'Brien SJ (2007) Genome Annotation Resource Fields--
837 GARFIELD: a genome browser for Felis catus. The Journal of heredity 98: 386–
838 389.

839 33. Gnirke A, Melnikov A, Maguire J, Rogov P, LeProust EM, et al. (2009) Solution
840 hybrid selection with ultra-long oligonucleotides for massively parallel targeted
841 sequencing. Nature Biotechnology 27: 182–189.

842 34. Depristo MA, Banks E, Poplin R, Garimella K V, Maguire JR, et al. (2011) A
843 framework for variation discovery and genotyping using next-generation DNA
844 sequencing data. Nature Genetics 43: 491–498. doi:10.1038/ng.806.

845 35. Zhi D, Raphael BJ, Price AL, Tang H, Pevzner P a (2006) Identifying repeat
846 domains in large genomes. Genome Biology 7: R7.1-R7.14

847 36. Li H, Durbin R (2009) Fast and accurate short read alignment with Burrows-
848 Wheeler transform. Bioinformatics (Oxford, England) 25: 1754–1760.

849 37. Li H, Handsaker B, Wysoker A, Fennell T, Ruan J, et al. (2009) The Sequence
850 Alignment / Map (SAM) Format and SAMtools 1000 Genome Project Data
851 Processing Subgroup: 1–2.

852 38. Browning SR, Browning BL (2007) Rapid and accurate haplotype phasing and
853 missing-data inference for whole-genome association studies by use of localized
854 haplotype clustering. American Journal of Human Genetics 81: 1084–1097.

855 39. Librado P, Rozas J (2009) DnaSP v5: a software for comprehensive analysis of
856 DNA polymorphism data. Bioinformatics (Oxford, England) 25: 1451–1452.

857 40. Sabeti PC, Reich DE, Higgins JM, Levine HZP, Richter DJ, et al. (2002)
858 Detecting recent positive selection in the human genome from haplotype
859 structure. Nature 419: 832–837. doi:10.1038/nature01027.1.

860 41. Gautier M, Vitalis R (2012) rehh: an R package to detect footprints of selection
861 in genome-wide SNP data from haplotype structure. Bioinformatics (Oxford,
862 England) 28: 1176–1177.

863 42. McNulty JC, Jackson PJ, Thompson D a, Chai B, Gantz I, et al. (2005) Structures
864 of the agouti signaling protein. Journal of Molecular Biology 346: 1059–1070.

865 43. Perry L, Nakamura T, Swing DA, Secrest L, Eagleson B, et al. (1996) Coupled
866 Site-Directed Mutagenesis/Transgenesis Identifies Important Functional Domains
867 of the Mouse Agouti Protein. Genetics: 255–264.

868 44. Miltenberger RJ, Wakamatsu K, Ito S, Woychik RP, Russell LB, et al. (2002)
869 Alleles at the Mouse agouti Locus. Genetics: 659–674.

870 45. Kingsley EP, Manceau M, Wiley CD, Hoekstra HE (2009) Melanism in
871 peromyscus is caused by independent mutations in agouti. PloS One 4: e6435.

872 46. Vage D, Lu D, Klungland H, Lien S, Adalsteinsson S, et al. (1997) A non-
873 epistatic interaction of agouti and extension in the fox, Vulpes vulpes. Nature
874 Genetics 15: 311–315.

875 47. Sanderson J, Sunquist ME, Iriarte AW (2002) NATURAL HISTORY AND
876 LANDSCAPE-USE OF GUIGNAS (ONCIFELIS GUIGNA) ON ISLA
877 GRANDE DE CHILOÉ , CHILE. Journal of Mammalogy 83: 608–613.
878 doi:10.1644/1545-1542(2002)083<0608.

879 48. Tajima F (1989) Statistical method for testing the neutral mutation hypothesis by
880 DNA polymorphism. Genetics 123: 585–595.

881 49. Sunquist ME, Sunquist F (2002) Wild Cats of the World. Chicago: University
882 Chicago Press.

883

884 **Figure Legends**

885

886 **Figure 1. Coat color phenotypes of *Leopardus* spp.** (A) Adult *L. colocolo* bearing a
887 wild-type (non-melanistic) coat color, next to a melanistic kitten. (B) Typical non-
888 melanistic *L. guigna* individual. (C) Melanistic *L. guigna* individual. (D) *L. geoffroyi*
889 individual showing a gray/yellowish background pelage with solid black spots. (E) A
890 melanistic *L. geoffroyi* individual. Photo credits to Jaguar Conservation Fund (A), Jerry
891 Laker (B), Jim Sanderson (C), Tadeu G. de Oliveira (D), and Projeto Gatos do Mato -
892 Brasil (E).

893

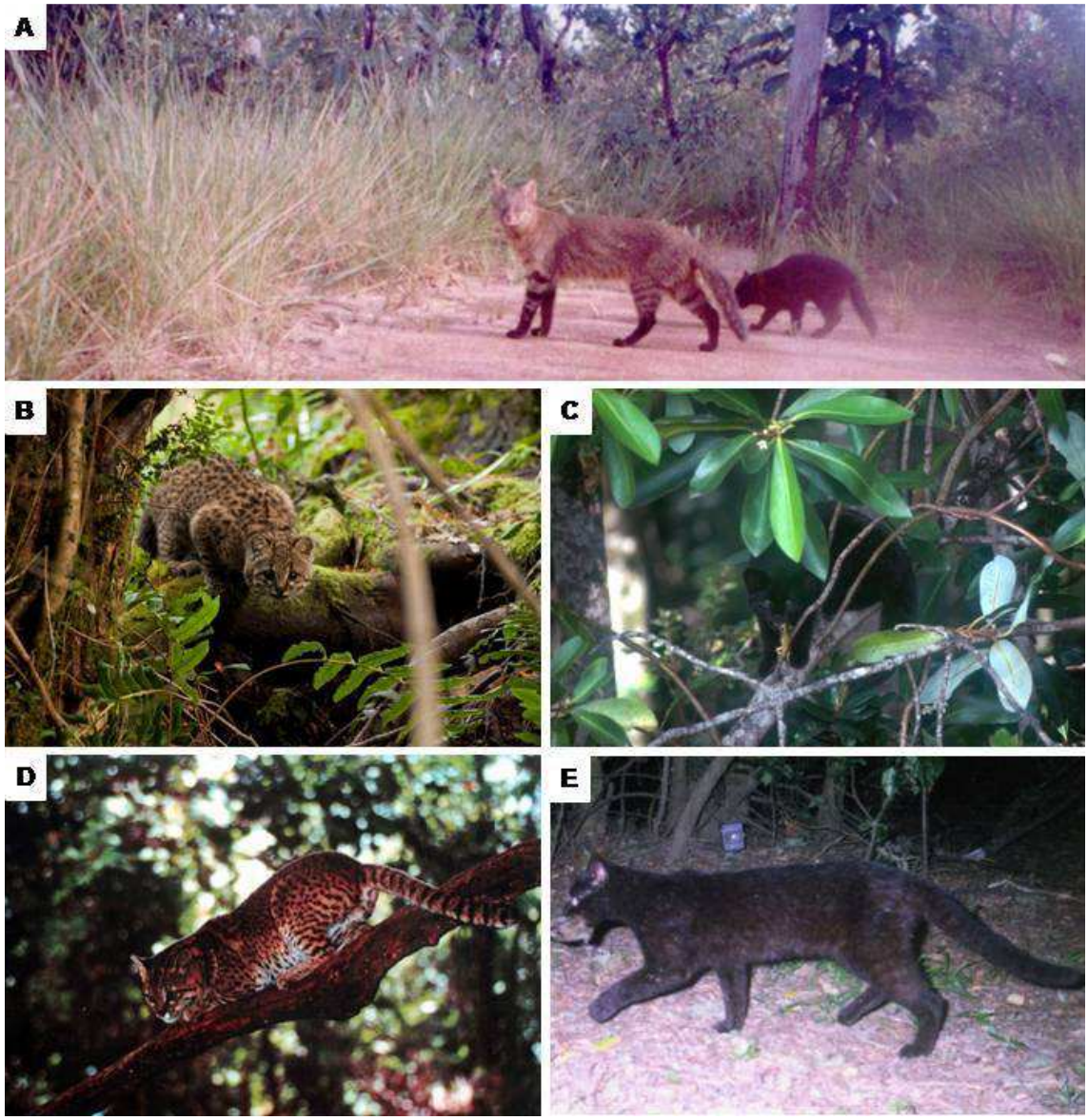
894 **Figure 2. Map depicting the geographic distribution of *Leopardus* spp.** Geographic
895 distribution of *Leopardus geoffroyi* (blue), *L. colocolo* (hatched area) and *L. guigna*
896 (orange) in South America (modified from [49]. Black circles represent the approximate

897 geographic origin of samples analyzed in this study for each species: (A) *L. geoffroyi*
898 from the southern portion of Rio Grande do Sul state, southernmost Brazil; (B) *L.*
899 *colocolo* from Emas National Park, Goiás state, central Brazil; and (C) *L. guigna* from
900 Chiloé Island, Chile. The inset at the top indicates the phylogenetic relations among the
901 species, with their estimated divergence times (as reported by JOHNSON et al., 2006)
902 shown on branches. *L. colocolo* (Lco) is the most divergent species (2.4 MA) and *L.*
903 *geoffroyi* (Lge) and *L. guigna* (Lgu) are sister-species whose divergence from a
904 common ancestral is estimated at 0.74 MA.
905
906 **Figure 3. Nucleotide diversity in *ASIP* and *MC1R* genomic segments.** (A,C,E)
907 Graphs comparing nucleotide diversity estimates of *ASIP* and *MC1R* segments for three
908 different phenotypic categories: melanistic individuals (M), non-melanistic individuals
909 (NM) and both subgroups combined (ALL), shown for each species: *L. colocolo* (A), *L.*
910 *guigna* (C) and *L. geoffroyi* (E). (B,D,F) Direct comparison of nucleotide diversity
911 estimates between melanistic (M) and non-melanistic (NM) individuals across the
912 different *ASIP* segments for *L. colocolo* (B) and *L. guigna* (D), and for the *MC1R*
913 segments of *L. geoffroyi* (F). The *ASIP* coding region (and implicated melanistic
914 mutations of *L. colocolo* and *L. guigna*) is located in segment AsipD, while the *MC1R*
915 coding region (including the *L. geoffroyi* melanistic mutation) is located in segment
916 Mc1rC.
917
918 **Figure 4. Results of neutrality tests for *Leopardus* spp.** Results of Tajima's D test, Fu
919 and Li's D test and Fu and Li's F test, respectively, shown for *L. colocolo* (A, B, C), *L.*
920 *guigna* (D, E, F), and *L. geoffroyi* (G-L). In the latter case, two versions of the test were
921 performed, one of them (G, I, K) considering individuals as units (*i.e.* coding
922 individuals as melanistic or not, regardless of their actual genotype), and the other one
923 (H, J, L) incorporating the haplotype information available for Mc1rC (i.e. moving the
924 non-melanistic haplotypes present in melanistic heterozygotes to the 'non-melanistic'
925 category). Asterisks above each bar indicate statistical significance ($p < 0.05$).
926
927 **Figure 5. Melanistic and non-melanistic haplotype structure.** (A,C,E) Extended
928 Haplotype Homozygosity (EHH) for melanistic and non-melanistic haplotypes as a
929 function of distance from the mutant (melanism-associated) allele in each species: *L.*

930 *colocolo* (A), *L. guigna* (C) and *L. geoffroyi* (E). (B,D,F) Haplotype bifurcation plots for
931 melanistic (left) and non-melanistic (right) haplotypes in *L. colocolo* (B), *L. guigna* (D)
932 and *L. geoffroyi* (F). The central dark blue dot represents the mutant allele associated
933 with melanism; branches represent haplotype divergence, and the thickness of the lines
934 is proportional to the number of chromosomes sampled per haplotype.
935
936 **Figure S1. Schematic representation of the domestic cat *ASIP* and *MC1R* genomic**
937 **regions**. A) Chromosome A3 region including the *ASIP* gene, depicting the relative
938 positions of the sequence contigs obtained from the fosmid probes, spanning *ca*. 930-kb.
939 B) Chromosome E2 region including the *MC1R* gene, depicting the relative positions of
940 the sequence contigs obtained from the fosmid probes, spanning *ca*. 488 kb. For both
941 (A) and (B), each box represents one contig; numbers above the boxes indicate contig
942 length (see Table S4 for details), while numbers below the line indicate the spacing
943 between contigs. Each horizontal arrow represents a gene, and indicates its relative
944 position and orientation relative to the chromosomal annotation. The three *ASIP* coding
945 exons are represented by vertical lines crossing the ASIP arrow.
946
947 **Figure S2. Haplotype structure across the *ASIP* and *MC1R* genomic segments in**
948 **each of the analyzed wild cat species.** Each row represents a sampled chromosome
949 (two per sampled individual, whose numbered ID is indicated on the left); blue and
950 yellow squares represent the major and minor alleles at each SNP site, respectively.
951 Horizontal red dashed lines indicate melanistic haplotypes (below) and non-melanistic
952 haplotypes (above) for the AsipD and Mc1rC segments (which contain the melanism-
953 associated mutations), and are extended arbitrarily to haplotypes of the same individuals
954 in the case of the other segments; vertical red lines indicate the position of the
955 melanism-associated mutation in each species.
956
957 **Figure S3. Haplotype networks constructed for *Leopardus* spp. using different data**
958 **sets of the *ASIP* and *MC1R* genes.** A) Network based on the AsipD genomic segment,
959 showing independent origins of melanism-associated alleles in *L. colocolo* and *L.*
960 *guigna*, as well as other relevant features (see main text). B) Network based on the *ASIP*
961 coding region. C) Network based on the *MC1R* coding region. Each distinct haplotype
962 is represented by a circle whose size is proportional to its frequency. Colors indicate the

963 frequency of the haplotype in each population group (green for *L. colocolo,* yellow for
964 *L. guigna,* blue for *L. geoffroyi*, black for melanistic animals and pink for the outgroup
965 *F. catus*). Bars or numbers placed on connecting branches indicate the number of
966 substitutions inferred to have occurred between related haplotypes; lines with no such
967 indication represent a single mutational step.

Figure 1.



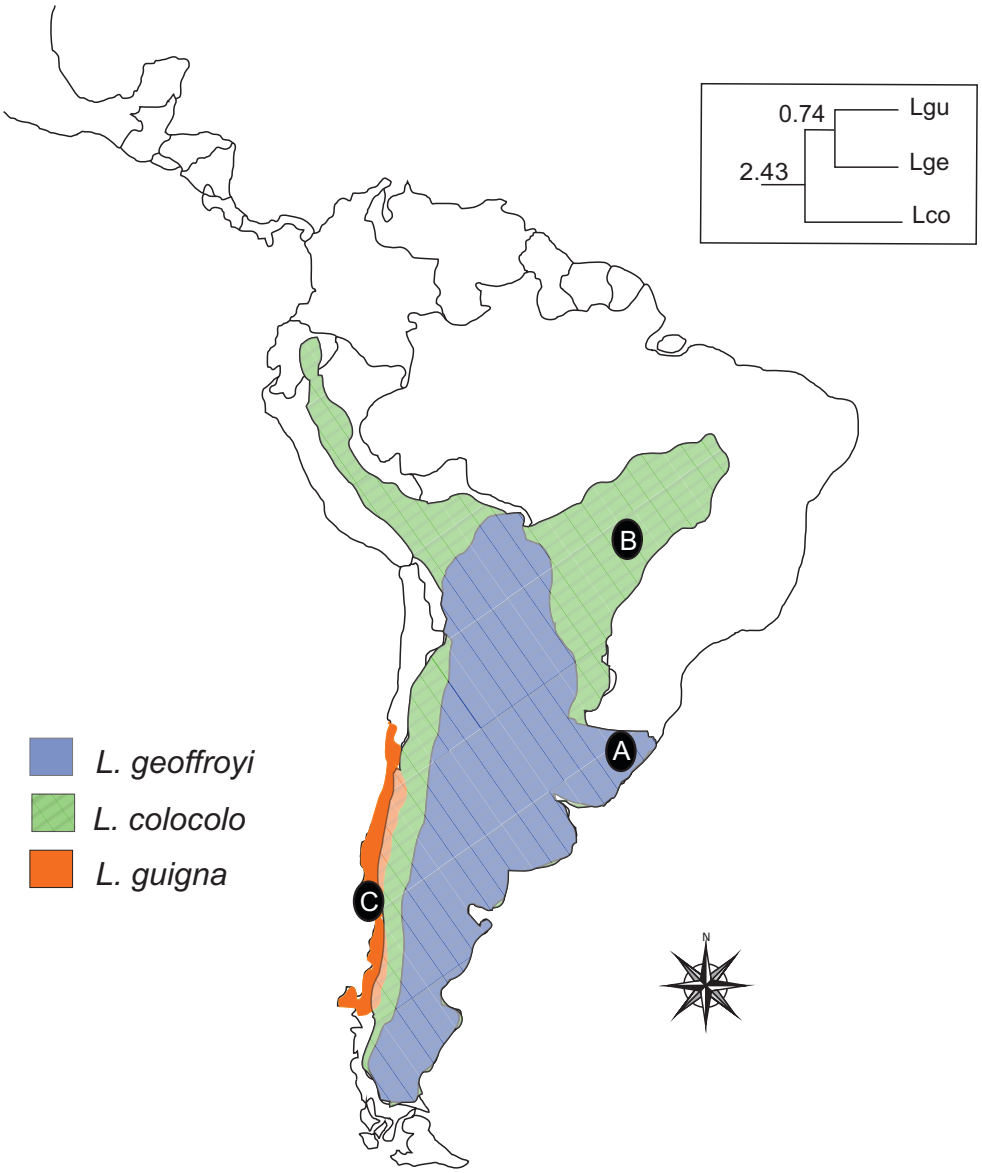
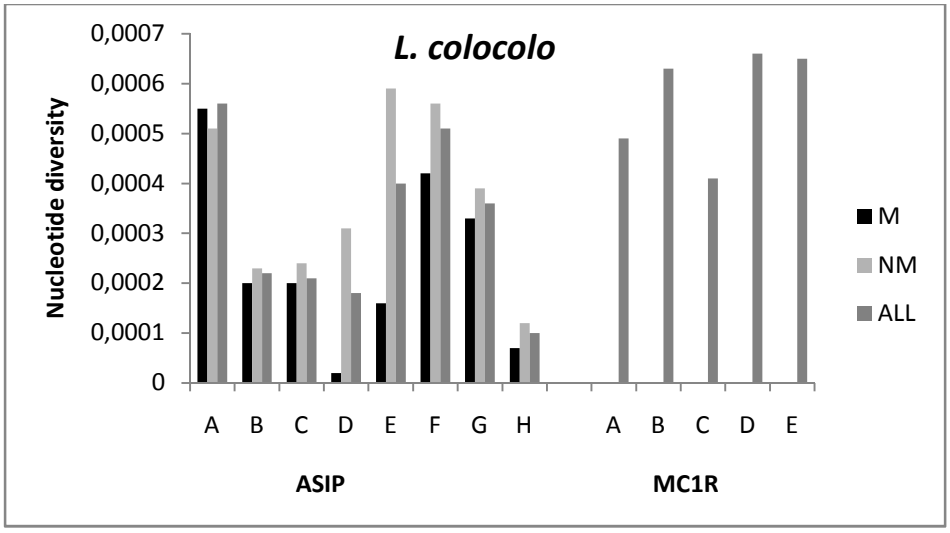

0.74
Lgu
2.43
Lge
Lco
B
A
C
L. geoffroyi
L. colocolo
L. guigna
N

Figure 3.

A)


L. colocolo
Nucleotide diversity
0,0007
0,0006
0,0005
0,0004
0,0003
0,0002
0,0001
0
A B C D E F G H
A B C D E
ASIP
MC1R
M
NM
ALL

B)

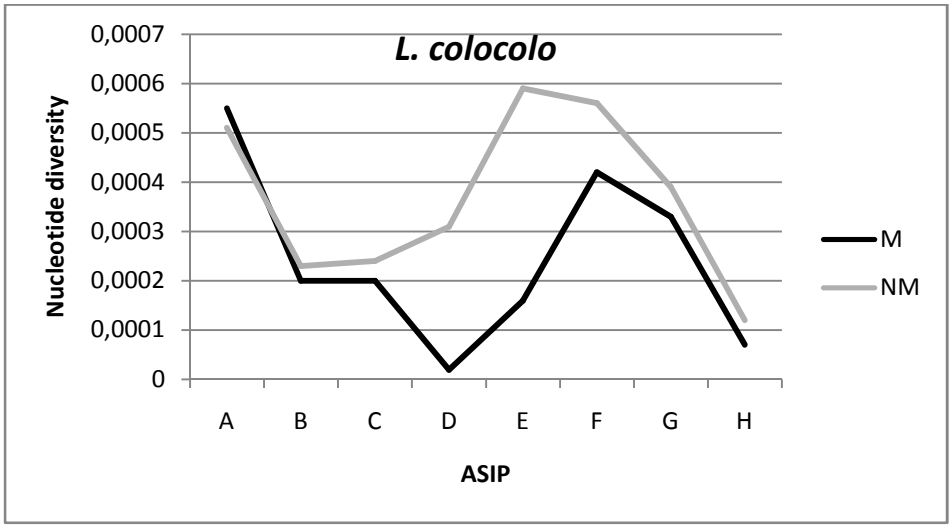
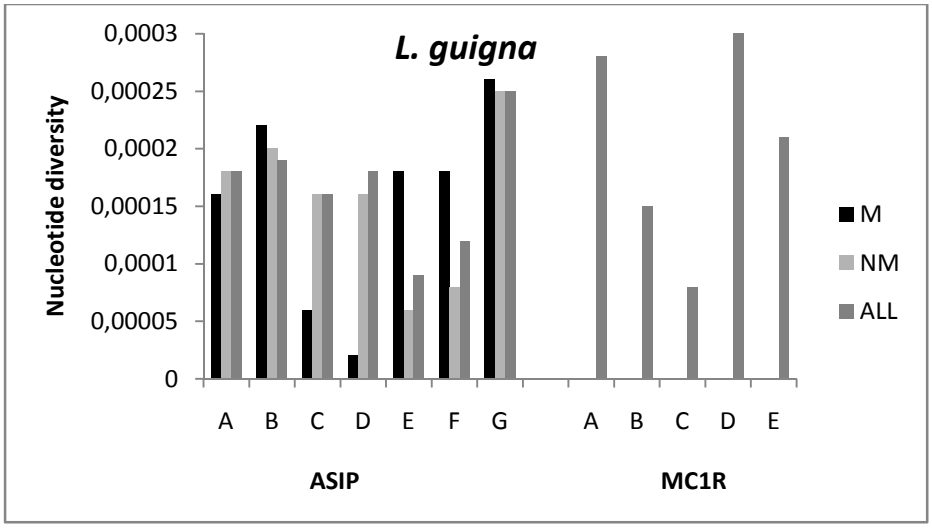

L. colocolo
Nucleotide diversity
0,0007
0,0006
0,0005
0,0004
0,0003
0,0002
0,0001
0
A B C D E F G H
ASIP
M
NM

Figure 3.

C)


L. guigna
Nucleotide diversity
0,0003
0,00025
0,0002
0,00015
0,0001
0,00005
0
A B C D E F G
A B C D E
ASIP
MC1R
M
NM
ALL

D)

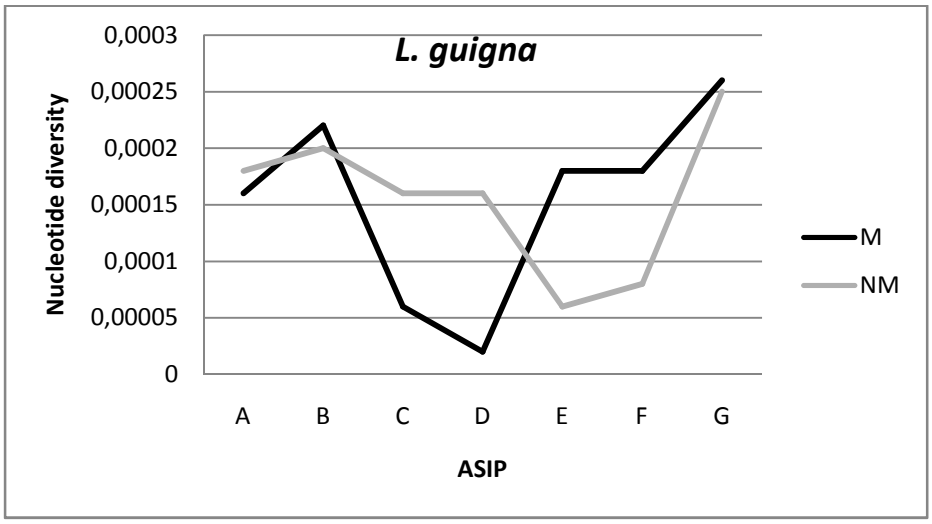
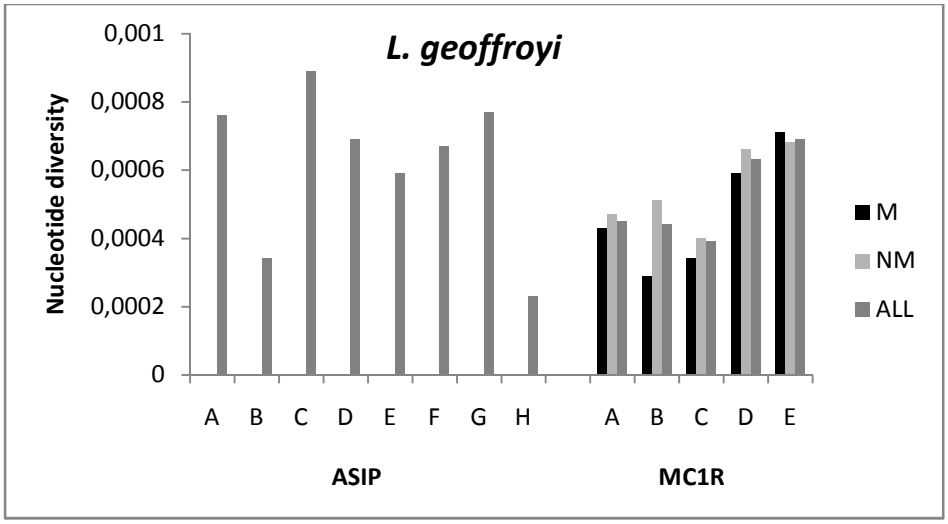

L. guigna
Nucleotide diversity
0,0003
0,00025
0,0002
0,00015
0,0001
0,00005
0
A B C D E F G
ASIP
M
NM

Figure 3.

E)


L. geoffroyi
Nucleotide diversity
0,001
0,0008
0,0006
0,0004
0,0002
0
A B C D E F G H
A B C D E
ASIP
MC1R
M
NM
ALL

F)

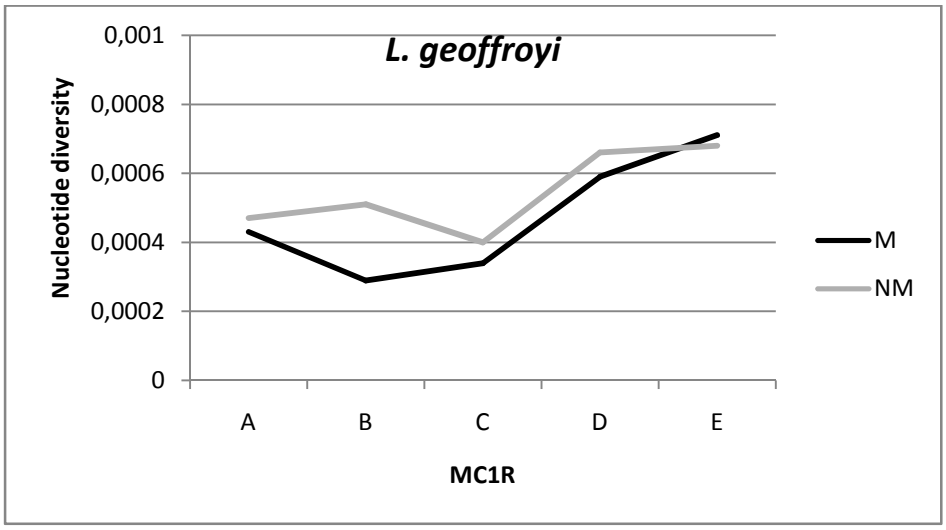
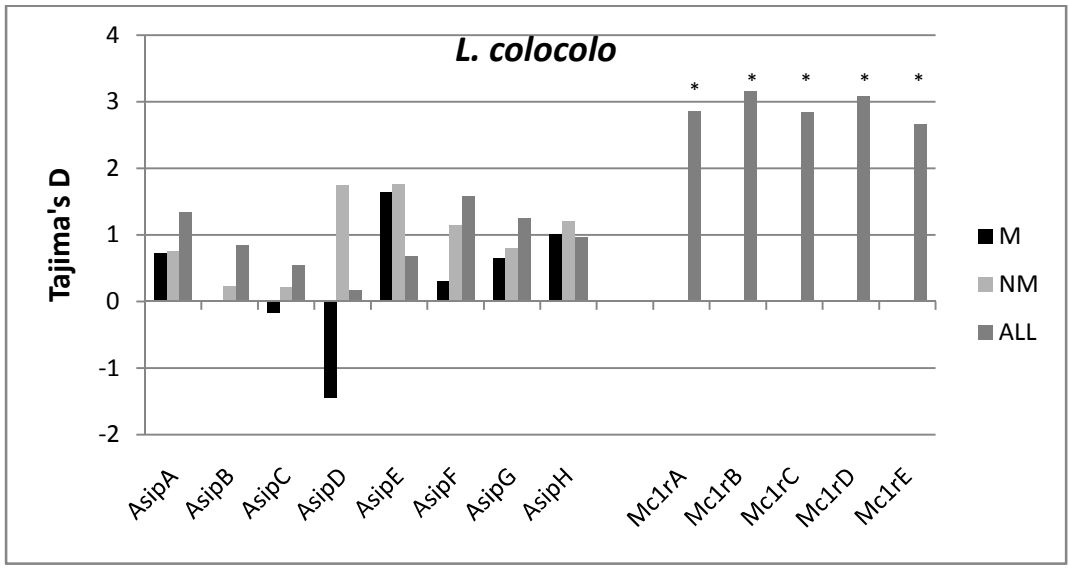
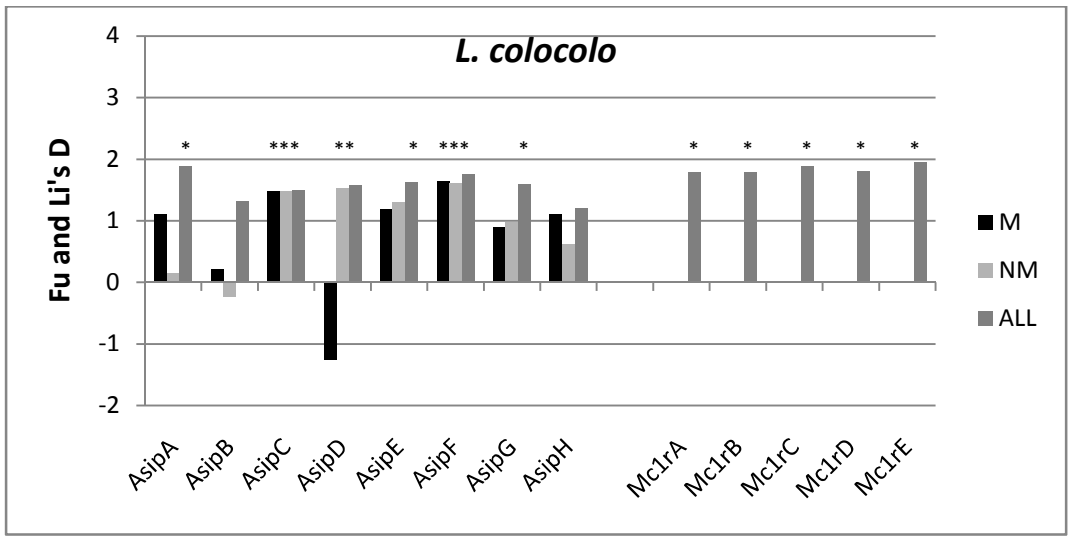

L. geoffroyi
Nucleotide diversity
0,001
0,0008
0,0006
0,0004
0,0002
0
A B C D E
MC1R
M
NM

Figure 4.

A)


L. colocolo
Tajima's D
4
3
2
1
0
-1
-2
*
*
*
*
*
M
NM
ALL
AsipA
AsipB
AsipC
AsipD
AsipE
AsipF
AsipG
AsipH
Mc1rA
Mc1rB
Mc1rC
Mc1rD
Mc1rE

B)


L. colocolo
Fu and Li's D
4
3
2
1
0
-1
-2
*
***
**
*
***
*
*
*
*
*
*
M
NM
ALL
AsipA
AsipB
AsipC
AsipD
AsipE
AsipF
AsipG
AsipH
Mc1rA
Mc1rB
Mc1rC
Mc1rD
Mc1rE

C)

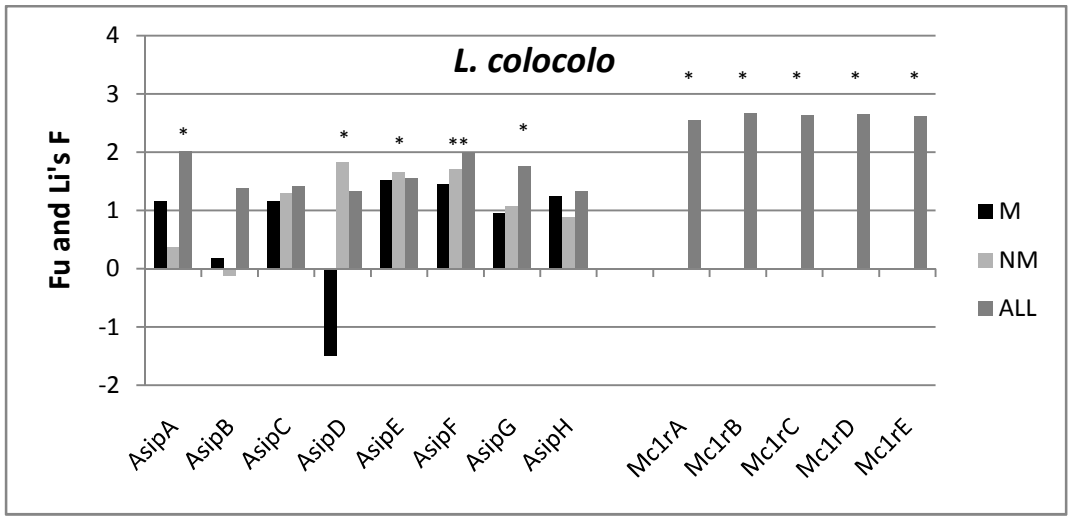
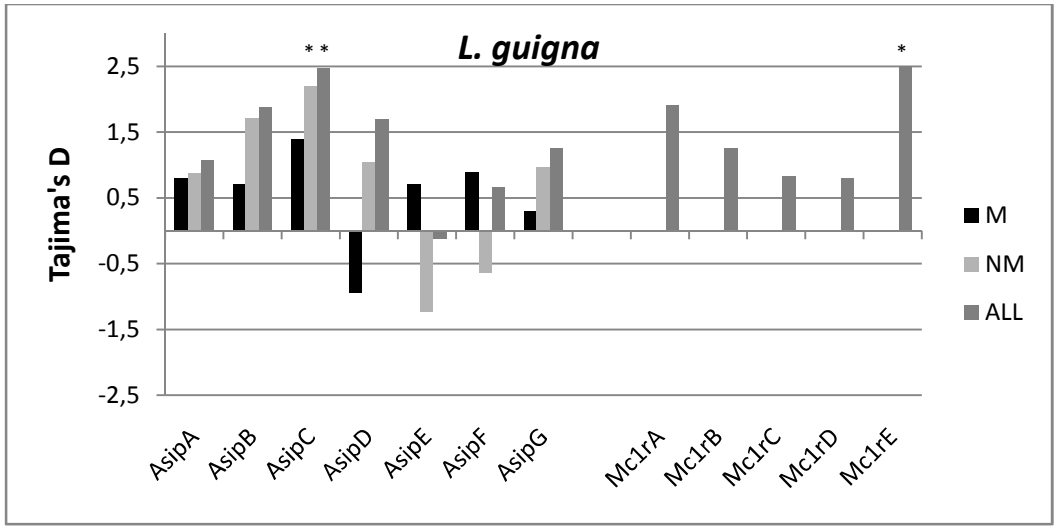
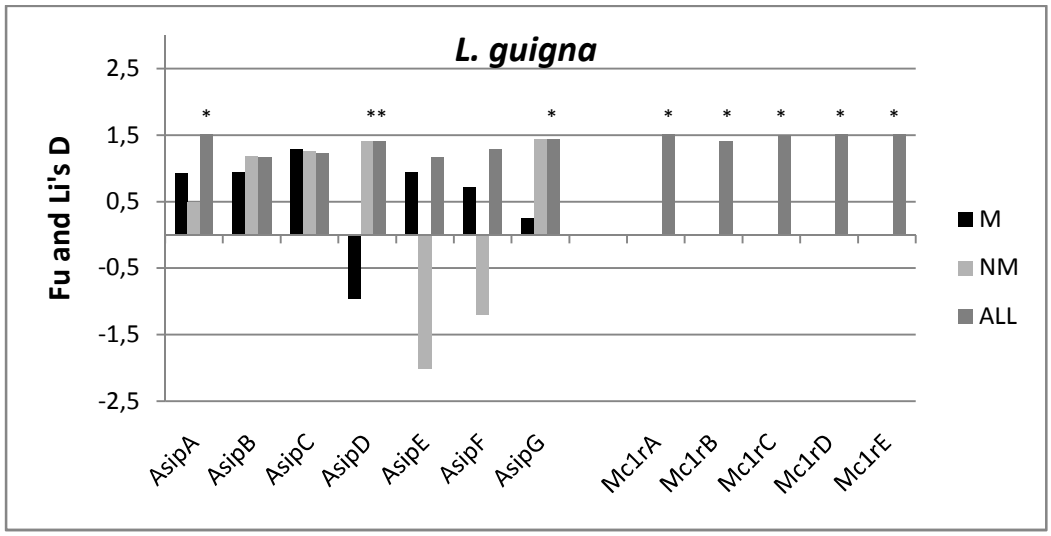

L. colocolo
Fu and Li's F
4
3
2
1
0
-1
-2
*
*
*
**
*
*
*
*
*
M
NM
ALL
AsipA
AsipB
AsipC
AsipD
AsipE
AsipF
AsipG
AsipH
Mc1rA
Mc1rB
Mc1rC
Mc1rD
Mc1rE

Figure 4.

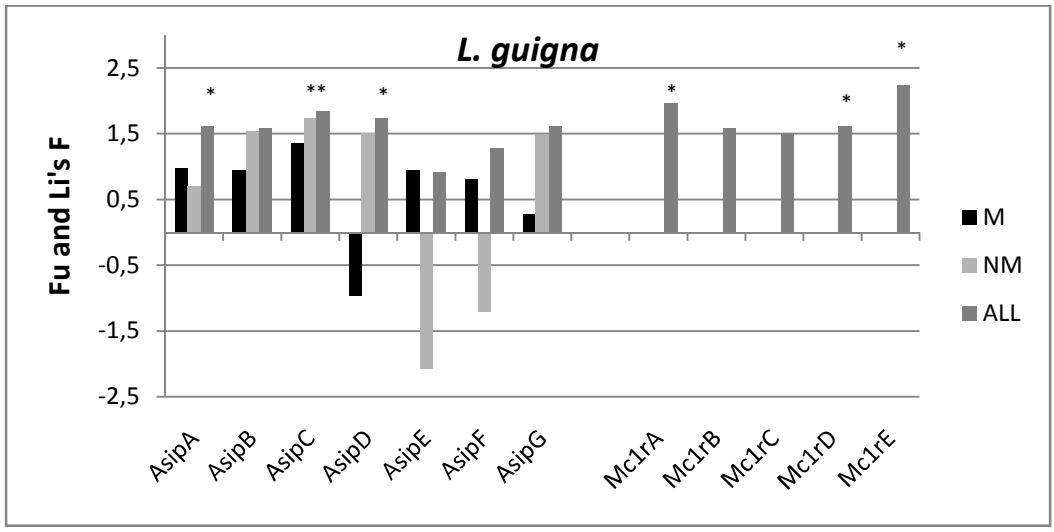
D)


L. guigna
Tajima's D
2,5
1,5
0,5
-0,5
-1,5
-2,5
* *
*
AsipA
AsipB
AsipC
AsipD
AsipE
AsipF
AsipG
Mc1rA
Mc1rB
Mc1rC
Mc1rD
Mc1rE
M
NM
ALL

E)


L. guigna
Fu and Li's D
2,5
1,5
0,5
-0,5
-1,5
-2,5
*
**
*
*
*
*
*
*
AsipA
AsipB
AsipC
AsipD
AsipE
AsipF
AsipG
Mc1rA
Mc1rB
Mc1rC
Mc1rD
Mc1rE
M
NM
ALL

F)


L. guigna
Fu and Li's F
2,5
1,5
0,5
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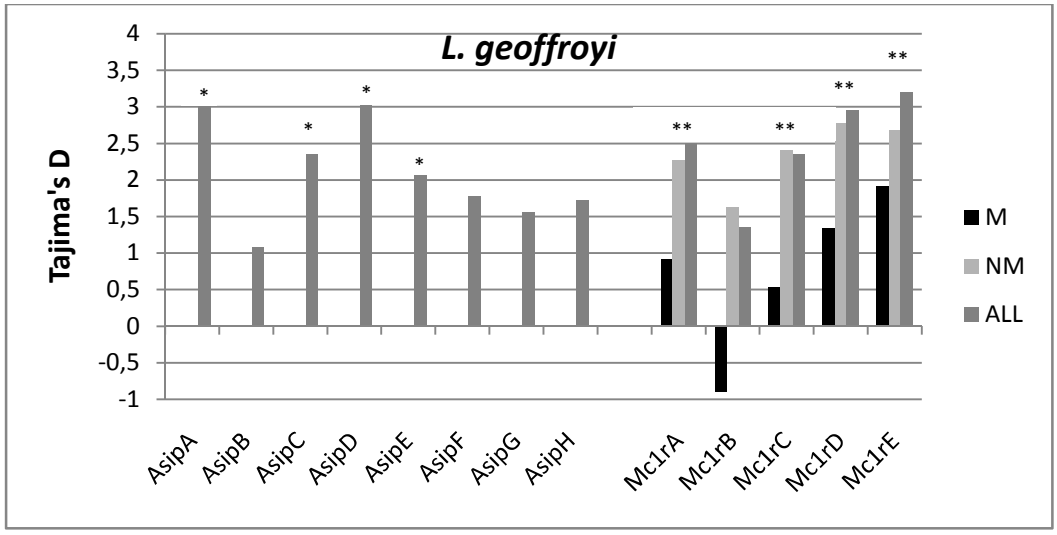
-1,5
-2,5
*
**
*
*
*
*
*
AsipA
AsipB
AsipC
AsipD
AsipE
AsipF
AsipG
Mc1rA
Mc1rB
Mc1rC
Mc1rD
Mc1rE
M
NM
ALL

Figure 4.

G)


L. geoffroyi
Tajima's D
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3,5
3
2,5
2
1,5
1
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0
-0,5
-1
AsipA AsipB AsipC AsipD AsipE AsipF AsipG AsipH Mc1rA Mc1rB Mc1rC Mc1rD Mc1rE
M
NM
ALL

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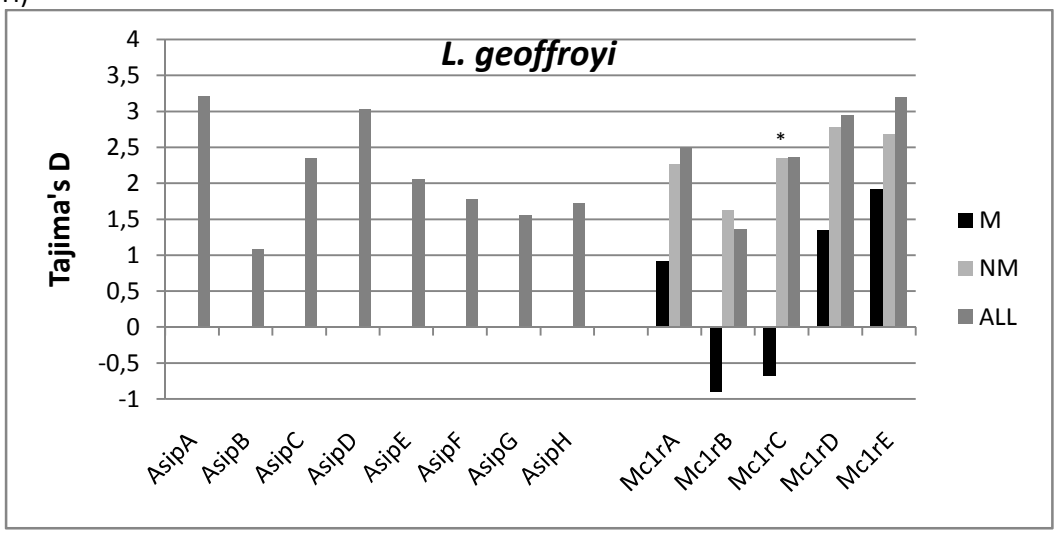
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Tajima's D
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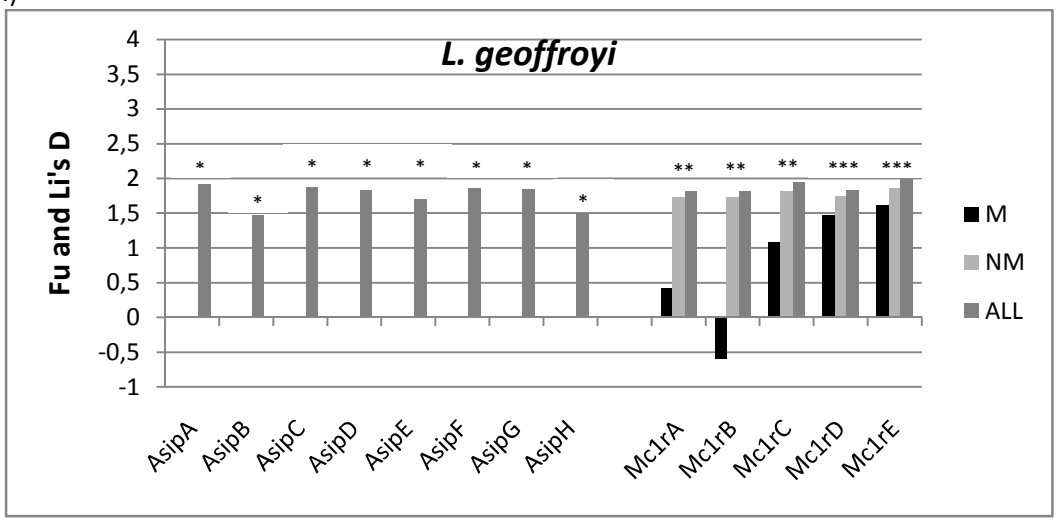
2,5
2
1,5
1
0,5
0
-0,5
-1
AsipA AsipB AsipC AsipD AsipE AsipF AsipG AsipH Mc1rA Mc1rB Mc1rC Mc1rD Mc1rE
M
NM
ALL

I)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Fu and Li's D
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1,5
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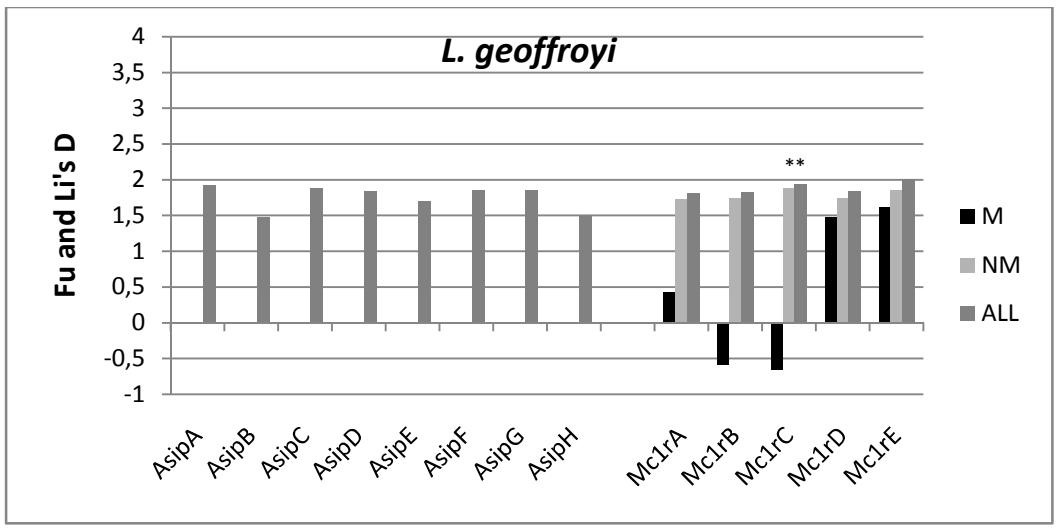
0
-0,5
-1
AsipA AsipB AsipC AsipD AsipE AsipF AsipG AsipH Mc1rA Mc1rB Mc1rC Mc1rD Mc1rE
M
NM
ALL

Figure 4.

J)


L. geoffroyi
Fu and Li's D
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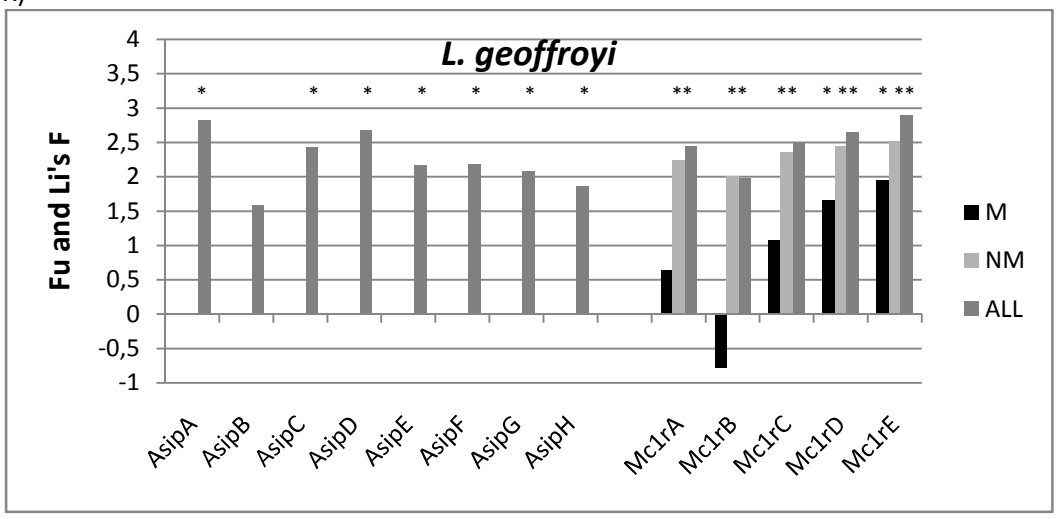
2,5
2
1,5
1
0,5
0
-0,5
-1
**
M
NM
ALL
AsipA
AsipB
AsipC
AsipD
AsipE
AsipF
AsipG
AsipH
Mc1rA
Mc1rB
Mc1rC
Mc1rD
Mc1rE

K)


L. geoffroyi
Fu and Li's F
4
3,5
3
2,5
2
1,5
1
0,5
0
-0,5
-1
* * * * * * * ** ** ** * ** * **
M
NM
ALL
AsipA
AsipB
AsipC
AsipD
AsipE
AsipF
AsipG
AsipH
Mc1rA
Mc1rB
Mc1rC
Mc1rD
Mc1rE

L)

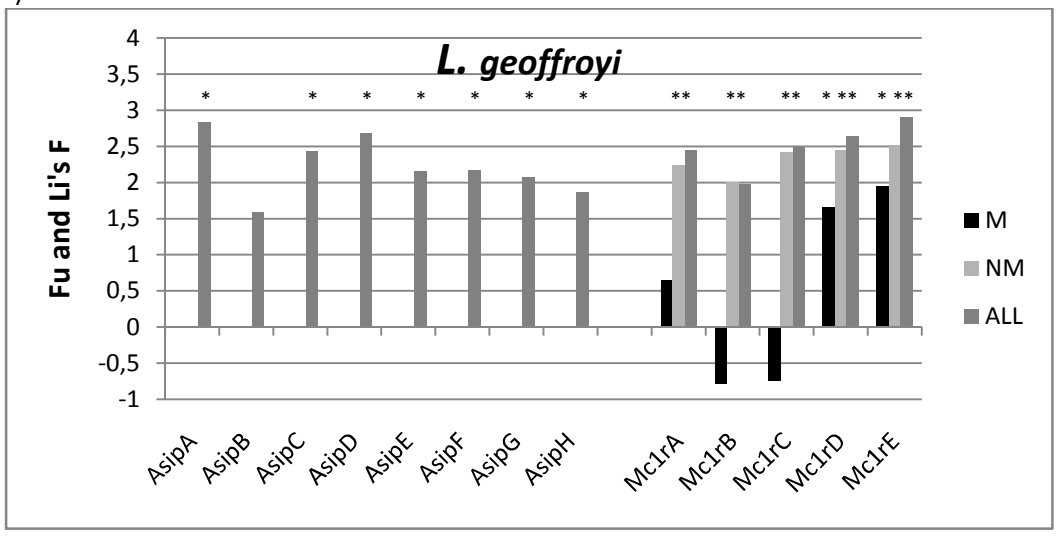

L. geoffroyi
Fu and Li's F
4
3,5
3
2,5
2
1,5
1
0,5
0
-0,5
-1
* * * * * * * ** ** ** * ** * **
M
NM
ALL
AsipA
AsipB
AsipC
AsipD
AsipE
AsipF
AsipG
AsipH
Mc1rA
Mc1rB
Mc1rC
Mc1rD
Mc1rE

Figure 5.

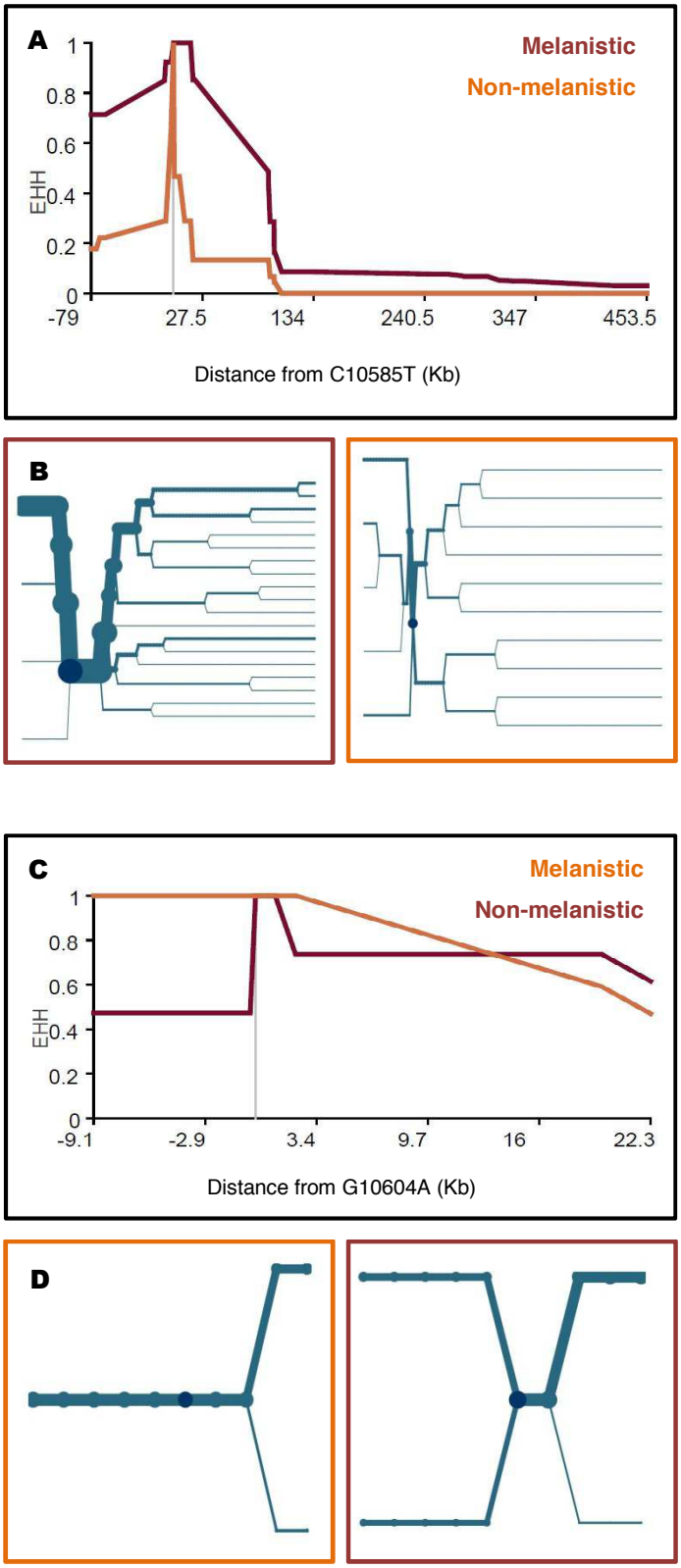
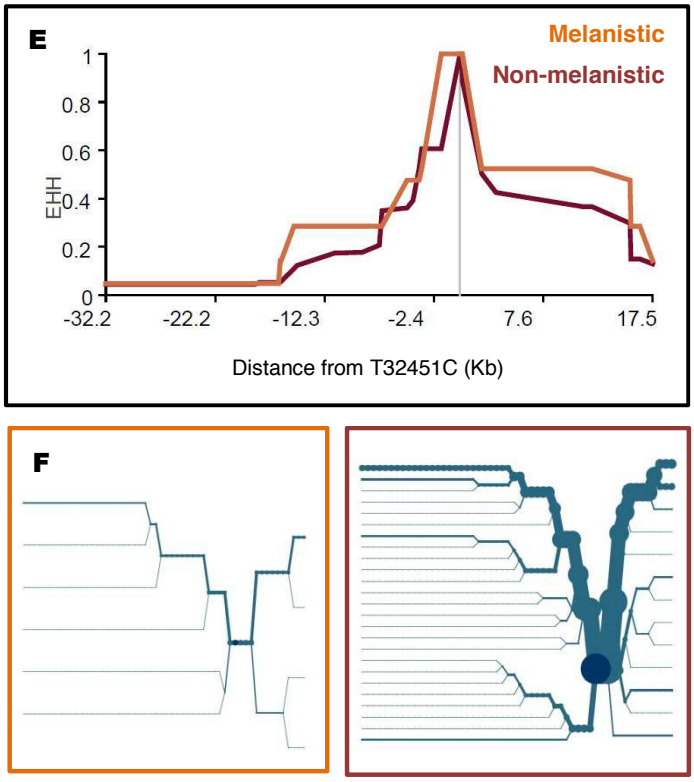
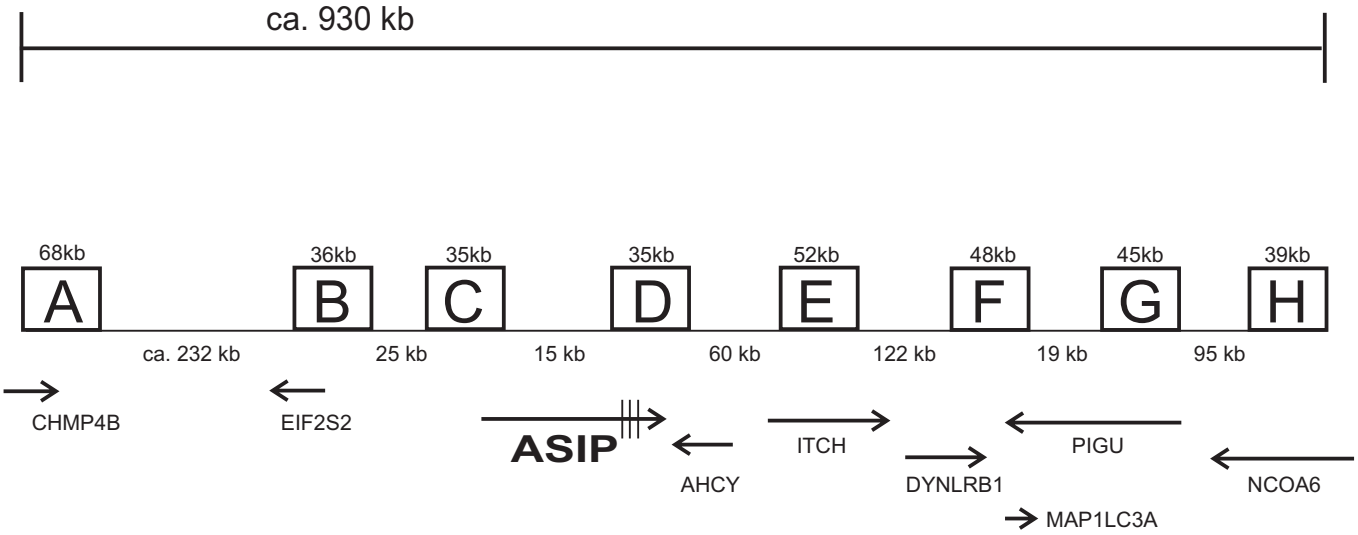

A
Melanistic
Non-melanistic
EHH
1
0.8
0.6
0.4
0.2
0
-79
27.5
134
240.5
347
453.5
Distance from C10585T (Kb)
B
C
Melanistic
Non-melanistic
EHH
1
0.8
0.6
0.4
0.2
0
-9.1
-2.9
3.4
9.7
16
22.3
Distance from G10604A (Kb)
D

Figure 5.


E
Melanistic
Non-melanistic
EHH
1
0.8
0.6
0.4
0.2
0
-32.2
-22.2
-12.3
-2.4
7.6
17.5
Distance from T32451C (Kb)
F

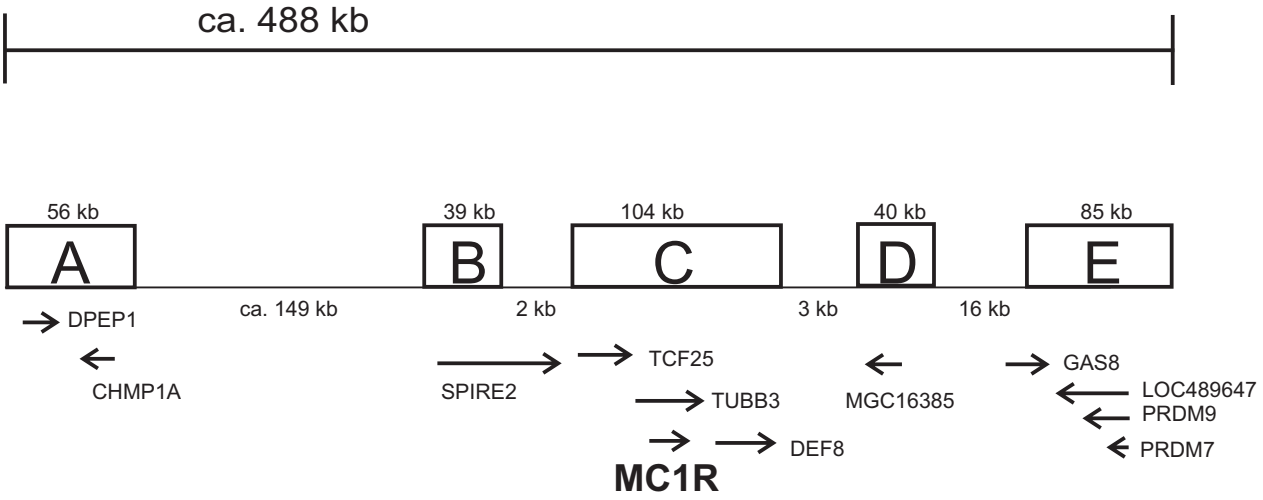
A)

chr A3

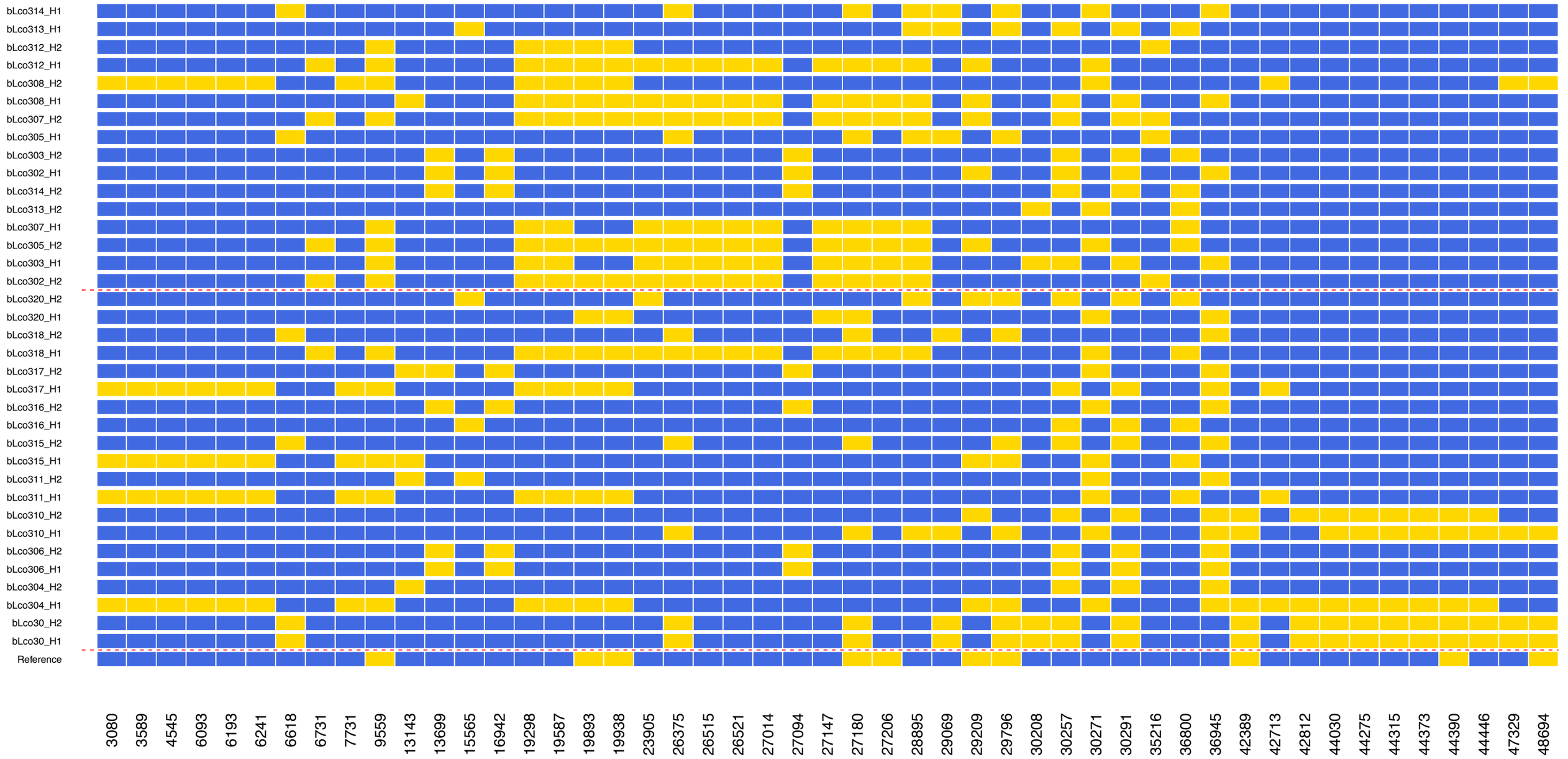


B)

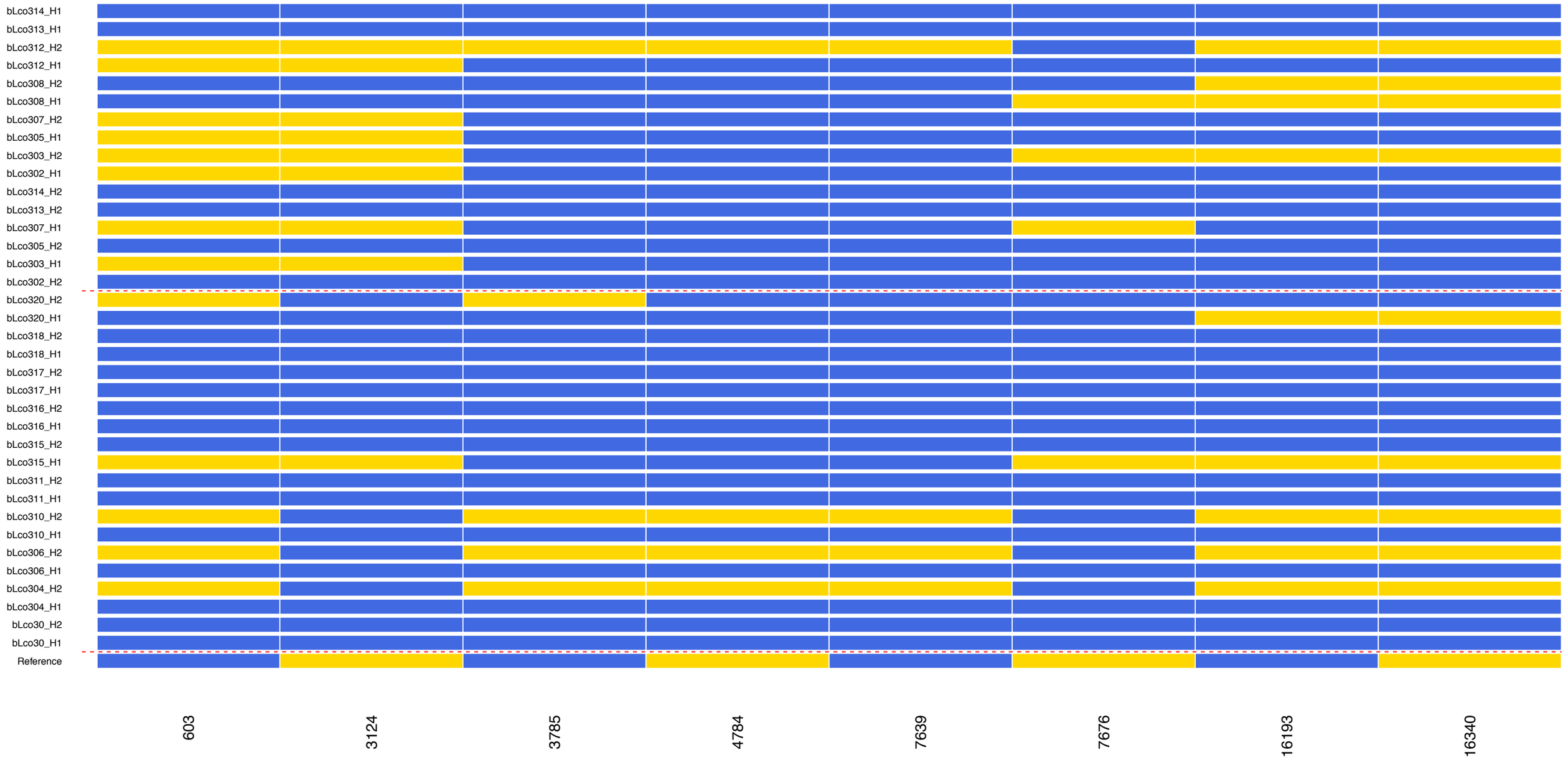
chr E2



bLco AsipA Haplotypes



bLco AsipB Haplotypes



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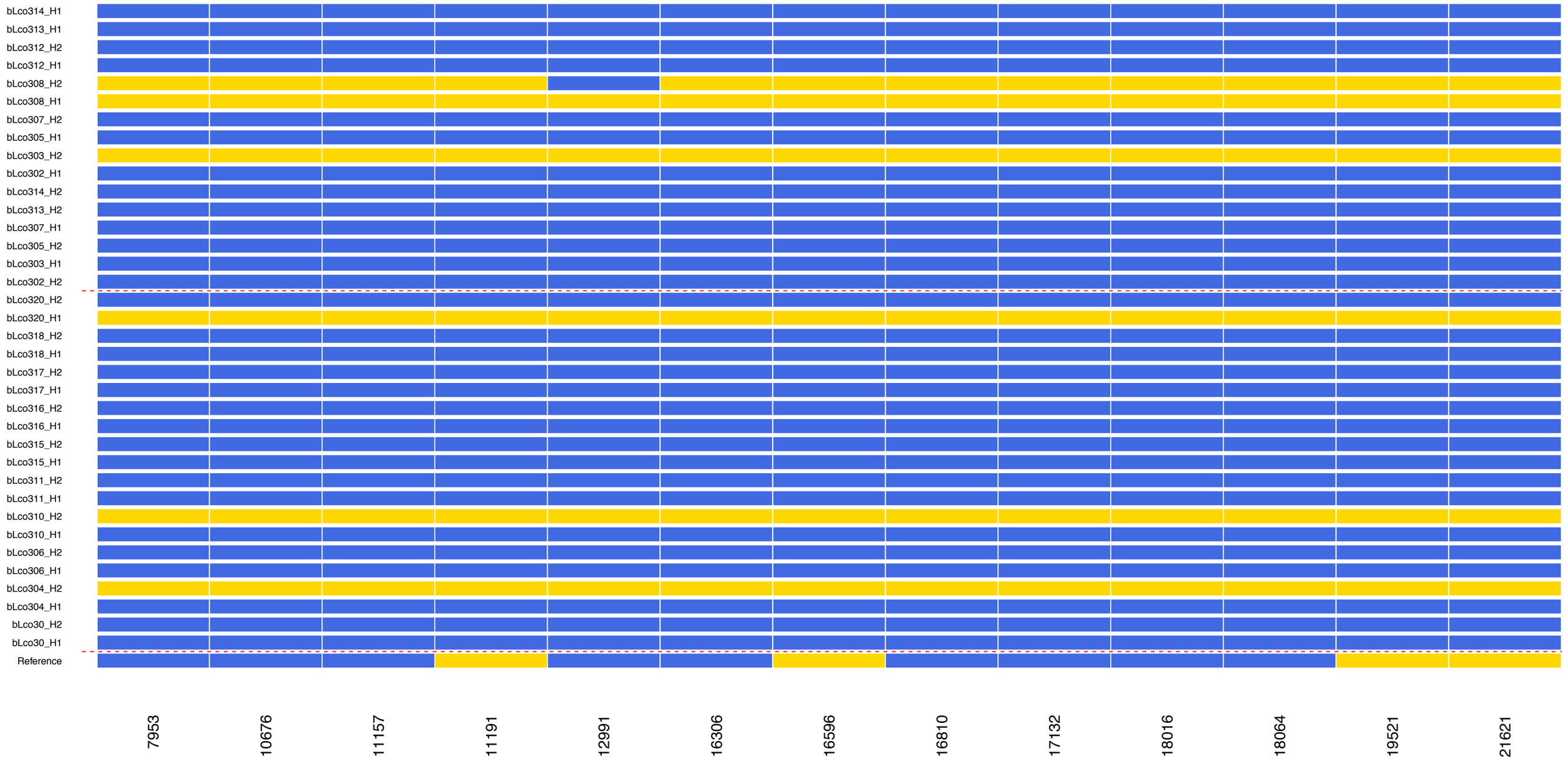

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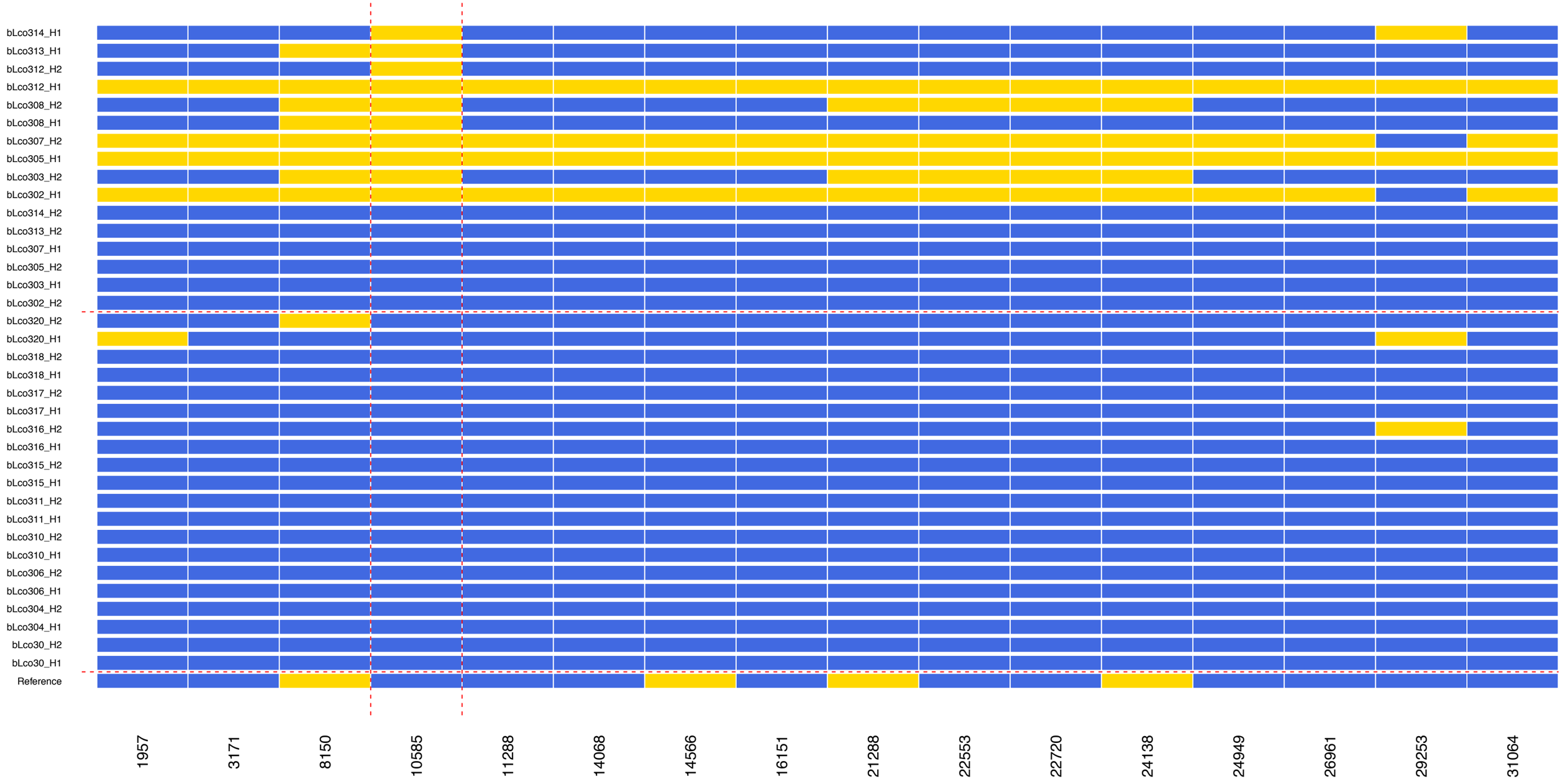

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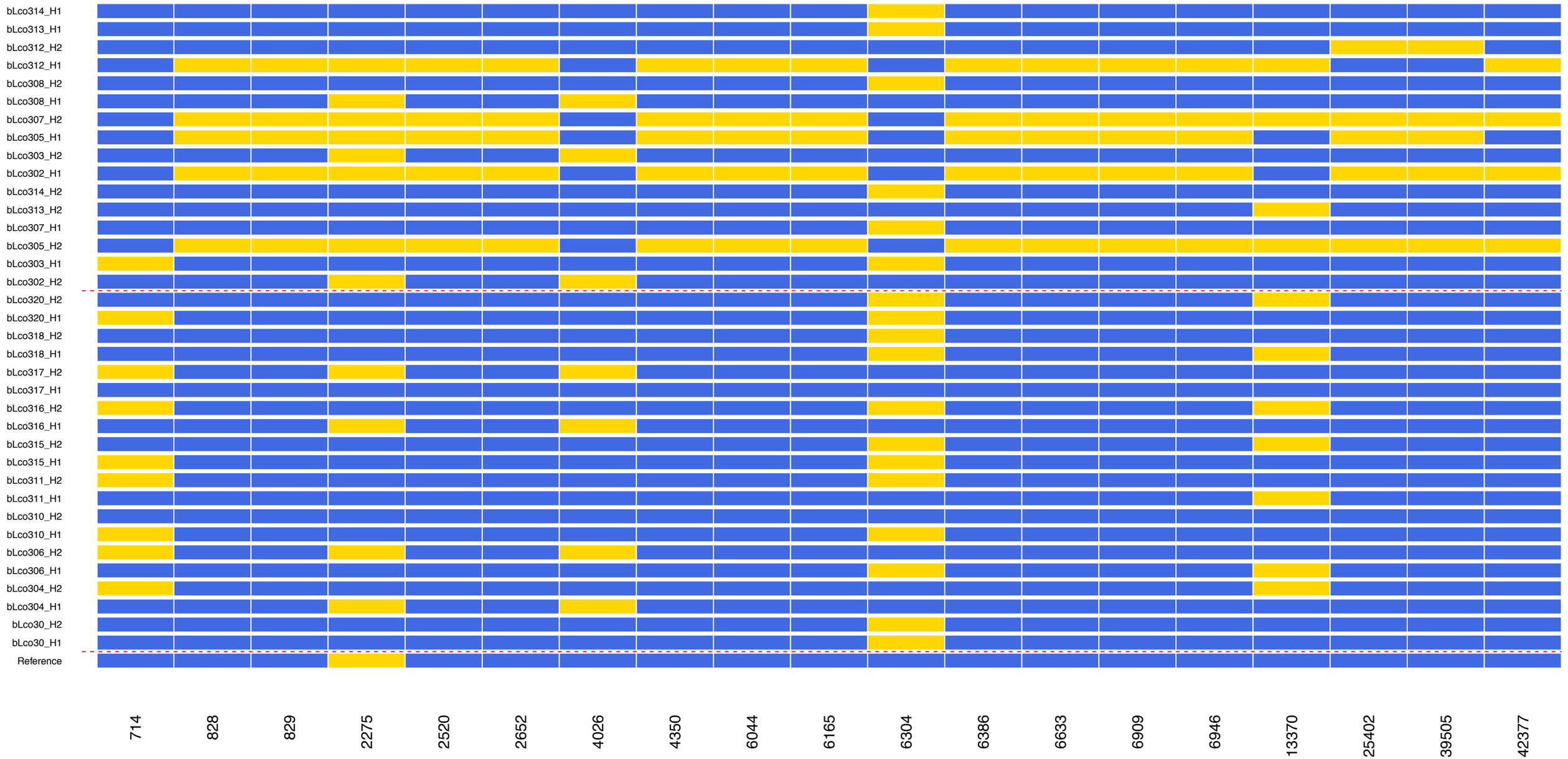

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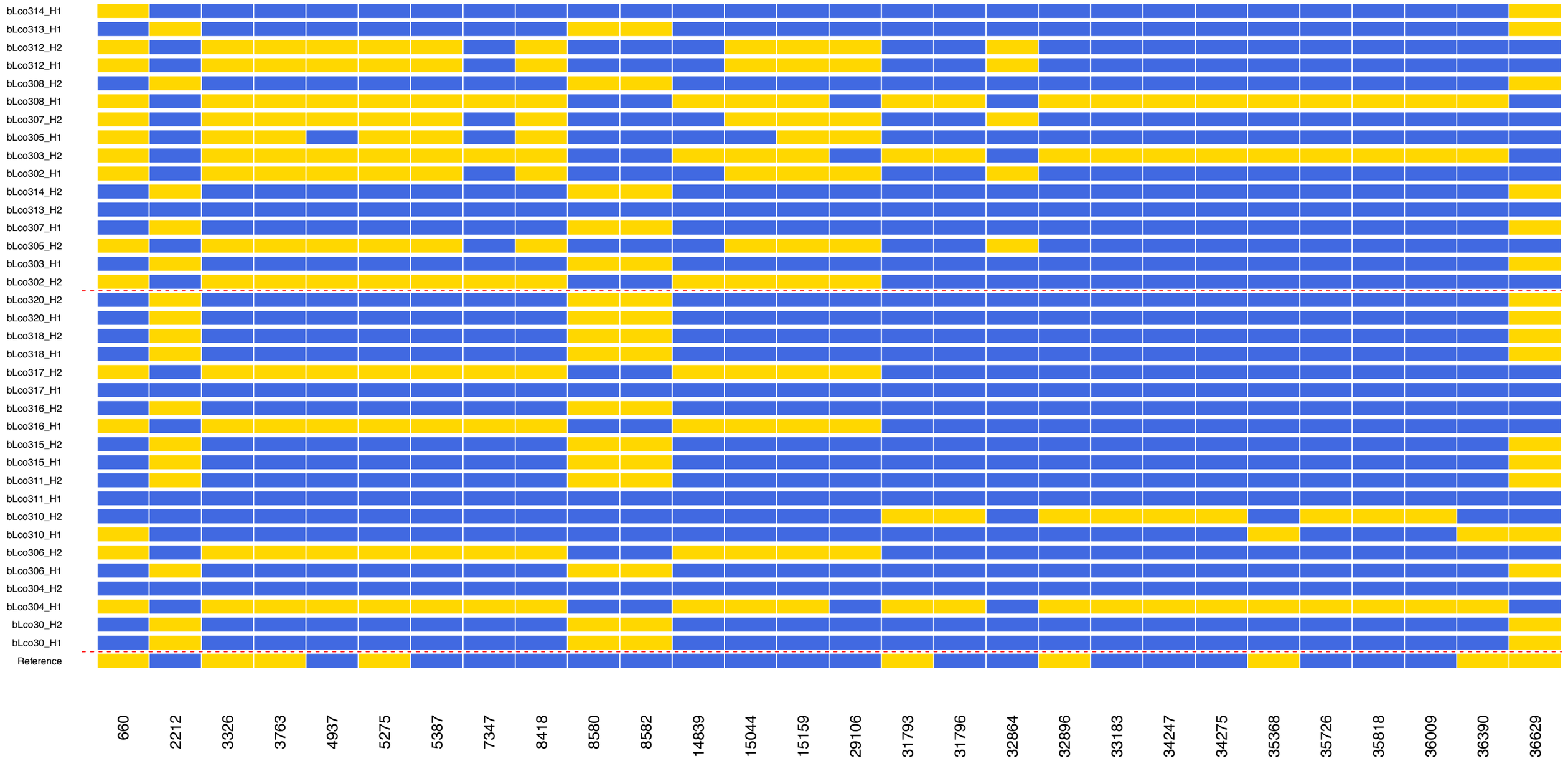

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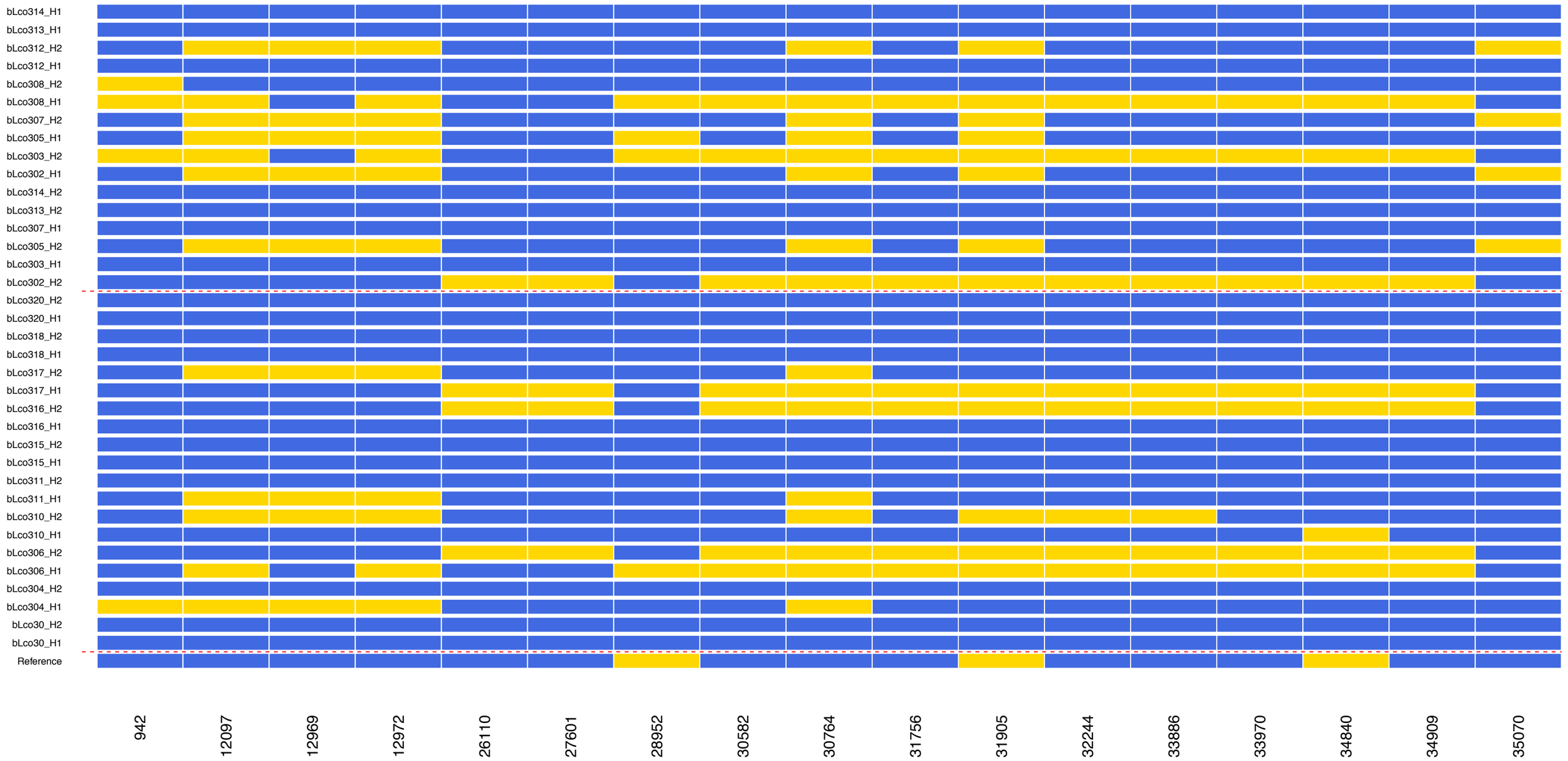

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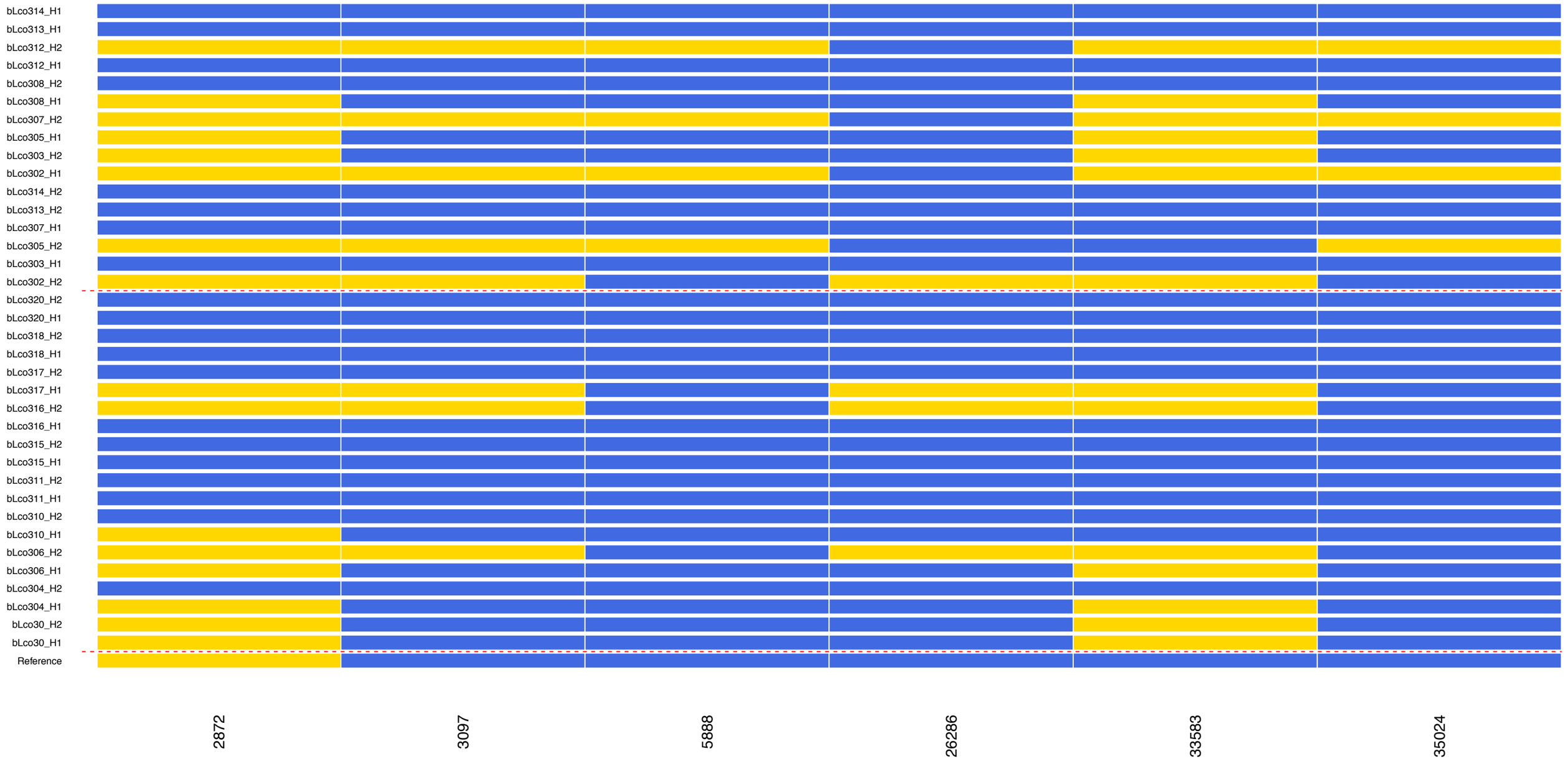

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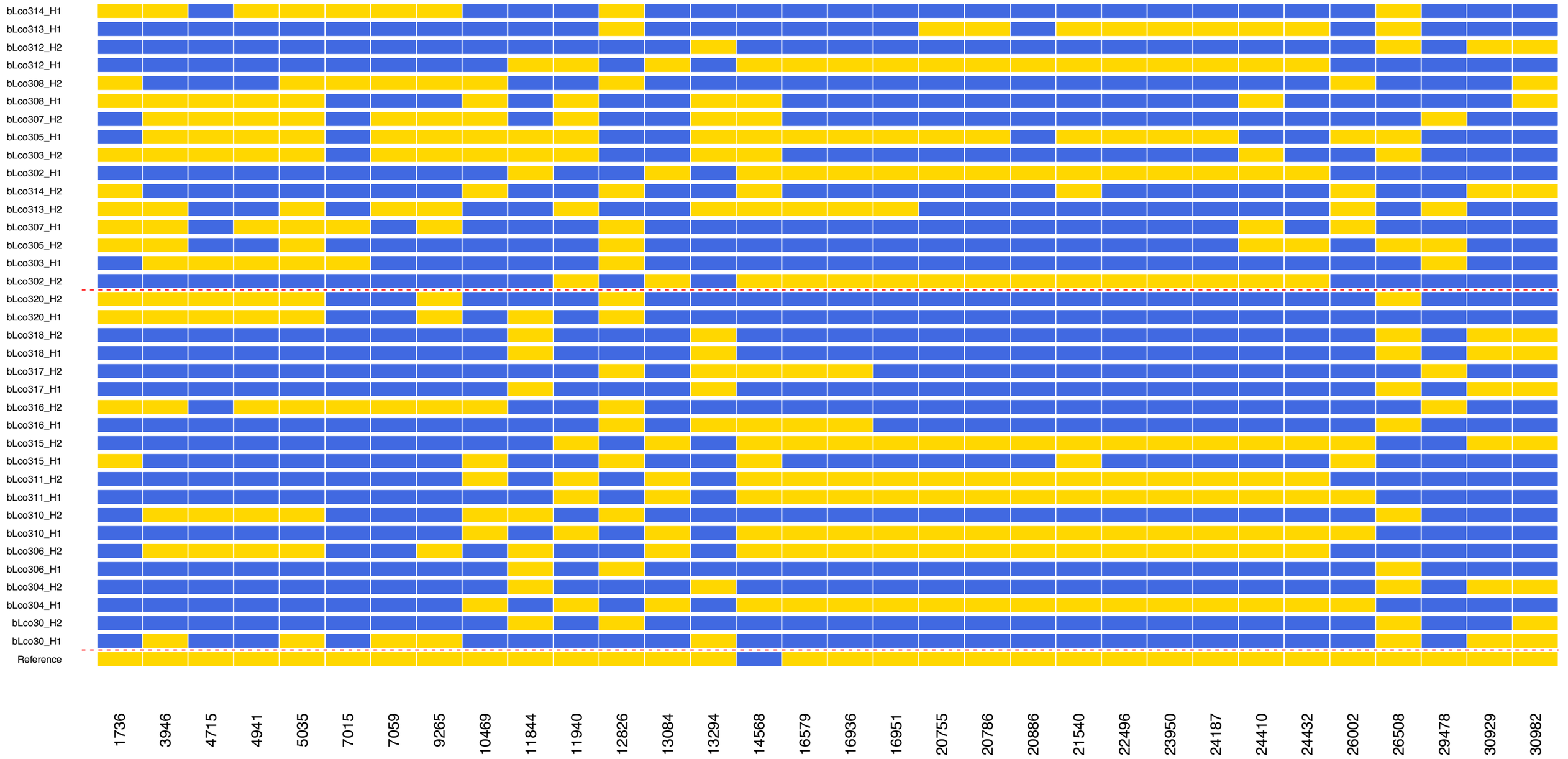

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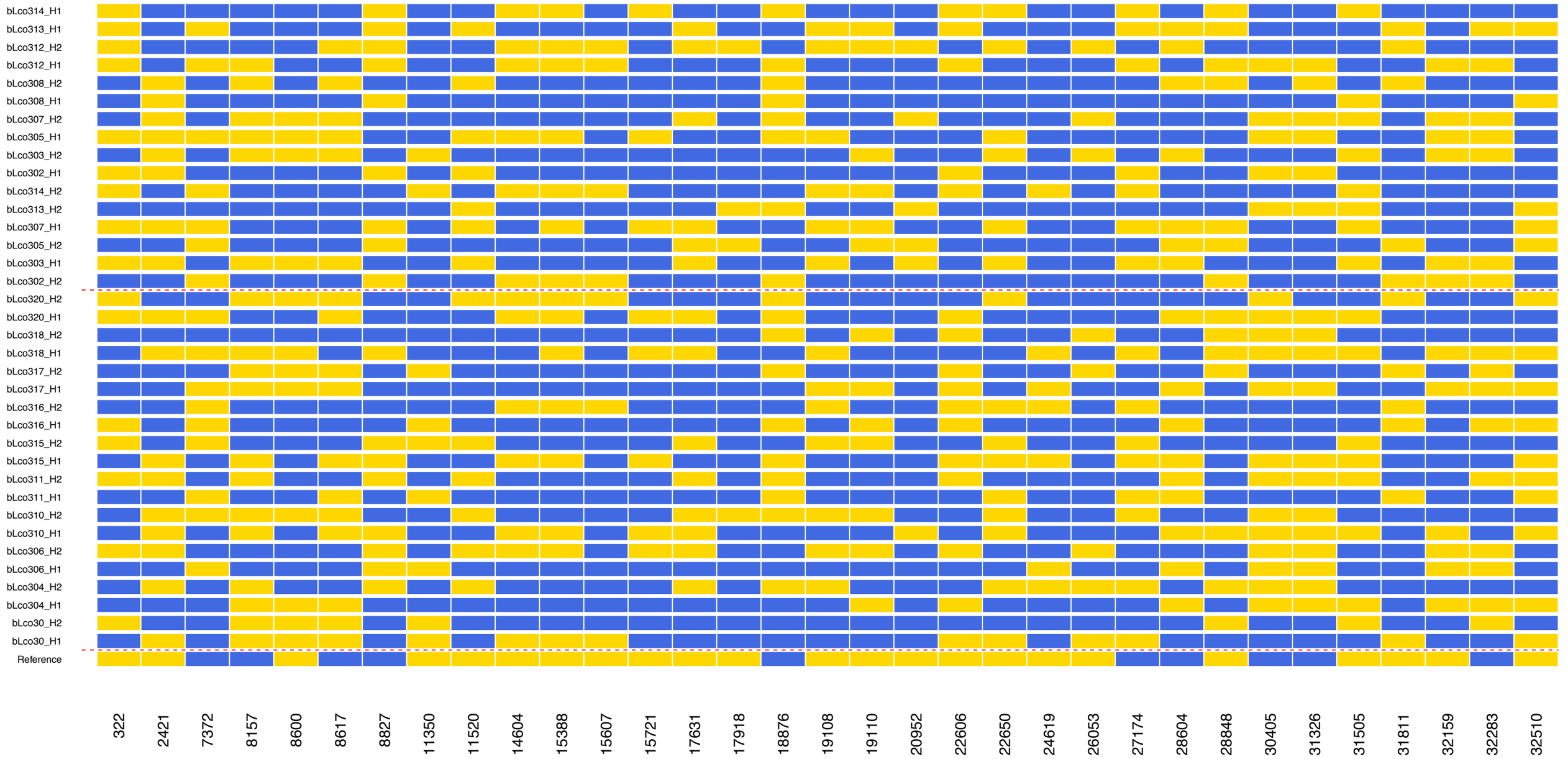

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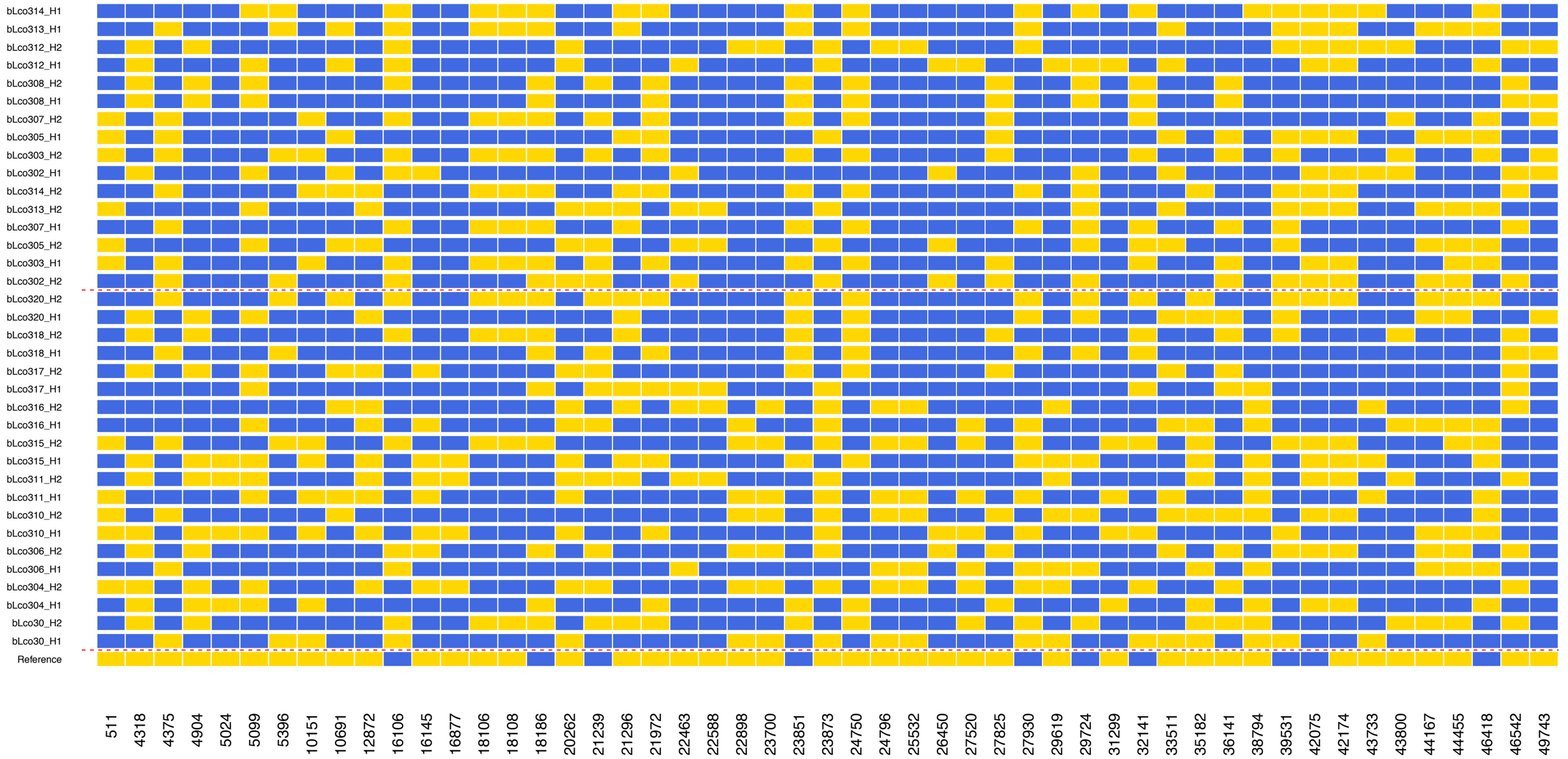

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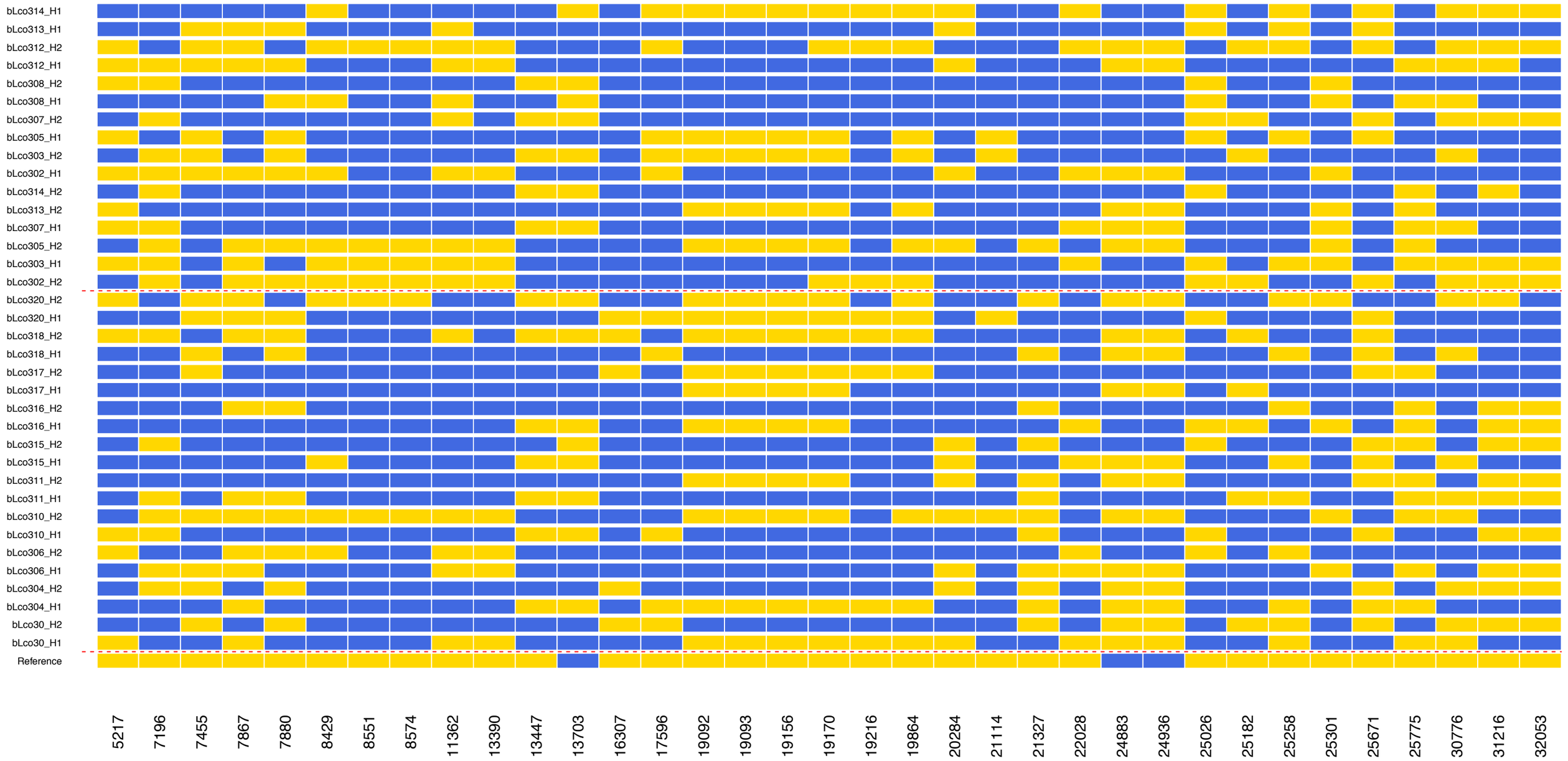

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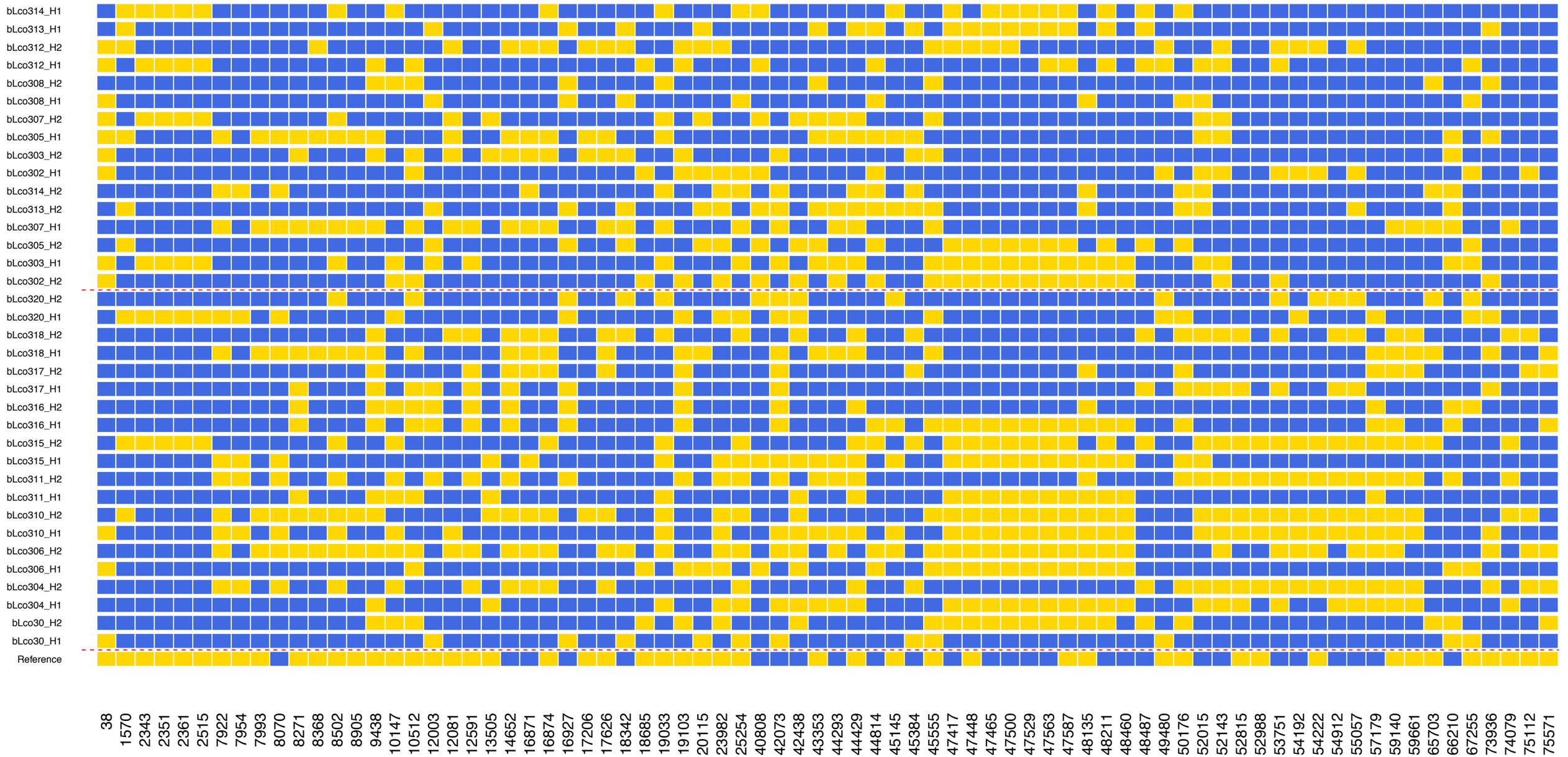

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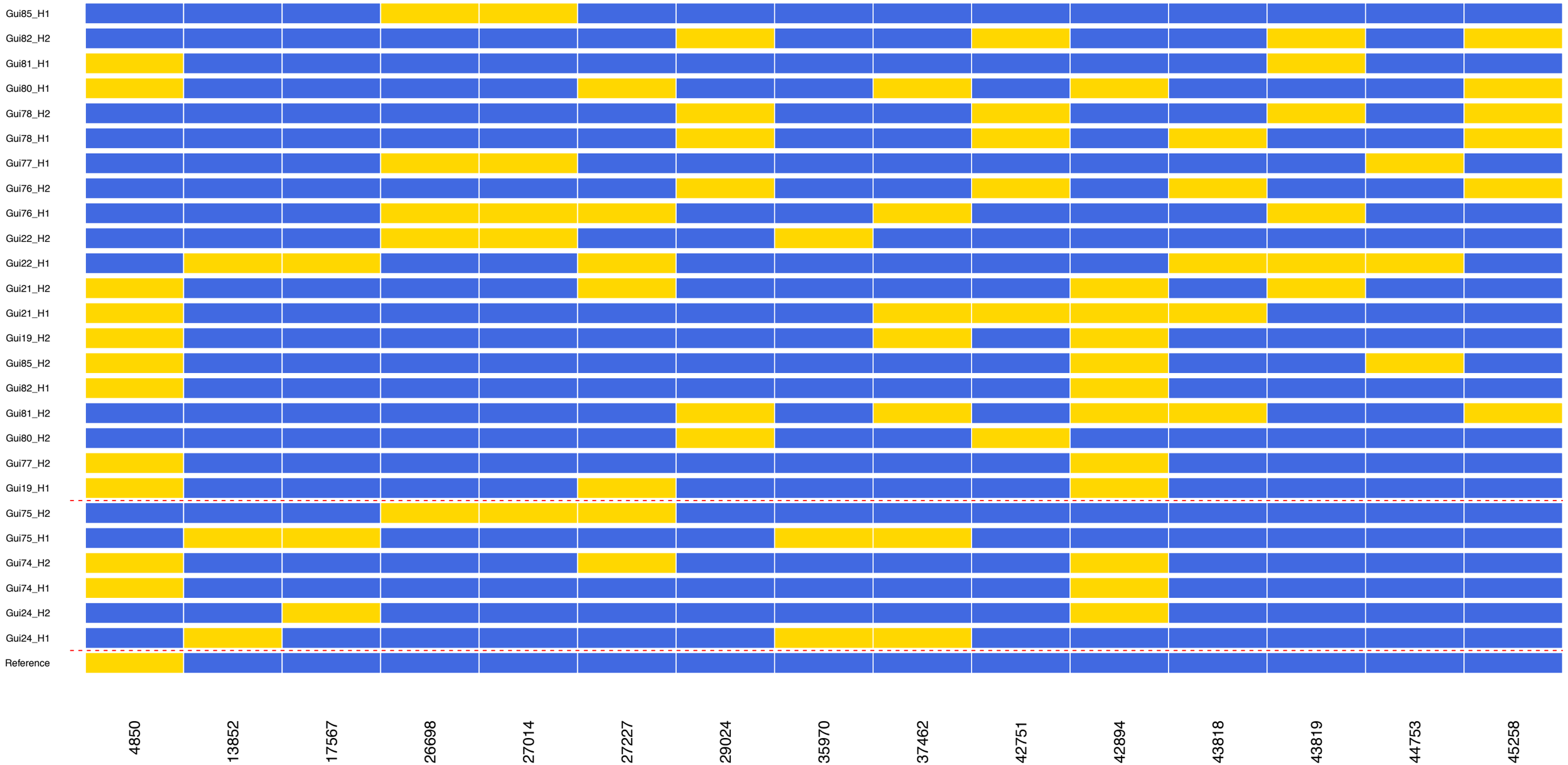

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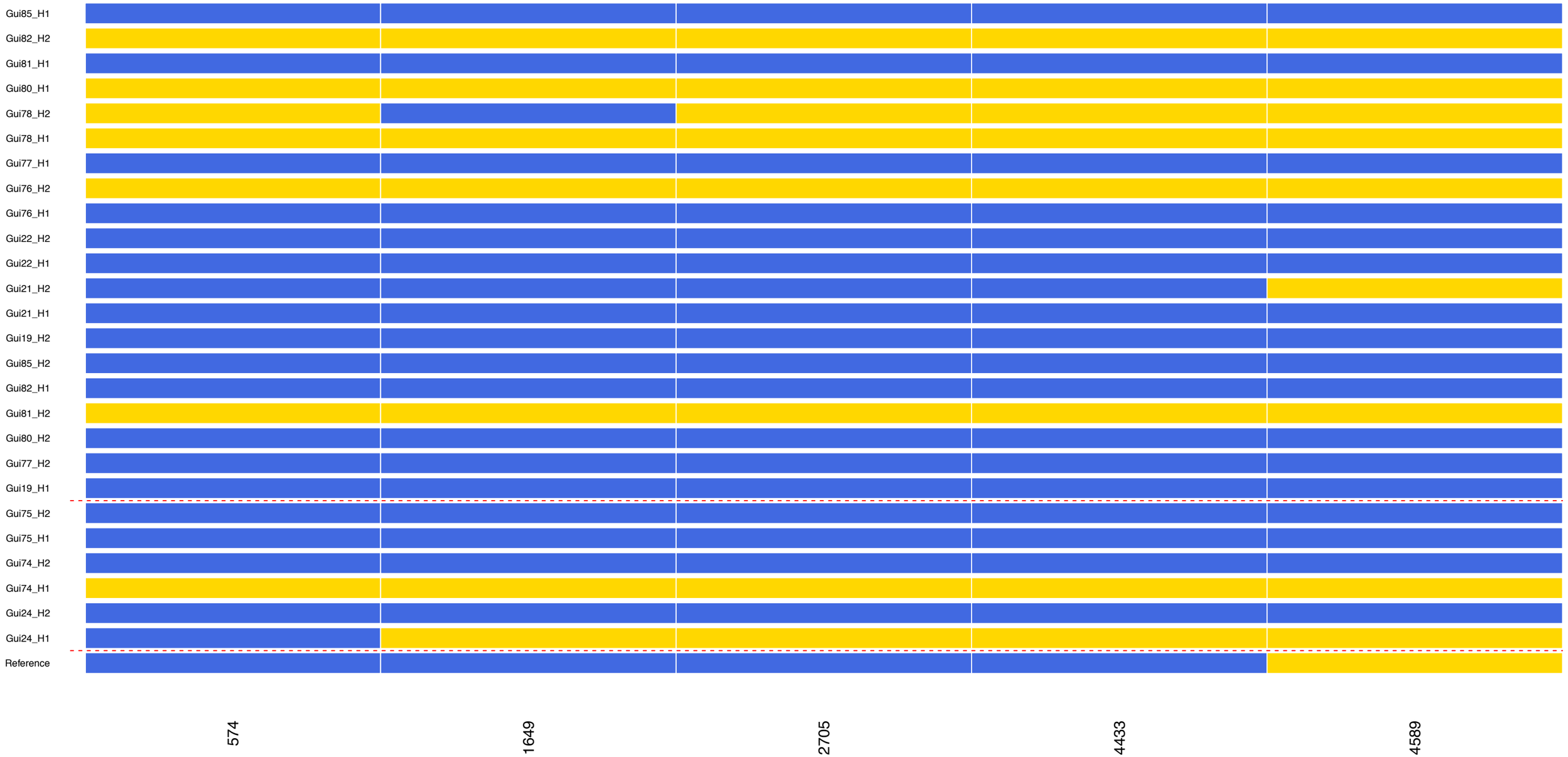

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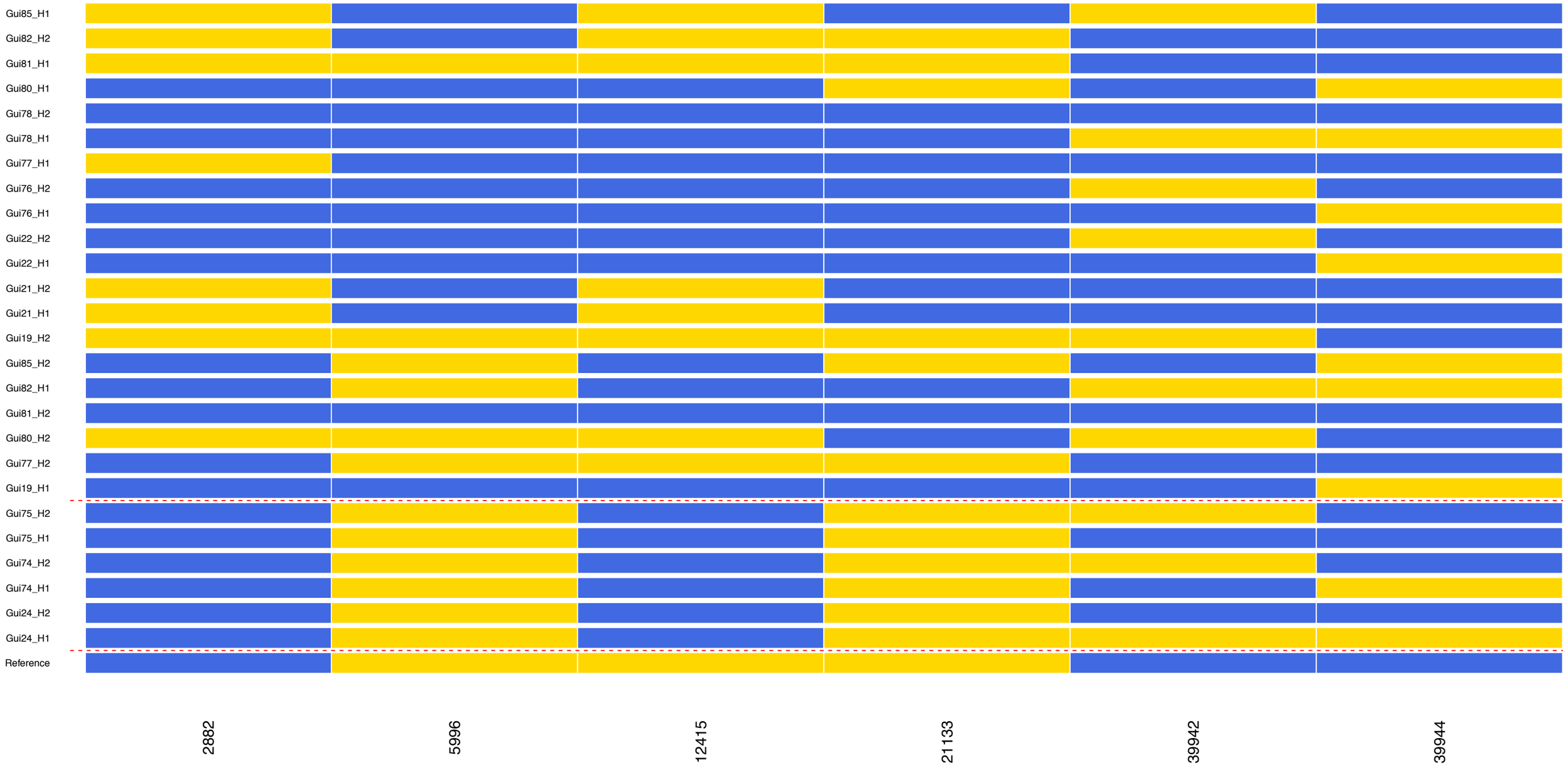

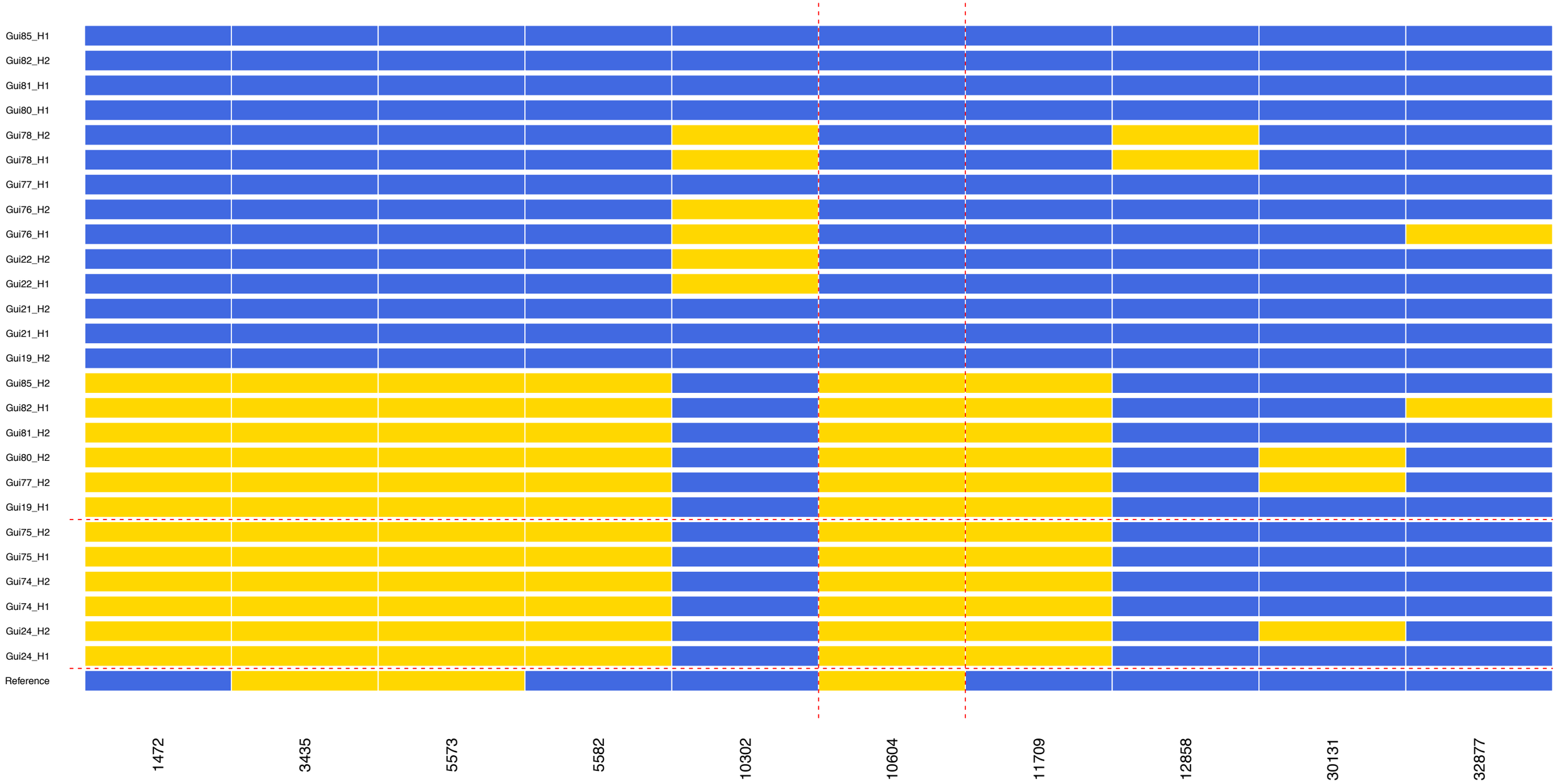
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Gui81_H1
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Gui76_H1
Gui22_H2
Gui22_H1
Gui21_H2
Gui21_H1
Gui19_H2
Gui85_H2
Gui82_H1
Gui81_H2
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Gui74_H2
Gui74_H1
Gui24_H2
Gui24_H1
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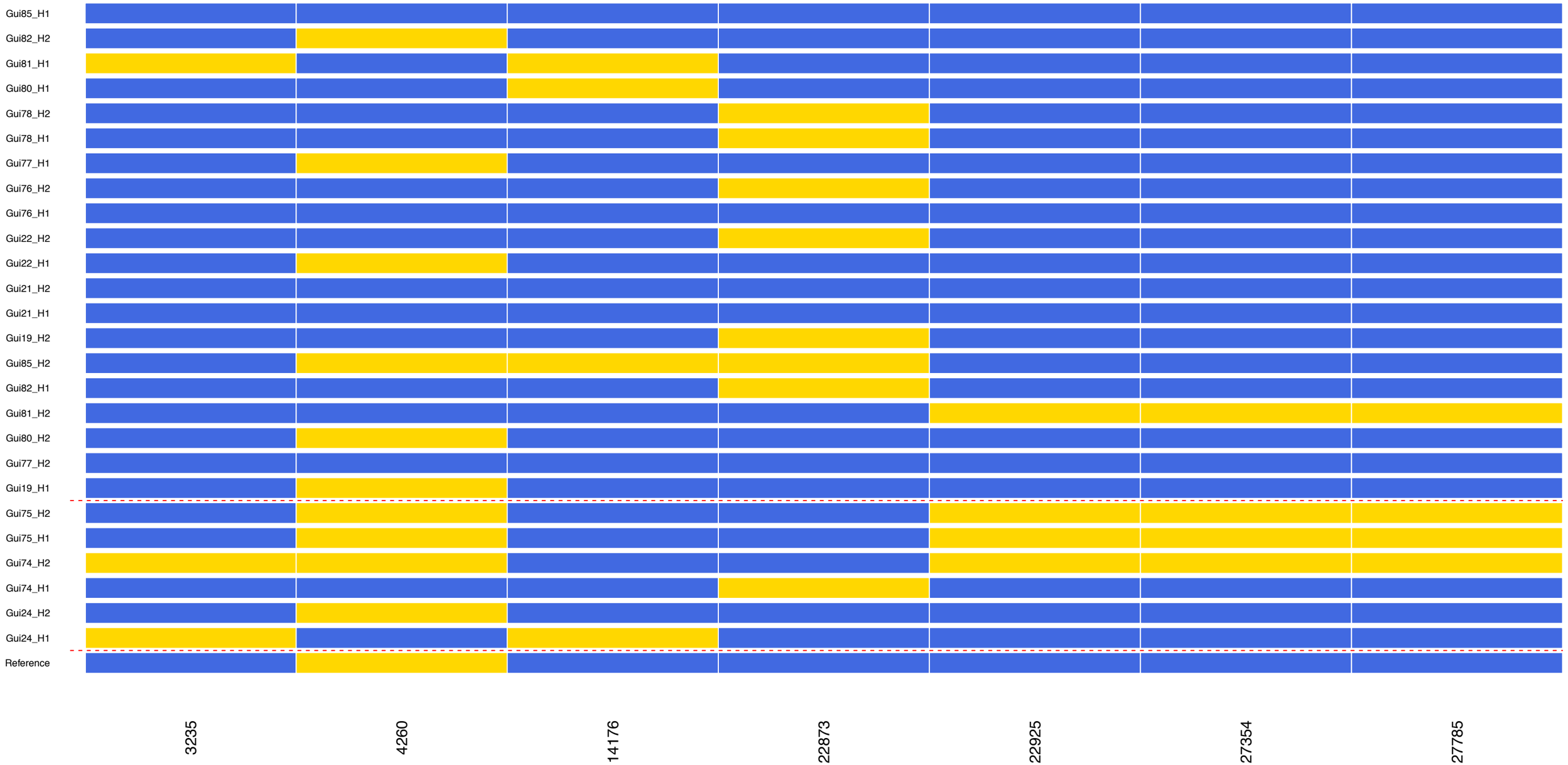
Gui AsipF Haplotypes



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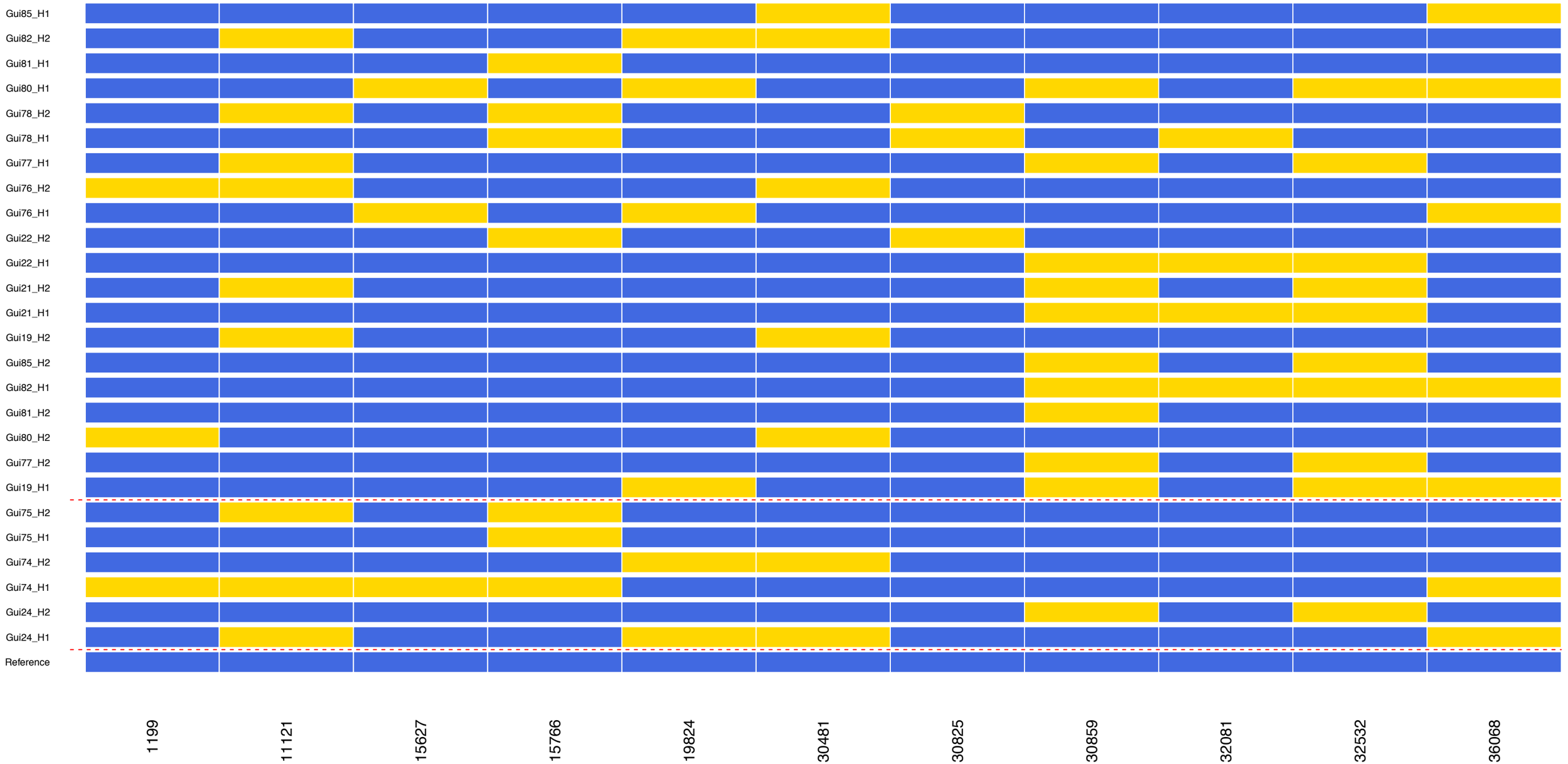

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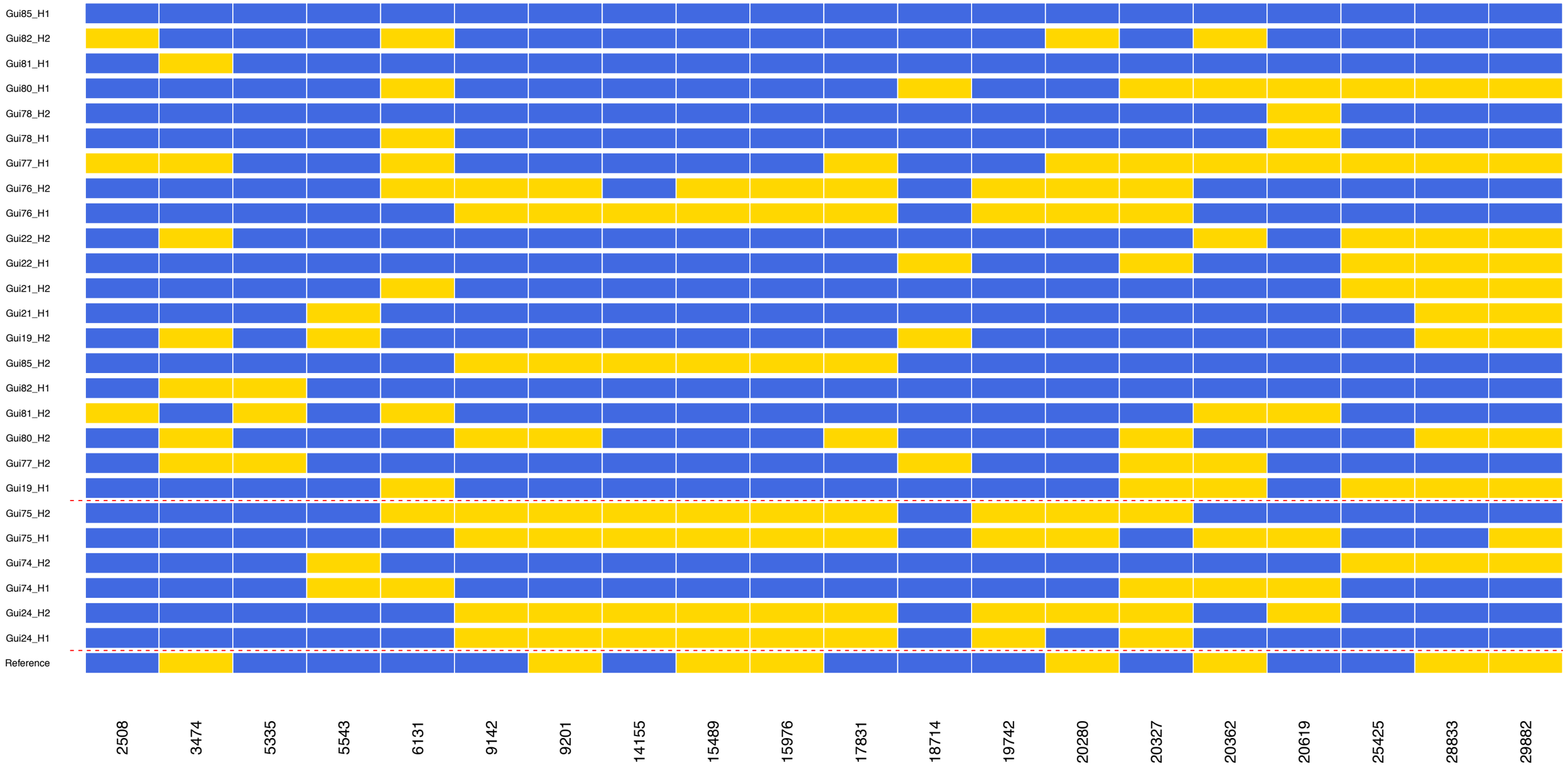

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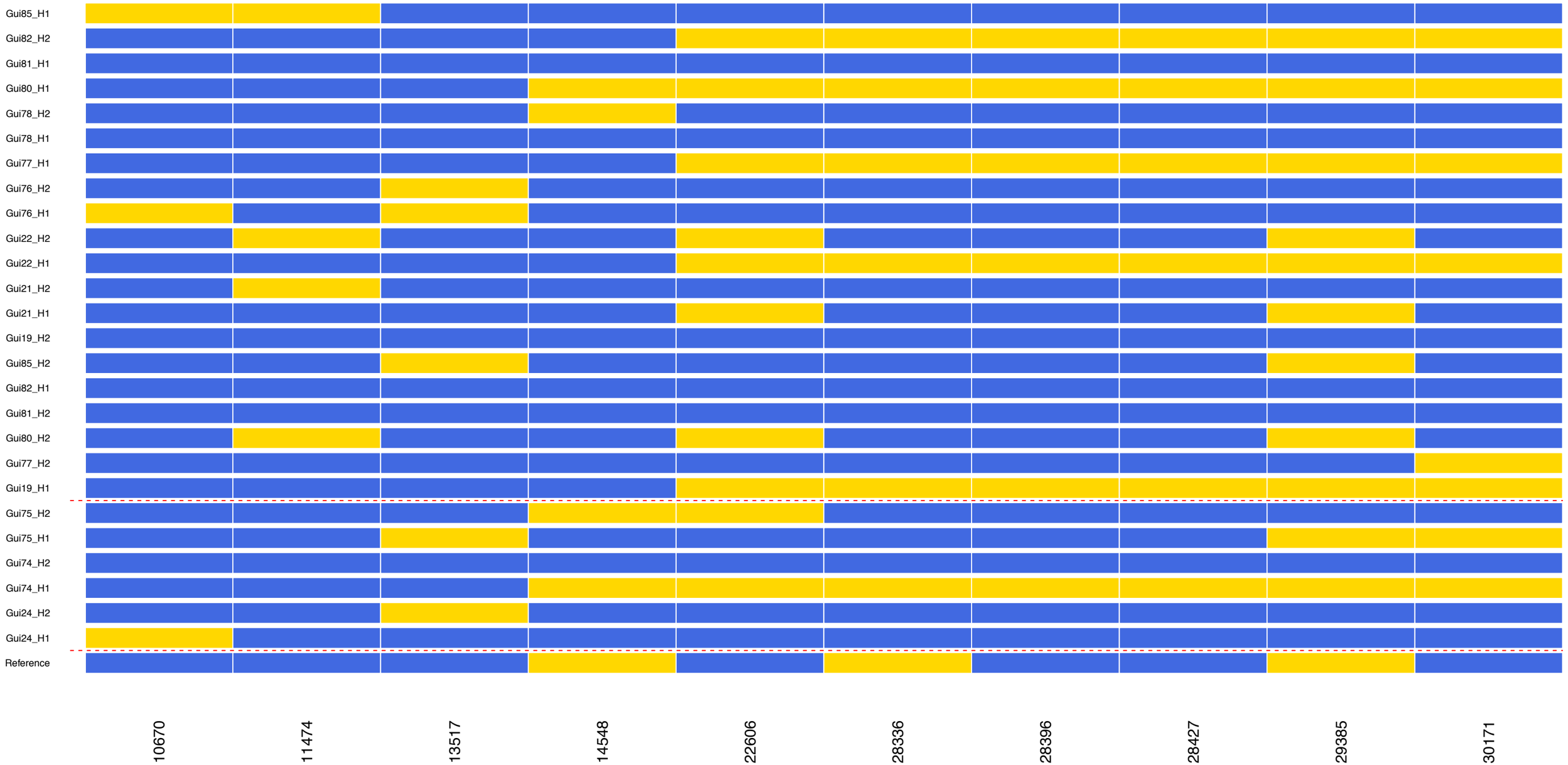
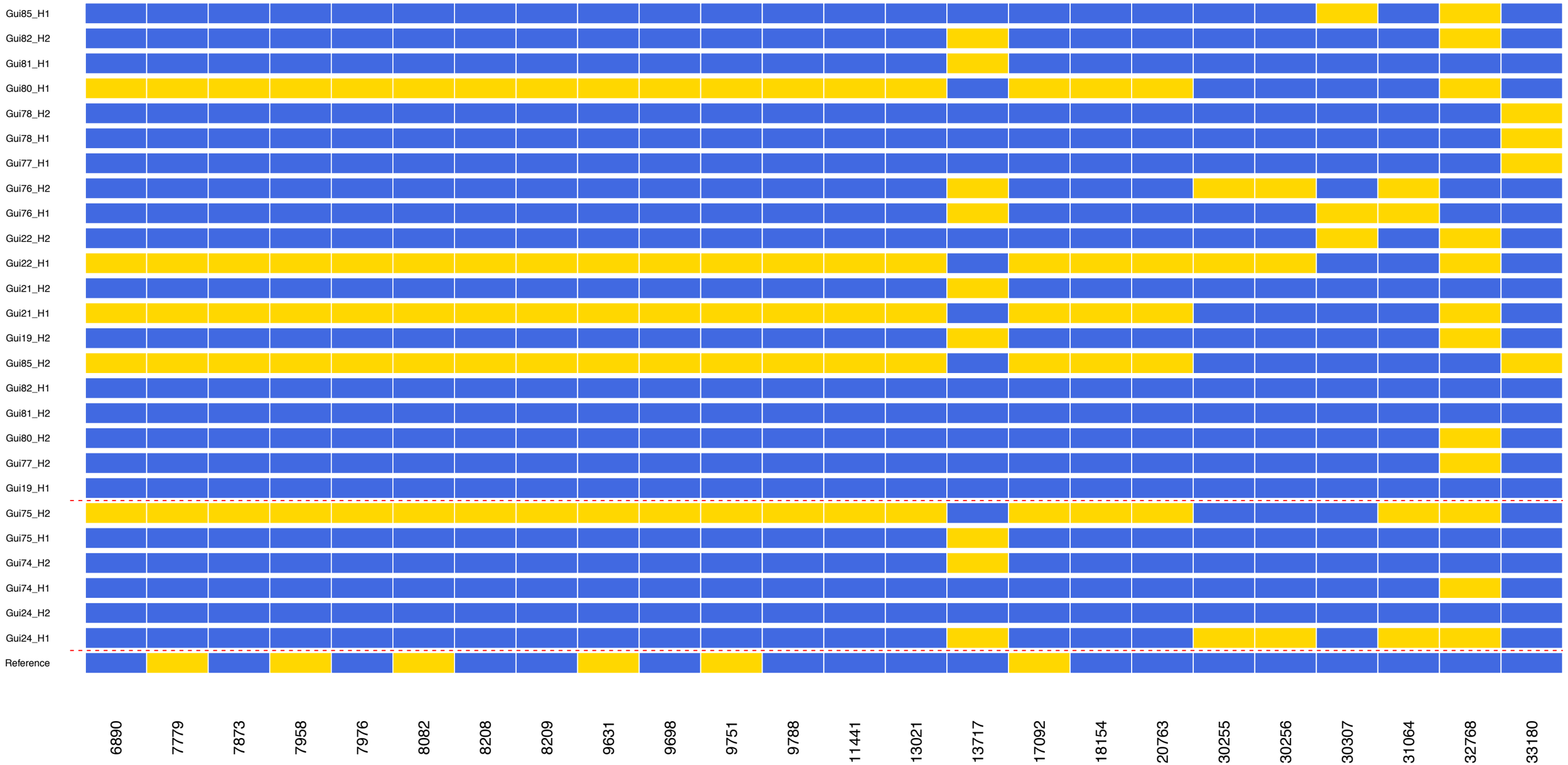


Gui Mc1rC Haplotypes

Gui Mc1rD Haplotypes



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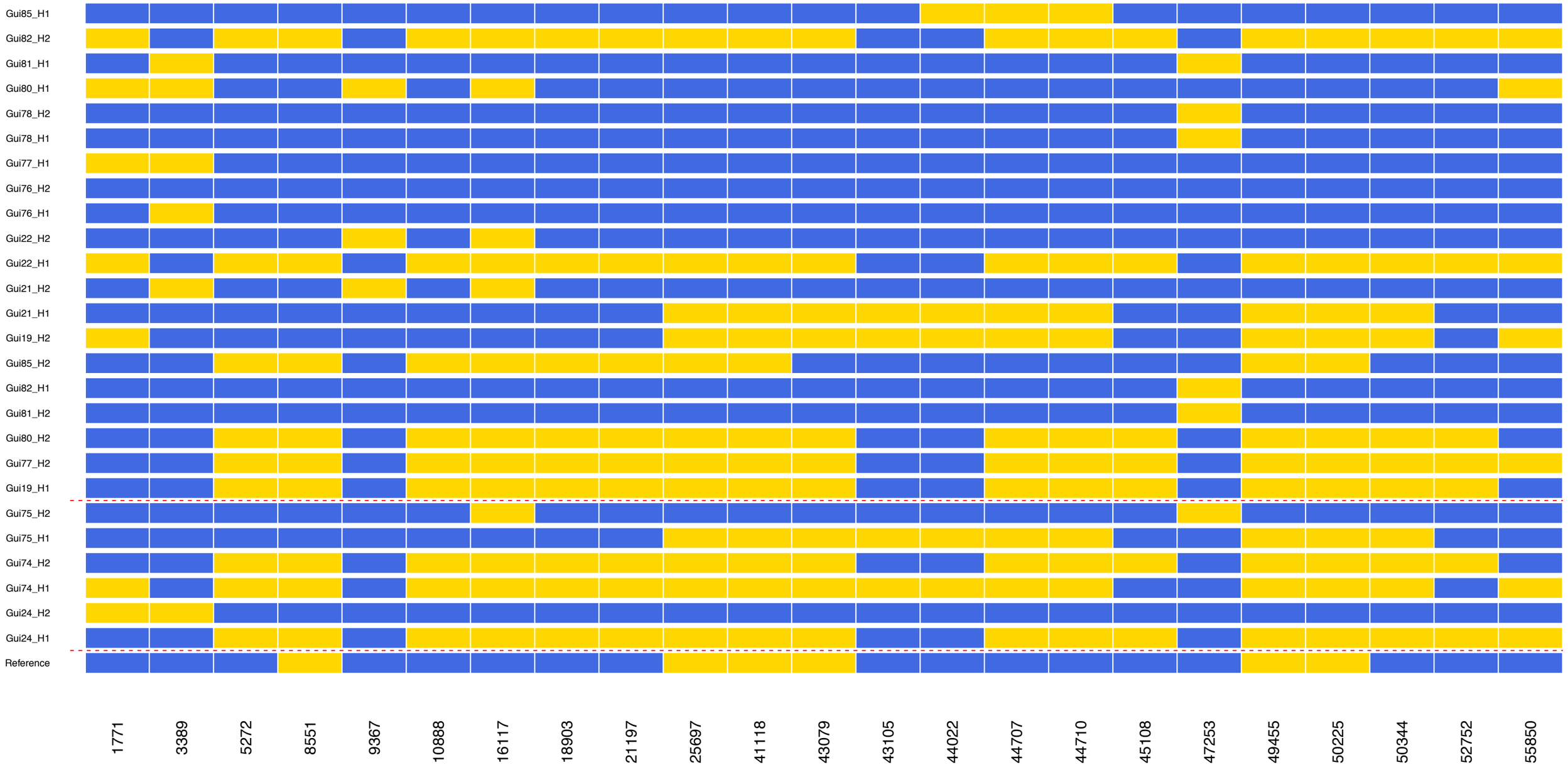

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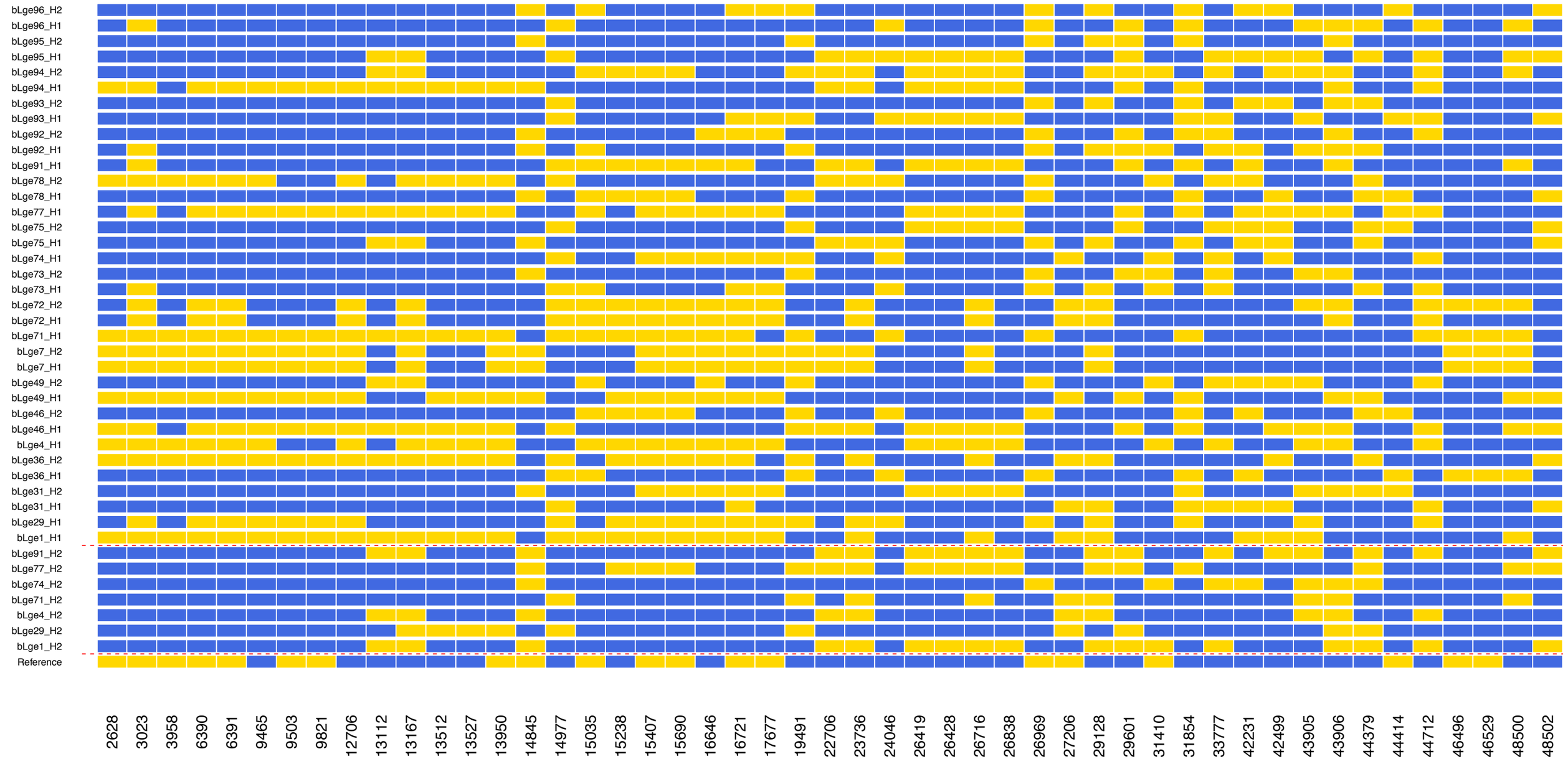

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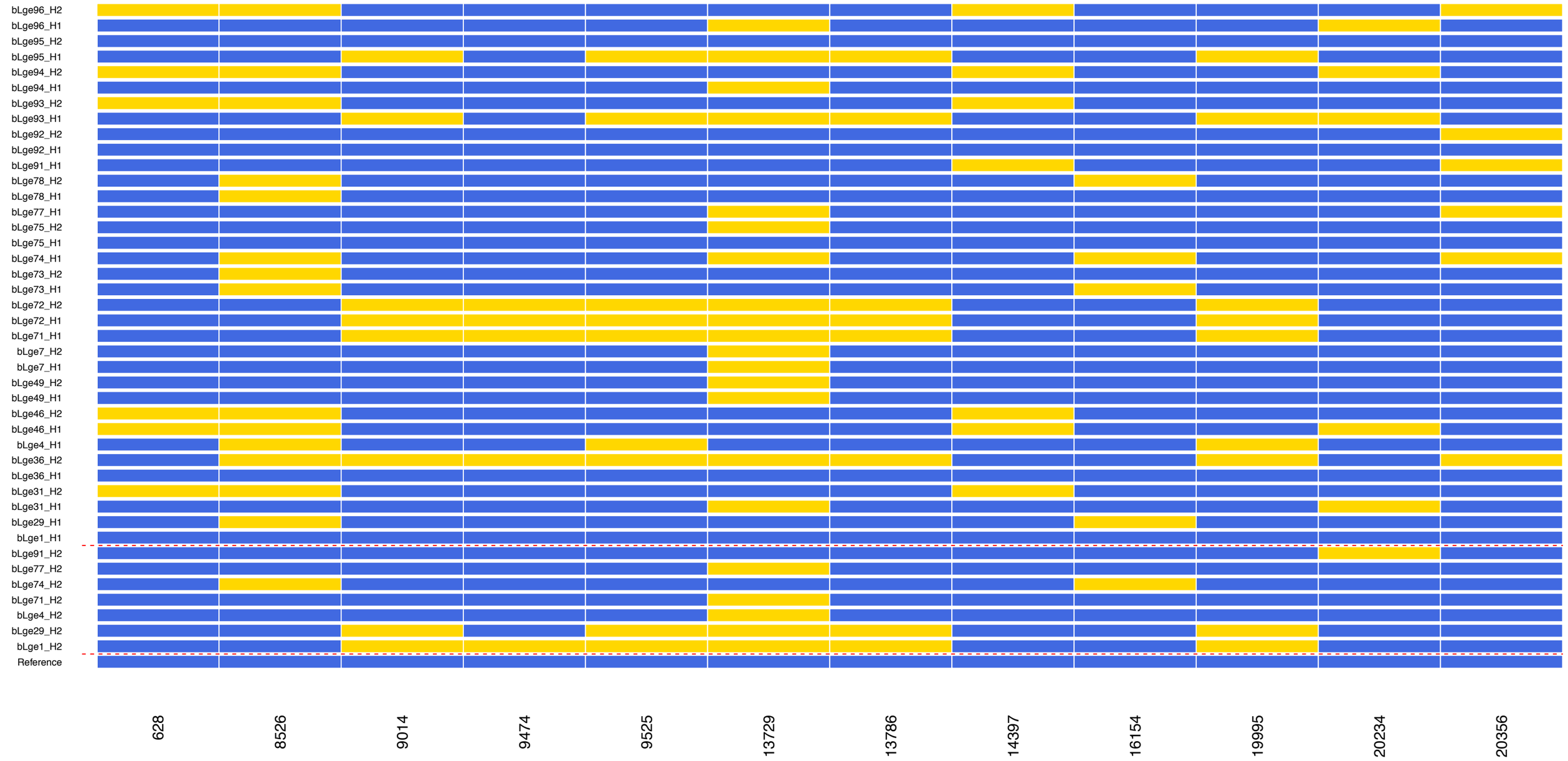

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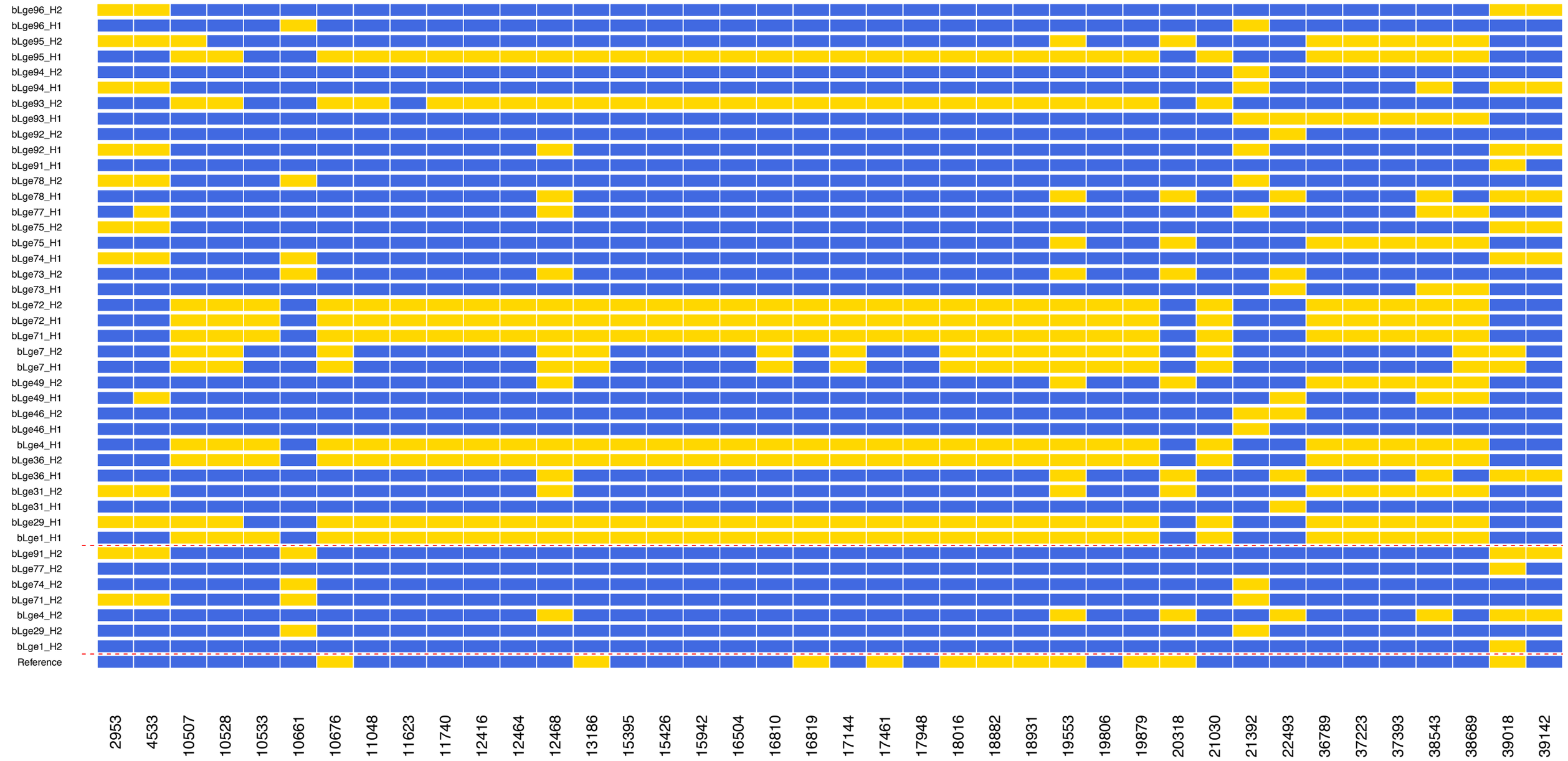

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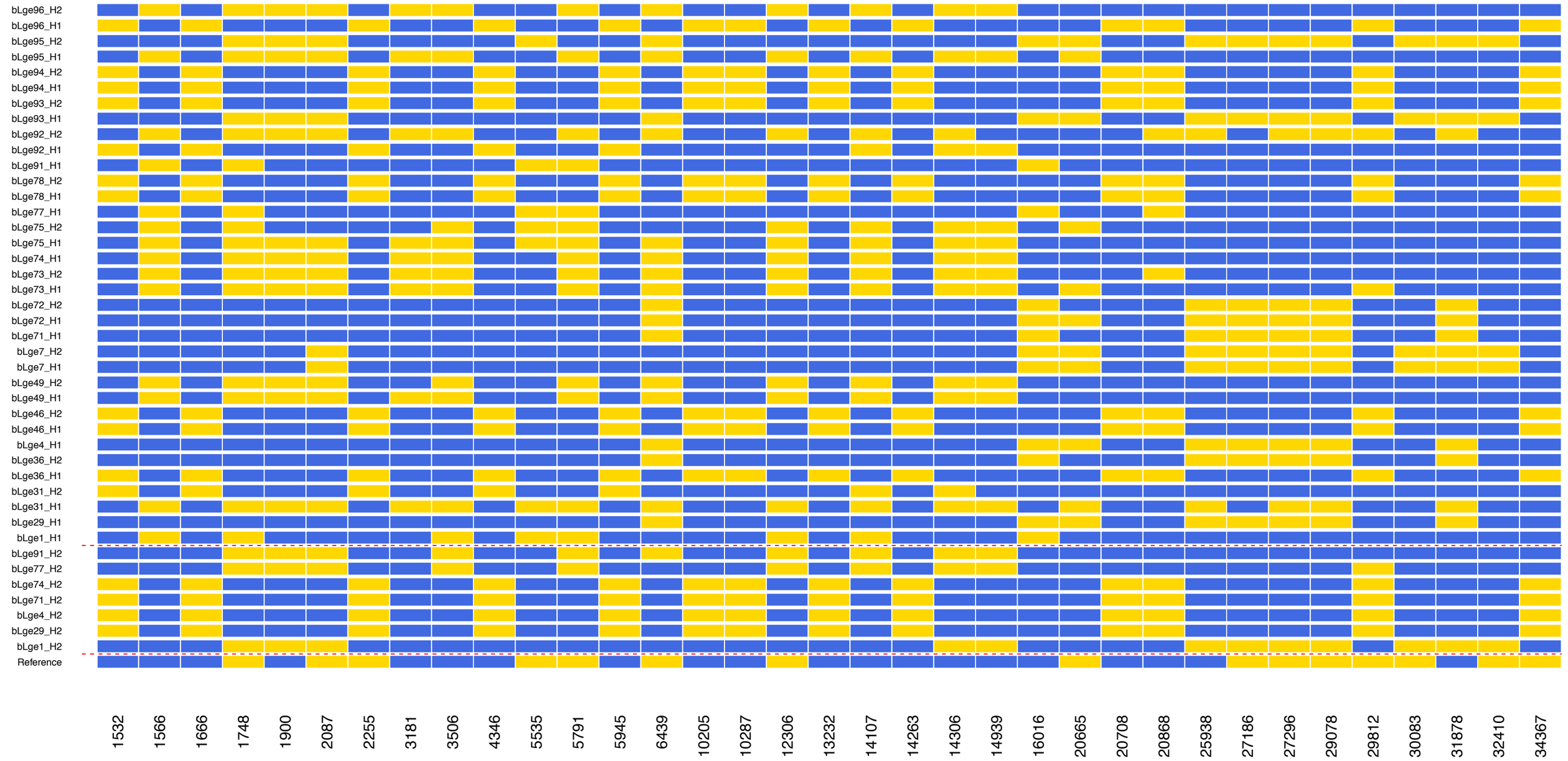

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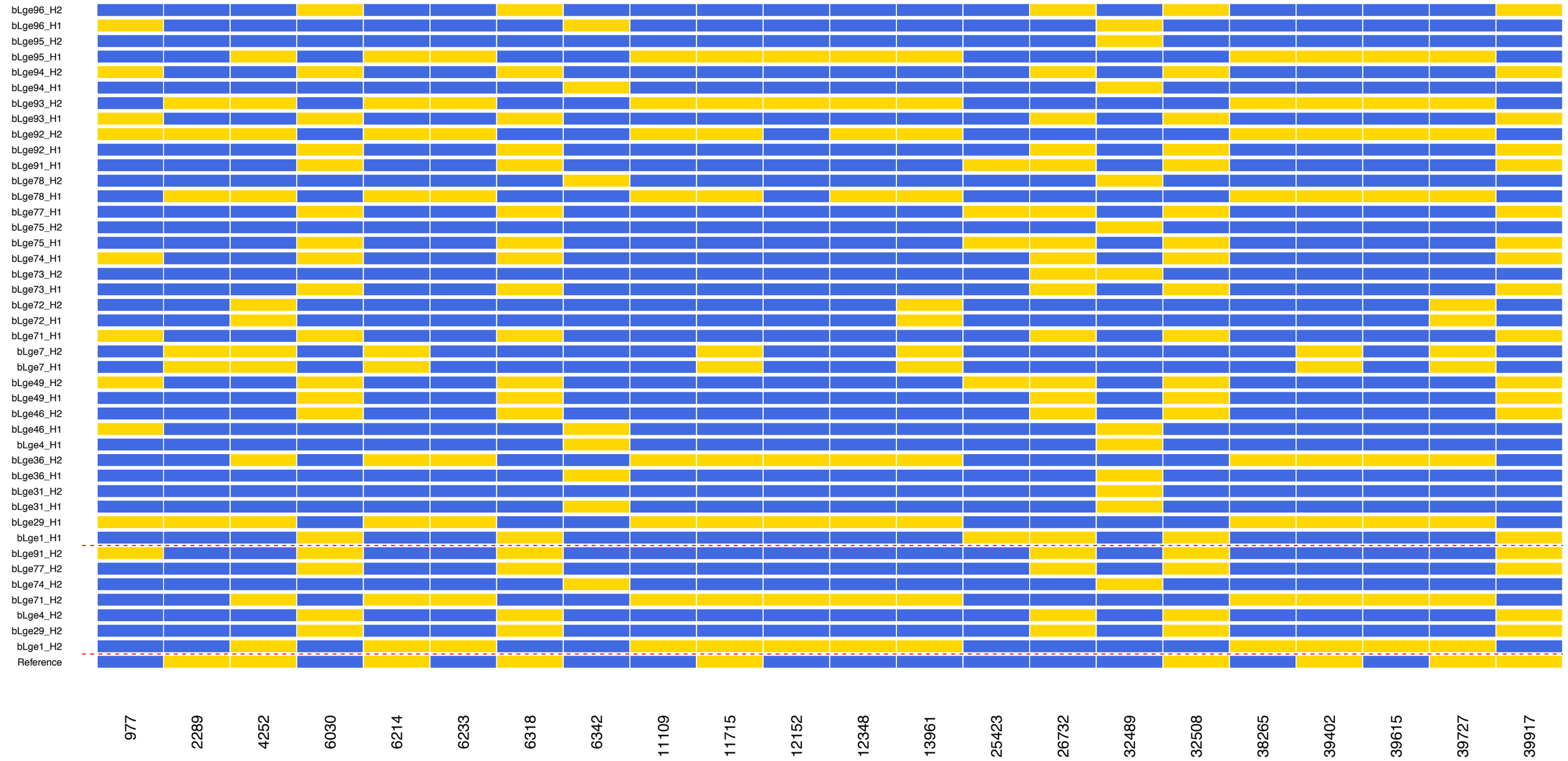

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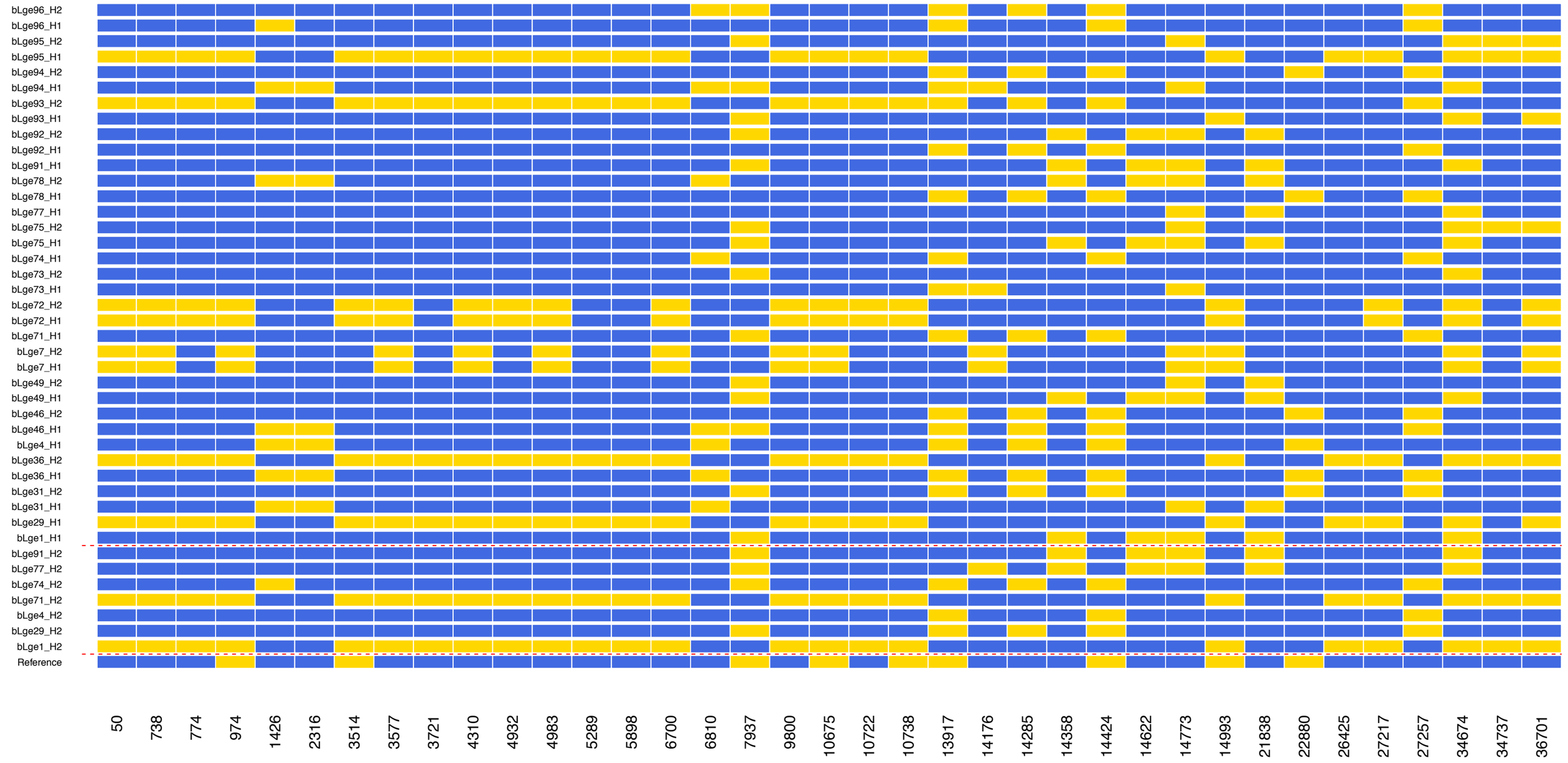

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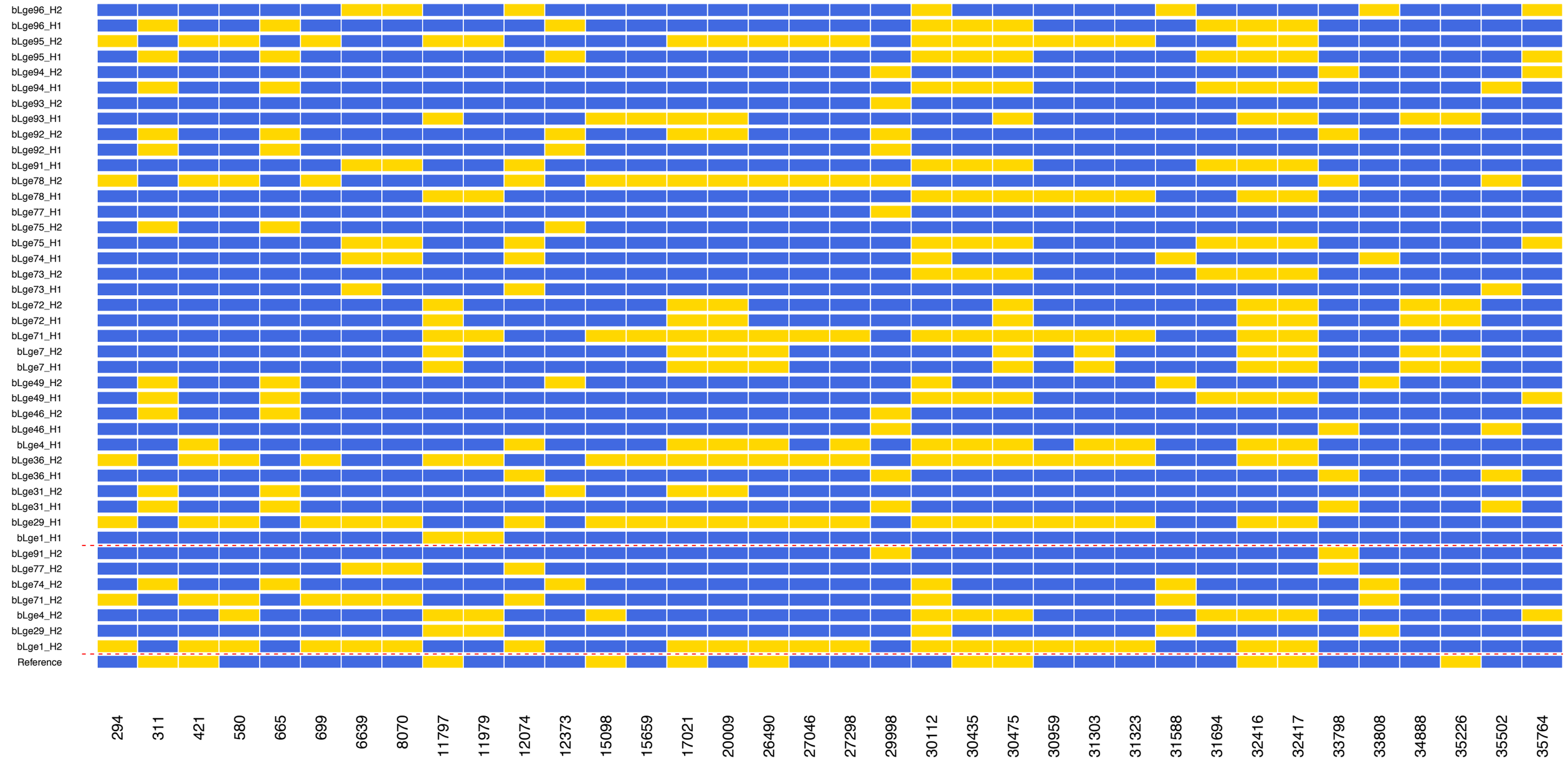

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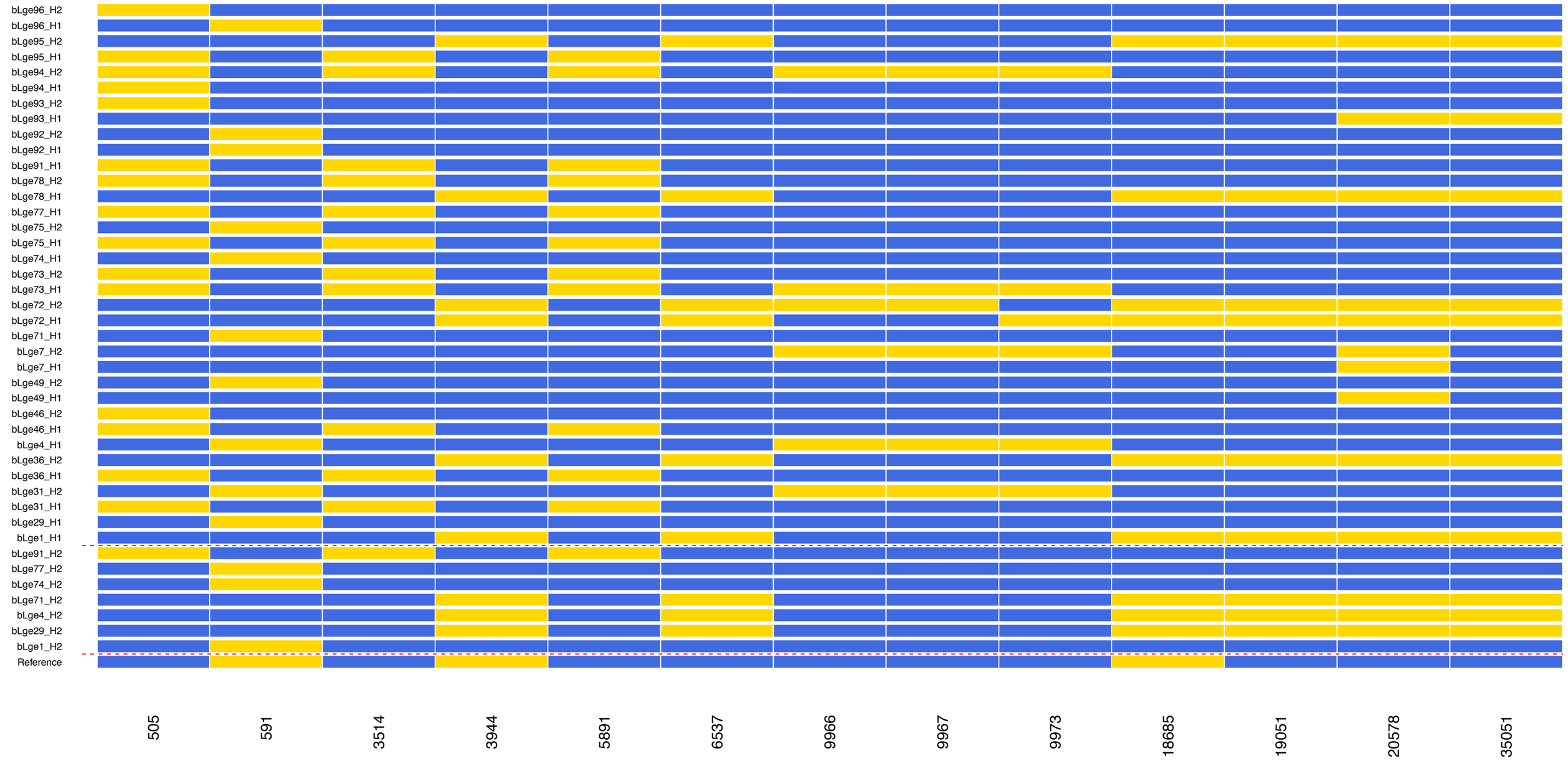

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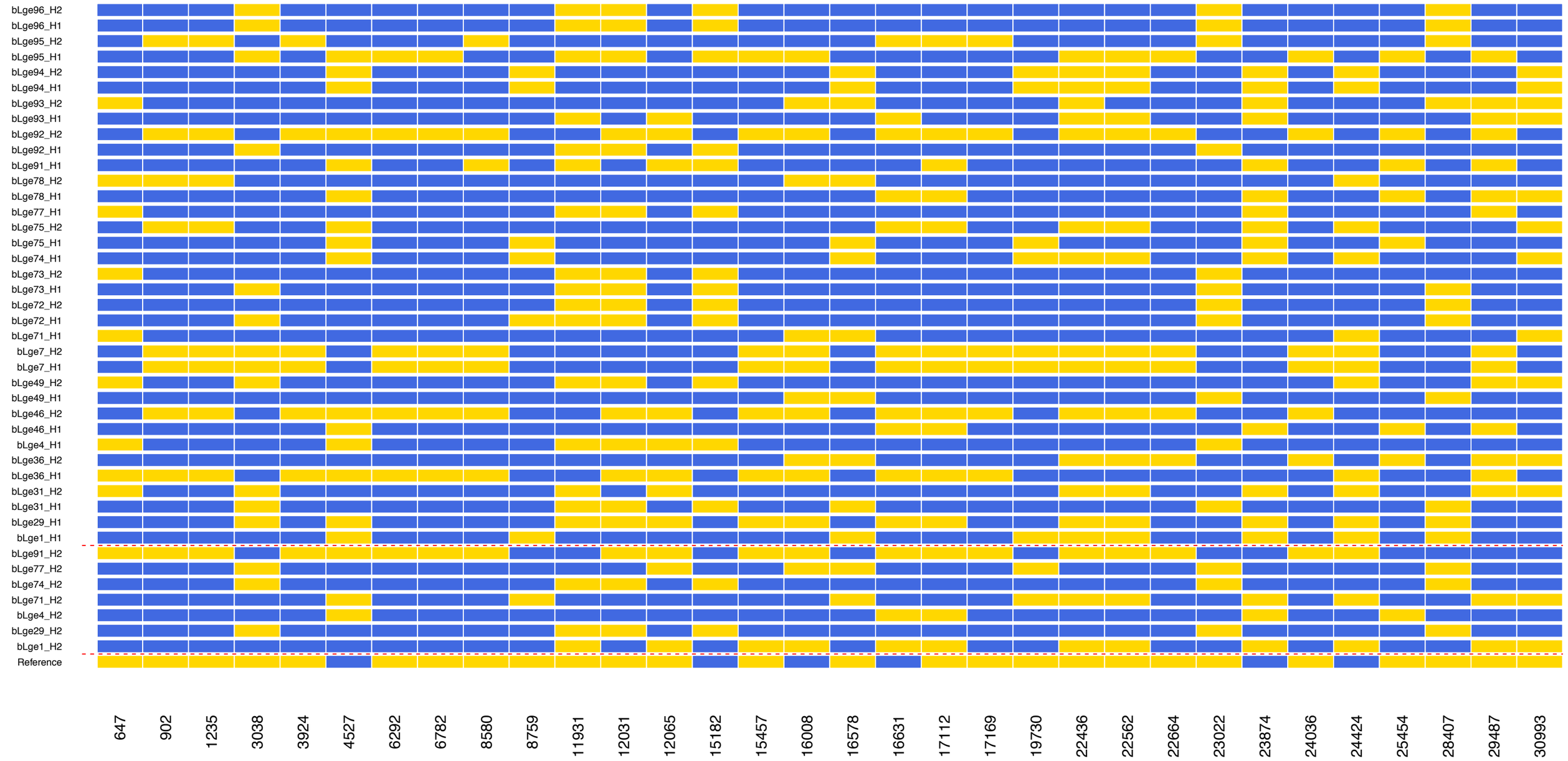

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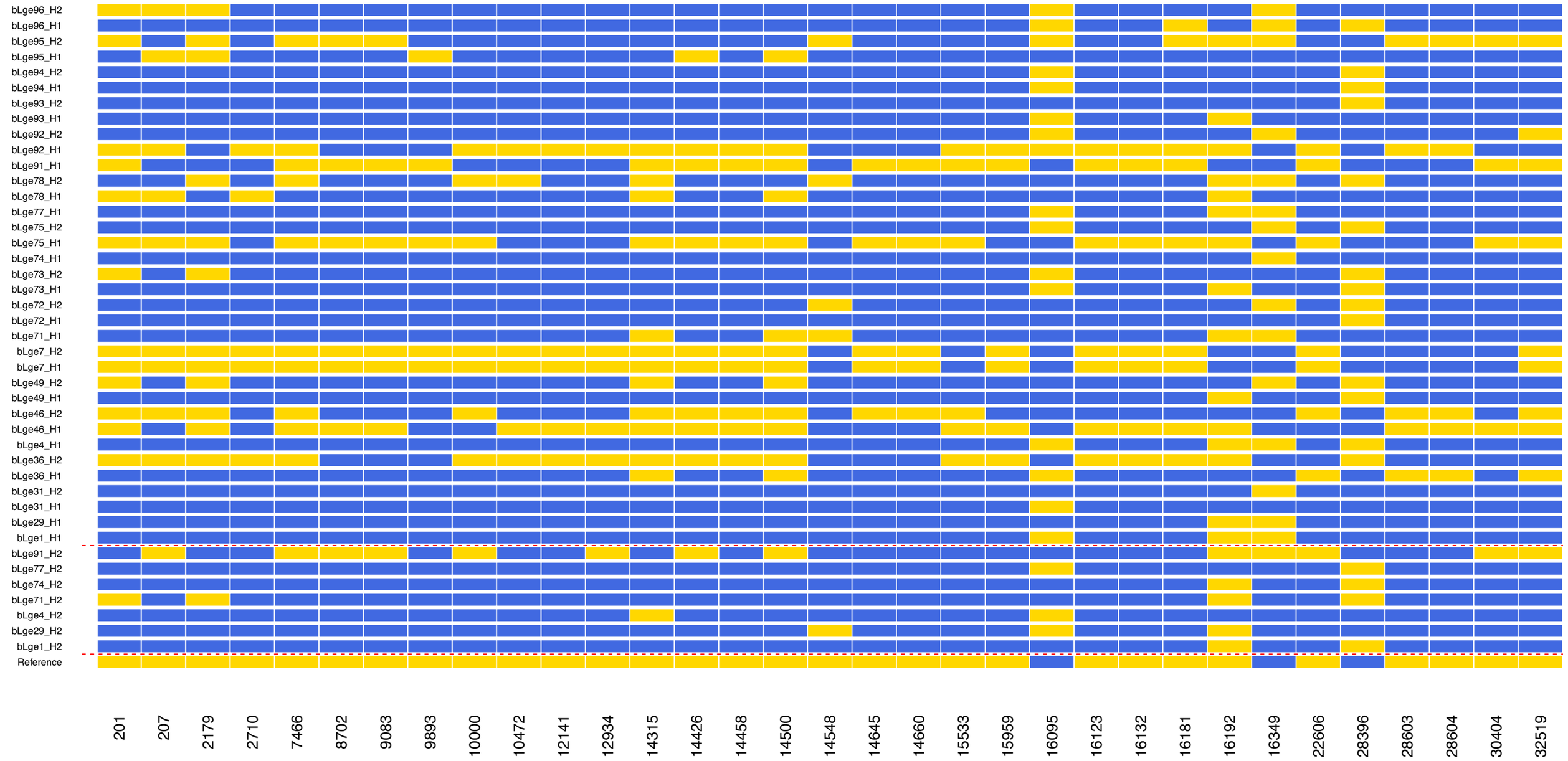

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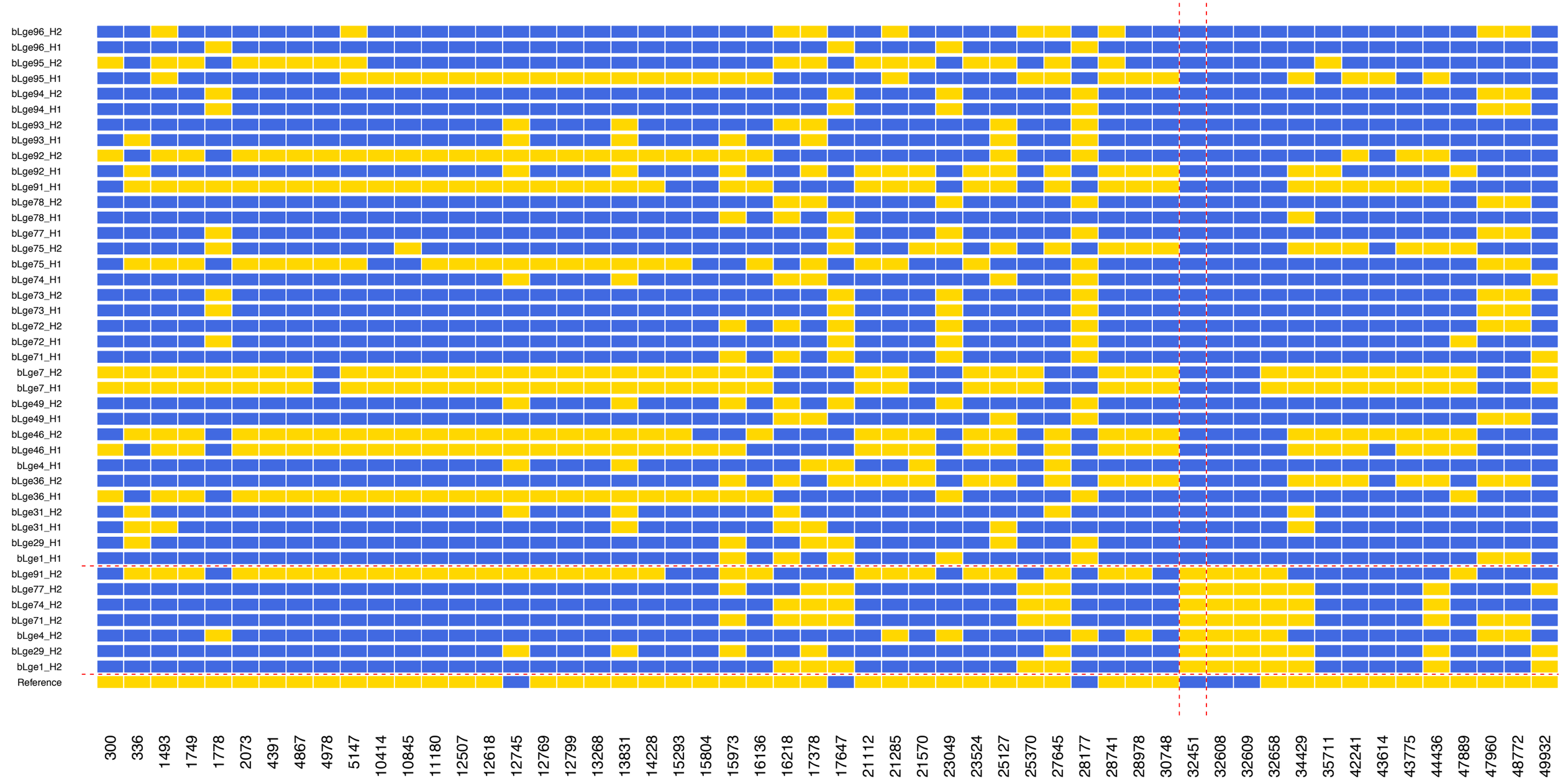

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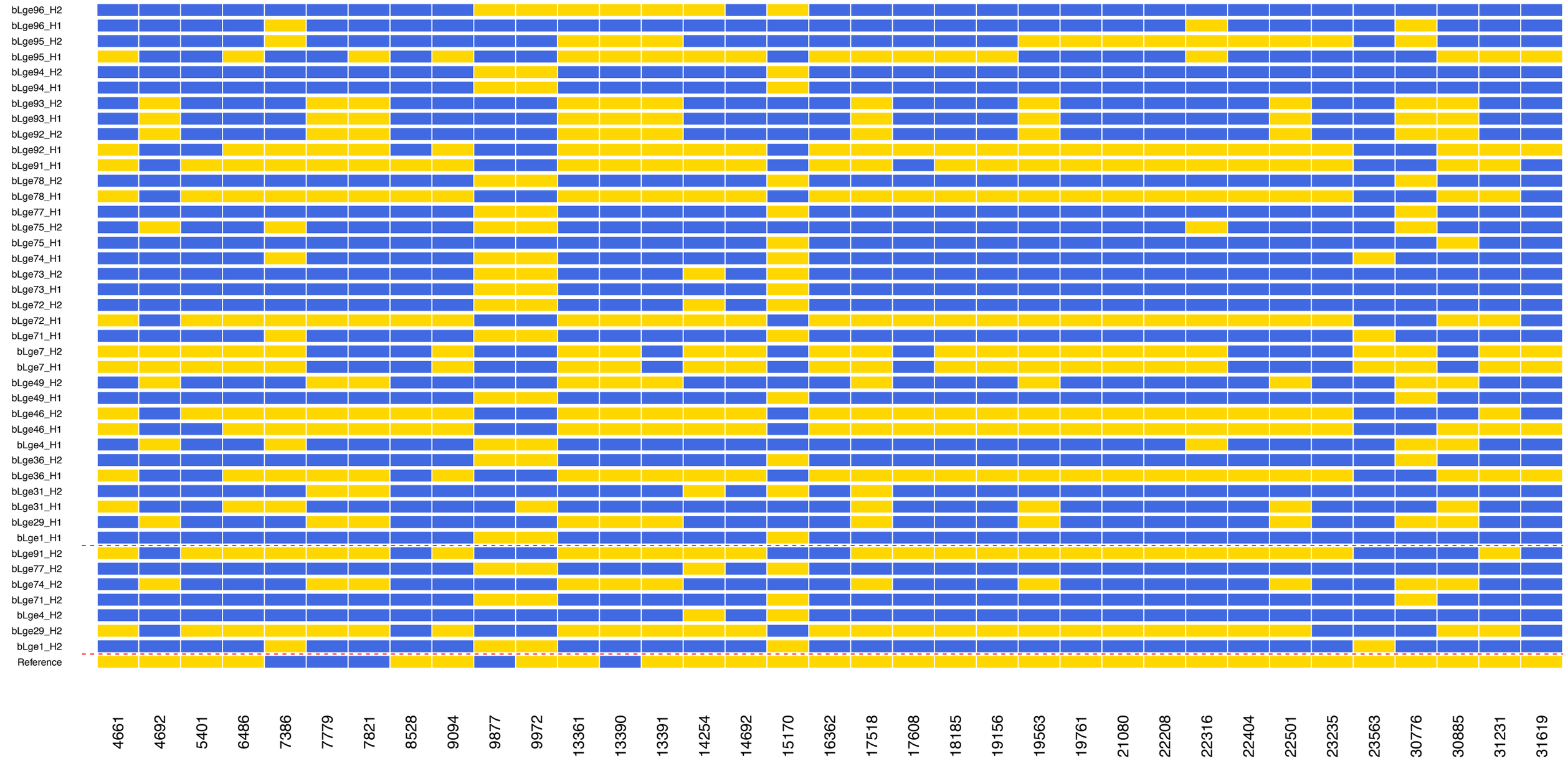

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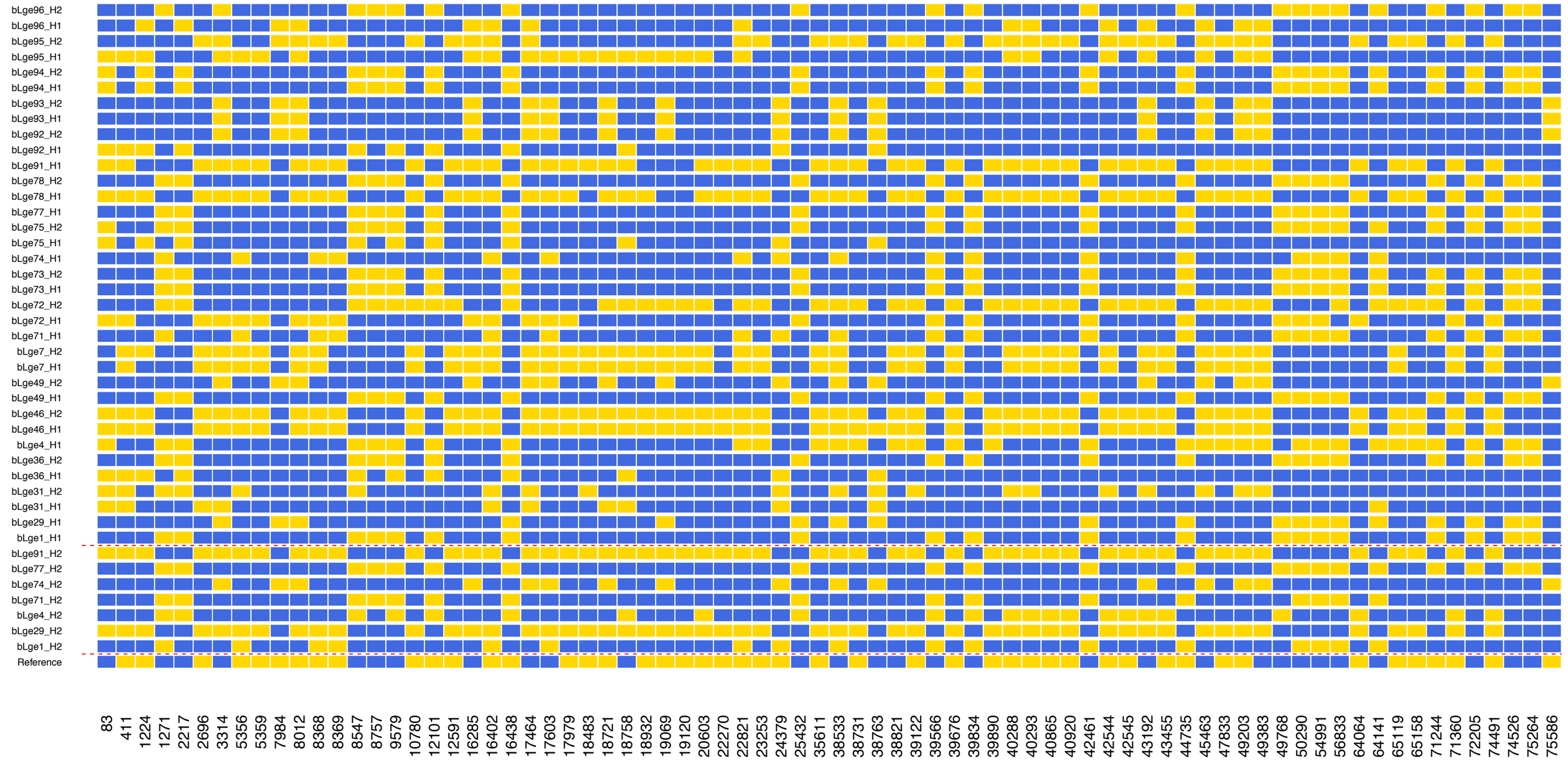

Figure S3.

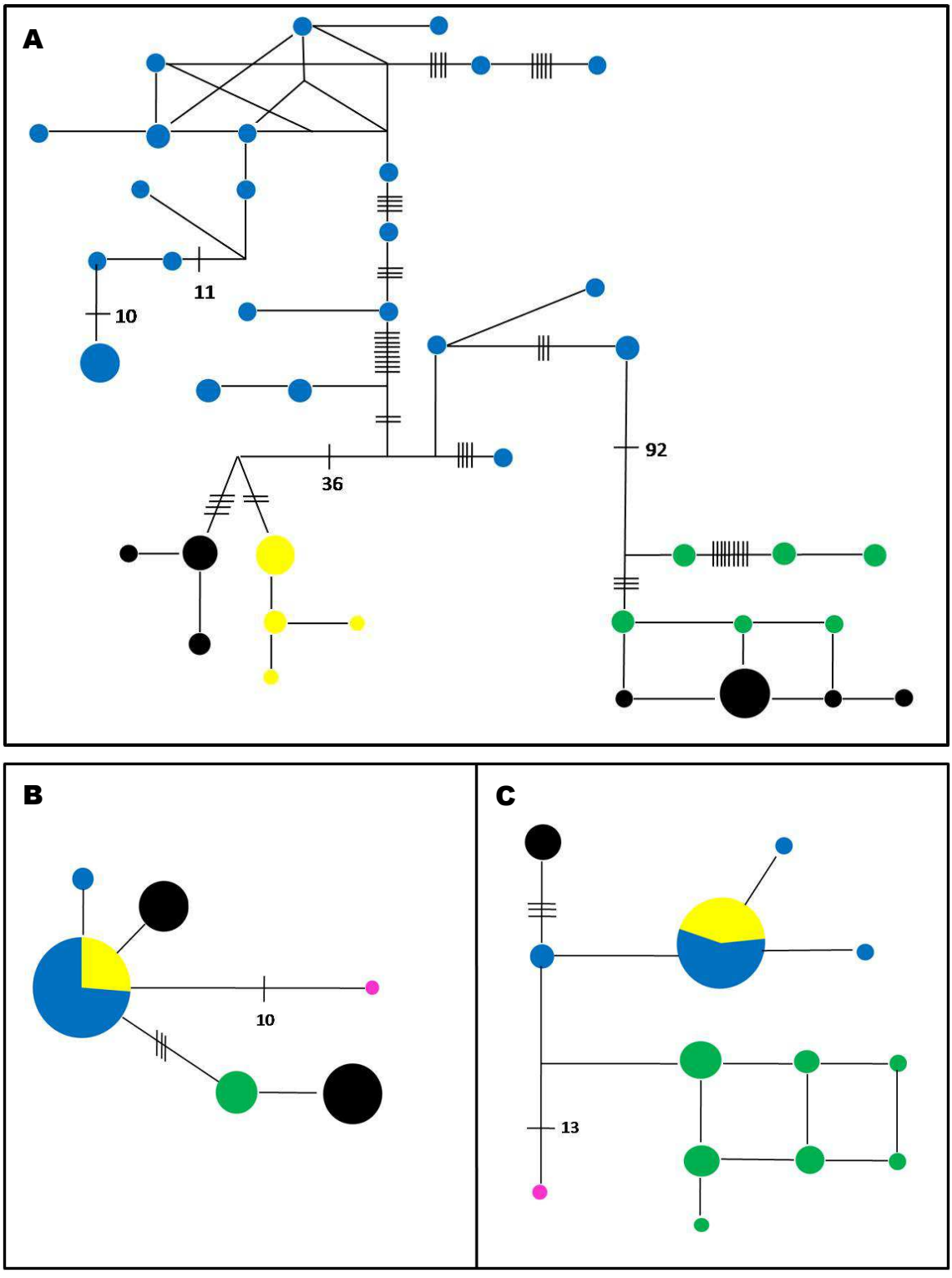

Table 1. Genotypes for melanism-related *ASIP* and *MC1R* genes, expected frequency of melanism assuming Hardy-Weiberg equilibrium (HWE) and field-based and molecular-derived estimates of melanism frequency in each species.

Species	*ASIP* genotype			Frequency of melanistic allele [a]	Expected Number of melanistic	Melanism frequency assuming HWE	Melanism frequency at population level (%)
	+/+	+/R120C	R120C/R120C				
L. colocolo	2	6	9*	0.71	8.57	0.50	30.7 [b]
	+/+	+/C126Y	C126Y/C126Y				
L. guigna	5	6	5	0.50	4.0	0.25	15.8 – 28.6 [c]
	MC1R genotype						
	+/+	+/C125R	C125R/C125R				
L. geoffroyi	16	7	0	0.15	6.38	0.28	20 [d]

[a] R120C is the melanistic allele in *L. colocolo*, C126Y in *L. guigna* and C125Y is the melanistic allele in *L. geoffroyi*.
[b] Melanism frequency based on wild-caught animals and camera trapping.
[c] Melanism frequency based on wild-caught animals and road-killed animals.
[d] Melanism frequency based on wild-caught, captive and road-killed animals.
*Lco30 was not taken in account in these estimates because it is not from Emas National Park.

Table S1. List of samples analyzed in the present study with genotypes for melanism-related *ASIP* and *MC1R* mutations in each species.

Sample ID	Geographic Origin	Institution/Contact	Coat Color	*ASIP* Genotype positions		*MC1R* Genotype positions
				10585	10604	32451
***Leopardus colocolo* (Pampas cat)**						
bLco-302	Emas National Park, Goiás – Brazil	Leandro Silveira/Anah Jacomo	Wild-type	C/T [+ ϕ]	G/G	T/T
bLco-303	Emas National Park, Goiás – Brazil	Leandro Silveira/Anah Jacomo	Wild-type	C/T [+ ϕ]	G/G	T/T
bLco-305	Emas National Park, Goiás – Brazil	Leandro Silveira/Anah Jacomo	Wild-type	C/T [+ ϕ]	G/G	T/T
bLco-307 [1]	Emas National Park, Goiás – Brazil	Leandro Silveira/Anah Jacomo	Wild-type	C/T [+ ϕ]	G/G	T/T
bLco-308 [1]	Emas National Park, Goiás – Brazil	Leandro Silveira/Anah Jacomo	Wild-type	C/C [+ ϕ]	G/G	T/T
bLco-312 [1]	Emas National Park, Goiás – Brazil	Leandro Silveira/Anah Jacomo	Wild-type	C/C [+ ϕ]	G/G	T/T
bLco-313	Emas National Park, Goiás – Brazil	Leandro Silveira/Anah Jacomo	Wild-type	C/T [+ ϕ]	G/G	T/T
bLco-314 [1]	Emas National Park, Goiás – Brazil	Leandro Silveira/Anah Jacomo	Wild-type	C/T [+ ϕ]	G/G	T/T
bLco-304	Emas National Park, Goiás – Brazil	Leandro Silveira/Anah Jacomo	Melanistic	T/T [+ ϕ]	G/G	T/T
bLco-306 [1]	Emas National Park, Goiás – Brazil	Leandro Silveira/Anah Jacomo	Melanistic	T/T [+ ϕ]	G/G	T/T
bLco-310	Emas National Park, Goiás – Brazil	Leandro Silveira/Anah Jacomo	Melanistic	T/T [+ ϕ]	G/G	T/T
bLco-311	Emas National Park, Goiás – Brazil	Leandro Silveira/Anah Jacomo	Melanistic	T/T [+ ϕ]	G/G	T/T
bLco-315	Emas National Park, Goiás – Brazil	Leandro Silveira/Anah Jacomo	Melanistic	T/T [+ ϕ]	G/G	T/T
bLco-316 [1]	Emas National Park, Goiás – Brazil	Leandro Silveira/Anah Jacomo	Melanistic	T/T [+ ϕ]	G/G	T/T
bLco-317	Emas National Park, Goiás – Brazil	Leandro Silveira/Anah Jacomo	Melanistic	T/T [+ ϕ]	G/G	T/T
bLco-318 [1]	Emas National Park, Goiás – Brazil	Leandro Silveira/Anah Jacomo	Melanistic	T/T [+ ϕ]	G/G	T/T
bLco-320	Emas National Park, Goiás – Brazil	Leandro Silveira/Anah Jacomo	Melanistic	T/T [+ ϕ]	G/G	T/T
Lco-30 [1]	Unkown	Cincinnati Zoo & Botanical Garden	Melanistic	T/T [+ ϕ]	G/G	T/T
***Leopardus guigna* (Kod kod)**						
Gui-18 [*]	Chiloé Island, Chile	Constanza Napolitano	Wild-type	C/C	G/G [+]	-
Gui-19 [1]	Chiloé Island, Chile	Constanza Napolitano	Wild-type	C/C	G/A [+ ϕ]	T/T

Sample ID	Geographic Origin	Institution/Contact	Coat Color	*ASIP* Genotype positions		*MC1R* Genotype positions
				10585	10604	32451
***Leopardus guigna* (Kod kod)**						
Gui-21	Chiloé Island, Chile	Constanza Napolitano	Wild-type	C/C	G/G $^{+\phi}$	T/T
Gui-22	Chiloé Island, Chile	Constanza Napolitano	Wild-type	C/C	G/G $^{+\phi}$	T/T
Gui-76	Chiloé Island, Chile	Constanza Napolitano	Wild-type	C/C	G/G $^{+\phi}$	T/T
Gui-77 [1]	Chiloé Island, Chile	Constanza Napolitano	Wild-type	C/C	G/A $^{+\phi}$	T/T
Gui-78 [1]	Chiloé Island, Chile	Constanza Napolitano	Wild-type	C/C	G/G $^{+\phi}$	T/T
Gui-80	Chiloé Island, Chile	Constanza Napolitano	Wild-type	C/C	G/A $^{+\phi}$	T/T
Gui-81	Chiloé Island, Chile	Constanza Napolitano	Wild-type	C/C	G/A $^{+\phi}$	TT
Gui-82	Chiloé Island, Chile	Constanza Napolitano	Wild-type	C/C	G/A $^{+\phi}$	T/T
Gui-85	Chiloé Island, Chile	Constanza Napolitano	Wild-type	C/C	G/A $^{+\phi}$	T/T
Gui-24 [1]	Chiloé Island, Chile	Constanza Napolitano	Melanistic	C/C	A/A $^{+\phi}$	T/T
Gui-73* [1]	Chiloé Island, Chile	Constanza Napolitano	Melanistic	C/C	A/A $^{+}$	T/T
Gui-74	Chiloé Island, Chile	Constanza Napolitano	Melanistic	C/C	A/A $^{+\phi}$	T/T
Gui-75 [1]	Chiloé Island, Chile	Constanza Napolitano	Melanistic	C/C	A/A $^{+\phi}$	T/T
Gui-79*	Chiloé Island, Chile	Constanza Napolitano	Melanistic	C/C	A/A $^{+}$	-
***Leopardus geoffroyi* (Geoffroy's cat)**						
bLge-07	Cachoeira do Sul, RS – Brazil	Cachoeira do Sul Zoo	Wild-type	C/C	G/G	T/T $^{\phi}$
bLge-31	Quaraí, RS – Brazil	Sapucaia do Sul Zoo	Wild-type	C/C	G/G	T/T $^{+\phi}$
bLge-36 [1]	Taim, RS – Brazil	Tatiane Trigo	Wild-type	C/C	G/G	T/T $^{+\phi}$
bLge-37*	São Lourenço do Sul, RS – Brazil	Sapucaia do Sul Zoo	Wild-type	-	-	T/T $^{+}$
bLge-46 [1]	Canela, RS – Brazil	Sapucaia do Sul Zoo	Wild-type	C/C	G/G	T/T $^{+\phi}$
bLge-47*	São Leopoldo, RS - Brazil	Sapucaia do Sul Zoo	Wild-type	-	-	T/T $^{+}$
bLge-49 [1]	unknown, RS - Brazil	Sapucaia do Sul Zoo	Wild-type	C/C	G/G	T/T $^{+\phi}$
bLge-72	Encruzilhada do Sul, RS – Brazil	Quinta da Estância Grande	Wild-type	C/C	G/G	T/T $^{\phi}$

Sample ID	Geographic Origin	Institution/Contact	Coat Color	*ASIP* Genotype positions		*MC1R* Genotype positions
				10585	10604	32451
bLge-73	Cachoeira do Sul, RS – Brazil	Cachoeira Zoo	Wild-type	C/C	G/G	T/T + ϕ
bLge-75	Arroio Grande, RS – Brazil	Fabio Mazim e José Bonifácio Garcia Soares	Wild-type	C/C	G/G	T/T + ϕ
bLge-78	Rio Grande, RS – Brazil	Fabio Mazim	Wild-type	C/C	G/G	T/T ϕ
bLge-92 [1]	Alegrete, RS – Brazil	Fundação Zoobotânica do Rio Grande do Sul	Wild-type	C/C	G/G	T/T ϕ
bLge-93	Arroio Grande, RS – Brazil	Fundação Zoobotânica do Rio Grande do Sul	Wild-type	C/C	G/G	T/T + ϕ
bLge-94	Vale do Quilombo, RS – Brazil	Fundação Zoobotânica do Rio Grande do Sul	Wild-type	C/C	G/G	T/T + ϕ
bLge-95	Cristal, RS – Brazil	Fundação Zoobotânica do Rio Grande do Sul	Wild-type	C/C	G/G	T/T ϕ
bLge-96	Pelotas, RS – Brazil	Fundação Zoobotânica do Rio Grande do Sul	Wild-type	C/C	G/G	T/T ϕ
bLge-01	Santa Cruz do Sul, RS – Brazil	Sapucaia do Sul Zoo	Melanistic	C/C	G/G	C/T + ϕ
bLge-04	Cachoeira do Sul, RS – Brazil	Cachoeira do Sul Zoo	Melanistic	C/C	G/G	C/T + ϕ
bLge-29 [1]	Quaraí, RS – Brazil	Dênis Sana, Tatiane Trigo, Cibele Indrusiak	Melanistic	C/C	G/G	C/T + ϕ
bLge-71	Pelotas, RS – Brazil	Thales R. O. de Freitas e José Stoltz	Melanistic	C/C	G/G	C/T + ϕ
bLge-74 [1]	Pinheiro Machado, RS – Brazil	Fabio Mazim	Melanistic	C/C	G/G	C/T + ϕ
bLge-77 [1]	Dom Pedrito, RS – Brazil	Fabio Mazim	Melanistic	C/C	G/G	C/T + ϕ
bLge-91 [1]	Itaqui, RS – Brazil	Fundação Zoobotânica do Rio Grande do Sul	Melanistic	C/C	G/G	C/T ϕ

[1] Samples included in the initial set of samples (four individual of each phenotype; Data set 1)

+ Genotype defined based on Sanger sequencing (Data set 2)

ϕ Genotype defined based on next-generation sequencing (Data set 3)

* Samples were not included in the hybrid capture assay due to low DNA quality for the library building.

Table S2. Primers developed in this study for PCR amplification and sequencing of *MC1R* in felids. F1, R1 refer to the first fragment of the coding exon and F2, R2 refer to the second fragment of the coding exon.

Primer	**Sequence (5' – 3')**
MC1R – F1	CCTGCTGGAAGCACCACT
MC1R – R1	GACGCTAGCCACCCAGATAG
MC1R – F2	GTGGACCGCTACATTTCCAT
MC1R – R2	GCCATAGGATATCCCCACCT

Table S3. GenBank accession number of mammalian sequences for comparative genomic analyses in the *ASIP* and *MC1R* genes.

	GenBank Accession Number	
Mammal species	***ASIP***	***MC1R***
Domestic cat (*Felis catus*)	NP_001009190.1	AY237395
Dog (*Canis familiaris*)	NP_001007264.1	AF064455
Human (*Homo sapiens*)	NM_001672	AF326275
Cow (*Bos taurus*)	X99692.1	GU982927
Mouse (*Mus musculus*)	NM_015770.3	AB306322
Red fox (*Vulpes vulpes*)	--	X90844
Horse (*Equus caballus*)	AF288358.1	--
Pig (*Sus scrofa*)	AJ427478.2	--
Rat (*Rattus rattus*)	NM_052979.1	--

Table S4. Chromosome coordinates and lengths of fosmid clones derived from *F. catus* selected for probe synthesis and targeted resequencing of *ASIP* and *MC1R* gene regions.[a]

Segment	Fosmid ID	Chromosome coordinates	Length (bp)
AsipA[b]	165060_F.CATUS_FOSMID-042_M13	chrA3: 3828195 - 3875337	47143
	161571_F.CATUS_FOSMID-095_K15	chrA3: 3845608 - 3896486	50879
AsipB	165209_F.CATUS_FOSMID-082_C20	chrA3: 4128427 - 4164971	36545
AsipC	162081_ F.CATUS_FOSMID-14_C16	chrA3: 4190631 - 4226080	35450
AsipD	162155_F.CATUS_FOSMID-036_A21	chrA3: 4241012 - 4276720	35709
AsipE	162683_F.CATUS_FOSMID-072_H5	chrA3: 4337331 - 4389470	52140
AsipF	161863_F.CATUS_FOSMID-013_F1	chrA3: 4511089 - 4559874	48786
AsipG	161280_F.CATUS_FOS-003_J17	chrA3: 4579022 - 4624358	45337
AsipH	161571_F.CATUS_FOSMID-075_I22	chrA3: 4718970 - 4757804	38835
Mc1rA[c]	162683_F.CATUS_FOSMID-039_H22	chrE2: 74693023 - 74732935	39912
	162298_F.CATUS_FOSMID-035_E17	chrE2: 74712132 - 74748688	36557
Mc1rB	161957_F.CATUS_FOSMID-032_P20	chrE2: 74897213 - 74935909	38696
Mc1rC[d]	165209_F.CATUS_FOSMID-058_N1	chrE2: 74937764 - 74988665	50901
	161795_F.CATUS_FOS-044_G13	chrE2: 74964001 - 75012055	48054
	162155_F.CATUS_FOSMID-093_J7	chrE2: 74989867 - 75042008	52141
Mc1rD	165232_F.CATUS_FOSMID-028_C14	chrE2: 75039343 - 75079289	39946
Mc1rE[e]	162683_F.CATUS_FOSMID-081_M17	chrE2: 75095713 - 75130034	34321
	162683_F.CATUS_FOSMID-044_K8	chrE2: 75132381 - 75180838	48457

[a] According to the *Garfield browser;* Pontius and O'Brien 2007.
[b] Composite contig length of 68291 bp.
[c] Composite contig length of 55665 bp
[d] Composite contig length of 104244 bp.
[e] Composite contig length of 85125 bp.

Table S5. Variable sites in the *ASIP* and *MC1R* coding sequences identified from the initial samples set of *Leopardus colocolo* (bLco), *L. guigna* (Gui) and *L. geoffroyi* (bLge) – Data set 1. Site numbers (vertical notation) refer to the aligned positions in the *ASIP* and *MC1R* relative to the domestic cat genome. Synonymous substitution is showed by "s" and non-synonymous substitution by "ns". Shaded positions refer to the potential melanism-inducing mutations in each species. Melanistic individuals and their respective genotype are shown in bold. "N" refers to genotypes which were not identified.

	***ASIP* Variable sites**						***MC1R* Variable sites**											
	Nucleotide position						Nucleotide position											
			1	1	1	1	3	3	3	3	3	3	3	3	3	3	3	3
	6	6	0	0	0	0	2	2	2	2	2	2	2	2	2	2	2	2
	2	2	5	5	5	6	0	1	1	1	4	4	6	6	6	6	9	9
	8	9	2	8	8	0	9	4	5	7	5	5	0	0	5	6	3	4
	9	9	1	0	5	4	8	1	9	5	1	3	8	9	8	9	7	5
F. catus	C	G	C	A	C	G	A	C	C	C	T	T	C	G	G	A	A	C
Substitution	s	ns	s	ns	ns	ns	ns	s	s	ns	ns	s	ns	ns	ns	s	ns	ns
L. colocolo																		
bLco 307	T	A	T	.	C	.	N	G	.	C/T	.	.	.	.	A	C	.	.
bLco 308	T	A	T	.	C/T	.	.	G	.	.	.	.	.	.	A	C	.	.
bLco 312	T	A	T	.	C	.	N	.	.	.	.	.	.	.	A	C	.	.
bLco 314	T	A	T	.	C/T	.	C	.	T	.	.	.	.	.	A	C	.	.
Lco 30	T	A	T	.	**T**	.	C	.	.	.	.	.	.	.	A	C	.	.
bLco 306	T	A	T	.	**T**	.	C/A	.	.	.	.	.	.	.	A	C	.	.
bLco 316	T	A	T	.	**T**	.	C/A	.	.	.	.	.	.	.	A	C	.	.
bLco 318	T	A	T	.	**T**	.	C/A	G	.	.	.	.	.	.	A	C	.	.
L. guigna																		
Gui 19	.	.	.	.	.	G/A	.	.	.	.	.	.	.	.	.	.	G	.
Gui 77	.	.	.	.	.	G/A	.	.	.	.	.	.	.	.	.	.	G	.
Gui 78	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	G	.
Gui 81	.	.	.	.	.	G/A	.	.	.	.	.	.	.	.	.	.	G	.
Gui 24	.	.	.	.	.	**A**	.	.	.	.	.	.	.	.	.	.	G	.
Gui 73	.	.	.	.	.	**A**	.	.	.	N	.	.	.	.	.	.	G	.
Gui 75	.	.	.	.	.	**A**	.	.	.	.	.	.	.	.	.	.	G	.
Gui 79	.	.	.	.	.	**A**	.	.	.	.	.	.	.	.	.	.	G	.
L. geoffroyi																		
bLge 36	.	.	.	A/G	.	.	.	.	.	.	.	C/T	.	.	.	.	G	C/T
bLge 46	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.
bLge 49	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.
bLge 92	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	C/T
bLge 29	.	.	.	.	.	.	.	.	.	.	**C/T**	.	C/T	A/G	A/G	.	G	.
bLge 74	.	.	.	.	.	.	.	.	.	.	**C/T**	.	N	N	N	.	G	.
bLge 77	.	.	.	.	.	.	.	.	.	.	**C/T**	.	C/T	A/G	A/G	.	G	.
bLge 91	.	.	.	.	.	.	.	.	.	.	**N**	.	C/T	A/G	A/G	.	G	.

Table S6. Variable sites on *ASIP* exon 4 between *Leopardus colocolo* and *L. guigna* (Data set 2). Melanistic individuals and the melanism-inducing mutations are shown in bold. Synonymous substitution is showed by "s" and non-synonymous substitution by "ns".

ASIP Variable sites			
Nucleotide position			
	1	1	1
	0	0	0
	5	5	6
	2	8	0
	1	5	4
Felis catus	C	C	G
Substitution	s	ns	ns
L. colocolo			
bLco 302	T	C/T	.
bLco 303	T	C/T	.
bLco 305	T	C/T	.
bLco 307	T	C/T	.
bLco 308	T	C	.
bLco 312	T	C	.
bLco 313	T	C/T	.
bLco 314	T	C/T	.
Lco 30	T	**T**	.
bLco 304	T	**T**	.
bLco 306	T	**T**	.
bLco 310	T	**T**	.
bLco 311	T	**T**	.
bLco 315	T	**T**	.
bLco 316	T	**T**	.
bLco 317	T	**T**	.
bLco 318	T	**T**	.
bLco 320	T	**T**	.
L. guigna			
Gui 18	.	.	.
Gui 19	.	.	A/G
Gui 21	.	.	.
Gui 22	.	.	.
Gui 76	.	.	.
Gui 77	.	.	A/G
Gui 78	.	.	.
Gui 80	.	.	A/G
Gui 81	.	.	A/G
Gui 82	.	.	A/G
Gui 85	.	.	A/G
Gui 24	.	.	**A**
Gui 73	.	.	**A**
Gui 74	.	.	**A**
Gui 75	.	.	**A**
Gui 79	.	.	**A**

Table S7. Variable sites in the *MC1R* coding exon of *L. geoffroyi* (Data set 2). Variant fixed within-species were excluded. Melanistic individuals and the melanism-inducing mutation are shown in bold. Synonymous substitution is showed by "s" and non-synonymous substitution by "ns". "N" refers to genotypes which were not identified.

	MC1R Variable sites					
	Nucleotide position					
	3	3	3	3	3	3
	2	2	2	2	2	2
	4	4	6	6	6	9
	5	5	0	0	5	4
	1	3	8	9	8	5
Felis catus	T	T	C	G	G	C
Substitution	ns	s	ns	ns	ns	ns
L. geoffroyi						
bLge 31	.	.	N	N	N	.
bLge 36	.	C/T	.	.	G	C/T
bLge 37	.	.	.	.	G	.
bLge 46	.	.	.	.	G	.
bLge 47	.	.	.	.	G	.
bLge 49	.	.	.	.	G	.
bLge 73	.	.	.	.	G	.
bLge 75	.	.	.	.	G	.
bLge 92	N	N	.	.	G	C/T
bLge 93	.	.	.	.	G	.
bLge 94	.	.	.	.	G	.
bLge 95	N	N	N	N	N	.
bLge 01	**C/T**	.	C/T	A/G	A/G	.
bLge 04	**C/T**	.	N	N	N	.
bLge 29	**C/T**	.	C/T	A/G	A/G	.
bLge 71	**C/T**	.	N	N	N	.
bLge 74	**C/T**	.	N	N	N	.
bLge 77	**C/T**	.	C/T	A/G	A/G	.
bLge 91	**N**	N	C/T	A/G	A/G	.

Table S8. Length of *ASIP* and *MC1R* genomic segments in *Leopardus* spp. and the corresponding number of SNPs identified in each species.

Segment	Length (bp)	Number of SNPs			
	Lco/Lgu/Lge	Lco	Lgu	Lge	Lco/Lgu/Lge
AsipA	49437	49	15	49	113
AsipB	20667	8	5	12	25
AsipC	40003	13	6	40	59
AsipD	34797	16	10	35	61
AsipE	42609	19	5	22	46
AsipF	37265	28	7	37	72
AsipG	36185	17	11	36	64
AsipH	38260 *	6	---	13	19
Total	---	156	59	244	459
Mc1rA	31838	32	20	32	84
Mc1rB	33790	33	10	33	76
Mc1rC	74574	51	13	54	118
Mc1rD	35012	35	24	35	94
Mc1rE	76173	76	23	76	175
Total		227	90	230	547

*No SNPs could be called for Lgu in AsipH.

Table S9. Nucleotide diversity in *ASIP* and *MC1R* genomic segments estimated for three different phenotypes subgroups in *Leopardus* spp: non-melanistic individuals (NM), melanistic individuals (M) and both phenotypes together (ALL). The segment implicated in melanism of each species is shaded. Values are indicated as percentage (%).

	L. colocolo			*L. guigna*		
Segment	NM	M	ALL	NM	M	ALL
ASIP						
A	0.051 (±0.004)	0.055 (± 0.006)	0.056 (±0.004)	0.018 (±0.001)	0.016 (±0.002)	0.018 (±0.001)
B	0.023 (±0.005)	0.020 (±0.006)	0.022 (±0.004)	0.020 (±0.004)	0.022 (±0.007)	0.019 (±0.003)
C	0.024 (±0.009)	0.020 (±0.008)	0.021 (±0.006)	0.016 (±0.001)	0.006 (±0.001)	0.016 (±0.001)
D	0.031 (±0.006)	0.002 (±0.001)	0.018 (±0.005)	0.016 (±0.003)	0.002 (±0.001)	0.018 (±0.001)
E	0.059 (±0.009)	0.016 (±0.002)	0.040 (±0.008)	0.006 (±0.002)	0.018 (±0.004)	0.009 (±0.002)
F	0.056 (±0.008)	0.042 (±0.010)	0.051 (±0.006)	0.008 (±0.002)	0.018 (±0.004)	0.012 (±0.002)
G	0.039 (±0.007)	0.033 (±0.007)	0.036 (±0.005)	0.025 (±0.002)	0.026 (±0.005)	0.025 (±0.002)
H	0.012 (±0.002)	0.007 (±0.002)	0.010 (±0.001)	--	--	--
MC1R						
A	0.052 (±0.004)	0.047 (±0.004)	0.049 (±0.002)	0.025 (±0.002)	0.029 (±0.007)	0.028 (±0.002)
B	0.063 (±0.003)	0.063 (±0.002)	0.063 (±0.001)	0.015 (±0.002)	0.017 (±0.004)	0.015 (±0.002)
C	0.037 (±0.002)	0.043 (±0.001)	0.041 (±0.001)	0.008 (±0.001)	0.008 (±0.001)	0.008 (±0.001)
D	0.066 (±0.004)	0.065 (±0.003)	0.066 (±0.002)	0.033 (±0.008)	0.034 (±0.016)	0.032 (±0.007)
E	0.059 (±0.003)	0.067 (±0.003)	0.065 (±0.002)	0.020 (±0.002)	0.024 (±0.004)	0.021 (±0.001)

	L. geoffroyi		
Segment	NM	M	ALL
ASIP			
A	0.074 (±0.004)	0.080 (±0.005)	0.076 (±0.003)
B	0.034 (±0.004)	0.034 (±0.005)	0.034 (±0.003)
C	0.085 (±0.012)	0.099 (±0.018)	0.089 (±0.010)
D	0.071 (±0.003)	0.066 (±0.005)	0.069 (±0.002)
E	0.058 (±0.006)	0.060 (±0.014)	0.059 (±0.005)
F	0.067 (±0.008)	0.071 (±0.013)	0.067 (±0.007)
G	0.072 (±0.007)	0.081 (±0.008)	0.077 (±0.005)
H	0.023 (±0.003)	0.023 (±0.003)	0.023 (±0.002)
MC1R			
A	0.047 (±0.003)	0.043 (±0.004)	0.045 (±0.003)
B	0.051 (±0.007)	0.029 (±0.009)	0.044 (±0.006)
C	0.040 (±0.004)	0.034 (±0.007)	0.039 (±0.004)
D	0.066 (±0.005)	0.059 (±0.011)	0.063 (±0.005)
E	0.068 (±0.005)	0.071 (±0.010)	0.069 (±0.004)

CAPÍTULO IV:
DISCUSSÃO GERAL

A facilidade em observar a diversidade de cores em animais, incluindo variação na coloração de fundo e também presença de manchas no corpo (como pintas, listras, rosetas), acompanhada da crescente aplicação de técnicas moleculares, tem permitido a investigação da base molecular destas características fenotípicas e uma melhor compreensão da evolução da coloração em múltiplas espécies de animais.

O presente trabalho aborda o estudo de uma das diversas características que influencia a variação da coloração em felinos: o melanismo. O primeiro estudo investigando a base molecular do fenótipo melânico em múltiplas espécies de felídeos (EIZIRIK et al., 2003) gerou uma excelente oportunidade para o estudo da evolução do melanismo em Felidae. Extremamente comum neste grupo de mamíferos, o melanismo é um dos muitos polimorfismos de coloração de felídeos, sendo documentado em 13 das 37 espécies atuais (SCHNEIDER et al., 2012). No entanto, evidências moleculares associadas com a coloração negra foram descobertas previamente em apenas três destas espécies (EIZIRIK et al., 2003), possibilitando a continuação do estudo deste tema em uma perspectiva mais ampla.

Este estudo teve como objetivo geral investigar a associação de variantes moleculares ao fenótipo melânico em felinos e inferir seu potencial significado adaptativo. Os resultados obtidos geraram importantes e inéditas informações sobre os mecanismos genéticos e os processos evolutivos subjacentes a esta característica fenotípica em cinco diferentes espécies.

Neste sentido, no primeiro artigo desta tese (Capítulo II) documentamos a perfeita associação de duas mutações distintas com o fenótipo melânico em duas espécies de felinos asiáticos: o leopardo, popularmente conhecido como pantera negra (*Panthera pardus*), e o gato dourado asiático (*Pardofelis temminckii*). Ambas as mutações não-sinônimas foram identificadas no exon 4 do gene *ASIP*. A primeira mutação introduz um *stop codon* prematuro em indivíduos melânicos de *P. pardus*, provavelmente induzindo a completa perda de função da proteína ASIP. A mutação associada com o melanismo em *P. temminckii* causa a substituição de um dos sítios de aminoácidos altamente conservado em mamíferos, o que também afetaria negativamente a função da proteína. Um aspecto interessante acerca da identificação destas mutações é que ambas estão localizadas no domínio C-terminal da ASIP, região que contém dez resíduos de cisteína completamente conservados em mamíferos. A relevância funcional destes resíduos seria a de estabilizar a estrutura terciária da proteína através de pontes de dissulfeto (OLIVEIRA; RAMACHANDRAN; ADAMS, 1994), conformação extremamente importante também para o correto acoplamento ao receptor de membrana MC1R. Estudos prévios demonstraram experimentalmente que mutações não-sinônimas envolvendo cada um destes resíduos de cisteína causam a perda completa ou

parcial da função da proteína, resultando, consequentemente, em fenótipos melânicos em camundongos (MILTENBERGER et al., 2002; PERRY et al., 1996). Estas evidências facilitam a interpretação funcional sobre as mutações implicadas no melanismo destas espécies de felinos asiáticos. No caso de *P. temminckii*, a mutação inferida como implicada na coloração negra envolve a 9ª cisteína do domínio, um dos resíduos essenciais para a atividade da ASIP em camundongo. Portanto, os resultados reportados neste artigo fortemente sugerem que ambas as mutações identificadas nestes felinos selvagens causam melanismo devido à perda de função da ASIP.

No manuscrito que compõe o terceiro capítulo, continuamos investigando casos adicionais de envolvimento dos genes *ASIP* e *MC1R* no melanismo de populações naturais de felídeos. O estudo teve como foco três espécies de pequenos felinos endêmicos da América do Sul, pertencentes ao gênero *Leopardus: L. colocolo, L. guigna* e *L. geoffroyi*. Os resultados obtidos revelaram três mutações distintas fortemente associadas com o melanismo de cada espécie, sendo duas delas identificadas no exon 4 do gene *ASIP* e a terceira no gene *MC1R*. Desta forma, todas as oito mutações implicadas no melanismo de felídeos descobertas neste e em estudos prévios (EIZIRIK et al., 2003; SCHNEIDER et al., 2012) estão relacionadas ao gene *ASIP* ou ao *MC1R*. Cinco destas oito mutações estão localizadas no *ASIP* corroborando a conclusão do primeiro artigo desta tese de que o *ASIP* parece estar mais envolvido no melanismo de felinos do que o *MC1R*. Tal observação pode ser explicada devido a diferentes efeitos de seleção atuando sobre estes genes. Além disso, os resultados apoiaram a inferência de seleção natural atuando sobre estes locos durante a história evolutiva do fenótipo melânico nestes felídeos, com ao menos um caso indicativo de seleção positiva recente favorecendo a mutação.

Estudos moleculares e evolutivos sobre este fenótipo de coloração são bastante recentes e ainda escassos, limitando-se a identificar mutações associadas e, em alguns casos, investigar a importância de fatores ecológicos afetando esta característica em populações naturais. Neste contexto, o estudo apresentado no terceiro capítulo apresenta alguns avanços interessantes, pois, além de identificar três novas mutações associadas ao melanismo, realiza um estudo em nível genômico empregando técnicas de sequenciamento de DNA de nova geração. Assim sendo, consiste em um dos primeiros estudos a empregar tal abordagem em populações de animais selvagens, especialmente no contexto da investigação de regiões genômicas associadas à variação fenotípica.

Todos os resultados apresentados nesta tese indicam que o melanismo surgiu independentemente pelo menos oito vezes na família Felidae. De acordo com o conhecimento

atual sobre a filogenia deste grupo, é provável que a base molecular do fenótipo seja espécie-específica em todos os treze casos. Uma possível exceção é *L. tigrinus*, espécie que apresenta evidências de hibridação com *L. geoffroyi* (TRIGO et al., 2008), cuja variante de coloração negra pode ter sido introduzida nestas espécies através de eventos de introgressão, como é o caso de cães e lobos-cinza da América do Norte (ANDERSON et al., 2009).

Considerando a existência de vários genes envolvidos na produção de fenótipos melânicos, é surpreendente que mutações nos genes *ASIP* e *MC1R* sejam tão recorrentes em felídeos. Uma possível explicação é que os outros genes produziriam maiores efeitos pleiotrópicos e, portanto, estariam sob maiores efeitos seletivos negativos. Por outro lado, as mutações que causam perda de função da ASIP e ganho de função do MC1R produziriam efeitos mais brandos, se algum, uma vez que não há até o momento evidências de efeitos pleiotrópicos associados com o melanismo no gato doméstico ou felídeos selvagens.

Os resultados aqui obtidos revelam o potencial de estudos moleculares para investigar a diversidade de fenótipos de coloração, fornecendo informações acerca da base genética e história evolutiva de tal fenômeno. À medida que cresce a obtenção de dados genômicos de organismos-modelo e espécies domésticas, estudos de associação envolvendo populações naturais têm se tornado progressivamente uma realidade para muitos organismos. Há um considerável interesse no estudo da base molecular de características sofrendo efeitos de seleção natural em populações selvagens (FEDER; MITCHELL-OLDS, 2003). A aplicação destes estudos em escala genômica pode revelar as origens históricas e a dinâmica evolutiva de uma ampla variedade de características fenotípicas. Além disso, pode auxiliar em investigações ecológicas como estimativas de frequência e a dinâmica espacial de tais variantes a partir do uso de amostras não invasivas (p.ex. pelos e fezes), contribuindo também para a caracterização detalhada de populações evolutivamente diferenciadas e a definição de unidades evolutivas intra-específicas (EIZIRIK, E, JOHNSON, WE AND O'BRIEN; SJ, 2006).

Perspectivas

As inferências e conclusões procedentes deste estudo abrem novas perspectivas para a investigação detalhada da evolução do melanismo em Felidae, permitindo também que estudos envolvendo outros fenótipos possam ser iniciados, com ênfase na ocorrência e dinâmica destas características em populações naturais.

Uma questão que permanece em aberto para novas investigações é a identificação da base molecular envolvida nas cinco espécies adicionais de felídeos com ocorrência de melanismo. A perspectiva é promissora, considerando os resultados obtidos sobre o envolvimento dos genes *ASIP* e *MC1R* em induzir melanismo em felídeos.

Uma abordagem bastante interessante para futuros estudos envolvendo genes de coloração é a aplicação de ensaios funcionais para investigar as consequências biológicas das mutações identificadas no *ASIP* e *MC1R*. Isto contribuiria para verificar experimentalmente as implicações destas mutações em fenótipos de coloração e os mecanismos funcionais modificados pelas mesmas.

A condução de futuros estudos genômicos sobre o melanismo em felídeos, incluindo uma amostragem mais representativa das espécies provenientes das áreas de estudo e também de outras regiões, é fundamental para a verificação dos padrões moleculares e evolutivos aqui sugeridos, e poderá auxiliar em análises mais específicas de datação destas mutações. Além disso, a combinação destas abordagens com estudos ecológicos e comportamentais investigando a ocorrência de indivíduos melânicos em diferentes tipos de habitat seria também bastante interessante, contribuindo para uma compreensão mais abrangente do significado adaptativo deste fenótipo neste grupo de organismos.

REFERÊNCIAS BIBLIOGRÁFICAS

ANDERSON, T. M. et al. Molecular and evolutionary history of melanism in North American gray wolves. **Science (New York, N.Y.)**, v. 323, n. 5919, p. 1339-43, 6 mar. 2009.

AYOUB, N. A et al. Evolution and phylogenetic utility of the melanocortin-1 receptor gene (MC1R) in Cetartiodactyla. **Molecular phylogenetics and evolution**, v. 52, n. 2, p. 550-7, ago. 2009.

BACKSTRÖM, N. et al. Levels of linkage disequilibrium in a wild bird population. **Biology letters**, v. 2, n. 3, p. 435-8, 22 set. 2006.

BARRETT, R. D. H.; SCHLUTER, D. Adaptation from standing genetic variation. **Trends in ecology & evolution**, v. 23, n. 1, p. 38-44, jan. 2008.

BARSH, G. S. Pigmentation, pleiotropy, and genetic pathways in humans and mice. **Am J. Hum. Genet**, v. 57, p. 743-747, 1995.

BARSH, G. S. The genetics of pigmentation: from fancy genes to complex traits. **Trends in genetics : TIG**, v. 12, n. 8, p. 299-305, ago. 1996.

BASHIR, T. et al. Notable observations on the melanistic Asiatic Golden cat (Pardofelis temminckii) of Sikkim , India. **NeBIO**, v. 2, n. 1, p. 1-4, 2011.

BERNATCHEZ, L.; LANDRY, C. MHC studies in nonmodel vertebrates: what have we learned about natural selection in 15 years? **Journal of evolutionary biology**, v. 16, n. 3, p. 363-77, maio. 2003.

BULTMAN, S. J.; MICHAUD, E. J.; WOYCHIK, R. P. Molecular characterization of the mouse agouti locus. **Cell**, v. 71, n. 7, p. 1195-204, 24 dez. 1992.

CADIEU, E. et al. NIH Public Access. v. 326, n. 5949, p. 150-153, 2010.

CANDILLE, S. I. et al. A -defensin mutation causes black coat color in domestic dogs. **Science (New York, N.Y.)**, v. 318, n. 5855, p. 1418-23, 30 nov. 2007.

CARO, T. The Adaptive Significance of Coloration in Mammals. **BioScience**, v. 55, n. 2, p. 125-136, 2005.

CATANIA, F. et al. World-wide survey of an Accord insertion and its association with DDT resistance in Drosophila melanogaster. **Molecular Ecology**, v. 13, p. 2491-2504, 2004.

CHAN, Y. F. et al. Adaptive evolution of pelvic reduction in sticklebacks by recurrent deletion of a Pitx1 enhancer. **Science (New York, N.Y.)**, v. 327, n. 5963, p. 302-5, 15 jan. 2010.

CHARLESWORTH, B.; MORGAN, M. T.; CHARLESWORTH, D. The effect of deleterious mutations on neutral molecular variation. **Genetics**, v. 134, n. 4, p. 1289-303, ago. 1993.

CHARLESWORTH, B.; NORDBORG, M.; CHARLESWORTH, D. The effects of local selection , balanced polymorphism and background selection on equilibrium patterns of genetic diversity in subdivided populations. **Genetical research**, v. 70, p. 155-174, 1997.

CHARLESWORTH, D. Balancing selection and its effects on sequences in nearby genome regions. **PLoS genetics**, v. 2, n. 4, p. e64, abr. 2006.

CHIU, R. W. K. et al. Noninvasive prenatal diagnosis of fetal chromosomal aneuploidy by massively parallel genomic sequencing of DNA in maternal plasma. **PNAS**, v. 105, n. 51, p. 20458-20463, 2008.

CLARK, A. G. et al. Inferring Nonneutral Evolution from Human-Chimp-Mouse Orthologous Gene Trios. **Science (New York, N.Y.)**, v. 302, p. 1960-1963, 2003.

COLOSIMO, P. F. et al. Widespread parallel evolution in sticklebacks by repeated fixation of Ectodysplasin alleles. **Science (New York, N.Y.)**, v. 307, n. 5717, p. 1928-33, 25 mar. 2005.

DAVIES, K. **Decifrando o genoma: a corrida para desvendar o DNA humano**. [S.l.] Companhia das Letras, 2001. p. 468

DHIMAN, N.; SMITH, D. I.; POLAND, G. A. Next-generation sequencing: a transformative tool for vaccinology. **Expert review of vaccines**, v. 8, n. 8, p. 963-7, ago. 2009.

DIGUISTINI, S. et al. De novo genome sequence assembly of a filamentous fungus using Sanger, 454 and Illumina sequence data. **Genome biology**, v. 10, n. 9, p. R94, jan. 2009.

EDWARDS, R. A et al. Using pyrosequencing to shed light on deep mine microbial ecology. **BMC genomics**, v. 7, p. 57, jan. 2006.

EISENBERG, J.; REDFORD, K. **Mammals of the Neotropics: the Central Neotropics**. 3ª. ed. Illinois: University of Chicago Press, 1999.

EIZIRIK, E. et al. Molecular genetics and evolution of melanism in the cat family. **Current biology : CB**, v. 13, n. 5, p. 448-53, 4 mar. 2003.

EIZIRIK, E, JOHNSON, WE AND O'BRIEN; SJ. Definindo unidades evolutivamente significativas e unidades de manejo para a conservação de carnívoros neotropicais. In: MORATO RG, RODRIGUES FHG, EIZIRIK E, MANGINI PR, A. F. AND M.-F. J. (Ed.). **Manejo e Conservação de Carnívoros Neotropicais**. São Paulo: Ministério do Meio Ambiente, Instituto Brasileiro do Meio Ambiente e dos Recursos Naturais Renováveis, Centro Nacional de Pesquisas para a Conservação dos Predadores Naturais, 2006. p. 49-63.

ELLEGREN, H. Sequencing goes 454 and takes large-scale genomics into the wild. **Molecular ecology**, v. 17, n. 7, p. 1629-31, abr. 2008.

ELLEGREN, H.; SHELDON, B. C. Genetic basis of fitness differences in natural populations. **Nature**, v. 452, n. 7184, p. 169-75, 13 mar. 2008.

FAN, H. C. et al. Noninvasive diagnosis of fetal aneuploidy by shotgun sequencing DNA from maternal blood. **PNAS**, v. 105, n. 42, p. 16266-16271, 2008.

FAY, J. C.; WU, I. Hitchhiking under positive Darwinian selection. **Genetics**, v. 155, p. 1405-1413, 2000.

FEDER, M.; MITCHELL-OLDS, T. Evolutionary and ecological functional genomics. **Nature reviews. Genetics**, v. 4, p. 649-655, fev. 2003.

FIERER, N. et al. Metagenomic and small-subunit rRNA analyses reveal the genetic diversity of bacteria, archaea, fungi, and viruses in soil. **Applied and environmental microbiology**, v. 73, n. 21, p. 7059-66, nov. 2007.

FREER, R. A. **The Spatial Ecology of the Güiña (Oncifelis guigna) in Southern Chile by**. [S.l: s.n.]. p. 219

FU, Y. X.; LI, W. H. Statistical tests of neutrality of mutations. **Genetics**, v. 133, p. 693-709, 1993.

GARRIGAN, D.; HEDRICK, P. W. Perspective: detecting adaptive molecular polymorphism: lessons from the MHC. **Evolution; international journal of organic evolution**, v. 57, n. 8, p. 1707-22, ago. 2003.

GHIMIREY, Y.; PAL, P. First camera trap image of Asiatic golden cat in Nepal. **Cat News**, v. 51, p. 19, 2009.

GILBERT, W. DNA sequencing and gene structure. **Bioscience Reports**, v. 1, p. 353-375, 1981.

HARVEY, P. H.; PAGEL, M. D. **The comparative method in evolutionary biology.** Oxford, UK: Oxford Univ. Press, 1991.

HE, L. et al. A biochemical function for attractin in agouti-induced pigmentation and obesity. **Nature genetics**, v. 27, n. 1, p. 40-7, jan. 2001.

HIRSCHHORN, J. N.; DALY, M. J. Genome-wide association studies for common diseases and complex traits. **Nature reviews. Genetics**, v. 6, n. 2, p. 95-108, fev. 2005.

HOEKSTRA, H. E. Genetics, development and evolution of adaptive pigmentation in vertebrates. **Heredity**, v. 97, n. 3, p. 222-34, set. 2006.

HOEKSTRA, H. E.; NACHMAN, M. W. Different genes underlie adaptive melanism in different populations of rock pocket mice. **Molecular ecology**, v. 12, n. 5, p. 1185-94, maio. 2003.

HOEKSTRA, HOPI E., HIRSCHMANN, RACHEL J., BUNDEY, RICHARD A., INSEL, PAUL A. CROSSLAND, J. P. A single amino acid mutation contributes to adaptive beach mouse color pattern. **Science (New York, N.Y.)**, n. July, p. 101-104, 2006.

HOFREITER, M.; SCHÖNEBERG, T. The genetic and evolutionary basis of colour variation in vertebrates. **Cellular and molecular life sciences : CMLS**, v. 67, n. 15, p. 2591-603, ago. 2010.

HORI, M. Frequency-Dependent Natural Selection in the Handedness of Scale-Eating Cichlid Fish. **Science**, v. 260, p. 216-219, 1993.

HUANG, S. et al. The genome of the cucumber, Cucumis sativus L . **Nature genetics**, v. 41, n. 12, p. 1275-1283, 2009.

HUBBARD, J. K. et al. Vertebrate pigmentation: from underlying genes to adaptive function. **Trends in genetics : TIG**, v. 26, n. 5, p. 231-9, maio. 2010.

HUDSON, R. R.; KREITMAN, M.; AGUADE, M. A test of neutral molecular evolution based on nucleotide data. **Genetics**, v. 116, p. 153-159, 1987.

IMELFORT, M. et al. Discovering genetic polymorphisms in next-generation sequencing data. **Plant biotechnology journal**, v. 7, n. 4, p. 312-7, maio. 2009.

ISHIDA, Y. et al. A homozygous single-base deletion in MLPH causes the dilute coat color phenotype in the domestic cat. **Genomics**, v. 88, n. 6, p. 698-705, dez. 2006.

JACKSON, I. J. Evolution and expression of tyrosinase-related proteins. **Pigment cell research / sponsored by the European Society for Pigment Cell Research and the International Pigment Cell Society**, v. 7, p. 241-242, 1994.

JOHNSINGH, A. J. T.; PANWAR, H. S.; RODGERS, W. A. Ecology and conservation of large felids in India. In: **Wildlife conservation: present trends and perspectives for the 21 st century**. Yokohoma, Japan: Proc. V Int. Congr. Ecol, 1991. p. 160-165.

JOHNSON, W. E. et al. The late Miocene radiation of modern Felidae: a genetic assessment. **Science (New York, N.Y.)**, v. 311, n. 5757, p. 73-7, 6 jan. 2006.

KAWANISHI, K. et al. Near fixation of melanism in leopards of the Malay Peninsula. **Journal of Zoology**, v. 282, n. 3, p. 201-206, 13 nov. 2010.

KAYSER, M.; BRAUER, S.; STONEKING, M. A genome scan to detect candidate regions influenced by local natural selection in human populations. **Molecular biology and evolution**, v. 20, n. 6, p. 893-900, jun. 2003.

KIJAS, J. M. H. et al. Melanocortin Receptor 1 (MC1R) Mutations and Coat Color in Pigs. **Genetics**, v. 150, p. 1177-1185, 1998.

KIMURA, M. Evolutionary rate at the molecular level. **Naure**, v. 217, p. 624, 1968.

KIMURA, M. Recent development of the neutral teory viewed from the Wrightian tradition of theoretical population genetics. **Proceedings of the National Academy of Sciences U S A**, v. 88, n. 14, p. 5969-5973, 1991.

KINGSLEY, E. P. et al. Melanism in peromyscus is caused by independent mutations in agouti. **PloS one**, v. 4, n. 7, p. e6435, jan. 2009.

KLUNGLAND, H. et al. The role of melanocyte-stimulating hormone (MSH) receptor in bovine coat color determination. **Mammalian genome : official journal of the International Mammalian Genome Society**, v. 6, n. 9, p. 636-9, set. 1995.

KOHN, M. H.; PELZ, H.; WAYNE, R. K. Natural selection mapping of the warfarin-resistance gene. **Proceedings of the National Acad- emy of Sciences**, v. 97, n. 14, p. 7911-7915, 2000.

KURAMOTO, T. et al. Cloning of the rat agouti gene and identification of the rat nonagouti mutation. **Mammalian genome : official journal of the International Mammalian Genome Society**, v. 12, n. 6, p. 469-71, jun. 2001.

LEWONTIN, R. C.; KRAKAUER, J. DISTRIBUTION OF GENE FREQUENCY AS A TEST OF THE THEORY O F THE SELECTIVE NEUTRALITY OF POLYMORPHISMS. **Genetics**, v. 74, n. 1, p. 175-195, 1973.

LI, R. et al. The sequence and de novo assembly of the giant panda genome. **Nature**, v. 463, n. 7279, p. 311-7, 21 jan. 2010.

LING, M. K. et al. Association of feather colour with constitutively active melanocortin 1 receptors in chicken. **European Journal of Biochemistry**, v. 270, n. 7, p. 1441-1449, abr. 2003.

LUCHERINI, M.; VIDAL, E. L.; BELDOMENICO, P. First record of sympatry of Guigna and Geoffroyi's cat. **Cat News**, v. 35, p. 20-21, 2001.

LUIKART, G. et al. The power and promise of population genomics: from genotyping to genome typing. **Nature reviews. Genetics**, v. 4, n. 12, p. 981-94, dez. 2003.

MAJERUS, M. E. N. **Melanism: Evolution in Action**. Oxford: Oxford Univ. Press, 1998.

MANCEAU, M. et al. Convergence in pigmentation at multiple levels: mutations, genes and function. **Philosophical transactions of the Royal Society of London. Series B, Biological sciences**, v. 365, n. 1552, p. 2439-50, 27 ago. 2010.

MARDIS, E. R.; WILSON, R. K. Cancer genome sequencing: a review. **Human molecular genetics**, v. 18, n. R2, p. R163-8, 15 out. 2009.

MAYNARD SMITH, J.; HAIGH, J. The hitchhiking effect of a favorable gene. **Genetic Research**, v. 23, p. 23-35, 1974.

MCDONALD, J. H.; KREITMAN, M. Adaptive protein evolution at the Adh locus in Drosophila. **Nature**, v. 351, p. 652-654, 1991.

MCROBIE, H.; THOMAS, A.; KELLY, J. The genetic basis of melanism in the gray squirrel (Sciurus carolinensis). **The Journal of heredity**, v. 100, n. 6, p. 709-14, 2009.

MILTENBERGER, R. J. et al. Alleles at the Mouse agouti Locus. **Genetics**, n. 160, p. 659-674, 2002.

MOROZOVA, O.; MARRA, M. A. Applications of next-generation sequencing technologies in functional genomics. **Genomics**, v. 92, n. 5, p. 255-64, nov. 2008.

MOUNTJOY, K. G. et al. The cloning of a family of genes that encode the melanocortin receptors. **Science (New York, N.Y.)**, v. 257, n. 5074, p. 1248-51, 28 ago. 1992.

MUNDY, N. I.; KELLY, J. Evolution of a pigmentation gene, the melanocortin-1 receptor, in primates. **American journal of physical anthropology**, v. 121, n. 1, p. 67-80, maio. 2003.

NACHMAN, M. W.; HOEKSTRA, H. E.; D'AGOSTINO, S. L. The genetic basis of adaptive melanism in pocket mice. **Proceedings of the National Academy of Sciences of the United States of America**, v. 100, n. 9, p. 5268-73, 29 abr. 2003.

NADEAU, N. J.; MINVIELLE, F.; MUNDY, N. I. Association of a Glu92Lys substitution in MC1R with extended brown in Japanese quail (Coturnix japonica). **Animal genetics**, v. 37, n. 3, p. 287-9, jun. 2006.

NAIR, S. et al. A Selective Sweep Driven by Pyrimethamine Treatment in Southeast Asian Malaria Parasites. **Molecular Biology and Evolution**, v. 20, p. 1526-1536, 2002.

NIELSEN, R. Molecular signatures of natural selection. **Annual review of genetics**, v. 39, p. 197-218, jan. 2005.

NOWELL, K.; JACKSON, P. Status Survey and Conservation Action Plan Wild Cats. **IUCN/SSC Cat Specialist Group, Gland, Switzerland**, p. 383, 1996.

OLIVEIRA, B.; RAMACHANDRAN, G.; ADAMS, M. CALCIUM CHANNEL NEUROTRANSMITTER RELEASE : The w-Conotoxins and w-Agatoxins. **Annual Reviews Biochem.**, v. 63, p. 823-867, 1994.

OLIVEIRA, T. G. **Neotropical Cats: Ecology and Conservation**. São Luís: EDUFMA, 1994.

OLIVEIRA, T. G.; CASSARO, K. **Guia de Identificação de Felinos Brasileiros**. 2nd. ed. São Paulo: Sociedade de Zoológicos do Brasil, 1999.

ORTOLANI AND CARO, T. The adaptative significance of color patterns in carnivores: Phylogenetic test of classic hypotheses. In: JL, G. (Ed.). **Carnivore Behavior, Ecology and Evolution**. Vol. 2 ed. New York: Cornell Univ. Press, 1996. p. 132-188.

OSGOOD, W. H. The mammals of Chile. **Zoological Series, Field Museum of Natural History**, v. 30, p. 1-268, 1943.

PALAISA, K. et al. Long-range patterns of diversity and linkage disequilibrium surrounding the maize Y1 gene are indicative of an asymmetric selective sweep. **Proceedings of the National Academy of Sciences of the United States of America**, v. 101, n. 26, p. 9885-90, 29 jun. 2004.

PASVOL, G.; WEATHERALL, D. J.; WILSON, R. J. M. Cellular mechanism for the protective effect of haemoglobin S against P. falciparum malaria. **Nature**, v. 274, p. 701-703, 1978.

PERRY, L. et al. Coupled Site-Directed Mutagenesis/Transgenesis Identifies Important Functional Domains of the Mouse Agouti Protein. **Genetics**, n. 144, p. 255-264, 1996.

RIEDER, S. et al. Mutations in the agouti (ASIP), the extension (MC1R), and the brown (TYRP1) loci and their association to coat color phenotypes in horses (Equus caballus). **Mammalian genome : official journal of the International Mammalian Genome Society**, v. 12, n. 6, p. 450-5, jun. 2001.

RITLAND, K.; NEWTON, C.; MARSHALL, H. D. Inheritance and population structure of the white-phased "Kermode" black bear. **Current biology : CB**, v. 11, n. 18, p. 1468-72, 18 set. 2001.

ROBBINS, L. S. et al. Pigmentation phenotypes of variant extension locus alleles result from point mutations that alter MSH receptor function. **Cell**, v. 72, n. 6, p. 827-34, 26 mar. 1993.

ROBINSON, R. Inheritance of the black form of the leopard Panthera pardus. **Genetica**, v. 41, p. 190-197, 1970.

ROSENBLUM, E. B. Convergent evolution and divergent selection: lizards at the White Sands ecotone. **The American naturalist**, v. 167, n. 1, p. 1-15, jan. 2006.

RUBIN, C.-J. et al. Whole-genome resequencing reveals loci under selection during chicken domestication. **Nature**, v. 464, n. 7288, p. 587-91, 25 mar. 2010.

RÖMPLER, H. et al. Nuclear gene indicates coat-color polymorphism in mammoths. **Science (New York, N.Y.)**, v. 313, n. 5783, p. 62, 7 jul. 2006.

SABETI, P. C. et al. Detecting recent positive selection in the human genome from haplotype structure. **Nature**, v. 419, n. October, p. 832-837, 2002.

SANDERSON, J.; SUNQUIST, M. E.; IRIARTE, A. W. NATURAL HISTORY AND LANDSCAPE-USE OF GUIGNAS (ONCIFELIS GUIGNA) ON ISLA GRANDE DE CHILOÉ , CHILE. **Journal of Mammalogy**, v. 83, n. 2, p. 608-613, 2002.

SCHLÖTTERER, C. Towards a molecular characterization of adaptation in local populations. **Current opinion in genetics & development**, v. 12, n. 6, p. 683-7, dez. 2002.

SCHNEIDER, A. et al. How the Leopard Hides Its Spots: ASIP Mutations and Melanism in Wild Cats. **PloS one**, v. 7, n. 12, p. e50386, jan. 2012.

SEARLE, A. G. **Comparative Genetics of Coat Color in Mammals**. London: Logos Press, 1968. p. 308

SILVERS, W. K. **The coat Colors of Mice: a Model for Mammalian Gene Action and Interaction**. New York: Springer-Verlag, 1979.

SLATE, J. O. N. Quantitative trait locus mapping in natural populations : progress , caveats and future directions. **Molecular Ecology**, v. 14, p. 363-379, 2005.

SMITH, A G. et al. The human melanocortin-1 receptor locus: analysis of transcription unit, locus polymorphism and haplotype evolution. **Gene**, v. 281, n. 1-2, p. 81-94, 27 dez. 2001.

STEPHAN, W.; WIEHE, T. H. E.; LENZ, M. W. The effect of strongly selected substitutions on neutral polymorphism: analytical results based on diffusion theory. **Theoretical Population Biology**, v. 41, p. 237-254, 1992.

STORZ, J. F. Using genome scans of DNA polymorphism to infer adaptive population divergence. **Molecular ecology**, v. 14, n. 3, p. 671-88, mar. 2005.

STUMPF, M. P. H.; MCVEAN, G. A T. Estimating recombination rates from population-genetic data. **Nature reviews. Genetics**, v. 4, n. 12, p. 959-68, dez. 2003.

SUNQUIST, M. E.; SUNQUIST, F. **Wild cats of the World**. Chicago: University Chicago Press., 2002.

TABOR, H. K.; RISCH, N. J.; MYERS, R. M. Candidate-gene approaches for studying complex genetic traits: practical considerations. **Nature Reviews Genetics**, v. 3, n. May, p. 1-7, 2002.

TAJIMA, F. Statistical method for testing the neutral mutation hypothesis by DNA polymorphism. **Genetics**, v. 123, p. 585-595, 1989.

TAKEUCHI, S. et al. A possible involvement of melanocortin 1-receptor in regulating feather color pigmentation in the chicken. **Biochimica et Biophysica Acta**, v. 1308, p. 164-168, 1996.

TAYLOR, K. H. et al. Ultradeep bisulfite sequencing analysis of DNA methylation patterns in multiple gene promoters by 454 sequencing. **Cancer research**, v. 67, n. 18, p. 8511-8, 15 set. 2007.

THERON, E. et al. The molecular basis of an avian plumage polymorphism in the wild: a melanocortin-1-receptor point mutation is perfectly associated with the melanic plumage morph of the bananaquit, Coereba flaveola. **Current biology : CB**, v. 11, n. 8, p. 550-7, 17 abr. 2001.

TRIGO, T. C. et al. Inter-species hybridization among Neotropical cats of the genus Leopardus, and evidence for an introgressive hybrid zone between L. geoffroyi and L. tigrinus in southern Brazil. **Molecular ecology**, v. 17, n. 19, p. 4317-33, out. 2008.

VAGE, D. et al. A non-epistatic interaction of agouti and extension in the fox, Vulpes vulpes. **Nature genetics**, v. 15, p. 311-315, 1997.

VARSHNEY, R. K. et al. Next-generation sequencing technologies and their implications for crop genetics and breeding. **Trends in biotechnology**, v. 27, n. 9, p. 522-30, set. 2009.

VASEMÄGI, A; PRIMMER, C. R. Challenges for identifying functionally important genetic variation: the promise of combining complementary research strategies. **Molecular ecology**, v. 14, n. 12, p. 3623-42, out. 2005.

VELASCO, R. et al. A High Quality Draft Consensus Sequence of the Genome of a Heterozygous Grapevine Variety. **Plos One**, n. 12, p. 1-18, 2007.

VIGOUROUX, Y. et al. Identifying genes of agronomic importance in maize by screening microsatellites for evidence of. **PNAS**, v. 99, n. 15, p. 9650-9655, 2002.

VÅGE, D. I. et al. Molecular and pharmacological characterization of dominant black coat color in sheep. **Mammalian genome : official journal of the International Mammalian Genome Society**, v. 10, n. 1, p. 39-43, jan. 1999.